Arsenic exposure and glutamate-induced gliotransmitter release from astrocytes

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Abstract
The present study used cultures of primary astrocytes, isolated from neonatal rats, to verify the hypothesis that arsenite-induced neurotoxicity can influence neuronal function by altering glutamate-induced gliotransmitter release. Primary astrocytes were exposed to 0, 2.5, 5, 10, 20 or 30 µM arsenite for 24 hours. Cell viability and morphological observations revealed that 5 µM arsenic exposure could induce cytotoxicity. Cells were then cultured in the presence of 0, 2.5, 5, or 10 µM arsenite for 24 hours and stimulated with 25 µM glutamate for 10 minutes. Results showed that [Ca²⁺]ᵢ in astrocytes exposed to 5 and 10 µM arsenite was significantly increased and levels of D-serine, γ-aminobutyric acid and glycine in cultures exposed to 2.5–10 µM arsenite were also increased. However, glutamate levels in the media were significantly increased only after treatment with 10 µM arsenite. In conclusion, our findings suggest that arsenic exposure may affect glutamate-induced gliotransmitter release from astrocytes and further disturb neuronal function.

Key Words
arsenite; astrocyte; glutamate; neuron; cell viability; intracellular free calcium; gliotransmitter; neurotoxicity; neural regeneration

Research Highlights
(1) Arsenite has toxic effects on primary cultured astrocytes and decreases cell viability.
(2) Glutamate (25, 50, 100 µM) increases astrocytic calcium levels independent of the dose and time of exposure.
(3) Arsenite significantly increases glutamate-induced astrocytic calcium concentrations.
(4) Arsenite can promote glutamate-induced astrocytic release of D-serine, γ-aminobutyric acid and glycine.
(5) Arsenite may affect gliotransmitter release, and further disturb neuronal function.

Abbreviations
GABA, γ-aminobutyric acid; HBSS, Hanks’ balanced salt solution; GFAP, glial fibrillary acidic protein
INTRODUCTION

Inorganic arsenic is one of the most common contaminants in the natural and occupational environment. Chronic exposure to inorganic arsenic is known to be associated with an increased risk of cancers and noncancer outcomes such as skin lesions, vascular diseases, and neurotoxicity[1-3]. A significant association between chronic arsenic exposure and cognitive dysfunction has been revealed in three epidemiological studies performed in children and adolescents from different regions of the world[4-6]. These findings raise concern over neurotoxicity induced by low levels of arsenic in drinking water. However, the toxicological mechanisms involved remain poorly understood.

Neuronal damage has long been recognized to play a crucial role in toxin-induced loss of brain function. However, within the past decade, studies have focused on the role of astrocytes in neurotoxic processes[7]. Astrocytes, which have been regarded as passive elements, fulfill nutritive and structural functions for neurons, and are now thought to be indispensable for neuronal survival and function[8]. They contribute to the formation and preservation of a secure blood-brain barrier, and their tight organization around the micro-vasculature provides anatomical evidence for the necessity of chemicals in the blood to enter astrocytes on their way to neurons[9]. Therefore, astrocytes are likely to be the first target cells of arsenite in the brain.

The classical view of astrocytes as simple supportive cells for neurons is being replaced by a new vision in which astrocytes are active elements involved in the physiology of the nervous system[10]. This new vision is based on the fact that astrocytes are stimulated by synaptically released neurotransmitters, which increase the concentration of intracellular free calcium ([Ca$^{2+}$]), and in turn, stimulate the release of gliotransmitters that regulate synaptic efficacy and plasticity[10]. Consequently, astrocytes are able to communicate with neurons. Our understanding of synaptic function, previously thought to exclusively result from signaling between neurons, has also changed to include bidirectional signaling between neurons and astrocytes[11]. Hence, astrocytes have been revealed as integral elements involved in synaptic physiology, therefore contributing to the processing, transfer and storage of information by the nervous system. Reciprocal communication between astrocytes and neurons is therefore part of the intercellular signaling process involved in brain function[12-14].

Thus, toxin-induced changes of gliotransmitter release may affect neuronal function. However, until now, few studies have focused on the effects of arsenite on gliotransmitter release from astrocytes. In the present study, exogenous glutamate was used to simulate cross-talk between neurons and astrocytes to explore the effects of arsenite on glutamate-induced gliotransmitter release from primary cultured astrocytes.

RESULTS

Arsenite-induced cytotoxicity in primary cultured astrocytes from neonatal rats

To determine the toxic effects induced by arsenite, cell viability and morphological observations were examined. As shown in Figure 1, the number of detached cells increased in cultures treated with 20 and 30 μM arsenite, and gaps among cells were widened. Moreover, networks among cell bodies almost disappeared in cultures treated with 30 μM arsenite.

Figure 1 Effects of arsenite on morphology of astrocytes from neonatal rats (inverted phase-contrast microscope, ×200).

(A) 0 μM arsenite (control); (B) 2.5 μM arsenite; (C) 5 μM arsenite; (D) 10 μM arsenite; (E) 20 μM arsenite; (F) 30 μM arsenite. Arsenite at 20 and 30 μM significantly altered morphology of astrocytes, as manifested by increased detached cells, widened intercellular space and shortened or lack of processes.
In contrast, cell viability determined by the MTT assay was inhibited by arsenite in a dose-dependent manner (Figure 2). Compared with control cultures, cell viability decreased significantly in cultures treated with 5 to 30 μM arsenite ($P < 0.05$). Exposure to 2.5 μM arsenite did not result in obvious changes in cell viability. Results of morphological changes and cell viability suggested that exposure to 20 and 30 μM arsenite could result in serious damage in astrocytes. Thus, exposure to 20 and 30 μM arsenite was not adopted in subsequent experiments.

**Effects of arsenite on glutamate induced $[\text{Ca}^{2+}]_i$ in primary cultured astrocytes**

As shown in Figure 3, the intensity of intracellular calcium fluorescence increased after exposure to 25, 50 or 100 μM glutamate, and reached a maximum after 25 to 30 seconds, and gradually diminished thereafter. The maximal change in intracellular fluorescence intensity was not significantly different among the treatments following 25, 50 and 100 μM glutamate exposure (Figure 3G). Therefore, 25 μM glutamate was used in all subsequent experiments. Glutamate induced an increase in $[\text{Ca}^{2+}]_i$ following arsenite exposure (Figure 4). Compared with the control group, $[\text{Ca}^{2+}]_i$ in cultures treated with 5 and 10 μM arsenite increased significantly ($P < 0.05$).

**Effects of arsenite on gliotransmitter release from primary cultured astrocytes**

At first, levels of glutamate, D-serine, glycine and γ-aminobutyric acid (GABA) in the media after induction with 25 μM glutamate for 10, 20 or 30 minutes were determined and compared. However, no significant difference was observed (data not shown). Therefore, 10 minutes was selected as the induction time in subsequent experiments.

As shown in Figure 5, levels of D-serine, glycine and GABA in the media of cultures treated with arsenite were significantly higher than those in the control group ($P < 0.05$). However, glutamate levels in the media of cultures were significantly higher after treatment with 10 μM arsenite.
arsenite only \( (P < 0.05) \). In addition, D-serine levels in the media of cultures treated with 10 µM arsenite were also significantly higher than in cultures treated with 2.5 and 5 µM arsenite \( (P < 0.05) \). In contrast, levels of glutamate in the media were much lower.

**DISCUSSION**

Findings from this study demonstrated that arsenite could increase glutamate-induced calcium concentrations, which suggested that arsenite could disturb calcium signals by affecting the astrocytic response to extracellular stimuli. To date, astrocytic calcium signaling was thought to play a pivotal role in the bidirectional communication between astrocytes and neurons because it is triggered by neurotransmitters released during synaptic activity\[15-17\], and, in turn, it triggers the release of gliotransmitters\[18-19\]. Gliotransmitters are chemicals released from glial cells in response to physiological or pathological stimuli and play a very important role in physiological and pathological processes in the brain. It was reported that astrocytes can release a variety of gliotransmitters, such as glutamate, D-serine, GABA, and glycine, into the extracellular space\[20-22\]. Glutamate is the major excitatory neurotransmitter released from neurons, and it is also an important gliotransmitter released from astrocytes in the brain. Recent evidence showed that astrocytes are also replete with glutamate sensors, such as ionotropic and metabotropic glutamate receptors\[23\], which can combine with glutamate released from glutamatergic neurons and evoke the astrocytic calcium signal\[24\]. To date, studies have shown that the gliotransmitter glutamate has an excitatory effect on synaptic transmission\[25-26\]; however, GABA performs an inhibitory effect on synaptic function. D-serine and glycine, as the endogenous coagonist of N-methyl-D-aspartate receptors, contribute to the activation of postsynaptic N-methyl-D-aspartate receptors\[27-28\]. Results from this study showed that exposure to arsenite could promote release of D-serine, GABA and glycine; however, the enhanced release of glutamate was shown only after treatment of 10 µM arsenite. Furthermore, levels of glutamate in the media were much lower. In summary, a possible explanation for this result may be related to the presence of high affinity glutamate transporters in astrocytes, which clear the extracellular glutamate rapidly. Findings from our previous study\[29\] showed that exposure to 10 µM arsenite could markedly suppress the activities of astrocytic glutamate transporters. Therefore, enhanced glutamate levels in the media after exposure to 10 µM arsenite could be attributed partly to the inhibitory effects of arsenite on astrocytic glutamate transporters. Interestingly, D-serine was the only gliotransmitter released from astrocytes affected by arsenite in a dose-dependent manner, which suggests that D-serine release may be more selective to arsenite-induced toxicity.
In conclusion, results from the present study suggested that arsenite may affect glutamate-induced astrocytic calcium signaling, and in turn, disturb gliotransmitter release. However, whether toxin-induced changes of gliotransmitter release ultimately affect neuronal function requires further investigation. Our results give insights into the complex signaling that results from the coordinated neuron-astrocyte network. Because of the diversity of possibilities and contradictions concerning the mechanisms of arsenite-induced changes, more re-search is required to clarify the exact mechanism of arsenite-induced damage in astrocytes and the mecha-nisms that contribute to neurotoxicity induced by arsenite in astrocytes.

MATERIALS AND METHODS

Design
An in vitro comparative observation of neurotoxicity.

Time and setting
Experiments were conducted at the China Medical University, China from April 2009 to February 2010.

Materials
Neonatal (0–3 days) Wistar rats were provided by the Center of Laboratory Animals of China Medical University (license No. SYXK (Liao) 2003-0013). All experiments were carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Methods

Cell culture and identification
Primary astrocyte cultures were prepared as described previously[20]. Briefly, brains of neonatal Wistar rats were isolated. Cerebral hemispheres were separated from the brain stem and chopped into pieces less than 1 mm on each side. The tissues were washed three times with ice-cold Hanks’ balanced salt solution (HBSS) without Ca²⁺ or Mg²⁺, and then subjected to enzymatic digestion in 10 mL of 0.125% (w/v) trypsin solution (pH 7.4; Invitrogen, Carlsbad, CA, USA) at 37°C for 20 minutes. After digestion, they were transferred to Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA) and 1% (w/v) penicillin-streptomycin, followed by vigorous mixing for 1 minute to yield a mixed glial cell suspension. The solution containing dissociated cells was filtered through stainless steel (200 meshes), and the cell viability was determined using Trypan Blue exclusion assay. Cells that were more than 95% viable were plated at a density of 1 × 10⁶/mL, and maintained in culture dishes pre-coated with poly-L-lysine at 37°C in 5% CO₂ and maintained in 100% humidified atmosphere. The media was changed every three days. On the 10th day, the culture dishes were shaken for 15 hours at 250 r/min, using an orbital shaker, to remove oligodendrocytes. Following shaking, the media was changed immediately, and a nearly pure layer of astrocytes remained in the culture dishes. The target cells were grown for 5 days on the culture dishes, and then resuspended by trypsin and re-plated in the culture dishes at 1 × 10⁵/mL. After 5 days in culture, primary cultures yielded more than 95% astrocytes, as determined by glial fibrillary acidic protein (GFAP; BioGenex Company, San Ramon, CA, USA) immunofluorescence staining (Figure 6).
camera and associated software. The percentage of positively immunostained cells relative to the total number of cells (diamidino-phenylindole labeled cells) in 200 cells was calculated.

**MTT detection of cell viability**

Astrocytes were seeded in 96-well flat-bottom culture plates and exposed to 0, 2.5, 5, 10, 20, or 30 µM arsenite (sodium arsenite (NaAsO$_2$, ≥ 99.0%; Invitrogen) for 24 hours in media containing 5% (v/v) fetal bovine serum until a confluent layer was formed. After exposure to arsenite, the cells were incubated with MTT (0.5 g/L, 200 µL per well; Sigma, St. Louis, MO, USA) at 37°C for 4 hours. Then the media was carefully aspirated, and 150 µL dimethylsulfoxide (Sigma) per well was added to dissolve the blue formazan product. The absorbance was measured at 570 nm using a microplate reader (Multiscan Ascent, Labsystem, Espoo, Finland). The results were expressed as the percentage of control.

**Morphological assessment**

Astrocyte morphology was observed with an Olympus BX51 inverted phase-contrast microscope equipped with an Olympus SC35 digital camera system. The cells were plated in 24-well flat-bottom culture plates and were exposed to 0, 2.5, 5 or 10 µM arsenite in media containing 5% (v/v) fetal bovine serum for 24 hours until a confluent layer was formed. At the end of exposure, morphological changes were observed and their images were captured.

**Measurement of intracellular free calcium concentrations**

Briefly, cells seeded in dishes specifically for the laser confocal scanning analysis (Olympus), were exposed to 0, 2.5, 5 or 10 µM arsenite in media containing 5% (v/v) fetal bovine serum for 24 hours. Cells were preloaded with 1 mL Fluo 3-AM (5 µM; Dojindo, Kumamoto, Japan) and 0.05% (v/v) Pluronic F-127 (Dojindo) at 37°C for 45 minutes in the dark, as previously described by Kurose et al.$^{[31]}$ with some modifications. The media was replaced with 0.9 mL fresh HBSS. At first, the fluorescence intensity, excited at 488 nm and observed at 526 nm, was detected under quiescent conditions, and then, 0.1 mL 250, 500 or 1 000 µM glutamate solution was added to achieve final concentrations of 25, 50 or 100 µM glutamate in the media. Finally, the fluorescent images were captured and analyzed with MetaMorph software (Universal Imaging, West Chester, PA, USA). Fluorescent differences were calculated as the mean intensity over a defined region of interest containing the cell body of each cell. Calcium variations were estimated as changes of the fluorescence signal over baseline, $(F_{\text{max}}-F_o)/F_o$, where $F_o$ was the baseline fluorescence and $F_{\text{max}}$ was the maximal fluorescence intensity after addition of glutamate.

**Determination of gliotransmitter levels**

Cells plated in 6-well flat-bottom culture plates were exposed to 0, 2.5, 5 or 10 µM arsenite in media containing 5% (v/v) fetal bovine serum for 24 hours. Afterwards, cells were washed twice with HBSS, and then treated with 25 µM glutamate in HBSS at 37°C for 10, 20 or 30 minutes. The cells were further rinsed twice with HBSS, and incubated with fresh HBSS (1 mL/well) at 37°C for 6 hours. The media was then harvested and centrifuged at 1 200 r/min for 10 minutes. The supernatants were filtered using a 0.22 µm filter, and stored at −40°C until ready for analysis. The standards of glutamate, D-serine, GABA and glycine were purchased from Sigma.

Levels of glutamate, D-serine, GABA and glycine in the media were measured by high performance liquid chromatography (Waters Corporation, Milford, MA, USA). Briefly, pre-column derivatization with o-phthalaldehyde was used. Elution was carried out at room temperature with a Waters C18 column and a mobile phase of 0.1 M potassium acetate (pH 5.89)-methanol at a flow rate of 1 mL/min. Fluorescence detector conditions were excitation 250 nm with detection at emission 410 nm.

**Statistical analysis**

SPSS for Windows, version 11.5 (SPSS, Chicago, IL, USA) was used for statistical analysis. Results were expressed as mean ± SD. Differences among the means were evaluated by one-way analysis of variance followed by Student-Newman-Keuls test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

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REFERENCES

[1] Sun G, Xu Y, Li X, et al. Urinary arsenic metabolites in children and adults exposed to arsenic in drinking water in inner Mongolia, China. Environ Health Perspect. 2007;115(4):648-652.

[2] Wasserman GA, Liu X, Parvez F, et al. Water arsenic exposure and intellectual function in 6-year-old children in Araihazar, Bangladesh. Environ Health Perspect. 2007;115(2):285-289.

[3] Rocha RA, Gimeno-Alcainiz JV, Martin-Ibanez R, et al. Arsenic and fluoride induce neural progenitor cell apoptosis. Toxicol Lett. 2011;203(3):237-244.

[4] Calderon J, Navarro ME, Jimenez-Capdeville ME, et al. Exposure to arsenic and lead and neuropsychological development in Mexican children. Environ Res. 2001;85(2):69-76.

[5] Tsai SY, Chou HY, The HW, et al. The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. Neurotoxicology. 2003;24(4-5):747-753.

[6] Wasserman GA, Liu X, Parvez F, et al. Water arsenic exposure and children's intellectual function in Araihazar, Bangladesh. Environ Health Perspect. 2004;112(13):1329-1333.

[7] Lee M, Cho T, Jantaratnotai N, et al. Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J. 2010;24(7):2533-2545.

[8] Perea G, Araque A. Astrocytes potentiate transmitter release at single hippocampal synapses. Science. 2007;318(5841):1083-1086.

[9] LoPachin RM, Aschner M. Gial-neuronal interactions: relevance to neurotoxic mechanisms. Toxicol Appl Pharmacol. 1993;118(2):141-158.

[10] Verkhratsky A, Anderova M, Chvatal A. Differential calcium signaling in neuronal-glia networks. Front Biosci. 2009;14:2004-2016.

[11] Todd KJ, Serrano A, Lacaille JC, et al. Glial cells in synaptic plasticity. J Physiol Paris. 2006;99(2-3):75-83.

[12] Bergersen LH, Gundersen V. Morphological evidence for vesicular glutamate release from astrocytes. Neuroscience. 2009;158(1):253-259.

[13] Fellin T. Communication between neurons and astrocytes: relevance to the modulation of synaptic and network activity. J Neurochem. 2009;108(1):260-265.

[14] Pirttimaki TM, Hall SD, Parii HR. Sustained neuronal activity generated by glial plasticity. J Neurosci. 2011;31(21):7637-7647.

[15] Fellin T, Carmignoto G. Neurone-to-astrocyte signaling in the brain represents a distinct multifunctional unit. J Physiol. 2004;559(Pr 1):3-15.

[16] Schipke CG, Kettenmann H. Astrocyte responses to neuronal activity. Glia. 2004;47(3):226-232.

[17] Perea G, Araque A. Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. J Neurosci. 2005;25(9):2192-2203.

[18] Araque A. Astrocytes process synaptic information. Neuron Glia Biol. 2008;4(1):3-10.

[19] Nag S. Morphology and properties of astrocytes. Methods Mol Biol. 2011;686:69-100.

[20] Volterra A, Meldolesi J. Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci. 2005;6(8):626-640.

[21] Fellin T, Sul JY, D'Ascenzo M, et al. Bidirectional astrocyte-neuron communication: the many roles of glutamate and ATP. Novartis Found Symp. 2006;276:208-217.

[22] Barker AJ, Ullian EM. New roles for astrocytes in developing synaptic circuits. Commun Integr Biol. 2008;1(2):207-211.

[23] Gallo V, Ghiani CA. Glutamate receptors in glia: new cells, new inputs and new functions. Trends Pharmacol Sci. 2000;21(7):252-258.

[24] Xu J, Peng H, Kang N, et al. Glutamate-induced exocytosis of glutamate from astrocytes. J Biol Chem. 2007;282(33):24185-24197.

[25] Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev. 2006;86(3):1009-1031.

[26] Santello M, Volterra A. Synaptic modulation by astrocytes via Ca2+-dependent glutamate release. Neuroscience. 2009;158(1):253-259.

[27] Yang CR, Svensson KA. Allosteric modulation of NMDA receptors by astrocytic D-serine. Neuroscience. 2009;158(1):253-259.

[28] Oliet SH, Mohet JP. Regulation of N-methyl-D-aspartate receptors by astrocytic D-serine. Neuroscience. 2009;158(1):275-283.

[29] Zhao F, Liao Y, Jin Y, et al. Effects of arsenite on glutamate metabolism in primary cultured astrocytes. Toxicol In Vitro. 2012;26(1):24-31.

[30] Ronaldson PT, Bendayan M, Gingras D, et al. Cellular localization and functional expression of P-glycoprotein in rat astrocyte cultures. J Neurochem. 2004;89(3):788-800.

[31] Kurose I, Saito H, Miura S, et al. CD18/ICAM-1-dependent cell adhesion of rat hepatoma cells. J Clin Invest. 1997;99(5):867-878.

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