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Metabolic processes

Proposal for novel metabolic pathway of highly toxic dimethylated arsenics accompanied by enzymatic sulfuration, desulfuration and oxidation

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ABSTRACT

The International Agency for Research on Cancer (IARC) has concluded that dimethylarsinic acid [$(CH_3)_2AsO(OH)$, DMA^V], a main metabolite of inorganic arsenic, is responsible for carcinogenesis in urinary bladder and lung in rodents, and various modes of carcinogenic action have been proposed. One theory concerning the mode of action is that the biotransformation of dimethylarsinous acid [$(CH_3)_2AsOH$, DMA^{III}] from DMA^V plays an important role in the carcinogenesis by way of reactive oxygen species (ROS) production. Furthermore, dimethylmonothioarsinic acid [$(CH_3)_2AsS(OH)$, $DMMTA^V$], a metabolite of DMA^V, has also been noted because of its higher toxicity. However, the metabolic mechanisms of formation and disappearance of DMA^{III} and $DMMTA^V$, and their toxicity are not fully understood. Thus, the purpose of the present study was to clarify the mechanism of metabolic formation of $DMMTA^V$ and DMA^V from DMA^{III}. The *in vitro* transformation of arsenicals by treatment with liver homogenate from rodents and sulfur transferase was detected by HPLC-ICP-MS and HPLC-tandem MS. $DMMTA^V$ is produced from DMA^{III} but not DMA^V by cellular fractions from mouse liver homogenates and by rhodanese from bovine liver in the presence of thiosulfate, a sulfur donor. Not only $DMMTA^V$ thus produced but also DMA^{III} are re-converted into DMA^V by an *in vitro* addition of S9 mix. These findings indicate that the metabolic process not only of DMA^{III} to DMA^V or $DMMTA^V$ but also of $DMMTA^V$ to DMA^V consists of a complicated mode of interaction between monooxygenase including cytochrome P450 (CYP) and/or sulfur transferase.

Introduction

In humans and animals, consumption of inorganic arsenic leads to the excretion of dimethylarsinic acid [$(CH_3)_2AsO(OH)$, DMA^V] as the major metabolite of inorganic arsenic [1,2] and, therefore, DMA^V has been utilized as a biological marker of inorganic arsenic exposure. While the metabolic process that converts inorganic arsenic to DMA^V was once considered as a detoxification mechanism in response to the acute toxicity of inorganic arsenic [3], a more recent common view on the mechanism of arsenic toxicity based upon experimental data is that DMA^V has carcinogenic action in rodents, and that the metabolic methylation pathway for inorganic arsenic to DMA^V might be a toxicity-enhancing process rather than a detoxification process from the standpoint of carcinogenesis [4].

DMA^V is viewed as a contributor to the overall higher toxicity of its reduced metabolites, trivalent dimethylated arsenics such as dimethylarsinous acid [$(CH_3)_2AsOH$, DMA^{III}] and dimethylarsenic–glutathione conjugate [$(CH_3)_2As^{III}$-SG, DMA^{III}-SG] by the reaction with GSH [5]. DMA^V but not inorganic arsenic and monomethylarsonic acid induces higher toxicity with GSH. Therefore, the trivalent dimethylated arsenics are considered to be important candidates as active metabolic intermediates for arsenic toxicity and carcinogenesis. Previous studies have reported that DMA^{III} leads to DNA damage, cellular toxicity, chromosomal abnormality, apoptosis and gene mutation *via* the induction of oxidative stress [6–9]. Our previous studies [10,11] indicated that DMA^{III}, which is formed by reduction of DMA^V with GSH, reacts readily with oxygen and then changes into dimethylated arsenic peroxide

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[$(CH_3)_2AsOO(OH)$], which is a reactive oxygen species (ROS) and has an oxidizing potential for DNA by the addition reaction of an oxygen atom.

In the last decade, metabolites containing sulfur atoms produced metabolically from DMA^V have attracted considerable attention from the standpoint of arsenic toxicity and carcinogenesis [12,13]. Sulfur-containing metabolites of DMA^V produced by intestinal flora of rats were predicted to be dimethylated and trimethylated thioarsinic acids [14] and were further identified in biological systems [15,16]. Among their sulfur-containing metabolites, dimethylmonothioarsinic acid [$(CH_3)_2AsS(OH)$, $DMMTA^V$], but not dimethyldithioarsinic acid [$(CH_3)_2AsS(SH)$] or trimethylarsine sulfide [$(CH_3)_3AsS$], has both genotoxicity and cytotoxicity that are similar to those of DMA^{III} [17]. The toxicity was increased by the presence of GSH [18]. $DMMTA^V$, as well as DMA^{III}, is taken up efficiently in various organs in hamster *in vivo* and then changed metabolically into DMA^V [19]. Another study using a theoretical chemistry approach reported that $DMMTA^V$ is hydrolyzed enzymatically into DMA^V and consecutively, DMA^V is converted into DMA^{III} by undergoing further reduction under physiological conditions [20]. Finally, the toxicity of $DMMTA^V$ is due to DMA^{III} produced by the reduction of DMA^V with GSH, and is presumed to be mainly responsible for toxicological events in conjunction with ROS [21]. Since $DMMTA^V$, one of the toxic urinary metabolites of arsenics, had higher toxicity toward a bladder cancer cell line *via* the production of ROS [21,22], $DMMTA^V$ might bring about increased risk of cancer of the urinary bladder as a target organ of arsenic carcinogenesis. On the other hand, $DMMTA^V$ is considered to be a minor metabolite in urine after exposure of arsenic to humans: $DMMTA^V$ has been identified as a trace arsenical present in urine after ingestion of arsenosugars [23], a minor arsenical in the urine of Japanese males [24], and a trace arsenical in the urine of women who reside in an area of arsenic contamination in Bangladesh [25].

$DMMTA^V$ is believed to be synthesized through DMA^{III} formation by the reduction of DMA^V followed by the addition of a sulfur atom by hydrogen sulfide [26]. With regard to the metabolic production of thioarsenicals, hydrogen sulfide produced by anaerobic flora could directly convert DMA^V and trimethylarsine oxide to dimethylated and trimethylated thioarsenicals, respectively, *via* chemical processes [27]. Only one study concerning transformation of $DMMTA^V$ from arsenite with a possible involvement of intestinal bacteria flora has been reported so far [28]. However, several have reported on the metabolic production of $DMMTA^V$ in liver and blood. In an earlier study, Naranmandura et al. reported that $DMMTA^V$ and dimethyldithioarsinic acid might be produced from DMA^{III} in the presence of sulfur donors such as HS-, R-S-SH, and R-S-S-S-R in red blood cells and also in liver [29]. In such a scenario, the estimated value of urinary $DMMTA^V$ may be lower than the actual amount present and there is still insufficient evidence indicating enzymatic formation in the metabolic process of arsenic. Accordingly, further study of the metabolic pathways of methylated thioarsenicals, particularly of the highly toxic $DMMTA^V$, and the relevance of the toxic mechanism would be important to understand the mechanisms of toxicity of methylated arsenic.

It is necessary to clarify the involvement of enzymatic formation of $DMMTA^V$ from DMA^{III} and it is also important to examine those of DMA^V from $DMMTA^V$ with substitution reactions of sulfur and oxygen atoms because very little is known about this metabolism. The aim of the present study was to elucidate the metabolic processes of three highly toxic metabolites of inorganic arsenic, DMA^V, DMA^{III} and $DMMTA^V$. First, we attempted to elucidate possible metabolic processes in an *in vitro* system for these dimethylated arsenics using high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) or HPLC-tandem MS.

Materials and methods

Chemicals

DMA^{III}-SG and $DMMTA^V$ were synthesized in our laboratory according to the reports of Cullen et al. [30] and Fricke et al. [31], respectively. Diarsenic trioxide (As_2O_3, purity 99.99%) was obtained from Soekawa Chemicals Co., Ltd. (Tokyo, Japan). DMA^V, sodium thiosulfate pentahydrate ($Na_2S_2O_3{\cdot}5H_2O$), acetic acid and methanol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DMA^V was recrystallized twice from methanol before use. Ammonium acetate (CH_3COONH_4) and rhodanese that originated from bovine liver were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other general chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). S9 mix, which was prepared from liver of rats with cytochrome P450 (CYP) induced by i.p. injection of phenobarbital and 5, 6-benzoflavone, for Ames assay was purchased from Kikkoman Biochemifa Company (Tokyo, Japan).

Animals

Five week-old male ICR mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). The animals were housed under a 12-h light/dark cycle with free access to food and water under specific-pathogen free (SPF) conditions for one week. Animal experiments were performed in compliance with the Ethical Guidelines for Animal Experiments of Nihon University.

Preparation of cellular fractions from mouse liver and blood

Liver and blood from six week-old ICR male mice acclimated for one week as described above were prepared as follows. The liver was homogenized in the same amount of 15 mmol/L ammonium acetate at pH 7.0, and then centrifuged at 1000 rpm for 10 min. The supernatants were further centrifuged at 9000 × *g* for 20 min; the pellets and supernatants obtained were used as the mitochondrial fraction and microsomal and soluble fractions, respectively. Blood (0.5 mL) withdrawn from the heart by a heparinized syringe was separated into plasma and cells by centrifugation at 2000 rpm for 10 min. The cells obtained were washed 3 times with Krebs-Ringer phosphate buffer (pH 7.4). Stroma fraction of the cells was obtained by hemolysis by resuspension in 0.5 mL of ultrapure water. Each fraction sample was temporarily stored in ice until the start of experiments.

Production of $DMMTA^V$ from DMA^{III} using liver homogenate fractions and blood samples from mouse, and commercial-grade rhodanese

An aliquot (90 μL) containing DMA^{III}-SG and sodium thiosulfate dissolved in 15 mmol/L ammonium acetate buffer (pH 7.0) was pre-incubated at 37 °C for 5 min, and then further incubated for 30–180 min after starting the reaction by adding each cellular fraction (10 μL) prepared from mouse liver homogenates and blood, or commercial-grade rhodanese (10 μL, 6 units/mL). The final concentrations of DMA^{III}-SG and sodium thiosulfate in the reaction mixture were 10 μmol/L and 15 μmol/L, respectively. After centrifugation at 12,000 rpm for 1 min, the supernatants obtained were filtrated by a syringe filter (Millex®-LG, pore size 0.22 μm, Merck Millipore, Darmstadt, Germany) and then analyzed by HPLC-ICP-MS or HPLC-tandem MS spectrometry.