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Role of aquaporin 9 in cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes

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ABSTRACT

Aquaporin (AQP) 9 is a member of the aquaglyceroporin subfamily of AQPs in the transfer of water and small solutes such as glycerol and arsenite. It is well recognized that arsenic toxicity is associated with intracellular accumulation of this metalloid. In the present study, we examined the contribution of AQP9 to the uptake of inorganic arsenite, thereby increasing arsenic-induced cytotoxicity in primary mouse hepatocytes. Pretreatment with sorbitol as a competitive inhibitor of AQP9 and siRNA-mediated knockdown of AQP9 resulted in a significant decrease of arsenite uptake in the cell and its cytotoxicity. Furthermore, overexpression of AQP9 in HEK293 cells led to the enhancement of intracellular arsenic concentration, resulting in enhanced cytotoxicity after arsenite exposure. These results suggest that AQP9 is a channel to define arsenite sensitivity in primary mouse hepatocytes.

Introduction

Arsenic is a naturally occurring metalloid that is regarded as a ubiquitous worldwide contaminant (Nordstrom, 2002). Arsenic toxicity caused by drinking naturally polluted water from wells is a global health problem affecting many millions of people. The symptoms of chronic arsenic toxicity are characterized by skin lesions, increased risk for cancer, cardiovascular disease, diabetes, and liver damage (Ratnaike, 2003; Rossman, 2003; Navas-Acien et al., 2006, 2008; Guha Mazumder, 2001; Liu and Waalkes, 2008). On the other hand, arsenic trioxide is used as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia (Mayorga et al., 2002; Sanz et al., 2005). Although the exact mechanisms underlying the considerable variation in individual responses to arsenic in both cases are unclear, a possible explanation for this variation is differences in arsenic efflux and uptake, which are related with the cellular burden of arsenic.

Liver is one of the target organs for arsenic and the prime site for the conversion of inorganic arsenic into the methylated or glutathione-conjugated metabolites responsible for detoxification. It has been established that inorganic arsenic undergoes reduction and oxidative methylation, thereby forming mono- and di-methylated metabolites that are excreted into urine (Styblo et al., 2002). Alternatively, inorganic arsenite (iAsIII) reacts readily with glutathione to yield arsenic triglutathione ($As(GS)_3$), leading to excretion into extracellular space through multidrug resistance-associated protein (MRP) (Rosen, 2002; Kumagai and Sumi, 2007). In mammals, both $As(GS)_3$ and methylarsenite diglutathione are transported into bile via MRP2 (Kala et al., 2000). In contrast to studies on arsenic metabolism and efflux, few studies have examined arsenic uptake.

Aquaporin (AQP) 3, 7, and 9, members of the aquaglyceroporins subfamily of AQPs, have been shown to transport iAsIII into mammalian cells (Liu et al., 2004; Agre and Kozono, 2003; Bhattacharjee et al., 2004; Lee et al., 2006). AQP9 is found to be an important membrane protein that serves as a channel in the transfer of water and a large variety of small solutes (Tsukaguchi et al., 1998). This channel is highly expressed in the liver, which represents its main expression site, and also in the epidermis, lung, spleen, leukocytes, and adipose tissue (Rojek et al., 2007; Tsukaguchi et al., 1999; Ishibashi et al., 1998; Kuriyama et al., 1997). Among 13 AQPs cloned in mammals, hepatocytes express AQP 0, 8, 9, and 11 (Masyuk and LaRusso, 2006), indicating that AQP9 is the sole aquaglyceroporin subfamily expressed in liver (Kishida et al., 2000). To date, AQP9 has been shown to restore arsenic sensitivity to a *Saccharomyces cerevisiae* mutant that lacked FPS1, a homologue of AQP9 (Liu et al., 2004), and AQP9 transfectant accumulated higher levels of intracellular arsenic than the control after exposure to arsenic trioxide, resulting in a significant increase of arsenic-induced cytotoxicity in leukemia cells (Leung et al., 2007). Although these results suggested that AQP9 facilitates iAsIII uptake, thereby increasing cytotoxicity in yeast and leukemia cells, further studies are required to investigate the participation of AQP9 in arsenic-induced cytotoxicity in other mammalian cells.

We previously reported that sulforaphane, an activator of Nrf2, suppresses arsenic-induced cytotoxicity through the up-regulation of

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drug-metabolizing enzymes associated with arsenic efflux in primary mouse hepatocytes (Shinkai et al., 2006). Thus, we hypothesized that a substantial reduction in cellular accumulation of arsenic by decreasing iAsIII uptake would suppress the cytotoxicity caused by arsenic exposure. To test such a possibility, we examined whether AQP9 is implicated in iAsIII uptake and arsenic-induced cytotoxicity in hepatocytes.

Materials and methods

Materials. Sodium arsenite was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Anti-AQP9 (AQP91-A) and anti-5′-nucleotidase (5′-NT) were from Alpha Diagnostic (San Antonio, TX) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. HRP-conjugated anti-rabbit IgG was from Cell Signaling Technology (Beverly, MA). [U-^{14}C]glycerol (150 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other reagents and chemicals were of the highest grade available.

Cells and cell culture. Primary hepatocytes were isolated from 6- to 10-week-old C57BL/6J male mice by two-step collagenase perfusion. Parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 ×*g* for 3 min. Dead parenchymal hepatocytes were removed by density gradient centrifugation in Percoll. Final preparations were suspended at 4.0×10^5 cells/ml in Williams medium E supplemented with 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and then seeded at a density of 8×10^4 cells/cm^2 in culture plates coated with pig type I collagen (Iwaki, Tokyo, Japan). The human embryo kidney cell line HEK293 was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine, and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cultured cells were maintained at 37 °C in a humidified incubator under an atmosphere of 5% CO_2/95% air. Before treatment, cells were cultured in serum-free medium overnight and then exposed to iAsIII in serum-free medium.

Assay of cytotoxicity. 3-(4,5-dimethylthiazol-2-yl)-2,5-triphenyl tetrazolium bromide (MTT) assay was used to estimate cell viability, as described previously (Denizot and Lang, 1986). Briefly, cells were exposed to iAsIII in 96-well plates and then treated with 5 mg/ml MTT (1/20 volume) for another 4 h at 37 °C. After removing the medium, DMSO (100 μl/well) was added to dissolve MTT formazan. Absorbance at 540 nm was measured by an ImmunoMini NJ-2300 plate reader (Nippon InterMed, Tokyo, Japan). The LC_{50} values were calculated using a nonlinear regression program (Prism version 4.0; GraphPad Software Inc., San Diego, CA).

Western blotting. After treatment, cells were washed twice with ice-cold PBS(−). A crude membrane fraction was prepared from cultured hepatocytes by differential centrifugation, as described previously (Germann et al., 1989). Briefly, cells were collected by scraping into PBS, resuspended in hypotonic lysis buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$) and incubated on ice for 15 min. Swollen cells were ruptured with 20 strokes in a tightly fitting Dounce homogenizer, and the nuclei were removed by centrifugation at 400 ×*g* for 10 min at 4 °C. The pellet obtained by subsequent centrifugation at 30,000 ×*g* for 30 min at 4 °C was used as the crude membrane fraction. HEK293 cells were solubilized with SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol), incubated at 95 °C for 5 min, and stored at −20 °C until use. Protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL), with bovine serum albumin as a standard. After marker dye and 2-mercaptoethanol were added to the specimen, proteins were separated by SDS-PAGE and electro-transferred onto PVDF membrane at 2 mA/cm^2 for 60 min, according to the method of Kyhse-Andersen (1984). Blots were blocked with 5% skim milk in TTBS (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) and then incubated with primary antibodies. To detect immunoreactive proteins, we used HRP-conjugated anti-rabbit IgG and an ECL system (GE Healthcare Bioscience, Piscataway, NJ), and X-ray exposure. Quantitative analysis of band intensity was performed using the NIH image system.

siRNA transfection. Predesigned siRNA against mouse AQP9 (catalog no. SI02694909) and control siRNA (catalog no. 1022076) were purchased from QIAGEN (Valencia, CA). Cells were transiently transfected with double-stranded siRNAs for 48 h by Lipofectamine 2000 in serum-free medium according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA).

Plasmids and transient transfection of cells. To construct a His-tagged mouse AQP9 expression plasmid, the complete coding sequence of AQP9 was cloned by RT-PCR using total RNA obtained from RAW264.7 cells. Using sequences obtained from the NCBI database, we designed two primers (forward, 5′-TCTGGATCCGATGCCTTCTGAGAAGGACCGAGC-3′, reverse: 5′-TCTGGATCCCTACATGATGACGCTGAGTTCGTG -3′) containing the BamHI restriction site, respectively. The PCR product was cloned into a pcDNA3.1/His B vector (Invitrogen) to yield the plasmid pcDNA3.1-His/AQP9. The nucleotide sequence of AQP9 in pcDNA3.1-His/AQP9 was analyzed by DNA sequencing to confirm the correct nucleotide sequence and in-frame insertion. Plasmid pcDNA3.1-His/LacZ, containing the β-galactosidase gene, was purchased from Invitrogen. For transfection experiments, HEK293 cells grown to subconfluence were transiently

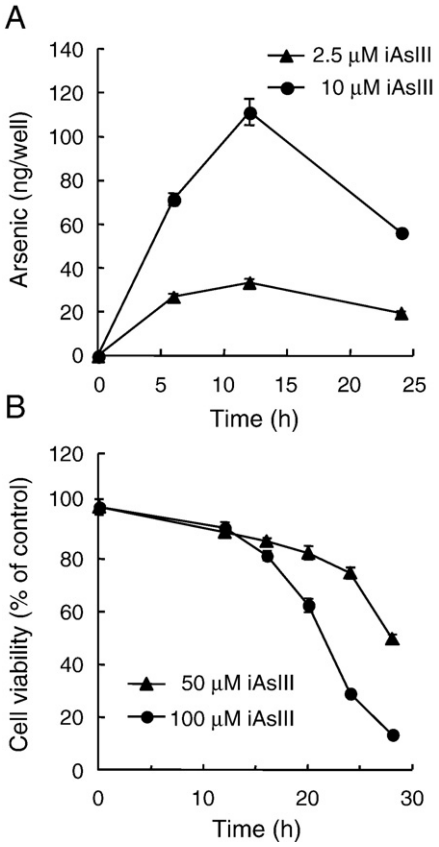

Fig. 1. Time-dependent arsenic accumulation and cell death induced by iAsIII exposure in primary mouse hepatocytes. (A) Cells were incubated with iAsIII (2.5 or 10 μM) for 6, 12, or 24 h and then arsenic accumulation was determined by ICP-MS. Each value represents the mean ± SE ($n=3$). (B) Cells were incubated with iAsIII (50 or 100 μM) for 12, 16, 20, 24, or 28 h and then MTT assay was performed. Each value represents the mean ± SE ($n=3$).