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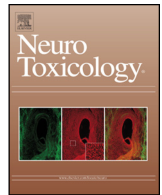

Effects of sodium arsenite on neurite outgrowth and glutamate AMPA receptor expression in mouse cortical neurons

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ABSTRACT

There has been broad concern that arsenic in the environment exerts neurotoxicity. To determine the mechanism by which arsenic disrupts neuronal development, primary cultured neurons obtained from the cerebral cortex of mouse embryos were exposed to sodium arsenite ($NaAsO_2$) at concentrations between 0 and 2 μM from days 2 to 4 in vitro and cell survival, neurite outgrowth and expression of glutamate AMPA receptor subunits were assessed at day 4 in vitro. Cell survival was significantly decreased by exposure to 2 μM $NaAsO_2$, whereas 0.5 μM $NaAsO_2$ increased cell survival instead. The assessment of neurite outgrowth showed that total neurite length was significantly suppressed by 1 μM and 2 μM $NaAsO_2$, indicating that the lower concentration of $NaAsO_2$ impairs neuritogenesis before inducing cell death. Immunoblot analysis of AMPA receptor subunit expression showed that the protein level of GluA1, a specific subunit of the AMPA receptor, was significantly decreased by 1 μM and 2 μM $NaAsO_2$. When immunocytochemistry was used to confirm this effect by staining for GluA1 expression in neuropeptide Y neurons, most of which contain GluA1, GluA1 expression in neuropeptide Y neurons was found to be significantly suppressed by 1 μM and 2 μM $NaAsO_2$ but to be increased at the concentration of 0.5 μM. Finally, to determine whether neurons could be rescued from the $NaAsO_2$-induced impairment of neuritogenesis by compensatory overexpression of GluA1, we used primary cultures of neurons transfected with a plasmid vector to overexpress either GluA1 or GluA2, and the results showed that GluA1/2 overexpression protected against the deleterious effects of $NaAsO_2$ on neurite outgrowth. These results suggest that the $NaAsO_2$ concentration inducing neurite suppression is lower than the concentration that induces cell death and is the same as the concentration that suppresses GluA1 expression. Consequently, the suppression of GluA1 expression by $NaAsO_2$ seems at least partly responsible for neurite suppression induced by $NaAsO_2$.

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1. Introduction

The developing brain is vulnerable to toxic chemical compounds such as heavy metals and persistent organic pollutants during gestation and early childhood, and exposure to them permanently affects brain functions (Costa et al., 2004; Grandjean and Landrigan, 2006; Winneke, 2011; Goulet et al., 2003; Costa et al., 2004; Farina et al., 2011; Winneke, 2011). Among those toxic chemicals, arsenic has been known to have neurotoxic effects during development. Follow-up studies of the victims of arsenic poisoning in the Morinaga milk incident in Japan have shown that oral exposure to arsenic during infancy increases the prevalence of brain disorders (Dakeishi et al., 2006). Epidemiological studies from Mexico, Bangladesh, China and Taiwan have shown that chronic exposure to arsenic decreased cognitive performances of children (Calderón et al., 2001; Wang et al., 2007; Hamadani et al., 2011; Wasserman et al., 2004, 2007; Tsai et al., 2003).

Studies have been performed on experimental animals to determine how arsenic exposure impairs normal brain development. Rats and mice exposed to arsenic during gestation and early childhood exhibit behavioral deficits such as changes in locomotor activity, learning, memory, depression-like behavior and neuromotor reflex (Rodríguez et al., 2002; Xi et al., 2009; Martinez-Finley et al., 2009). The behavioral changes caused by arsenic seem to be accompanied by neurochemical abnormalities and loss of nerve fibers (Nagaraja and Desiraju, 1993; Martinez et al., 2008;

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Ríos et al., 2009). The inhibitory effects of arsenic on neuritogenesis have been monitored in in vitro experiments, and the results have been consistent with the results observed in animals in vivo. Studies conducted on immortalized cell lines have shown that arsenic impairs neurite outgrowth and complexity (Frankel et al., 2009). Arsenic has been shown to exert its neurotoxic effects by inducing apoptotic cell death as well as by inhibiting neuritogenesis (Koike-Kuroda et al., 2010). We recently reported the finding that neurite elongation in the Neuro2A cell line was suppressed by sodium arsenite at a lower concentration than the concentration that induced apoptosis (Aung et al., 2013). Thus, neuritogenesis would seem to be more sensitive to arsenic toxicity than cell viability. Arsenic may exert its toxic effects through several mechanisms, including some distinct mechanisms that are responsible for either the suppression of neurite growth or for the induction of apoptosis. One of the possible mechanisms underlying arsenic-induced neurite suppression is thought to be oxidative stress. It has been reported that treatments with an antioxidant, a superoxide scavenger or a H_2O_2 scavenger effectively reverse the inhibitory effect of arsenic on neurite outgrowth of Neuro2A cell line (Wang et al., 2010). Based on the results that the treatment with an AMPK activator or the overexpression of a constitutively active AMPK-α1 reversed arsenic-induced inhibition of neurite outgrowth (Wang et al., 2010), the authors suggested that the LKB1-AMPK signaling pathway is involved in the arsenic-induced neurite suppression. However, the precise mechanisms underlying neurite suppression by arsenic are not completely understood.

Glutamate transmission plays essential roles in a variety of brain functions and in development. AMPA receptors, a major subtype of glutamate receptors, are composed of four types of subunits, i.e., GluA1, 2, 3 and 4, and play a critical role in excitatory glutamate transmission (Kumar and Mayer, 2013). AMPA receptors are also involved in the formation of neuronal networks and connectivity. Pharmacological potentiation of AMPA receptors and overexpression of either AMPA receptor subunit GluA1 or GluA2 by transient transfection promotes neurite outgrowth (Voss et al., 2007; Prithviraj et al., 2008; Chen et al., 2009). These results suggest that modifications of AMPA receptor subunit expression and subsequent increases in excitability due to enhanced glutamate transmission are closely related to the regulation of neuritogenesis.

In the present study, we investigated the effects of arsenic on AMPA receptors in primary cultures of mouse cortical neurons to determine the mechanism by which arsenic exposure suppresses neuritogenesis. Immunoblot and immunocytochemistry analyses showed that arsenic reduced the level of GluA1 expression in parallel with the suppression of neurite outgrowth. We also transfected GluA1/2 into primary neurons with plasmid vectors to determine whether overexpression of AMPA receptors would rescue neurite outgrowth suppressed by arsenic.

2. Materials and methods

2.1. Mice

Male and female C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and were bred at the National Institute for Environmental Studies. They were acclimatized to the environment for about 1 week prior to use. Throughout the experiments, animals were maintained in a controlled environment at a temperature of 24 ± 1 °C and humidity of $50 \pm 10\%$ and under a 12/12 h light/dark cycle (light, ZT0–12; dark, ZT12–24). Food (CE-2, CLEA) and water were available ad libitum unless otherwise indicated. Adult males and females were kept in couples to obtain pregnant females. Vaginal plug was checked each day to estimate the gestational age.

For the experiment focusing on neuropeptide Y (NPY) neurons, NPY-hrGFP transgenic mice generated by Prof. Bradford B. Lowell (Beth Israel Deaconess Medical Center) were purchased from the Jackson Laboratory (B6.FVB-Tg(Npy-hrGFP)1Lowl/J, Stock#006417, Bar Harbor, ME) (van den Pol et al., 2009). For culture of primary NPY-hrGFP neurons, the brain at embryonic day 17 was used and green fluorescence in the cerebral cortex observed with a IX70 fluorescence microscope (Olympus, Tokyo, Japan).

2.2. Ethics statement

Mice were handled in a humane manner in accordance with the National Institute for Environmental Studies guidelines.

2.3. Primary culture of neurons and transfection of plasmids

Primary cultures of mouse cortical neurons were prepared following the method described previously (Chenal and Pellerin, 2007) with slight modifications. Briefly, the pregnant mouse at embryonic day 17 was sacrificed by decapitation and the cerebral cortices of embryos were collected under dissection microscope. The cortices were washed with 10 mM HEPES-buffered Krebs–Ringer bicarbonate buffer (HKRB) containing 10 mM glucose. They were incubated in HKRB supplemented with 20 U/ml papain (Sigma Chemical Co., St. Louis, MO), 0.015 mg/ml deoxyribonuclease (Sigma), 0.75 mg/ml bovine serum albumin (Sigma) and 1 mM cysteine (Sigma) for 10 min at 37 °C in a shaking water bath, followed by gentle mechanical trituration. The obtained cell suspension was centrifuged at $100 \times g$ for 5 min and the cell pellet collected. In the case that transfection of plasmids was required, we used the Amaxa Nucleofector with mouse neuron nucleofector® kit (#VPG-1001, Lonza, Basel, Switzerland) following manufacturer's instruction. For tracing neurites, we used the pmStrawberry plasmid expressing red fluorescent protein mStrawberry under the control of CMV promoter (Maekawa et al., 2009) in Figs. 1C–J, 4, and Supplementary Fig. 1. For overexpressions of GluA1 and 2, we used pGFP-GluA1 expressing GFP-fused GluA1 under the control of CMV promoter (kindly provided by Dr. Jeremy M. Henley, University of Bristol) and pVenus-GluA2 expressing GluA2-fused with Venus, a GFP variant, under the control of CMV promoter. Correct band sizes and surface expressions of fused proteins in primary cultures of neurons have been already determined previously (Perestenko and Henley, 2003; Maekawa et al., 2009). We used pEGFP (Clontech, Takara, Japan) expressing GFP as a control instead of pGFP-GluA1 and pVenus-GluA2. Cells undergoing or not transfection were resuspended in Neurobasal medium (Invitrogen, Carlsbad, CA) containing B-27 supplement (50×, Invitrogen), 0.5 mM L-glutamine (Invitrogen) and antibiotics (Pen/Strep, #15140-148, 100×, Invitrogen). Cells were seeded onto poly-L-ornithine (15 mg/L)-precoated coverslips (12 mm, Matsunami glass, Osaka, Japan) in 24 wells cell culture plates (Falcon, Becton Dickinson AG, Basel, Switzerland). The cell density of primary cultures of neurons was 478 ± 41 cells/mm^2 (mean ± S.E.M.). Sodium arsenite ($NaAsO_2$) was added on the 2nd day in vitro (DIV2) and maintained up to DIV4 at different concentrations. Transfection efficiency was determined by counting the cells positive for both mStrawberry and 4′,6-diamidino-2-phenylindole (DAPI), a nucleus marker, in the images of fixed and DAPI-stained samples, which were obtained by confocal fluorescence microscopy (details are described below). The transfection efficiency was found to be 25.9%.

2.4. Cell survival assay by the WST-1 method

The effect of $NaAsO_2$ on cell survival of neurons was determined using a colorimetric assay based on the reduction of tetrazolium

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