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Inhibition of neurite outgrowth and alteration of cytoskeletal gene expression by sodium arsenite

Kyaw Htet Aung [a], Ryohei Kurihara [a], Shizuka Nakashima [a], Fumihiko Maekawa [b], Keiko Nohara [b], Tetsuya Kobayashi [a], Shinji Tsukahara [a,*]

[a] Division of Life Science, Graduate School of Science and Engineering, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama City, Saitama 338-8570, Japan
[b] Molecular Toxicology Section, Center for Environmental Health Sciences, National Institute for Environmental Studies, Tsukuba, Ibaraki 305-8506, Japan

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ABSTRACT

Arsenic compounds that are often found in drinking water increase the risk of developmental brain disorders. In this study, we performed live imaging analyses of Neuro-2a cells expressing SCAT3, a caspase-3 cleavage peptide sequence linking two fluorescent proteins; enhanced cyan fluorescence protein (ECFP) and Venus, to determine whether sodium arsenite ($NaAsO_2$; 0, 1, 5, or 10 μM) affects both neurite outgrowth and/or induces apoptosis with the same doses and in the same cell cultures. We observed that the area ratio of neurite to cell body in SCAT3-expressing cells was significantly reduced by 5 and 10 μM $NaAsO_2$, but not by 1 μM, although the emission ratio of ECFP to Venus, an endpoint of caspase-3 activity, was not changed. However, cytological assay using apoptotic and necrotic markers resulted in that apoptosis, but not necrosis, was significantly induced in Neuro-2a cells when $NaAsO_2$ exposure continued after the significant effects of $NaAsO_2$ on neurite outgrowth were found by live imaging. These results suggested that neurite outgrowth was suppressed by $NaAsO_2$ prior to $NaAsO_2$-induced apoptosis. Next, we examined the effects of $NaAsO_2$ on cytoskeletal gene expression in Neuro-2a cells. $NaAsO_2$ increased the mRNA levels of the light and medium subunits of neurofilament and decreased the mRNA levels of tau and tubulin in a dose-dependent manner; no significant effect was found in the mRNA levels of the heavy subunit of neurofilament, microtubule-associated protein 2, or actin. The changes in cytoskeletal gene expression are likely responsible for the inhibitory effects of $NaAsO_2$ on neurite outgrowth.

1. Introduction

Developmental neurotoxicity of several environmental chemicals may result in cognitive deficits and behavioral changes in children, which may persist throughout life (Grandjean and Landrigan, 2006). Several lines of evidence have shown that low-level exposure to inorganic lead and polychlorinated biphenyls during neurodevelopment may be related to the increased prevalence of mental retardation, cerebral palsy, and attention-deficit hyperactivity disorder in children (Winneke, 2011; Costa et al., 2004). Moreover, exposure to inorganic methyl mercury is known to cause neurodevelopmental disorders such as learning disabilities and abnormal locomotor activity in animal models (Goulet et al., 2003; Sakamoto et al., 2002). These results indicate that developmental exposure to neurotoxic chemicals can exert deleterious effects on the normal development and functions of the brain.

Arsenic, a heavy metal compound, is a neurotoxic chemical (Grandjean and Landrigan, 2006) found naturally in drinking water throughout the world. Epidemiological studies from Bangladesh and China showed that chronic exposure to drinking water containing arsenic decreases cognitive performance in children (Wang et al., 2007; Hamadani et al., 2011; Wasserman et al., 2007, 2004). In vitro studies have shown that sodium arsenite ($NaAsO_2$) induces apoptosis in neurons (Namgung and Xia, 2001; Wong et al., 2005) and neuronal cell lines (Keim et al., 2012; Koike-Kuroda et al., 2010), suggesting that $NaAsO_2$ exhibits neurotoxicity by inducing apoptosis. However, measurements of cell viability or cytotoxicity alone is not sufficient to provide accurate information for determining the toxicity of the chemical, because it has been reported that $NaAsO_2$ disrupts neurite production, outgrowth, and complexity in newly differentiated PC12 cells (Frankel et al., 2009). Moreover, there is a relationship between damage to neurites and cell death by apoptosis, because several lines of evidence have demonstrated that neurite damage is a cue for the induction of apoptosis (Berliocchi et al., 2005; Volbracht et al., 2001).

Neurite outgrowth is an indispensable process for proper development of the nervous system. Abnormal formation of

* Corresponding author at: Tel.: +81 48 858 3420; fax: +81 48 858 3419.
E-mail address: stsuka@mail.saitama-u.ac.jp (S. Tsukahara).

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neurites during development is correlated with developmental disabilities and impaired behavioral functions in rodents and primates, including humans (Berger-Sweeney and Hohmann, 1997). Neurite outgrowth is mainly orchestrated by three cytoskeletal components: microtubules, microfilaments, and neurofilaments. The coordinated rearrangement of cytoskeletal components, especially microtubules and microfilaments, plays a key role in the transition of neurons from an undifferentiated state to neurite-bearing morphology (Dehmelt and Halpain, 2004). Therefore, these cytoskeletal components involved in neuritogenesis may be a target of arsenic compounds exhibiting developmental neurotoxicity, although this has not been completely determined.

We previously reported that live cell imaging analysis of cells expressing SCAT3 is useful for evaluating the effects of chemicals on apoptosis (Koike-Kuroda et al., 2010). SCAT3 is a caspase-3 cleavage peptide linking two fluorescent proteins, enhanced cyan fluorescence protein (ECFP) and Venus, which function as the donor and acceptor, respectively, for fluorescence resonance energy transfer (FRET) (Takemoto et al., 2003). Live imaging with FRET technology can therefore be used to monitor the life and death of SCAT3-expressing cells (Takemoto et al., 2003, 2007). In order to demonstrate live imaging can be applied more efficiently to evaluation of developmental neurotoxicity, we investigated the effects of $NaAsO_2$ on neurite outgrowth in SCAT3-expressing Neuro-2a cells, a mouse neuroblastoma cell line, simultaneously with monitoring of apoptosis with live imaging. Furthermore, to understand the mechanisms by which $NaAsO_2$ disrupts neurite outgrowth, we examined whether $NaAsO_2$ alters the expression levels of cytoskeletal components involved in neuritogenesis. In addition, we also tested whether decreased cell viability of Neuro-2a cells by $NaAsO_2$ is caused by cell death of apoptosis and/or necrosis to determine the difference in $NaAsO_2$ doses, at which disruption of neuritogensis, apoptosis, and necrosis can be observed.

2. Materials and methods

2.1. Cell passaging

Neuro-2a cells (Collection No. IFO50081; the Health Science Research Resources Bank, Kobe, Japan) were maintained in minimum essential medium (MEM, Catalogue number: 11095, Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 0.1 mM non-essential amino acids (NEAA; Gibco-Invitrogen) and 10% fetal bovine serum (FBS; Gibco-Invitrogen), which is hereafter referred to as cell maintenance medium, in 75 cm^2 flasks in an atmosphere containing 5% CO_2 at 37 °C. Passaging of Neuro-2a cells was performed every 2–3 days, and passage numbers 10–17 were used for the following experiments.

2.2. Live imaging analyses of Neuro-2a cells

2.2.1. Transfection of SCAT3

Neuro-2a cells were seeded onto an 8-well chamber slide (10^4 cells/well) (LAB-TEK™ Chambered Coverglass; Nalge Nunc International, Rochester, NY, USA) that was pre-coated with 0.25% polyethyleneimine (Sigma–Aldrich, St. Louis, MO, USA). Neuro-2a cells were then transfected with the SCAT3 expression vector pcDNA-SCAT3, provided by Dr. M. Miura (The University of Tokyo, Tokyo, Japan), using FuGENE HD transfection reagents (Promega, Madison, WI, USA). According to the manufacturer's protocol for the transfection reagents, we transfected Neuro-2a cells with 2.0 μg pcDNA-SCAT3 in 300 μl cell maintenance medium in an atmosphere containing 5% CO_2 at 37 °C for 8 h. After transfection, Neuro-2a cells were washed with Dulbecco's phosphate-buffered saline (Nacalai Tesque, Inc., Kyoto, Japan) to remove transfection reagent and were kept in 300 μl cell maintenance medium in an atmosphere containing 5% CO_2 at 37 °C until starting live imaging.

2.2.2. Live imaging and $NaAsO_2$ exposure

One or two days after transfection, Neuro-2a cells in a well of the 8-well chamber slide were cultured in 300 μl MEM (without phenol red and L-glutamine, Catalogue number: 51200, Gibco-Invitrogen) containing 0.1 mM NEAA (Gibco-Invitrogen), 2 mM L-glutamine (Gibco-Invitrogen), insulin–transferrin–selenium–X supplement (Gibco-Invitrogen), and 0.3 mM N6, 2′-O-dibutyryladenosine cyclic monophosphate sodium salt (dibutyryl cyclic AMP; Sigma–Aldrich), which is hereafter referred to as experimental medium, to promote neuronal cell differentiation. Neuro-2a cells in some wells were exposed to $NaAsO_2$. Crystalline $NaAsO_2$ (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in sterile-filtered water (Sigma–Aldrich) at a concentration of 100 mM, and the $NaAsO_2$ solution was diluted with the experimental medium at concentrations of 1, 5, and 10 μM. In this study, we selected the above-mentioned doses of $NaAsO_2$ in accordance with the previous studies of our and other research groups (Frankel et al., 2009; Wang et al., 2010; Koike-Kuroda et al., 2010).

Live imaging of SCAT3-expressing Neuro-2a cells was carried out using a fluorescence time-lapse microscope BioZero 8100 (Keyence Co., Osaka, Japan). After culturing in the experimental medium with or without $NaAsO_2$ treatment was stared, the well plate containing Neuro-2a was immediately placed in an incubation chamber assembled in the microscope (Microscope Incubation System INU-KI-F1, Tokai Hit, Shizuoka, Japan) in which the temperature and gas concentration were controlled at 37 °C and 5% CO_2, respectively. The well plate was kept for 2 h to stabilize culture condition for Neuro-2a cells in the incubation chamber. Live imaging was then performed by capturing digital photomicrographic images of Venus and ECFP in SCAT3-expressing Neuro-2a cells at 2 h after initiation of $NaAsO_2$ treatment. A 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters of 480AF30 for ECFP and 535AF25 for Venus (Opto Science Inc., Tokyo, Japan) were used for imaging of SCAT3-expressing cells. Images were captured with an objective lens (Plan Fluor ELWD DM 20× C, NA 0.45; Nikon, Tokyo, Japan) and a CCD camera on the fluorescence microscope every 30 min for 9 h. Four independent live imaging experiments were carried out in this study.

2.2.3. Quantification of neurite outgrowth

In this experiment, we measured the areas of neurites and cell bodies in the digital photomicrographs obtained with live imaging using KEYENCE BZ II software (Keyence) and then calculated the ratio of neurite area to cell body area as an endpoint to evaluate the effects of $NaAsO_2$ on neurite outgrowth. The areas of the neurite and cell body of SCAT3-expressing Neuro-2a cells (number of cells analyzed: 100–140 per well) can be measured separately, because there was a difference in the fluorescence intensities of Venus between cell bodies and neurites with the fluorescent intensity of cell bodies being stronger than that of neurites (Fig. 1). To measure the area of neurites, we measured the area of the regions, the brightness of which was 20–30 on a scale of 256. In addition, we measured the area of regions with brightness of 31–255 on a scale of 256, which corresponded to the area of the cell bodies. After measuring both areas in each image, the area ratio of the neurites to the cell body was calculated at each time point and then calibrated using the ratio of the same areas 120 min after initiation of $NaAsO_2$ exposure, which was set at a value of 1.