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Mitochondria Are the Main Target Organelle for Trivalent Monomethylarsonous Acid (MMA^{III})-Induced Cytotoxicity

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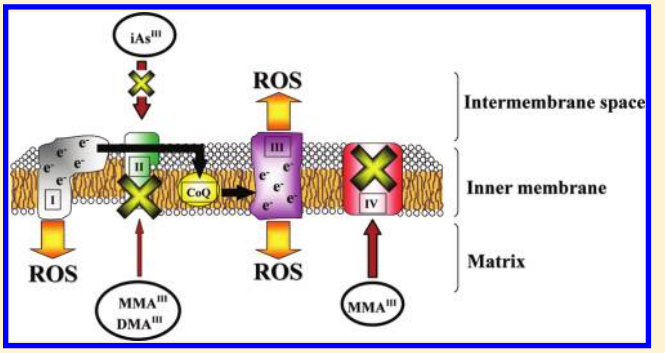
Supporting Information

ABSTRACT: Excessive generation of reactive oxygen species (ROS) is considered to play an important role in arsenic-induced carcinogenicity in the liver, lungs, and urinary bladder. However, little is known about the mechanism of ROS-based carcinogenicity, including where the ROS are generated, and which arsenic species are the most effective ROS inducers. In order to better understand the mechanism of arsenic toxicity, rat liver RLC-16 cells were exposed to arsenite (iAs^{III}) and its intermediate metabolites [i.e., monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III})]. MMA^{III} ($IC_{50} = 1\ \mu M$) was found to be the most toxic form, followed by DMA^{III} ($IC_{50} = 2\ \mu M$) and iAs^{III} ($IC_{50} = 18\ \mu M$). Following exposure to MMA^{III}, ROS were found to be generated primarily in the mitochondria. DMA^{III} exposure resulted in ROS generation in other organelles, while no ROS generation was seen following exposures to low levels of iAs^{III}. This suggests the mechanisms of induction of ROS are different among the three arsenicals. The effects of iAs^{III}, MMA^{III}, and DMA^{III} on activities of complexes I−IV in the electron transport chain (ETC) of rat liver submitochondrial particles and on the stimulation of ROS production in intact mitochondria were also studied. Activities of complexes II and IV were significantly inhibited by MMA^{III}, but only the activity of complexes II was inhibited by DMA^{III}. Incubation with iAs^{III} had no inhibitory effects on any of the four complexes. Generation of ROS in intact mitochondria was significantly increased following incubation with MMA^{III}, while low levels of ROS generation were observed following incubation with DMA^{III}. ROS was not produced in mitochondria following exposure to iAs^{III}. The mechanism underlying cell death is different among As^{III}, MMA^{III}, and DMA^{III}, with mitochondria being one of the primary target organelles for MMA^{III}-induced cytotoxicity.



INTRODUCTION

Exposure to arsenic is associated with cancer of the skin, liver, lungs, and urinary bladder.[1,2] In mammals, ingested inorganic arsenite (iAs^{III}) or inorganic arsenate (iAs^{V}) can be biotransformed through a series of enzymatically mediated methylation reactions, producing methylated trivalent and pentavalent metabolites,[3] and the final metabolites that are excreted in the urine are mostly mono- and dimethylated pentavalent species.[3,4] However, it is not known which of these forms (inorganic arsenic or its metabolites) is responsible for arsenic's carcinogenicity, and the mechanism is not well understood.

A few studies have indicated that the arsenic-induced cancer associated with chronic exposure to inorganic arsenic may be mediated by its intermediate metabolites such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}).[5−7] In addition, the formation of these products may result in the generation of reactive oxygen species (ROS) and free radicals that could exert carcinogenic effects.[8] In fact, recent evidence suggests that an increase in oxidative stress in cells treated with arsenicals may be the molecular mechanism behind arsenic-induced carcinogenesis.[9,10]

Generally speaking, trivalent arsenicals are more cytotoxic and genotoxic than their pentavalent counterparts.[11,12] In addition, trivalent arsenicals are able to induce oxidative DNA damage in animals as well as a greater frequency of chromosomal aberrations in cells.[13−15] Previous studies from our laboratory suggest that iAs^{III} methylation occurs in the liver, and the trivalent arsenicals are bound to proteins throughout the methylation process ($iAs^{III} \rightarrow MMA^{III} \rightarrow DMA^{III}$).[16] The liver is also one of the identified target organ for arsenic toxicity and carcinogenicity, but it remains unclear if the cytotoxcity of these trivalent arenicals, or the ROS production associated with their formation may be responsible for this toxic effect.

Kitchin et al. has indicated that the generation of ROS is likely to play an important role in the early stages of arsenic carcinogenesis, as well as risk factor for cancer development in target organs in humans.[17] In other studies, high levels of products of DNA oxidation, such as oxidation8-oxo-2′-deoxyguanosine

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(8-oxodG), were detected in arsenic-induced human skin cancer in China, as well as in human urine after exposure to cadmium and arsenic.[18,19] Similar results were also obtained from rats after long-term exposure to DMA^V, with the generation of 8-OHdG being increased in the liver and bladder.[20,21] Thus, understanding of the mechanism of arsenic induced ROS generation is important for better understanding the mechanism of carcinogenicity and could also aid in the development of strategies to counteract these effects.

Mitochondria are known to be one of the major generators of ROS in cells. Dysfunction of electron transfer through the mitochondrial respiratory chain may result in increased ROS formation, including the formation of the superoxide anion radical ($O_2\bullet-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\bullet$).[22,23] Moreover, complexes I and III in the electron transport chain (ETC) are thought to be the main leak sites for ROS production, as some of the electrons passing through the mitochondrial respiratory chain (i.e., complex I and III) leak out to molecular oxygen (O_2) to form superoxide radicals and then dismutate to H_2O_2.[24]

The present study explores the mechanism of toxicity and localization of ROS generated by iAs^{III} and its intermediate metabolites such as MMA^{III} and DMA^{III} in rat liver RLC-16 cells. Although these trivalent arsenicals are highly toxic to RLC-16 cells, the induction mechanism of cell death seems to vary with species. The majority of ROS were generated in mitochondria following exposure to MMA^{III}, while other organelles produced lower amounts of ROS following exposure to DMA^{III}. No ROS were detected in cells following exposure to low levels iAs^{III}. This study also investigated how the complexes of the electron transfer chain (ETC) within the mitochondria were inhibited by As^{III}, MMA^{III}, and DMA^{III}. Activities of complex II and IV were strongly inhibited by exposure to MMA^{III}, but only the activity of complexes II was inhibited by exposure to DMA^{III}. No inhibitory effects were observed following exposure to iAs^{III}, suggesting ROS are generated mainly through the inhibition of complex IV due to exposure to MMA^{III}.

MATERIAL AND METHODS

Reagents. All reagents were of analytical grade or better. 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, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). Nitric acid, hydrogen peroxide (30%), cytochrome *c* (Horse Heart), L-glutamic acid, L-(−)-malic acid, L-cysteine, succinic acid, sodium hydrosulfite, 10% formalin neutral buffer solution, potassium cyanide, 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 ICPMS arsenic standard solution (1,000 μg/mL) was purchased from SPEX CentiPrep (Metuchen, NJ, USA). Stock solutions of all arsenic compounds (10 mmol/L) were prepared from their respective compounds and were stored in the dark at 4 °C. The stock solutions were diluted daily as necessary.

Preparation of Monomethylarsonous Acid (MMA^{III}) and Dimethylarsinous Acid (DMA^{III}). MMA^{III} and DMA^{III} were prepared by reducing monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V), respectively, with 5 mol equiv of L-cysteine in distilled water at 90 °C for 1 h. The identities of the trivalent forms were confirmed by comparison to their iodide forms using gel filtration HPLC−ICP MS.[25]

Isolation of Subcellular Fractions from Rat Livers and Cells and the Preparation of Submitochondrial Particles. All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH version, revised 1996) and the Guidelines of the Animal Investigation Committee, Showa Pharmaceutical University, Japan.

Six week old male Wistar rats were purchased from Clea Japan (Tokyo). The rats were housed in a humidity-controlled room, maintained at 22−25 °C with a 12 h light−dark cycle. The animals were fed a commercial diet (MF; Clea Japan) and tap water ad libitum. Following a one-week acclimatization period, rats at 7 weeks of age (body weight, 180−220 g) were used for experiments.

Whole liver perfusion was performed following the method developed in our laboratory. Briefly, rats under sodium pentobarbital anesthesia were dissected to expose the heart, and then 0.2 mL of heparin was injected into the left ventricle. The remaining blood was perfused through the portal vein by a roller pump with phosphate buffered saline (PBS) at a flow rate of 60 mL/min. Blood-free liver (or cells) was minced in ice-cold homogenization buffer A (230 mM Mannitol, 70 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA·2Na, and 0. 5% bovine serum albumin (BSA), pH 7.4) by using a Dounce homogenizer to make 20% (w/v) liver (or cells) homogenates and then kept on ice for 5 min to remove unbroken cells and connective tissues. After removing the unbroken cells, the supernatant was centrifuged twice at 700*g* for 10 min at 4 °C to obtain the crude cell nuclear fractions. Cytoplasmic and nuclear marker proteins (i.e., GAPDH and p84, respectively) were used to determine the purification of nuclear fractions.

Mitochondria were isolated by subjecting the supernatant to centrifugation at 12,000*g* for 10 min at 4 °C to obtain the pellet. The pellet was washed twice and resuspended in isolation buffer B (230 mM Mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA·2Na) at a protein concentration of 10 mg/mL. Mitochondria activity was determined by the cytochrome *c* oxidase (CCO) assay kit (Sigma, USA). Moreover, the pooled supernatant was further centrifuged at 105,000*g* for 10 min at 4 °C to obtain the pellet fraction as the crude microsome.

Submitochondrial particles were prepared from frozen and thawed mitochondria (10−20 mg proteins/mL) and disrupted by sonication for three 15 s periods with 30 s intervals at an output of 40 W using an ultrasonic homogenizer (Bio rupter UCD-200, Cosmo BioCo., Ltd., Tokyo, Japan) and then centrifuged at 10,000*g* for 10 min (4 °C). The supernatant was decanted and centrifuged at 105,000*g* for 30 min. The pellet (i.e., submitochondrial particles) was washed once and resuspended in isolation buffer B, and the protein concentration was determined according to Lowry's method using BSA as the standard.

Culture of RLC-16 Cells. Rat liver cell line RLC-16 was 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.

Effect of *N*-Acetylcysteine (NAC) on the Viability of RLC-16 Cells. To investigate the effect of *N*-acetyl-L-cysteine (NAC) on RLC-16 cells, the cells were cultured in a 96-well culture dish in the presence or absence of 2 mM NAC for 24 h and then exposed to various concentrations of arsenic compounds (i.e., iAs^{III}, MMA^{III}, and DMA^{III}) for 24 h. Then, 10 μL of an MTT solution was added to each well (at the final concentration of 0.5 mg/mL), and the plates were incubated for an additional 3 h at 37 °C. Afterward, the cell cultures were washed with PBS, and 150 μL of DMSO was added to each well. Cell viability was measured as the absorbance at 570 nm with a microplate reader and expressed as a percentage of the control level ($n = 5$).

MTT Assay for Cellular Viability. RLC-16 cells were seeded at a density of 2×10^4 cells/100 μL/well in 96-well microtiter plates (Promega Corporation). Twenty-four hours postseeding, the cultures were washed twice with PBS and then exposed to various concentrations of trivalent arsenic compounds for 24 h. Then, 10 μL of an MTT solution was added to each well (at the final concentration of 0.5 mg/mL), and the plates were incubated for an additional 3 h at 37 °C. Afterward, cell cultures were washed with PBS, and 150 μL of DMSO was added to each well. Cell viability was measured as the absorbance at 570 nm with a microplate reader and expressed as a percentage of the control level.

Arsenic Measurements. Cells were seeded at a density of 1.0×10^6 cells in a 10 cm culture dish ($n = 3$) and then exposed to arsenic compounds in fresh medium. After exposure, the cell monolayer was washed twice with PBS. The cells were collected and then suspended in 300 μL of a 100 mM ammonium acetate solution (pH 6.5 at 25 °C; dissolved oxygen was purged by bubbling with nitrogen gas). The suspended cells were disrupted on ice with an ultrasonic homogenizer (Biorupter UCD-200, Cosmo Bio Co., Ltd., Tokyo, Japan) operating at 200 W and 20 kHz for 30 s. This was done three times with intervals of 45 s, followed by centrifugation at 105,000*g* for 1 h at 4 °C to yield the supernatant (soluble fraction) and insoluble sediment fractions (i.e., precipitates). The concentrations of arsenic in the supernatant and insoluble fractions of cells were determined with an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction system (ORS) with an He flow of 3.0 mL/min to prevent molecular interference by $^{40}Ar^{35}Cl^+$ (signal at m/z 75). Prior to analysis, liver samples were wet-ashed with a mixture of concentrated nitric acid and 30% H_2O_2 (1:1, v/v) at 150 °C for 2 days.

Removing Unbound Arsenic Compounds in Supernatants of Cells. The supernatants (500 μL) were dialyzed two times for 6 h each in a Slide-A-Lyzer Dialysis Cassette against 500 mL of 50 mM ammonium acetate buffer (pH 7.4) at 4 °C to remove the unbound arsenicals (i.e., free arsenicals) according to the method reported elsewhere.[16] The cell supernatants and the protein-bound arsenic in the remaining dialyzed supernatant were analyzed using ICPMS following wet-digesting with a mixture of concentrated nitric acid and 30% H_2O_2 (1:1, v/v) at 150 °C for 2 days. Concentration of free arsenic was calculated as supernatant minus dialyzed supernatant, while the concentration of arsenic bound to proteins was calculated as total arsenic minus unbound free arsenic.

Preparation of Decylubiquinol and Reduced Cytochrome *c*. The preparation of decylubiquinol was carried out according to Fisher's method[26] with slight modifications. Briefly, 100 μL of 500 mmol/L decylubiquinone was diluted using absolute ethanol to a concentration of 25 mmol/L, followed by the addition of 6 mL of a solution consisting of 0.1 mol/L potassium phosphate buffer, pH 7.4, and 0.25 mol/L sucrose. Then, 1 mL of cyclohexane and 0.1 g of solid sodium dithionite were added to the solution. The resulting mixture was shaken until colorless. The upper layer of cyclohexane was decanted into a separate vial. The aqueous solution was extracted twice with a 1 mL portion of cyclohexane twice, with each organic phase being decanted off and then combined with the initial organic layer. The combined organic phase was evaporated under vacuum until a light-yellow syrup remained in the bottom of the tube. This syrup was dissolved in 900 μL of absolute ethanol and 100 μL of 0.1 mol/L HCl, and stored in aliquots at −80 °C.

Reduced cytochrome *c* was prepared by reducing the cytochrome *c* with ascorbic acid in 10 mM potassium phosphate buffer (pH 7.0). After reduction, the ascorbic acid was removed by dialysis with the Slide-A-Lyzer Dialysis cassette (cutoff molecular weight, MW, <5K) in 10 mM potassium phosphate buffer (pH 7.0) at 4 °C for 24 h. The dialysis buffer was changed every 8 h. The reduced cytochrome *c* was stored in an atmosphere of nitrogen at −20 °C.

Measurement of Reactive Oxygen Species (ROS) in Cells and Mitochondria. To assess the production of total ROS, RLC-16 cells were seeded on 6-well culture plates with cover glasses at a density of 2×10^5 cells for 24 h, then preincubated with 2 μmol/L of CM-DCFHFDA for 30 min at 37 °C. After the incubation, cells were exposed to iAs^{III}, MMA^{III}, and DMA^{III} at the respective IC_{50} dose for 24 h. Following incubation, the cells were washed twice with PBS to remove the medium and fixed with 10% formalin neutral solution (pH 7.4) for 2 h, and a few drops of Crystal/Mount were applied to the cover glass. Cellular images were captured by confocal microscopy using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc.). Fluorescence intensity of ROS was analyzed by Image J (Display DICOM software) ($n = 10$).

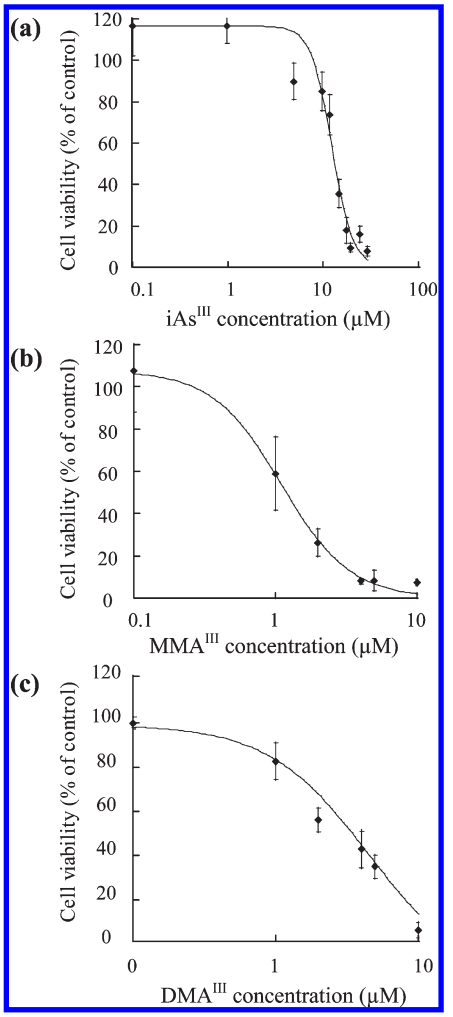

Figure 1. Viability of RLC-16 cells after exposure to iAs^{III}, MMA^{III}, and DMA^{III}. Cells were exposed to various concentrations of iAs^{III} (a), MMA^{III} (b), and DMA^{III} (c) for 24 h. The IC_{50} values for iAs^{III}, MMA^{III}, and DMA^{III} were calculated to be 18 μM, 1 μM, and 2 μM, respectively. Data are expressed as the mean ± SD ($n = 4$).

Generation of ROS in isolated rat liver mitochondria was measured by monitoring ROS-induced fluorescence of 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) (λ_{ex} = 510 nm, λ_{em} = 525 nm). Mitochondria (0.1 mg protein/mL) were incubated at 37 °C in a mixture of the KCl buffer (125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.4), 1 mM DCFH-DA, and 6 mM substrate (succinate, malate, and glutamate). The fluorescein was used to prepare a standard curve for the quantitation of the fluorescence signal (i.e., ROS), which was detected by an F-2500 fluorescence spectrophotometer.

Enzyme Assays for Electron Transport Chain Complex Activities (I−IV). Activities of complexes I−IV were determined using spectrophotometry according to published methods,[27] with a U-3000

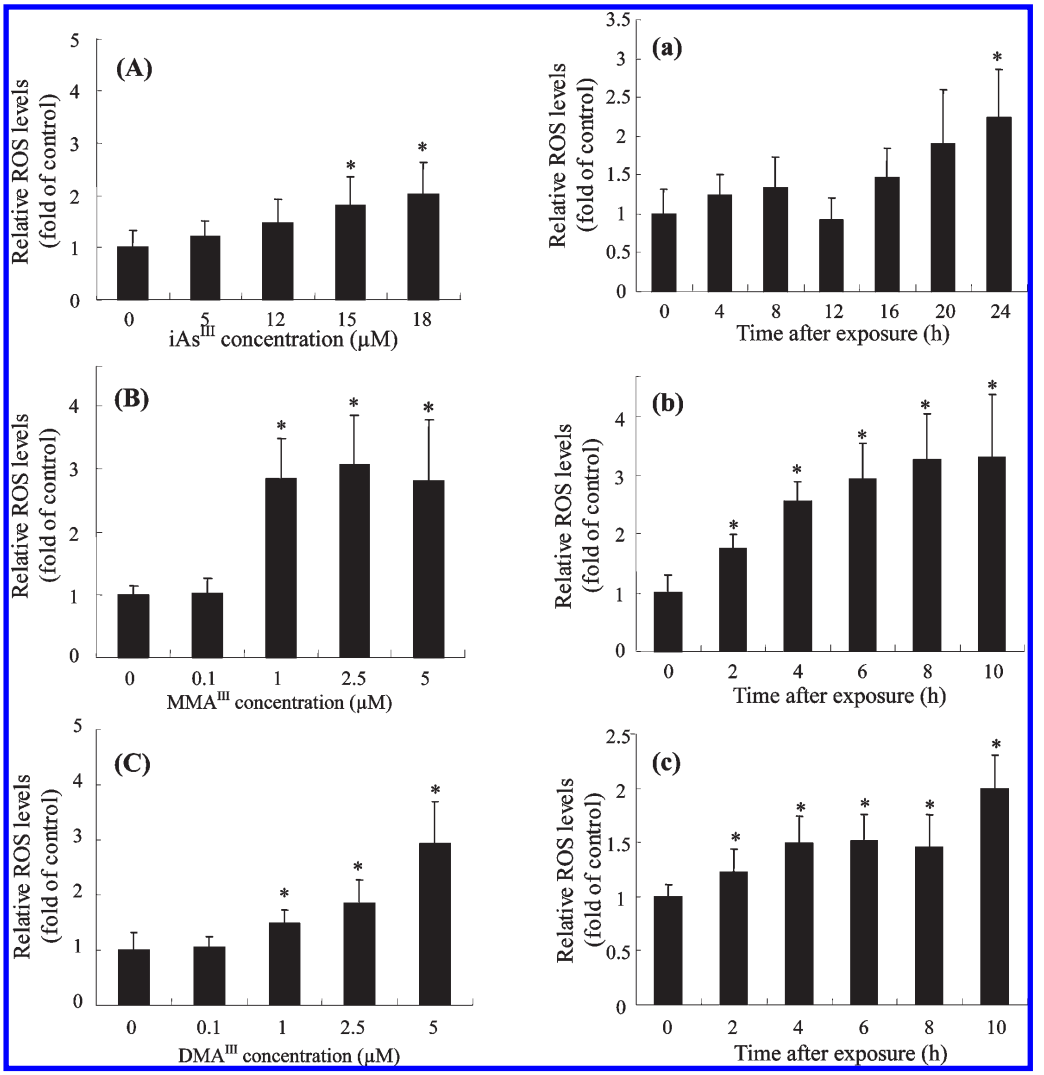

Figure 2. Dose- and time-dependent generation of reactive oxygen species (ROS) in RLC-16 cells after exposure to iAs^{III}, MMA^{III}, and DMA^{III}. RLC-16 cells were seeded on 6-well culture plates with cover glasses, and then cultured for 24 h. Cells were pretreated with 2 μM CM-H_2DCFDA for 1 h and then exposed to various concentrations of iAs^{III} (A), MMA^{III} (B), and DMA^{III} (C) for 24 h or exposed to iAs^{III} (a), MMA^{III} (b), and DMA^{III} (c) at their IC_{50} values for different time points. Following exposure, cells were fixed with 10% formalin and then ROS generation was determined using a confocal laser scanning microscope. Fluorescence intensity of ROS was analyzed by Image J (Display DICOM software). Data are expressed as the means ± SD (n = 10). Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

spectrophotometer being used throughout (for details, see Supporting Information).

Statistical Analysis. Each experiment was performed at least three times. Statistical analysis of data was carried out using Student's t-test, and the data were expressed as the mean ± SD. Differences between the two groups were regarded as significant at $p < 0.05$, and significant differences were labeled by asterisks (*).

RESULTS

Viability of RLC-16 Cells after Exposure to Trivalent iAs^{III}, MMA^{III}, and DMA^{III}. Figure 1 shows the effects of arsenic compounds, i.e., iAs^{III}, MMA^{III}, and DMA^{III}, on the survival of RLC-16 cells determined by an MTT assay. MMA^{III} was found to be the most cytotoxic form, followed by DMA^{III} and iAs^{III}, respectively. The IC_{50} values for MMA^{III}, DMA^{III}, and iAs^{III} were calculated to be 1, 2, and 18 μM, respectively.

Aldehyde dehydrogenase 2 (ALDH2), a mitochondrial-specific enzyme, has been proved to be involved in the MTT assay. Thus, we examined whether the aldehyde dehydrogenase (ALDH) activity is affected by its trivalent arsenic compounds directly; the isolated RLC-16 cells mitochondria were incubated with its different arsenic species, as shown in Supporting Information, Figure 1. However, no appreciable inhibition effects were observed after incubation with different arsenicals.

Dose- and Time-Dependent Generation of ROS in RLC-16 Cells after Exposure to iAs^{III}, MMA^{III}, and DMA^{III}. In order to understand the differences in cytotoxicity among iAs^{III}, MMA^{III}, and DMA^{III}, the generation of ROS was assessed on the basis of dose- and time-dependent exposure in RLC-16 cells.

Figure 2 (A, B, and C) shows the dose-dependent exposure effect of iAs^{III}, MMA^{III}, and DMA^{III} on ROS generation in RLC-16 cells. The intracellular ROS levels were increased only by exposure to iAs^{III} at high concentrations (18−24 μM) (Figure 2A). By contrast, the intracellular ROS levels were considerably increased by exposure to MMA^{III} at low concentrations (e.g., 1 μM) (Figure 2B). Low concentrations of DMA^{III} (1 μM) also

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induced intracellular ROS, and this induction increased in a dose-dependent manner, though with lower overall levels than those recorded for MMA^{III}-exposed cells (Figure 2C). Figure 2 (a, b, and c) shows the time-dependent generation of ROS in RLC-16 cells after exposure to iAs^{III}, MMA^{III}, and DMA^{III} at their IC_{50} doses for 24 h. Interestingly, the generation of ROS was observed at a later time for the iAs^{III}-exposed cells (Figure 2a) than for MMA^{III}- and DMA^{III}-exposed cells (Figure 2b and c), which showed increased levels of ROS that began shortly after exposure and increased up until 24 h after exposure. These results imply that the methylated trivalent arsenicals, MMA^{III} and DMA^{III}, are much more potent in inducing ROS than iAs^{III}. To demonstrate that cell death is mainly caused by the induction of ROS by exposure to MMA^{III} and DMA^{III}, but not by iAs^{III}, *N*-acetylcysteine (NAC) was used as an ROS scavenger in this experiment. As expected, NAC did not appreciably prevent the loss of cellular viability caused by exposure to iAs^{III}, but it was effective in preventing the loss of cellular viability by exposure to either MMA^{III} or DMA^{III} (Figure 3). This suggests that oxidative stress may be an important cause of MMA^{III}- and DMA^{III}-induced cells death.

Determination of the Localization of ROS Generation and Arsenic Distribution in RLC-16 Cells after Exposure to Arsenicals. The cellular location (organelles) of the iAs^{III}-, MMA^{III}-, and DMA^{III}-induced ROS generation was investigated. A ROS fluorescent probe CM-DCFHDA and mitochondria fluorescent probe Mito-Tracker were used to detect ROS generation in organelles (e.g., mitochondria). Figure 4A shows the microscopy images of ROS generation in RLC-16 cells by exposure to different arsenicals at the respective LC_{50} values. The green fluorescence indicates ROS generation, while the red fluorescence indicates mitochondria (Mito-Tracker). The presence of yellow fluorescence (green and red fluorescent overlap) indicates that ROS were generated in the mitochondria.

Substantial yellow fluorescence was observed in mitochondria exposed to MMA^{III} (Figure 4A), while orange fluorescence was detected by exposure to iAs^{III} at IC_{50} values (white arrow). In addition, even though the yellow fluorescence was detected in mitochondria by following exposure to DMA^{III}, it seemed to be more in other organelles, as indicated in green fluorescence (yellow arrow). These results suggest that although the ROS can be induced by treatment with iAs^{III}, MMA^{III}, and DMA^{III}, the locations of the ROS generation seem to be different from methylated species (i.e., MMA^{III} and DMA^{III}). Our findings suggest that the generation of ROS mainly takes place in mitochondria following treatment by trivalent MMA^{III}, and DMA^{III}-induced ROS seems to take place in alternative organelles such as the ribosome, endoplasmic reticulum, golgi apparatus, etc. No attempt was made to identify the location of ROS following exposure to DMA^{III}.

The uptake of arsenic compounds was also examined in cultures of RLC-16 cells following exposure to the trivalent arsenic compounds at their IC_{50} dose for 24 h, as shown in Figure 4B. Arsenic accumulation was the highest in iAs^{III}-exposed cells (Figure 4B). Interestingly, in the MMA^{III}-exposed cells, most of the arsenic was found bound to proteins, while in the iAs^{III}- and DMA^{III}-exposed cells, much higher levels of free arsenic were found (approximately 40% and 65% respectively). This finding suggests that MMA^{III} may have a higher binding affinity to proteins than iAs^{III} and DMA^{III}, which is consistent with our previous study.[16]

In addition, subcellular distributions of arsenicals were determined after exposure to three arsenicals at the same dose (i.e., 1 μM) for

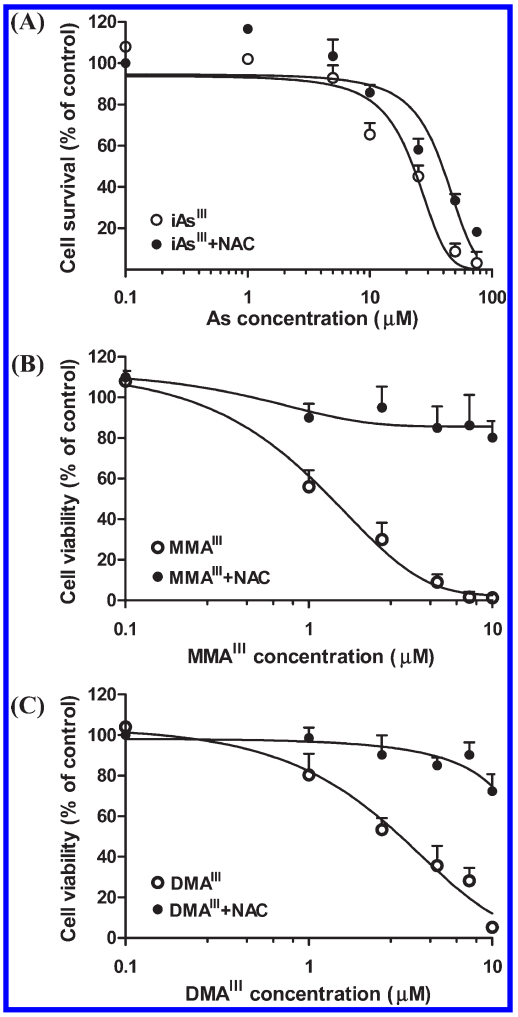

Figure 3. Effect of *N*-acetylcysteine (NAC) on the viability of RLC-16 cells exposed to iAs^{III}, MMA^{III}, and DMA^{III}. Cells were exposed to increasing concentrations of iAs^{III} (A), MMA^{III} (B), and DMA^{III} (C) with (○) or without 2 mM NAC (●) for 24 h. Results are expressed as the mean values ± standard deviation from five independent experiments (n = 5).

24 h. Interestingly, arsenic was accumulated more in mitochondria than in nuclei and microsomes after exposure to MMA^{III}, while arsenic was distributed more in microsomes than in mitochondria and nuclei after exposure to DMA^{III}, as shown in Figure 5. Furthermore, only low levels of arsenic were determined in three organelles after exposure to iAs^{III}, suggesting the distribution of arsenic in organelles is different between its methylated species.

Effects of Trivalent Arsenic Compounds, iAs^{III}, MMA^{III}, and DMA^{III}, on Complexes I−IV in Rat Liver Submitochondrial Particles. Experimental evidence indicates that the mitochondria are the major source of ROS production in cells following exposure to MMA^{III} (Figure 4A). It was theorized that the inhibitions of the electron transport chain following exposure to MMA^{III} may be associated with the generation of ROS. To assess the effects of iAs^{III}, MMA^{III}, or DMA^{III} on the activities of complexes I−IV in mitochondria, submitochondrial particles of rat liver were exposed to varying levels of each arsenical.

Enzyme activities of complexes I were not inhibited by exposure to any of the trivalent arsenic compounds, but the activities were

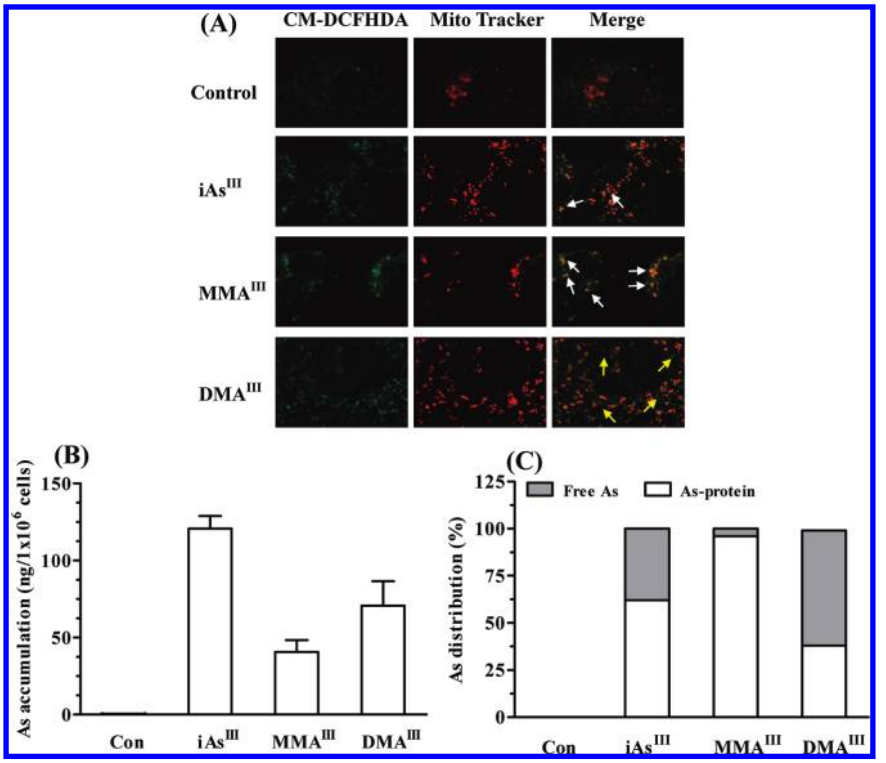

Figure 4. Determination of the localization of reactive oxygen species (ROS) generation and arsenic distribution in RLC-16 cells after exposure to iAs^{III}, MMA^{III}, and DMA^{III}. Cells were pretreated with 2 μM CM-H_2DCFDA and 500 nM Mito Tracker for 1 h, and then exposed to different arsenicals at the respective LC_{50} values. Localization of ROS was estimated by measuring CM-H_2DCFDA and Mito Tracker fluorescence (A). Arsenic accumulations (B) and distribution ratios (C) in RLC-16 cells were determined by ICP MS after exposure to arsenicals at IC_{50} values for 24 h. Data are expressed as the mean ± SD (n = 4).

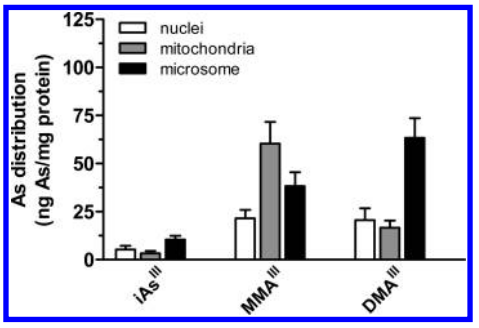

Figure 5. Subcellular distribution of arsenicals in RLC-16 cells after exposure to different arsenicals. Cells were exposed to three different arsenicals at the low dose (1 μM) for 24 h. After exposure, cells were washed twice with PBS, homogenized with a glass-Teflon homogenizer in an atmosphere of nitrogen, and then centrifuged at 700, 5000, and 15000g to obtain the fractions of nuclei, mitochondria, and microsome, respectively. Arsenic concentration in the different fractions was determined by HPLC-ICP MS after washing with HNO_3 and H_2O_2 at 135 °C for 2 days. Data are expressed as the mean ± SD (n = 3).

significantly inhibited (approximately 60%) by exposure to rotenone, suggesting that the three arsenic compounds had no effect on complex I, as shown in Supporting Information, Figure 2.

The effects of trivalent arsenicals on enzyme activities in complex II are shown in Figure 6. No inhibition was observed for complex II after exposure to inorganic iAs^{III}, while the enzyme activities were significantly inhibited following exposure to malonate, an inhibitor of complex II (approximately 90% of activities). In contrast, incubation with MMA^{III} at the high concentrations resulted in a 25% decrease in activity, while incubation with DMA^{III} at 100 μM (Figure 5) resulted in a 55% decrease in activity.

Figure 7 shows the effects of trivalent arsenicals on enzyme activities in complex III. Activities of complex III were not inhibited by the exposure of any of the trivalent arsenicals; in fact, an increase in activity was seen following exposure, whereas it was significantly inhibited (loss of 70% of activity) after exposure to antimycin A, a known inhibitor of complex III.

Enzyme activities in complex IV were not affected by exposure to iAs^{III} and DMA^{III}, but they were inhibited (loss of 50−100% of activity) following exposure to MMA^{III} at 10 and 100 μM, as shown in Figure 8. KCN, used as a positive control, completely inhibited the enzyme activities in complex IV (Figure 8). These data suggest that of the four complexes in the mitochondrial ETC, complex IV is the most significantly affected by the presence of MMA^{III}.

Generation of ROS in Rat Liver Mitochondria by Incubation with iAs^{III}, MMMA^{III}, and DMA^{III}. To determine whether this inhibition of the activity of complexes II and IV, following MMA^{III} exposure, results in an increase in ROS in mitochondria, intact rat liver mitochondria were incubated with iAs^{III}, MMA^{III}, or DMA^{III} at 10 μM. As expected, the generation of ROS was not detected in mitochondria following incubation with iAs^{III}, while a low amount of ROS was detected in mitochondria upon exposure to DMA^{III}. Conversely, ROS levels significantly increased (2-fold) in mitochondria following incubation with MMA^{III} (Figure 9), suggesting that MMA^{III} is the most potent of the

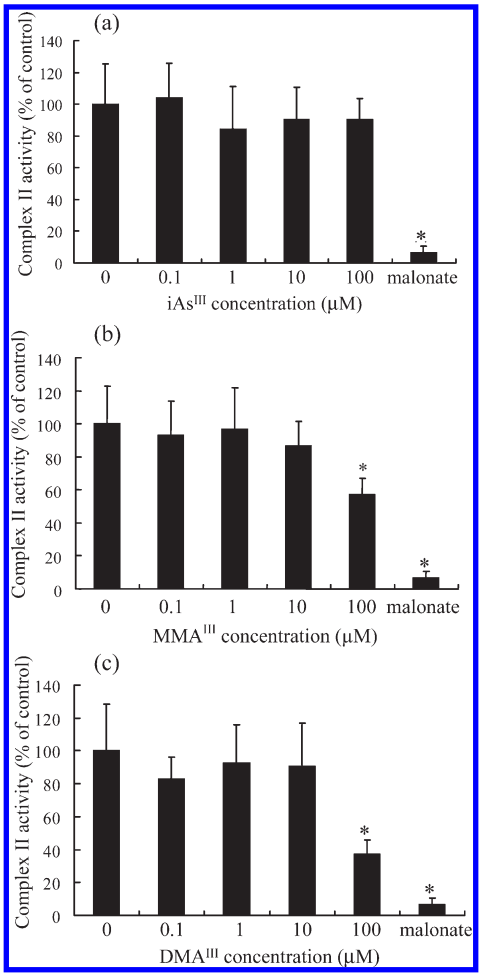

Figure 6. Effects of trivalent arsenicals on enzyme activities of complex II of the electron transport chain. Inhibition of complex II activity was measured according to the procedure described in the Materials and Methods section. The positive control was exposed to 1 mM malonate. Data are expressed as the mean ± SD (n = 5). Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

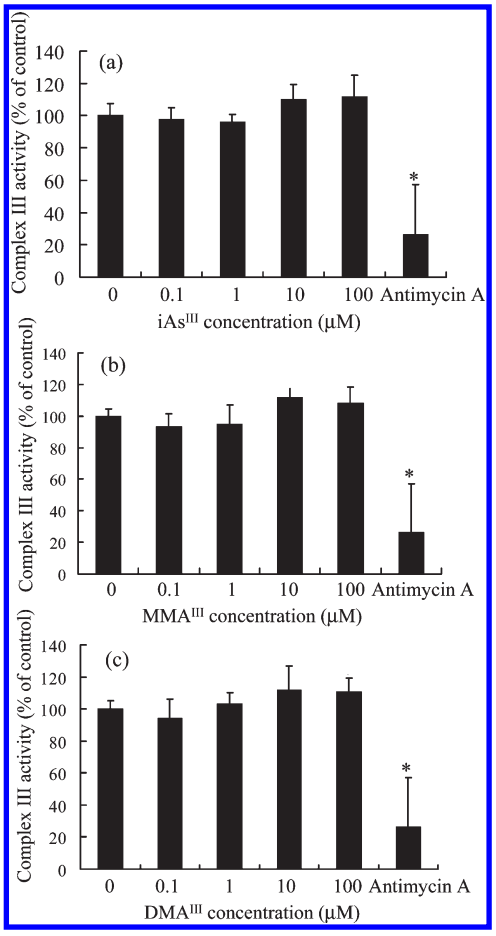

Figure 7. Effects of arsenicals on enzyme activities of complex III of the electron transport chain. Inhibition of complex III activity was measured according to the procedure described in the Materials and Methods section. The positive control was exposed to 2 mg/mL antimycin A. Data were expressed as the means ± SD (n = 5). Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

three arsenicals. These results support our hypothesis that the generation of ROS in mitochondria, at least in part, is due to the inhibition of activities of complexes II and IV by MMA^{III}. This is the first evidence that the mitochondria are a specific target organelle for MMA^{III}-induced cytotoxicity.

DISCUSSION

The liver is one of the target organs for arsenic-induced carcinogenicity, and arsenic metabolites have been suspected to be directly involved in carcinogenesis.[1,2,8,17] In general, inorganic arsenic can be methylated in the liver of most mammals, and the experimental evidence indicates that the trivalent methylated forms, MMA^{III} and DMA^{III}, are among the most toxic forms of arsenic.[12,28] Oxidative stress has been suggested to be a possible mode of carcinogenic action of arsenicals.[17−19] However, it remains unclear which arsenic species may be contributing to the toxicity and carcinogenicity of arsenic, and also which forms of arsenic may be contributing to the generation of ROS that lead to oxidative stress.

Although trivalent arsenicals are highly toxic to most cells lines, the difference among the toxicity mechanisms of iAs^{III}, MMA^{III}, and DMA^{III} are not well understood. This study shows, for the first time, that ROS are generated in the mitochondria following exposure to MMA^{III} (Figure 4A), while exposure to iAs^{III} and DMA^{III} induced ROS in other organelles (e.g., ribosome, endoplasmic reticulum, or golgi apparatus), though the definitive identification of the organelles would require further investigation. Overall, these findings suggest that the mechanisms of induction of cell death differ among iAs^{III}, MMA^{III}, and DMA^{III} (Figure 4).

Mitochondrial impairment is also related to the development of various cancers.[23,29,30] It has been reported that dysfunction of the enzyme activities of complexes I and III is the main source of ROS generation in mitochondria, and they are also considered to be the main leak sites for ROS.[22,29,31] However, our present study suggests that it was the activities of complexes II and IV, and not complexes I and III, that were inhibited following exposure to MMA^{III} (Figures 6 and 8) and that ROS generation was enhanced in intact mitochondria that were incubated with MMA^{III} (Figure 9). Although the concentrations required to inhibit complex II or IV are 10−100-fold higher than that of cytotoxic doses (i.e., IC_{50}), it is probably due to the mitochondrial activities differences between rat liver cell lines and rat primary hepatocytes

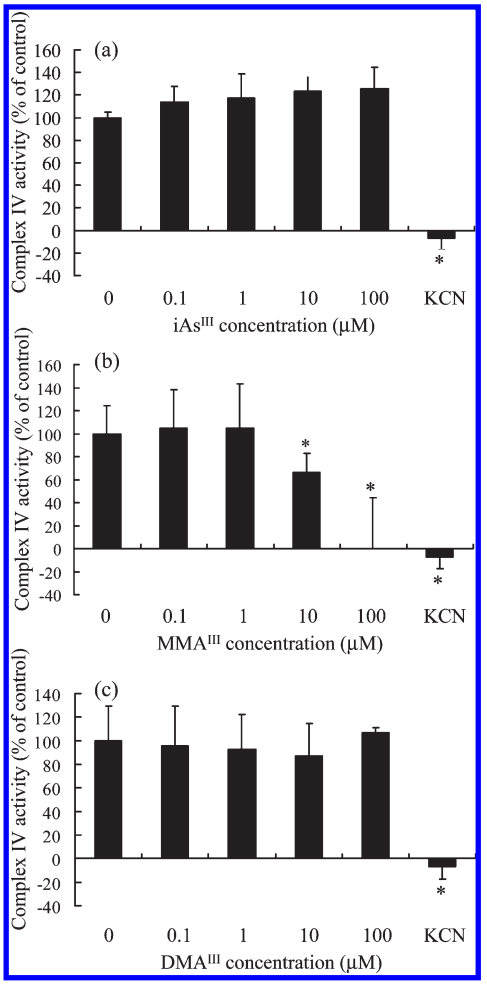

Figure 8. Effects of arsenicals on enzyme activities of complex IV of the electron transport chain. Inhibition of complex IV activity was measured according to the procedure described in the Materials and Methods section. The positive control was exposed to 2 mM KCN. Data are expressed as the mean ± SD (n = 5). Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

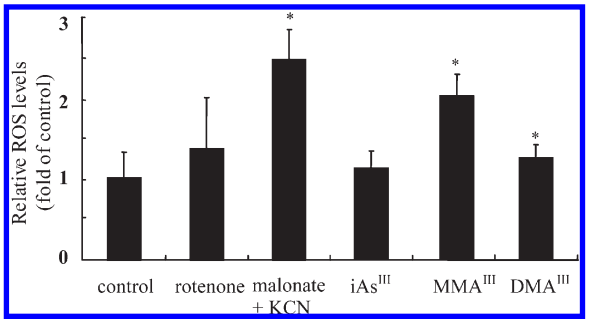

Figure 9. Reactive oxygen species (ROS) generation in intact mitochondria after incubation with arsenic compounds. Intact mitochondria were isolated from Wistar rat liver as described in the Materials and Methods section. ROS generation was measured by monitoring ROS-induced fluorescence of DCFH-DA. Mitochondria were incubated in the KCl buffer with 1 mM DCFH-DA at 37 °C for 30 min. After the incubation, the mitochondria substrates glutamate (6 mM) and malate (6 mM) were added along with the trivalent arsenic species at 10 μM. This mixture was allowed to incubate for 3 h. The amount of ROS was measured using an F-2500 fluorescence spectrophotometer. The positive control was exposed to 100 μM H_2O_2 for 2 h. Data were expressed as the mean ± SD (n = 5). Asterisks (*) indicate significant difference from the control (incubation with medium alone) at $P < 0.05$.

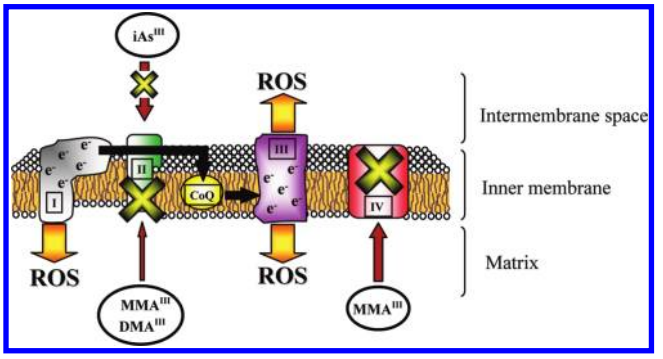

Figure 10. Proposed mechanisms underlying the MMA^{III}-induced cytotoxicity in cells.

and may also reflect differences in the kinetic behavior of cells that affect uptake and retention of arsenicals. In fact, Dopp et al.[32] have reported that the primary human hepatocytes exhibit greater resistance to trivalent MMA^{III} and DMA^{III} (IC_{50}: 12 and 9 μM, respectively) than other liver cell lines.

In addition, significant amounts of ROS were also generated in intact mitochondria (i.e., rat liver mitochondria) incubated with a known inhibitor of complexes II with IV (i.e., malonate and KCN) (Figure 9), suggesting that inhibitions of the activities of complexes II and IV could induce the generation of ROS in mitochondria. This ROS generation may be due to the fact that electrons were not able to be transferred from complexes I and III to complexes III and IV, respectively. This may result in electrons (e^-) leaking out from complex I and complex III, increasing ROS generation, as schematically represented in Figure 10. It has been reported that mitochondria are also the target organelle for heavy metals such as cadmium (Cd). In this case, the toxic effect was linked to the generation of ROS that resulted from the inhibition of complexes II and III.[22] In that study, cadmium was found to bind to the Q_o (ubiquinol oxidizing) site of complex III, which inhibited the electron transfer activity in mitochondria. However, in the present study, no appreciable effect was detected on complex III following exposure to MMA^{III} and DMA^{III} (Figure 7). This may suggest that the mechanisms of inhibitions of complexes I−IV of the ETC may be different between different metals/metalloids and species.

Mitochondria reactive oxygen species (mtROS) have been considered to play an important role in carcinogenicity because the generation of ROS is associated with DNA damage and deletion mutations.[33] Thus, mtROS can damage tumor suppressor genes or enhance the expression of proto-oncogenes, and cells may go on to become tumor cells.[34,35] However, following exposure to arsenicals it has been shown that ROS may be generated and that these ROS may lead to DNA damage.[17,21,34,36] It has been suggested that arsenic-induced carcinogenicity in target organs may be due to ROS generated following exposure to the intermediate trivalent methylated metabolites.[8,14] Arsenic toxicity is dependent on its chemical form.[37] Cellular uptake of the trivalent arsenicals is more efficient than that of the pentavalent arsenicals,[38,39] and the methylated trivalent arsenicals have bee reported to be more cytotoxic and genotoxic than iAs^{III}.[11,12,38,39] Furthermore, our previous studies have shown that MMA^{III} has a

higher affinity for cysteine residues in proteins than inorganic iAs^{III} and DMA^{III},[16] and this study showed that most of the MMA^{III} was bound to cellular proteins (Figure 3C).

This is the first time that mitochondria have been identified as a target organelle for MMA^{III}-induced cytotoxicity (Figure 4). Although ROS were also induced by DMA^{III} in intact mitochondria (Figure 9), the generation of ROS was found in other organelles than mitochondria of RLC-16 cells exposed to DMA^{III} (Figure 4). This indicates that mitochondria in the cell may not be a target organelle for DMA^{III} toxicity. Recent unpublished data from our lab indicates that following DMA^{III} exposure, ROS are predominantly generated in the endoplasmic reticulum (ER).

In the present study, no increase in ROS production was found in cells following exposure to low levels of iAs^{III}, but high level exposure did lead to an increase in ROS. Moreover, cellular viability was not prevented by exposure to iAs^{III} at the IC_{50} doses in the presence of an antioxidant, but cellular viability was affected following exposure to either MMA^{III} or DMA^{III} (Figure 3). This may indicate that the toxic mechanism of iAs^{III} may not involve ROS generation. Others have reported that ROS generation in response to inorganic iAs^{III} has increased, though this might have been due to different cell lines and exposure levels than those in the present study.[40−42] Though it has been reported that a potential mechanism for iAs^{III}-induced genotoxicity involves disruption of mitochondrial function resulting in an increase in intracellular ROS, it remains unclear as to how the ROS induction was caused.[34] Cohen et al. have indicated that arsenic species responsible for arsenic toxicity or carcinogenicity in cells should be clearly identified.[43]

The ROS detection reagent, H_2DCF, is widely used to evaluate cellular oxidative stress. However, a few researchers have recently reported that the cytosolic oxidation of H_2DCF to DCF depends on the combined effect of Fenton-type reactions and enzymatic activity of cytochrome *c*, namely, that DCF-induced fluorescence requires the simultaneous presence in the cytosol of H_2DCF, H_2O_2, and Fe (II) or H_2DCF and cytochrome *c*.[44,45] In the present study, we also used the CM-DCFHFDA probe for detecting the intracellular ROS, suggesting that the detection of ROS is probably through the same procedure, but we did not attempt to confirm the mechanism in the present study.

This study suggests that different arsenic species may have different mechanisms of toxicity. In particular, MMA^{III} was found to be the most toxic form in RLC-16 cells, which may in part be due to a reduction in activity of the mitochondrial ETC complexes II and IV, which in turn results in an increase in ROS production. Future studies need to further explore the interaction of MMA^{III} with mitochondrial proteins.

■ ASSOCIATED CONTENT

Supporting Information. Enzyme assays for electron transport chain complex activities (I-IV); determination of aldehyde dehydrogenase 2 (ALDH-2) activities; effect of iAs^{III}, MMA^{III} and DMA^{III} on enzyme activities of aldehyde dehydrogenase; and effects of trivalent arsenicals on enzyme activities of complex I of the electron transport chain. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

iAs^{III}, arsenite; iAs^{V}, arsenate; DMA^{V}, dimethylarsinic acid; DMA^{III}, dimethylarsinous acid; ETC, electron transport chain; 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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