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Monomethylarsonous Acid Induced Cytotoxicity and Endothelial Nitric Oxide Synthase Phosphorylation in Endothelial Cells

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Abstract Chronic arsenic poisoning is reported to be associated with peripheral and cardiovascular disease, arteriosclerosis, Raynaud's syndrome, hypertension, and Blackfoot disease. Monomethylarsonous acid (MMA^{III}) is a reactive metabolite of inorganic arsenic and a potent inhibitor of endothelial nitric oxide synthase (eNOS). Arsenic is also reported to phosphorylate eNOS in cultured keratinocyte and Human T cell leukemia Jurkat cells, respectively. In the present study, we examined the cytotoxicity and eNOS phosphorylation by MMA^{III} exposure in cultured bovine aortic endothelial cells (BAEC). Results showed that MMA^{III} is more toxic than arsenite in BAEC cells. The IC_{50} values for MMA^{III} and arsenite were determined to be approximately 1.7 and 24.1 μmol/L, respectively. Exposure of BAEC to MMA^{III} (0.75 μmol/L) caused a significant eNOS phosphorylation 15 min after MMA^{III} exposure. However, a complex of MMA^{III} with dithiothreitol (DTT) that lacks the reactivity with vicinal thiols unaffected eNOS phosphorylation. The present study shows that MMA^{III} generated during biomethylation of arsenic is highly toxic in BAEC. Our study also suggests that MMA^{III} could induce the eNOS phosphorylation through modification to cellular thiols of the eNOS enzyme. And the initial up-regulation of eNOS phosphorylation by MMA^{III} seems to be an adaptive response against disruption of eNOS bioactivity during arsenic exposure.

Keywords monomethylarsonous acid (MMA^{III}) · arsenic · endothelial nitric oxide synthase (eNOS) · phosphorylation · endothelial cell

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Chronic arsenic poisoning caused by drinking water contaminated with arsenic is now a major worldwide public health problem (Aposhian 2006). Epidemiological studies demonstrated that excess intake of inorganic arsenic is associated with peripheral and cardiovascular disease, arteriosclerosis, Raynaud's syndrome, hypertension, and Blackfoot disease (Engel et al. 1994). However, the exact mechanism of arsenic-related vascular diseases remains unknown.

Inorganic arsenic undergoes extensive reduction and oxidative methylation in the body to produce mono-, di-, and possibly trimethylated metabolites (Aposhian et al. 2004). Studies in recent years demonstrated that these trivalent methylated arsenicals, such as monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), have stronger cytotoxicity and genotoxicity than inorganic arsenic itself (Petrick et al. 2000; Sakurai et al. 2002). Therefore, these active metabolites are regarded to significantly contribute to the adverse effects associated with inorganic arsenic exposure.

Vascular endothelial cells have long been considered as the primary target in the process of vasculopathy induced by arsenic exposure (Engel et al. 1994). Decreased nitric oxide (NO) bioactivity in endothelium is also implicated in

the pathophysiology of arsenic poisoning (Kumagai et al. 2004). We previously found that prolonged exposure of humans and rabbits to inorganic arsenic in vivo caused a reduction in systemic NO levels (Pi et al. 2000, 2003), which is thought to be related to the inactivity of endothelial NO synthase (eNOS). Further studies demonstrated that MMA^{III} was a potent inhibitor of eNOS activity (Sumi et al. 2005). Arsenic is also reported to phosphorylate eNOS in cultured human keratinocyte and Human T cell leukemia Jurkat cells, respectively (Hossain et al. 2003; Souza et al. 2001). However, such phenomena caused by MMA^{III} are poorly understood yet. And studies of MMA^{III} on vascular endothelial cells are also very limited. Therefore, in the present study, we examined the cytotoxicity and eNOS phosphorylation by MMA^{III} exposure in cultured endothelial cells.

Materials and Methods

Methylarsine oxide (CH_3AsO), synthesized and characterized as described previously (Cullen et al. 1984), was used as MMA^{III} in this experiment, since it hydrolyzes to MMA^{III} when dissolved in aqueous solution. Sodium arsenite (iAs^{III}) was from Wako Pure Chemical Industries, Ltd (Japan); Dithiothreitol (DTT) was from Nacalai Ltd (Japan); a complex of MMA^{III} with DTT was prepared by mixing CH_3AsO and DTT at an equal mole ratio as reported previously (Hoffman and Lane, 1992).

Bovine aortic endothelial cells (BAEC, Dainippon Pharmaceutical Industrial, Tokyo, Japan) were maintained in Dulbecco's modified eagle's medium nutrient mixture F-12 Ham (DMEM-F12, Sigma, USA) supplemented with 10% fetal calf serum (Sanko Junyaku Co, Japan), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Sigma, USA), heparin (10 U/mL, Mochida Co, Japan) and fibroblast growth factor (5 ng/mL, Proprotech Inc, USA) in a humidified and 37°C incubator with 5% CO_2 and 95% air. Cells were subcultured with 0.25% trypsin/EDTA (Sigma, USA) at a ratio of 1:4 and passages 4–7 were used for experiments.

Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Gomez et al., 1997). BAEC were seeded into 96-well plates at a density of 1×10^5 cells/well in DMEM-F12 and cultivated overnight until cell attachment. After 20 h of incubation with MMA^{III} (0–2 μmol/L) or iAs^{III} (0–50 μmol/L), MTT was added as a final concentration of 0.25 mg/mL and cells were subsequently incubated for 4 h. The medium was then removed and cells were washed twice with dulbecco's phosphate buffered saline (DPBS). Then dimethyl sulfoxide (DMSO) 100 μL was added for another 1 h. The optical density (OD) was determined spectrophotometrically at a wavelength of 540 nm and the values were expressed as percentages of control. IC_{50} was calculated with a nonlinear regression program using PRISM V3.0 (Graph Pad Software, Inc., San Diego, CA, USA). Data from eight batches of cell were used for replication.

Table 1 Cell viability of cultured BAEC exposed to MMA^{III} (0–2 μmol/L, 24 h)

MMA^{III}	Exposure time (h)	OD value	Cell viability (%)
0 (control)	24	1.10 ± 0.07	100.0
0.5	24	1.08 ± 0.11	98.3 ± 10.3
1.0	24	0.88 ± 0.04	79.9 ± 3.6
1.5	24	0.67 ± 0.09*	61.3 ± 8.6
2.0	24	0.10 ± 0.01*	9.2 ± 0.6

* Compared with control $p < 0.01$

Table 2 Cell viability of cultured BAEC exposed to iAs^{III} (0–50 μmol/L, 24 h)

iAs^{III}	Exposure time (h)	OD value	Cell viability (%)
0 (control)	24	1.72 ± 0.09	100.0
1	24	1.73 ± 0.10	100.2 ± 6.5
10	24	1.48 ± 0.12	86.0 ± 7.2
20	24	1.00 ± 0.07*	58.3 ± 3.9
50	24	0.50 ± 0.09*	29.1 ± 5.5

* Compared with control $p < 0.01$

After exposure of MMA^{III} (0.75 μmol/L, 0–15 min), iAs^{III} (100 μmol/L, 0–60 min), or MMA^{III}-DTT compound (0.75 μmol/L, 0–15 min) at 37°C, cell lysates were harvested with lysis buffer (50 mmol/L Tris (PH7.4)/150 mmol/L NaCl/5 mmol/L EDTA/100 mmol/L NaF/2 mmol/L sodium orthovanadate/15 mmol/L sodium diphosphate decahydrate/5 μg/ml aprotinin/10 μg/ml leupeptin/10 μg/ml pepstatin A/1% phenylmethylsulphonyl fluoride (PMSF)/1% triton X-100/1% sodium deoxy cholate). An equal amount (60 μg) of protein for each sample was resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF nitrocellulose membranes (Amersham Bioscience, UK). The specific primary antibodies used were: eNOS (1:1000, BD Transduction Lab, Japan) or phospho-eNOS$_{Ser1179}$ (1:1000, Zymed Lab Inc, CA, USA). Films were densitometrically quantified and analyzed with an image analyzer (Image Gauge V3.4, Fuji Photo Film Co, Ltd, Japan), and mean data (mean ± SD) on MMA^{III}-induced eNOS phosphorylation normalized by eNOS protein expression were calculated. Representative western blots depicting the nature of the changes were described herein. Incubation, isolation and western blot analysis were performed 3 times for each condition.

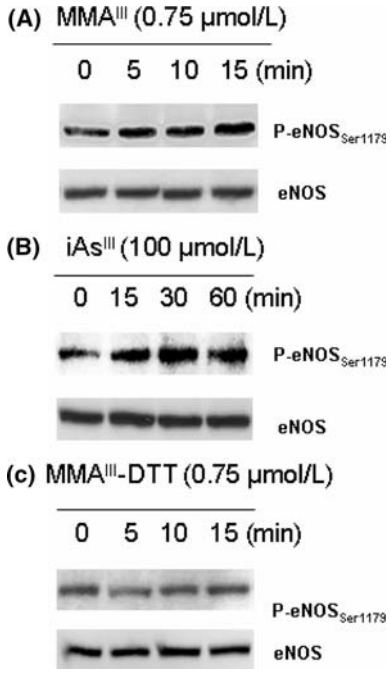

Fig. 1 eNOS$_{Ser1179}$ phosphorylation induced by MMA^{III}, iAs^{III}, or a complex of MMA^{III} with DTT in cultured BAEC. Cells were cultured overnight in serum-free medium and then incubated with **a** MMA^{III} (0.75 μmol/L, 0–15 min); **b** iAs^{III} (100 μmol/L, 0–60 min); **c** a complex of MMA^{III} with DTT (0.75 μmol/L, 0–15 min). Total protein was collected and 60 μg of protein was resolved by SDS-PAGE. Phosphorylation of eNOS$_{Ser1179}$ (P-eNOS$_{Ser1179}$) was detected by western blot analysis using phosphor-specific antibody of eNOS. Equal protein loading was confirmed by reprobing the original membranes for total eNOS. Panels are representative of 3 independent experiments

Data are expressed as mean ± SD. One way ANOVA process was used for analysis and differences with two-sided p values less than 0.05 were regarded as statistically significant.

Results and Discussion

Tables 1, and 2 showed the cell viability of BAEC exposed to MMA^{III} or iAs^{III} for 24 h, respectively. As to MMA^{III}, cell viability decreased markedly in a dose-dependent manner at treatment concentrations more than 1 μmol/L ($p < 0.01$), and cells were almost all died at 2 μmol/L. However, there was no effect on BAEC viability under such low levels of iAs^{III} exposure, more than 10 μmol/L of iAs^{III} showed significant cytotoxicity ($p < 0.01$). The IC$_{50}$ values for MMA^{III} and iAs^{III} were determined to be approximately 1.7 and 24.1 μmol/L, respectively. An earlier published study also found that MMA^{III} is more toxic than iAs^{III}. Their results showed that the IC$_{50}$ values of MMA^{III} in human hepatocytes, keratinocytes, bronchial cells, as well as UROtsa cells are 5.5, 2.6, 2.7, and 2.8 μmol/L, respectively (Styblo et al. 2000).

Table 3 Quantity results of eNOS$_{Ser1179}$ phosphorylation by MMA^{III}, or by a complex of MMA^{III} with DTT (0.75 μmol/L, 0–15 min) in cultured BAEC

Conditions	eNOS$_{Ser1179}$ phosphorylation (Fold Inc.)		
	5 min[a]	10 min[a]	15 min[a]
MMA^{III}	1.25 ± 0.19	1.41 ± 0.23	1.42 ± 0.08$
MMA^{III}-DTT	0.85 ± 0.20*	0.83 ± 0.06*	0.85 ± 0.11**

Bands of eNOS$_{Ser1179}$ Phosphorylation were densitometrically quantified and analysed with Image Gauge software from Fuji Photo Film Co, Ltd., and fold changes of phosphor-eNOS$_{Ser1179}$ (relative to that in control cells) normalized by eNOS protein expression are expressed as means ± SD of 3 independent experiments

[a] Time after exposure to different treatment

* $p < 0.05$, ** $p < 0.01$ versus MMA^{III}-treated cells, respectively

$ $p < 0.01$ versus control cells

In this study, a significant elevation of phosphorylated eNOS $_{Ser1179}$ was found 15 min after MMA^{III} exposure (1.42-fold increase versus control, Fig. 1a; Table 3). Under the same conditions, however, iAs^{III} didn't affect eNOS phosphorylation although a certain degree of eNOS$_{Ser1179}$ phosphorylation was seen 30 min following iAs^{III} exposure at 100 μmol/L (Fig. 1b). Other reports also show that iAs^{III} could induce the eNOS phosphorylation at high doses of 100–200 μmol/L (Hossain et al. 2003). The present study indicated that MMA^{III} is a reactive metabolite of inorganic arsenic that up-regulates eNOS phosphorylation in BAEC.

Modification of reactive sulfhydryl groups in many important enzymes has long been regarded as one of the possible mechanisms of arsenic poisoning (Sumi et al. 2005). It is well recognized that trivalent organoarsenicals exhibit high affinity for vicinal thiols (Hughes, 2002). Consistent with this notion, when we exposed a complex of MMA^{III} with DTT to BAEC, little appreciable phosphorylation of eNOS was detected (Fig. 1c; Table 3). The complex of MMA^{III} with DTT has no reactivity towards thiol groups, and our results suggested that covalent modification of MMA^{III} to cellular proteins via reactive thiols may be attributable to the enhancement of eNOS phosphorylation.

We previously found that prolonged exposure to inorganic arsenic by drinking water caused a reduction of systemic NO production in the residents of an endemic area of chronic arsenic poisoning (Pi et al. 2000). Further studies demonstrated that MMA^{III} was a potent inhibitor of eNOS activity (Sumi et al. 2005). However, reports also show that exposure of CHO-K1 or BAEC cells to iAs^{III} resulted in a transient increase of NO production in vitro (Liu and Jan 2000). Because eNOS phosphorylation is associated with increased NO bioactivity, the present study

may provide a possibility that up-regulation of eNOS phosphorylation by MMA^{III}, appears to account for the eNOS activation and serves as an acute cellular adaptive response against this toxic metalloid.

In conclusion, the present study shows that MMA^{III}, one of the active intermediate in inorganic arsenic methylation process, is highly toxic in BAEC. Our study also suggests that MMA^{III} generated during biomethylation of arsenic in endothelial cells could induce the eNOS phosphorylation through modification to vicinal thiols of the eNOS enzyme. And the initial up-regulation of eNOS phosphorylation by MMA^{III} seems to be an adaptive response against disruption of eNOS bioactivity during arsenic exposure.

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