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Chronic Methylated Arsenic-Exposure Induces Tolerance to the Acute Cytolethality of Inorganic Arsenate in Rat Liver Cells

Chikara KOJIMA, Seiichiro HIMENO and Teruaki SAKURAI*

Laboratory of Molecular Nutrition and Toxicology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan.

Summary

In this study, we examined the effects of chronic low-level exposure to methylated mammalian metabolites of inorganic arsenic on tolerance to an inorganic arsenate *in vitro* using the normal rat liver cell line TRL 1215. Cells were exposed to monomethylarsonic acid ($MMAs^V$; 1.3 mM), dimethylarsinic acid ($DMAs^V$; 0.7 mM), or trimethylarsine oxide ($TMAs^VO$; 10 mM), for 20 weeks. Cells chronically exposed to $MMAs^V$ or $TMAs^VO$ acquired tolerance to the acute cytolethality of inorganic arsenate, and the tolerance did not persist after arsenic-free incubation for an additional 8 weeks. In contrast, cells chronically exposed to $DMAs^V$ did not acquire tolerance to arsenate; however, the cells became arsenic tolerant after the arsenic-free incubation. Cellular arsenic accumulation was less in these arsenic-tolerant cells than in passage-matched control cells. Furthermore, cellular GSH depletion and inhibition of cell membrane transporters, such as multidrug resistance-associated protein, increased arsenic accumulation and decreased tolerance to the acute cytolethality of arsenate in these cells chronically exposed to methylated arsenic. These results indicate that chronic exposure to methylated arsenic compounds induces tolerance to inorganic arsenate, and that the tolerance is due to the induction of the arsenic excretion pathway.

Introduction

Inorganic arsenic is widely distributed in the environment as a natural component of soil and in water[1, 2]. Humans encounter inorganic arsenic in drinking water from wells drilled into arsenic-rich strata. In Asia and the Americas, chronic arsenic poisoning has occurred as a result of the consumption of high levels of arsenic-contaminated well water. Inorganic arsenic has a pronounced acute toxicity in humans and experimental animals[2]. Epidemiological studies have provided clear evidence that it is a human carcinogen with target sites including liver, skin, lung, kidney and urinary bladder[1, 2]. However, the mechanism of arsenic-induced impediment is not clear. Because animal models for inorganic arsenic toxicity are limited and equivocal, *in vitro* systems have been widely used to define the molecular event associated with inorganic arsenic carcinogenicity and toxicity. These approaches have provided useful informations on the genotoxic effects of inorganic arsenic, on inorganic arsenic-induced mutations, on inorganic arsenic-induced changes in methylation status of DNA, and on inorganic arsenic-induced expression of genes involved in the regulation of cellular growth and proliferation[3, 4]. It would appear that environmental exposure to arsenic will continue to be common.

In humans and numerous experimental animals, inorganic arsenic is enzymatically methylated into organic arsenic, such as monomethylarsonic acid ($MMAs^V$) and dimethylarsinic acid ($DMAs^V$)[5]. $MMAs^V$ and $DMAs^V$ are the

*To whom correspondence should be addressed. E-mail: teruaki@ph.bunri-u.ac.jp

major organic pentavalent arsenic metabolites in human urine after the exposure to inorganic arsenic[5, 6]. $DMAs^V$ is the ultimate metabolite in humans, while $DMAs^V$ is further methylated to trimethylarsine oxide ($TMAs^VO$) in some rodents (Fig. 1)[7]. It is believed that methylation of inorganic arsenic results in a reduction in general toxicity, as indicated by their increased *in vivo* lethal dose in 50% of a population (LD_{50}) and *in vitro* lethal concentration in 50% of a poplation (LC_{50})[8, 9]. However, recent studies have increasingly suggested that the methylation of inorganic arsenic is not a universal detoxification mechanism, because it has been often reported that $DMAs^V$ has a significant toxicity *in vitro* and *in vivo*. $DMAs^V$ is genotoxic *in vitro* and an effective tumor promoter or complete carcinogen in rodents[10-12]. We also reported that $DMAs^V$ primarily induces apoptosis and requires cellular reduced glutathione (GSH) to become cytotoxic[9, 13-16]. However, the details on the effects of chronic exposure to $DMAs^V$ have not yet been clarified. Furthermore, much less is known concerning the *in vitro* toxic potential or mechanisms of the other methylated arsenic metabolites, such as $MMAs^V$ and $TMAs^VO$. It has also been reported that methylated arsenic compounds accumulate during chronic arsenic poisoning in human body[17]. Thus, it would appear that defining the mechanisms of chronic arsenic poisoning, and perhaps carcinogenesis, requires further studies on the effects of the chronic exposure to methylated arsenic.

In this study, we demonstrated that chronic exposure to low-levels of $MMAs^V$, $DMAs^V$ and $TMAs^VO$ induced tolerance to the acute cytolethality of an inorganic arsenate using rat liver cells. This study may provide important information on the chronic arsenic poisoning.

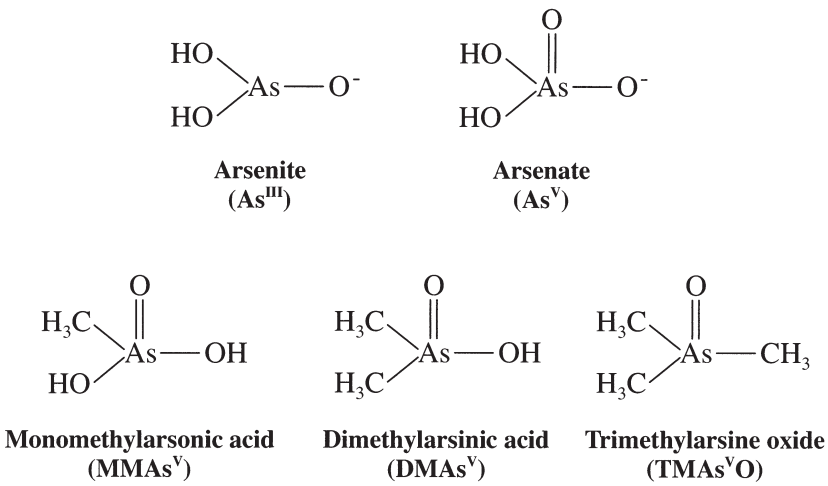

Fig. 1 Primary structure of inorganic and methylated arsenic

Materials and methods

Chemicals. Monomethylarsonic acid ($MMAs^V$) was obtained from Tri Chemical Laboratory Inc. (Yamanashi, Japan). Sodium arsenate and dimethylarsinic acid ($DMAs^V$) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Trimethylarsine oxide ($TMAs^VO$) was synthesized from trimethylarsine using hydrogen peroxide as described elsewhere[18]. These arsenic compounds were recrystallized twice and their purities were > 99.9 % as determined by gas chromatography-mass spectrometry[13]. Endotoxin contamination of these arsenic compounds was < 0.0000003 % (wt/wt) as determined by the endotoxin-specific limulus test (Seikagaku Co., Tokyo, Japan). Reduced glutathione (GSH), L-buthionine-(*S,R*)-sulfoximine (BSO; an inhibitor of γ-glutamylcysteine synthetase which decreases cellular GSH levels)[9, 13-16] and probenecid (an inhibitor of multidrug resistance-associated protein)[19, 20]

were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture condition. TRL 1215 cell line was generously provided by Dr. Michael P. Waalkes (National Cancer Institute at National Institute of Environmental Health Sciences, National Institute of Health, NC, USA). TRL 1215 cells are adhesive rat epithelial liver cells originally derived from the liver of 10-day old Fisher F344 rats[3] and were cultured in William's medium E (Sigma) supplemented with 10 % fetal bovine serum, 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under a humidified atmosphere of 5 % CO_2/95 % air at 37 ℃.

Chronic arsenic exposure. TRL 1215 cells were chronically exposed to $MMAs^V$ (1.3 mM), $DMAs^V$ (0.7 mM), or $TMAs^VO$ (10 mM) for 20 weeks in the 25 cm^2 tissue culture flasks. These concentrations were one-tenth of acute cytotoxic concentrations (LC_{50} values for 48 h *in vitro* incubation) of these methylated arsenic compounds in TRL 1215 cells[13-16, 21]. Control cells were incubated with medium alone for 20 weeks.

Assay for acute arsenic cytolethality. Control cells and cells chronically exposed to methylated arsenic were isolated by trypsinization, washed twice and resuspended in fresh medium. Samples of 2×10^4 cells/100 μl/well were plated in flat-bottomed 96-well tissue culture plates and allowed to adhere to the plate for 24 h at which time the medium was removed and replaced with fresh medium containing inorganic arsenate. Cells were then incubated with arsenate for an additional 48 h. After the incubation, cells were washed twice with warmed phosphate-buffered saline (pH = 7.4) to remove non-adherent dead cells, and cellular viability was determined by AlamarBlue assay, which is similar to MTT assay and measured metabolic integrity[13]. Briefly, after the incubation with arsenate and replacement with 100 μl fresh media, 10 μl/well AlamarBlue solution (Iwaki Grass Co., Chiba, Japan) was added directly to the wells, incubated at 37 ℃ for 4 h, and the absorbance at 570 nm (reference as 600 nm) was measured by a microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA, USA). Data are expressed as relative metabolic integrity using the values from control cells as 100 %.

Stability of arsenic tolerance. To define the stability of acquired arsenic tolerance in cells chronically exposed to methylated arsenic, the cells were passaged in arsenic-free medium for an additional 8 weeks, and then acute cytoleyhality of arsenate was measured.

Arsenic analysis. Control cells and cells chronically exposed to methylated arsenic that were grown in flat-bottomed 75 cm^2 tissue culture flask (8 x 10^7 cells/flask) were pre-incubated with or without 25 μM BSO for 24 h, and were further exposed to 50 μM arsenate for an additional 48 h in the presence or absence of 25 μM BSO or 500 μM probenecid. After the exposure, cellular arsenic amounts in these cells were analyzed by hydride generation coupled with atomic absorption spectrometry (AAS) using SpestraAA-220 (Varian Australia Pty Ltd., Mulgrave, Victoria, Australia)[21, 22]. The results are expressed as ng of the cellular arsenic contents per mg of cellular protein determined by BCA protein assay (Pierce Co., Rockford, IL, USA) with bovine serum albumin as a standard.

Statistics. Statistical evaluations in experiments were expressed as the arithmetic mean ± SEM and performed by ANOVA followed by Dunnett's multiple comparison test or the Student's *t*-test as appropriate. A value of $p < 0.05$ was considered significant in all cases.

Results

Chronic Methylated Arsenic Exposure Induced Tolerance to Acute Cytolethality of Inorganic Arsenate in Rat Liver TRL 1215 Cells.

TRL 1215 cells were incubated with $MMAs^V$ (1.3 mM), $DMAs^V$ (0.7 mM), $TMAs^VO$ (10 mM), or with medium alone (control) for 20 weeks. These concentrations of methylated arsenic compounds had no effect on the viability of TRL 1215 cells during the 20-week incubation. These cells that were chronically exposed to methylated arsenic were fur-

ther exposed to various concentrations of inorganic arsenate for an additional 48 h, and cell viability was then assessed by AlamarBlue assay. As shown in Table 1, chronic exposure to $MMAs^V$ or $TMAs^VO$ induced tolerance to the acute cytolethality of arsenate. Cells chronically exposed to $MMAs^V$ or $TMAs^VO$ had a greater than 17-fold or 4.5-fold increase in the LC_{50} for arsenate when compared with control cells. However, chronic $DMAs^V$ exposure did not induce arsenic tolerance.

We subsequently observed the effects of cellular GSH depletion on the arsenic tolerance induced by chronic exposure to methylated arsenic compounds following BSO treatment. BSO (25 μM) was cytotoxic to neither the control nor the cells chronically exposed to methylated arsenic, however, it significantly decreased the LC_{50} for arsenate in both control and cells chronically exposed to methylated arsenic (Table 1).

We also examined the effect of probenecid, which is a potent inhibitor of the function of multidrug resistance-associated protein, on the arsenic tolerance induced by chronic exposure to methylated arsenic compounds. Probenecid (500 μM) increased the cytolethality of arsenate in both the control cells and cells chronically exposed to methylated arsenic.

Effects of Arsenic Withdrawal from Cells Chronically Exposed to Methylated Arsenic on Acute Cytolethality of Arsenate

Cells chronically exposed to methylated arsenic were passaged in arsenic-free medium for an additional 8 weeks, and the acute cytolethality of arsenate in these cells was then measured. As shown in Table 2, the additional 8-week arsenic-free incubation decreased the arsenic tolerance in cells chronically exposed to $MMAs^V$. The LC_{50} for arsenate in the cells chronically exposed to $MMAs^V$ following the arsenic-free incubation was 3.1-fold higher than that of control cells; prior to the incubation it was 17-fold higher than that of the control cells. Furthermore, cells chronically exposed to $TMAs^VO$ lost the acquired arsenic tolerance after the arsenic-free incubation. On the other hand, cells chronically exposed to $DMAs^V$ showed significant tolerance to arsenate after the incubation in the arsenic-free medium for 8 weeks, while no arsenic tolerance was observe in the cells before the arsenic-free incubation.

Chronic Methylated Arsenic Exposure Decreased Cellular Arsenic Accumulation in Rat Liver TRL 1215 Cells

Cells chronically exposed to methylated arsenic were further incubated with 50 μM inorganic arsenate for 48 h in the presence or absence of BSO or probenecid, and the cellular arsenic contents of these cells were then measured by AAS. As shown in Fig. 2, chronic exposure to $MMAs^V$ or $TMAs^VO$ significantly decreased the cellular arsenic

Table 1 Acute *in vitro* cytolethality (LC_{50}) of arsenate in cells chronically exposed to methylated arsenic

cells were chronically exposed to;	LC_{50} of arsenate [Fold Increase†] (μM)		
	−	+ BSO	+ Probenecid
Medium	163.7 ± 15.2	20.2 ± 2.3†	62.0 ± 7.5†
$MMAs^V$ 1.3 mM	2827.0 ± 178.9 [17.3]*	122.0 ± 2.7†	800.1 ± 1.4†
$DMAs^V$ 0.7 mM	186.4 ± 26.7 [1.1]	52.0 ± 6.5††	100.4 ± 4.1†††
$TMAs^VO$ 10 mM	730.2 ± 35.3 [4.5]*	50.2 ± 0.3†	215.0 ± 1.9†

TRL1215 cells were incubated with $MMAs^V$ (1.3 mM), $DMAs^V$ (0.7 mM), $TMAs^VO$ (10 mM), or with medium alone (control) for 20 weeks. After the incubation, these cells were incubated with or without (control) 25 μM BSO for 24 h, and were further exposed to arsenate in the presence or absence (control) of 25 μM BSO or 500 μM probenecid for an additional 48 h, and cell viability was then assessed. Results are expressed as arithmetic mean ± SEM of three separate experiments performed in triplicate (n = 9). †The ratio of LC_{50} values of chronic methylated arsenic-exposed cells/control cells. $^{*}p < 0.001$ comparison with the control cells. $^{\dagger}p < 0.001$ comparison with the cells which were incubated with same arsenic or medium alone for 20 weeks and further exposed to arsenate in the absence of BSO or probenecid, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.05$.

Table 2 Acute *in vitro* cytolethality (LC_{50}) of arsenate in cells chronically exposed to methylated arsenic after the incubation in arsenic-free medium for 8 weeks

cells were chronically exposed to;	LC_{50} of arsenate [Fold Increase †] (μM)
Medium	176.5 ± 19.8
$MMAs^V$ 1.3 mM	548.3 ± 12.5 [3.1]*
$DMAs^V$ 0.7 mM	374.7 ± 9.3 [2.1]**
$TMAs^VO$ 10 mM	152.4 ± 30.0 [0.9]

TRL1215 cells were incubated with $MMAs^V$ (1.3 mM), $DMAs^V$ (0.7 mM), $TMAs^VO$ (10 mM), or with medium alone (control) for 20 weeks, and these cells were further incubated in arsenic-free medium for more 8 weeks. After the incubation, these cells were exposed to arsenate for an additional 48 h, and cell viability was then assessed. Results are expressed as arithmetic mean ± SEM of three separate experiments performed in triplicate (n = 9). †The ratio of LC_{50} values of chronic arsenic exposed cells/control cells after the arsenic-free incubation. *$p < 0.01$ comparison with the control cells after the arsenic-free incubation, **$p < 0.05$.

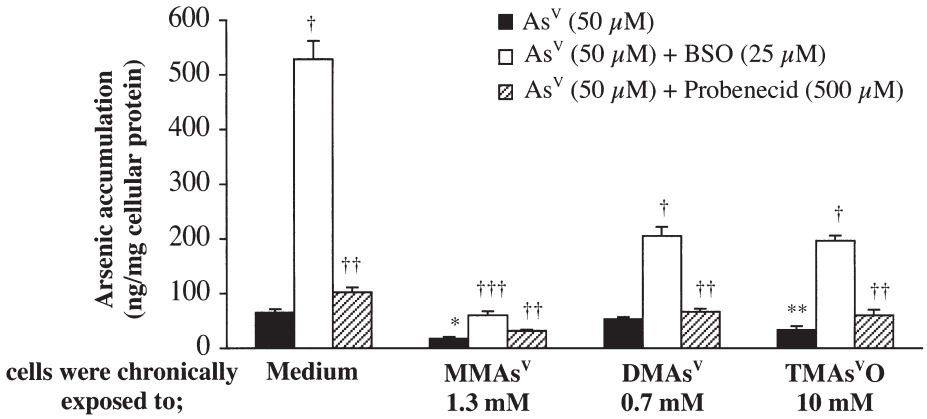

Fig. 2 Cellular arsenic accumulation in cells chronically exposed to methylated arsenic. TRL 1215 cells were incubated with $MMAs^V$ (1.3 mM), $DMAs^V$ (0.7 mM), $TMAs^VO$ (10 mM), or with medium alone (control) for 20 weeks. After the incubation, these cells were washed and incubated with or without (control) 25 μM BSO for 24 h, and were further exposed to 50 μM arsenate for an additional 48 h in the presence or absence (control) of 25 μM BSO or 500 μM probenecid. The cellular arsenic contents were then measured by AAS. Results are expressed as arithmetic mean ± SEM of three separate experiments performed in triplicate (n = 9).
*$p < 0.001$ comparison with the control cells, **$p < 0.01$.
†$p < 0.001$ comparison with the cells which were incubated with same arsenic or medium alone for 20 weeks and further exposed to arsenate without BSO or probenecid, ††$p < 0.05$, †††$p < 0.01$.

accumulation compared with the control cells, although cells chronically exposed to $DMAs^V$ did not have any effect (Fig. 2). Treatment with BSO or probenecid significantly augmented the cellular arsenic accumulation in both the control cells and cells chronically exposed to methylated arsenic (Fig. 2).

Discussion

The present study demonstrates that tolerance to the acute cytolethality of arsenate is induced in mammalian cells by chronic exposure to non-toxic levels of $MMAs^V$, $DMAs^V$, and $TMAs^VO$. We previously reported that the cellular arsenic contents of the cells that were exposed to micro- to millimolar levels of methylated arsenic compounds for 20 weeks were in the nanomolar range[21]. Thus, this experiment demonstrated the effects of chronic exposure to

nanomolar cellular levels of methylated arsenic compounds on arsenic sensitivity in rat liver cells. Methylated arsenic compounds are formed from inorganic arsenic by enzymatic methylation[23], and it has been reported that the urinary concentrations of methylated arsenic compounds in patients with chronic arsenic poisoning in Inner Mongolia, China were in the micromolar range[19]. This suggests that the use of nanomolar cellular arsenic concentrations for *in vitro* experiments may, at least partly, reflect the *in vivo* conditions.

Chronic exposure to $MMAs^V$ or $TMAs^VO$ induced significant tolerance to the acute toxicity of inorganic arsenate. This arsenic tolerance appears to be based on increased arsenic efflux because cells chronically exposed to $MMAs^V$ or $TMAs^VO$ had an enhanced ability to excrete cellular arsenic (Fig. 2). Cellular GSH depletion increased both the cytolethality and cellular accumulation of arsenate (Table 1, Fig. 2). Thus, cellular GSH may play a critical role in arsenic tolerance. We previously reported that chronic exposure to $MMAs^V$ or $TMAs^VO$ increased cellular GSH levels and cellular glutathione *S*-transferase (GST) activity[21]. GSH may decrease the cytolethality of arsenic through several processes, possibly through its role as an antioxidant, as a co-factor in the enzymatic methylation reaction of arsenic, by directly binding arsenic and thereby reducing the toxic potential, or through enhanced efflux of an arsenic-GSH conjugate[24]. GST catalyzes the formation of arsenic-GSH conjugates[3, 25]. It has been reported that these conjugates are excreted *via* some cell membrane transporters such as multidrug resistance-associated protein[3, 25]. In the present study, probenecid, which is an inhibitor of the multidrug resistance-associated protein function, augmented both the cytolethality and cellular accumulation of arsenate (Table 1, Fig. 2). Taken together, these data suggest that chronic exposure to $MMAs^V$ or $TMAs^VO$ might induce cellular GSH-dependent arsenic excretion mechanisms such as those involving the multidrug resistance-associated protein. On the other hand, these cells did not retain the arsenic tolerance after they were grown in an arsenic-free medium for a protracted period (Table 2). This result indicates that the arsenic tolerance induced by chronic exposure to $MMAs^V$ or $TMAs^VO$ is a temporary change.

Chronic exposure to $DMAs^V$ did not induce significant tolerance to the acute toxicity of inorganic arsenate (Table 1). However, cells chronically exposed to $DMAs^V$ became arsenic tolerant after they were grown in an arsenic-free medium for a protracted period (Table 2). This suggests that tolerance induced by chronic $DMAs^V$ exposure might be due to a protracted genotypic change in the cells. The reason for only $DMAs^V$ inducing this change is unclear. Further research is required in order to clarify the molecular mechanisms of the induction of arsenic tolerance by chronic exposure to $DMAs^V$ in mammalian cells.

In conclusion, the present study indicates that chronic exposure to $MMAs^V$, $DMAs^V$, and $TMAs^VO$ induces tolerance to acute inorganic arsenic cytolethality in rat liver cells. Chronic exposure to methylated arsenic compounds appears to induce GSH-dependent arsenic excretion pathways. This study may prove helpful in defining the mechanisms involved in chronic arsenic poisoning.

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