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Effects of arsenite on glutamate metabolism in primary cultured astrocytes

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ABSTRACT

The aim of this study was to explore the mechanisms that contribute to neurotoxicity induced by arsenite exposure focusing on the alteration of glutamate metabolism in primary cultured astrocytes. The cells were exposed to 0–30 μM arsenite for 24 h, and then cell viability, intracellular nonprotein sulfhydryl (NPSH) levels, mitochondrial membrane potential, activity of Na^+/K^+-ATPase, glutamine synthetase (GS) and glutamate transporter (GLAST and GLT-1), and protein expression of GS, GLAST and GLT-1 were examined. Compared with those in control, exposure to arsenite resulted in damages of astrocytes in a concentration dependent manner, which were shown by cell viabilities, and supported by morphological observation, mitochondrial membrane potential and intracellular NPSH levels. On the other hand, activities and protein expression of GS, GLAST and GLT-1 were significantly inhibited by arsenite exposure. Moreover, protein expression of GLAST and activities of GS were much more sensitive to arsenite. However, activities of Na^+/K^+-ATPase were not influenced obviously by arsenite exposure. In conclusion, findings from this study indicated that exposure to arsenite could inhibit glutamate metabolism in astrocytes, which might be related to arsenic-induced neurotoxicity.

1. Introduction

Chronic arsenic poisoning with high levels of arsenic may occur in occupationally exposed workers as well as in populations of certain areas of the world where drinking water contains exceedingly high levels of inorganic arsenic (iAs), especially in India, Bangladesh and China (Ahsan et al., 2000; Chen et al., 2005; Mazumder et al., 1998). It is well known that chronic exposure to iAs through drinking water is associated with various skin diseases, diabetes, cardiovascular diseases, peripheral neuropathy, and cancers of several organs (Bates et al., 1992; Chen et al., 2005; Gerr et al., 2000; Tondel et al., 1999; Tseng et al., 2000; Yoshida et al., 2004).

In recent years, there has been heightening concern over the effects of environmental arsenic exposure on neurological function in children and adolescents. Neurotoxic effects have been reported in clinical cases and animal experiments with chronic exposure to arsenic through drinking water (Chattopadhyay et al., 2002; Ratnaike, 2003; Rodriguez et al., 2001, 2002). In experimental studies, deficits in learning tasks (Nagaraja and Desiraju, 1994) as well as behavioral alterations (Rodriguez et al., 2001, 2002; Schulz, 2002) have been observed in rats treated with arsenite. Results of epidemiological studies (Calderón et al., 2001; Tsai et al., 2003; Wasserman et al., 2004) suggested that chronic exposure to arsenic through contaminated drinking water is associated with intellectual impairment. However, the toxicological mechanism involved arsenic induced neurotoxicity remains poorly understood.

Astrocytes, once considered little more than the glue that holds neurons in place, are now thought to be indispensable for neuronal survival and function. They contribute to the formation and preservation of a secure blood–brain barrier, and their tight organization around the microvasculature provides anatomical evidence for the necessity of chemicals in the blood to enter astrocytes on its way to neurons. On the other hand, astrocytes remove neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) from the synaptic cleft, which are then converted into metabolites (Anderson and Swanson, 2000). Glutamate is the major excitatory neurotransmitter in the brain. It acts at distinct subtypes of ionotropic and metabotropic receptors (iGluR and mGluR), respectively. Concentrations of extracellular glutamate are maintained exclusively within physiological levels by members of a family of sodium-dependent glutamate transporters (also known as excitatory amino acid transporters or EAAT), because no extracellular enzymes exist for the breakdown of glutamate. Glutamate transporters, located on both neurons and astrocytes, are responsible for the uptake of extracellular glutamate and permit normal excitatory transmission. EAAT on astrocytes also serve another vital role, where they provide glutamate for metabolic processes.

Up to 80% glutamate released into the synaptic cleft is taken-up by astrocytes through EAAT1 and EAAT2 (Rothstein et al., 1996). In rodents, the homologs for the human EAAT1 and EAAT2 are called

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glutamate–aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1). Glutamate taken up by astrocytes is amidated by the enzyme of glutamine synthetase (GS) to form glutamine. GS is localized almost exclusively in astrocytes (Bak et al., 2006). Glutamine is subsequently released by astrocytes for uptake by glutamatergic neurons, where it is deamidated for neurotransmission, when glutamate is required. Glutamate transporters are sodium-dependent proteins that rely on sodium and potassium gradients generated principally by Na^+/K^+-ATPase.

Because of the key roles of astrocytes in maintaining neuron's functions and many mechanisms controlling these functions, it is not surprising that dysfunction of astrocytes is suspected to play a primary role in the pathogenesis of neurotoxicity. Recent evidence has raised the possibility that alterations in glutamate metabolism may contribute to neuronal damage (Rae et al., 2000). Therefore, the main aim of this study was to explore the mechanisms that contribute to arsenic-induced neurotoxicity by focusing on the alteration of glutamate metabolism in primary cultured astrocytes.

2. Materials and methods

2.1. Reagents

Sodium arsenite ($NaAsO_2$, ⩾99.0%), Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Heat inactivated fetal bovine serum (FBS) was the product of Hyclone (Logan, UT, USA). Methyl thiazolyl tetrazolium (MTT), dimethylsulfoxide (DMSO), Rhodamine 123, poly-L-lysine and sodium arsenite were purchased from Sigma (Saint Louis, MO, USA). L-[3H]-Glutamate was the product of Amersham International (UK). Goat polyclonal antibodies developed against GS, GLAST, GLT-1 and actin against mouse proteins were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit monoclonal antibody developed against rat glial fibrillary acidic protein (GFAP) was purchased from BioGenex Company (San Ramon, CA, USA). The strept–avidinbiotin complex (SABC) reagent kit was the product of Boster Biological Technology Company (Wuhan, China). Enhanced chemiluminescence (ECL) plus kit was obtained from Amersham Life Science (Buckinghamsire, UK). Other chemicals of analytical grade were obtained from local chemical suppliers. They were prepared as stock solutions with sterile water, and diluted to the final concentrations with the extracellular solution before application. Water used in all the preparations was distilled and deionized.

2.2. Cell culture

The primary astrocyte culture was prepared as described previously (Ronaldson et al., 2004). Briefly, brains of neonatal (0–3 days) Wistar rats were isolated. After careful removal of the meninges, cerebral hemispheres were separated from the brain stem and chopped into pieces less than 1 mm on a side. The tissues were washed three times by ice-cold Hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}, and then subjected to enzymatic digestion in 10 ml of 0.125% (w/v) trypsin solution (pH 7.4) at 37 °C for 20 min. After digestion, they were transferred into DMEM with 10% (v/v) FBS and 1% (w/v) penicillin–streptomycin, and followed by vigorous mixing for 1 min to yield a mixed glial cell suspension. The solution containing dissociated cells was filtered through stainless steel (200 meshes), and then the cell viability was determined by Trypan Blue exclusion. Cells with viability more than 95%, was plated at a density of 1×10^6/ml, and maintained in the culture dishes pre-coated with poly-L-lysine at 37 °C in 5% CO_2 and 100% humidified atmosphere. The media was changed every three days. On the tenth day, the culture dishes were shaken for 15 h at 250 rpm in the orbital shaker for removing oligodendrocytes. Following the vibration, the media was changed immediately, and a nearly pure layer of astrocyte was remained in the culture dishes. The target cells were grown for five days in the culture dishes, and then resuspended by trypsin and re-plated in the culture dishes at 1×10^5/ml. A glass coverslip was placed beforehand in a culture dish and a part of cells was plated on it for GFAP staining by immunocytochemical method to identify the astrocyte. After five days in culture, primary cultures yielded more than 95% astrocytes, as determined by GFAP immunostaining (shown in Fig. 1).

All experiments were performed after the cells were in culture for five days. The experiment was carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the local authorities. All efforts were made to minimize the number of animals used and their suffering.

2.3. Immunocytochemical staining

GFAP immunostaining was performed by SABC method recommended by the manufacture. Briefly, cells attached on the glass coverslips were fixed with cold acetone for 10 min, and then incubated in 1% hydrogen peroxide in 0.1 M phosphate-buffered saline (PBS) for 20 min to quench endogenous activity of peroxidase. The cells were subsequently incubated with the blocking solution containing normal goat serum for 1 h, with primary rabbit anti-rat GFAP antibody at 4 °C overnight, with biotin-conjugated secondary antibody incubated for 30 min, and with streptavidin–biotin-peroxidase reagent for 30 min. After incubation in DAB reagent for 15 min, dehydrated processes were performed through alcohols and xylenes. The results were observed under an Olympus BX51 inverted phase-contrast microscope. All steps were carried out in a humidified chamber.

2.4. Quantification of cell viability

Cell viability was quantitatively evaluated by MTT assay. Astrocytes were seeded in the 96-well flat-bottom culture plates, and exposed to 0, 1, 3, 5, 10, 20, or 30 μM arsenite for 24 h in the media with 5% FBS when a confluent layer was formed. After exposure to arsenite, the cells were incubated with MTT (0.5 g/L, 200 μl per well) at 37 °C for 4 h. Then the media was carefully aspirated,

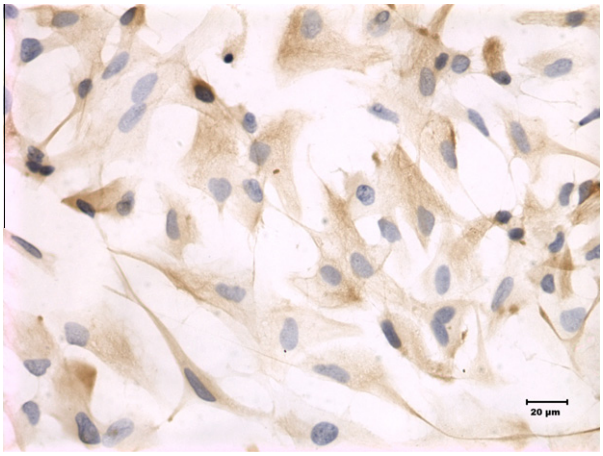

Fig. 1. Cells grown on the glass coverslips were stained with GFAP antibody. The images were collected with an Olympus BX51 inverted phase-contrast microscope (200×).