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Boric acid increases the expression levels of human anion exchanger genes *SLC4A2* and *SLC4A3*

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ABSTRACT. Boron is an important micronutrient in plants and animals. The role of boron in living systems includes coordinated regulation of gene expression, growth and proliferation of higher plants and animals. There are several well-defined genes associated with boron transportation and tolerance in plants and these genes show close homology with human anion exchanger genes. Mutation of these genes also characterizes some genetic disorders. We investigated the toxic effects of boric acid on HEK293 cells and mRNA expression of anion exchanger (*SLC4A1*, *SLC4A2* and *SLC4A3*) genes. Cytotoxicity of boric acid at different concentrations was tested by using the methylthiazolyldiphenyl-tetrazolium bromide assay. Gene expression profiles were examined using quantitative real-time PCR. In the HEK293 cells, the nontoxic upper concentration of boric acid was 250 µM; more than 500 µM caused cytotoxicity. The 250 µM boric acid concentration increased gene expression level of *SLC4A2* up to 8.6-fold and *SLC4A3* up to 2.6-fold, after 36-h incubation. There was no significant effect of boric acid on *SLC4A1* mRNA expression levels.

Key words: Boric acid; Anion exchanger; RT-PCR; Gene expression

INTRODUCTION

The role of boron in plants was described more than 80 years ago. Boron is an important micronutrient for plants and animals that may be toxic in high amounts. Increasing evidence suggests that boron is also an essential element for humans (Nielsen, 2000). Boron deficiency and toxicity also causes problems in agriculture. Boron is a widely used component of pharmaceuticals and has several industrial applications (Aviñó-Martínez et al., 2008). Additionally, it is used as a pesticide and fungicide and to protect food from microbes (Turkoglu, 2007; De Seta et al., 2009). Boron supplies the tissue antioxidant defenses via oxidative metabolism (Pawa and Ali, 2006). Boric acid is also a potential factor for genotoxicity in bacteria and human cells (Moore, 1997). The roles of boron in living organism include regulation of gene expression in mixed bacterial populations and the growth and proliferation of higher plants and lower animals. Deficiency and supplementation cause remarkable biological effects in human and animal tissues (in osteoporosis, arthritis, plasma lipid profiles, and brain function; Devirian and Volpe, 2003).

Several genes are associated with boron transportation and tolerance in plants (Sutton et al., 2007). *Arabidopsis thaliana BOR1* is a boron efflux transporter that regulates xylem loading and protects shoots from boron deficiency. Studies have demonstrated that homologs of BOR1 are found in various organisms including yeast, plants, and humans (Takano et al., 2002; Park et al., 2004; Miwa et al., 2007).

The sodium independent anion exchanger family plays an essential role in mediating anion transportation in mammalian tissues. These proteins are used in the regulation of intracellular pH, chloride, and cell volume. Specific members of this family also have roles in vectorial transepithelial base transport in several organ systems including the kidney, pancreas, and eye. The functionality of these transporters in mammalian cells is identified by observable abnormalities resulting from *SLC4A1-3* mutations in humans and targeted deletions in knockout models. *SLC4* gene products from various mammalian, marine invertebrates, fish, insects, and *Caenorhabditis elegans* have been cloned, localized, and functionally expressed (Romero et al., 2000; Sherman et al., 2005). *SLC4A1* mediates sodium-independent anion exchange in the erythrocytes and kidneys (Alper, 2002). Kidney-specific *SLC4A1* is expressed in collecting duct cells (Verlander et al., 1988), which plays an important role in bicarbonate reabsorption and urinary acidification. Some *SLC4A1* mutations have been described in humans and cause the familial erythroid disorders of spherocytic anemia, familial distal renal tubular acidosis, and ovalocytosis. In a knockout animal model, $SLC4A2^{-/-}$ mice develop achlorhydria and osteopetrosis and die before weaning. Other mutated $SLC4A2^{-/-}$ mice can survive, but males show infertility with defective spermatogenesis and a syndrome such as primary biliary cirrhosis. *SLC4A2* is expressed in several cell types and is involved in the regulation of cytoplasmic pH through the exchange of intracellular bicarbonate for extracellular chloride (Medina et al., 1997). *SLC4A2* has been observed in a broad pattern of expression and evaluated as a housekeeping regulator of intracellular pH (Medina et al., 2000). *SLC4A3* is a sodium-independent anion exchanger like *SLC4A1* and *SLC4A2*. Transcripts of *SLC4A3* in two variants have been found in the brain and heart (Kudrycki et al., 1990). *SLC4A3* expression in human embryonic kidney 239 (HEK293) cells has also been reported to mediate chlorine/bicarbonate exchange (Stewart et al., 2001; Fujinaga et al., 2003). *SLC4A1* is the only mammalian anion exchanger used in boron transportation. *SLC4A1* has been reported to mediate electrogenic sodium borate cotransportation (Park et al., 2004).

MATERIAL AND METHODS

Cell culture

HEK293 cells (ATTC, UK) were used. The cells were grown at 37°C in humid air containing 5% CO_2. The cell culture medium was Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) and 2.0 mM L-glutamine (Gibco). The medium was changed three times a week.

Toxicity tests

HEK293 cells were plated on 6-well plates at a density of 2.5 x 10^4 cells per well and grown in Dulbecco's modified Eagle's medium. The number of cells was counted using a hemacytometer. Plates were incubated for 36 h at 37°C with medium containing boric acid at various concentrations. Boric acid solutions were prepared using ultra-pure water. Experimental media were prepared by adding concentrated boric acid solutions to provide a range of concentrations: 50, 100, 250, 500, and 1000 µM. The boron-containing media were then removed and the cells were washed two times with normal culture medium without serum. Each experiment replicated four times.

After the boron exposure, the viability of cells was studied using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric staining method (Mosmann, 1983). Plates were incubated with medium containing 1 mg/mL MTT (Sigma-Aldrich). The medium from each well was replaced by isopropanol after 4 h of inoculation at 37°C with 5% CO_2. Isopropanol was allowed to solubilize the resulting formazan crystals for 1 h at 37°C with 5% CO_2. The color conversion was measured by an enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. Additionally, cell viability was determined with the Trypan blue exclusion test (Strober, 2001). Viable cells were detected based on the ability to exclude the dye. The percentage of viable cells was counted in a hemacytometer after 36 h of boron treatment and recorded as the number of adherent cells/well. Student *t*-tests were performed. P values less than 0.05 were considered to be statistically significant.

Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR analyses were performed to estimate the expression levels of *SLC4A1*, *SLC4A2*, *SLC4A3*, and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNAs (mRNAs) for the boron-treated HEK293 cells. Total RNA was extracted from cells using a High Pure RNA Isolation Kit (Roche) according to manufacturer instructions. RNA quality and quantity were determined using a NanoDrop 2000c spectrophotometer. A RevertAid^{TM} First-Strand cDNA Synthesis Kit (Fermentas) was used to synthesize cDNA from 2.5 µg total RNA using random hexamers with DNase-treated RNA samples according to the kit manual. To examine the expression level of *SLC4A1*, *SLC4A2*, and *SLC4A3* mRNA, we performed qRT-PCR using a SYBR Premix Ex *Taq* (Takara). The RT-PCR primers were as follows: for *SLC4A1* (forward primer 5'-CCGCATCTTGCTTCTGTTCA-3', reverse primer 5'-CACAGTGAGGATGAGGACGA-3'), *SLC4A2* (forward primer 5'-TGGGGACAA GCCCAAGATTC-3', reverse primer 5'-CAGATGCAGCCGCTCATAGA-3'), and *SLC4A3*

(forward primer 5'-GAGCAGCCCTATGTGACCAAG-3', reverse primer 5'-ATCCTGGCCA TCCTCATCGAA-3'). The housekeeping gene, GAPDH, was amplified using the following primer pairs: forward primer 5'-CACTCCTCCACCTTTGACG-3', reverse primer 5'-TCTTCCTCTTGTGCTCTTGC-3'. Twenty microliters of PCR mixture containing 2 µL template cDNA, 0.2 µM each of the primers, and 10 µL SYBR Premix Ex *Taq* 2X Mix (Takara) was placed in a 0.2-mL tube. qRT-PCR was carried out with a Corbett Rotor-Gene 6000 instrument, and the amplifications were analyzed using the Rotor-Gene Q software version 2. To confirm the amplification specificity, PCR products were subjected to a melting curve analysis. The optimized program involved denaturation at 95°C for 2 min followed by 40 cycles of amplification (at 95°C for 8 s, at 60°C for 10 s, and at 72°C for 12 s) for all reactions. For all cell culture experiments, four replicates of control and boric acid-treated cells were analyzed and the mean values reported. After amplification, the target gene amplification was calculated based on threshold cycle (Ct) value. Relative expression level of each group was calculated compared with housekeeping gene GAPDH. Student *t*-tests were performed. P values less than 0.05 were considered to be statistically significant.

RESULTS

In this study, we performed cytotoxicity tests and qRT-PCR analysis on total RNA samples from boric acid-treated HEK293 cells. The expression profiles of three human anion exchanger genes *SLC4A1*, *SLC4A2*, and *SLC4A3* were determined from boric acid-induced cells. To measure the viability of HEK293 cells, the MTT assay and the Trypan blue exclusion test were used. After the addition of boric acid in 5 concentrations (50, 100, 250, 500, and 1000 µM), HEK293 cells were incubated for 36 h. Results of the MTT test appear in Figure 1. The MTT test indicated no significant reduction in cell viability at concentrations of 50 to 250 µM boric acid compared to controls ($P < 0.05$). The half-maximal inhibitory concentration (concentration of boric acid to induce 50% cell mortality) was 500 µM, indicating the potency of toxicity. At concentrations of 500 to 1000 µM boric acid, cell viability dramatically decreases, which indicates a toxic effect on HEK293 cells. At the highest boron concentration, 1000 µM, significant toxicity was observed and nearly 80% of the cells were lost. Cell loss was primarily due to detachment from the bottom of the dishes during the incubation and the washes that followed.

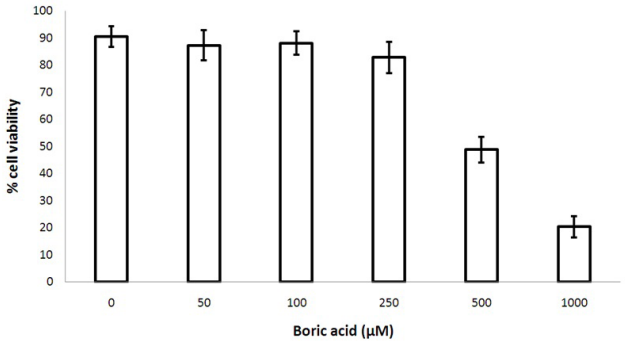

Figure 1. Dose-dependent toxicity of boric acid in HEK293 cells. Cells were cultured in serum-free DMEM, and incubated to a range of boron concentrations (0-1000 µM). Cells were counted after 36 h of exposure and recorded as the number of adherent cells/well.

After optimization, RT-PCR assays produced one specific peak on DNA melting analysis, and specificity was further confirmed by the presence of a single amplicon for the *SLC4A1*, *SLC4A2*, *SLC4A3*, and housekeeping gene GAPDH after agarose gel analyses. As shown in Figure 2, 250 µM boric acid had no observable effect on *SLC4A1* gene expression after 6, 12, 24, and 36 h of incubation. Compared to the controls, *SLC4A2* gene expression increased 2.72-, 3.71-, 4.85-, and 8.56-fold after 6, 12, 24, and 36 h of exposure to 250 µM boric acid, respectively. Under the same conditions, *SLC4A3* gene expression increased up to 2.6-fold compared to the starting point after 36 h of incubation.

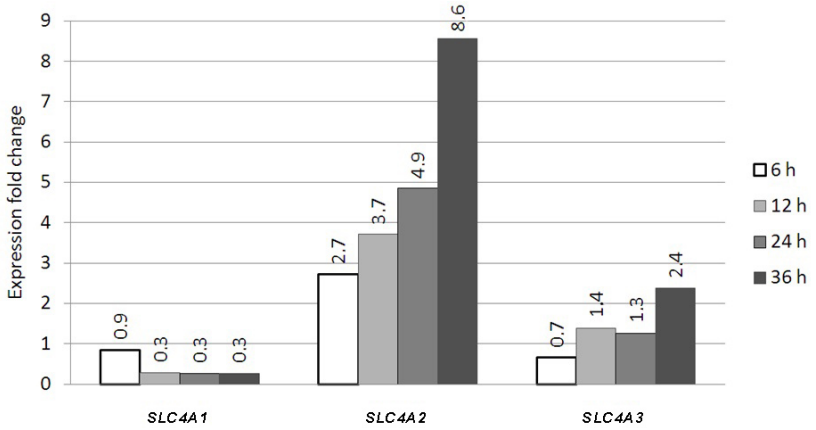

Figure 2. *SLC4A1*, *SLC4A2* and *SLC4A3* gene expression changes in HEK293 cells exposed to boric acid for 6, 12, 24, and 36 h using quantitative real-time PCR. Values represent the mean of four independent experiments. Data represent the mRNA fold changes relative to 0 h as control.

Relative gene expression was calculated using the ΔCt method, which was normalized against the GAPDH housekeeping control gene. At varying times (6, 12, 24, and 36 h), each group was compared to parallel control cell cultures without boric acid. The data represent the fold changes relative to 0 h control as means ± SEM. Differences between boric acid-treated samples at 0 and 36 h were tested with the pair's Student *t*-test using Excel 2007. Statistical significance was set at $P < 0.05$.

DISCUSSION

A review of the literature indicates that boron is an essential element for higher animals, including humans, for whom boron is nutritionally beneficial (Nielsen, 2000). Boron supports key enzymes involved in antioxidant processes and exhibits immunomodulatory functions that enhance the resistance of animals to mycotoxin. Boron is also implicated in calcium metabolism (Nielsen, 1994). Dietary deprivation of boron also causes suboptimal biological functioning, which can be reversed by boron supplementation. Boron deficiency-related health problems have been observed in rats, chickens, and humans (Nielsen, 1992; Devirian and Volpe, 2003).

The understanding of the molecular mechanisms of boron transportation across membranes, especially those in plants, has advanced greatly since 2000. The first boron transporter

gene in plants, *BOR1*, was identified in *Arabidopsis* mutants (Tanaka et al., 2008). Later, several boron membrane transporter genes in plant cells were characterized. Alignment analyses of the human genome against the plant *BOR1* protein sequence demonstrate high similarity of the plant sequence with the human *SLC4A* anion exchanger gene family. These similarities range between 35 and 39% at the protein level. Structural similarity may imply some functional homology. It has been reported that a member of the human *SLC4A* anion exchanger family, *SLC4A1*, is used for boron membrane transportation. *SLC4A1* has been cloned on the basis of the sequence homology with the plant boron transporter *BOR1* (Romero et al., 2004; Lopez et al., 2009).

Until now, no information about the relationship between the boron membrane transportation mechanism and *SLCA1*-3 anion exchangers in humans has been available. The purpose of this study was to evaluate the effect of boric acid on the gene expression of three subtypes of human *SLC4A* anion exchangers at the mRNA level. In humans, the *SLC4A* family contains sodium bicarbonate cotransporters, sodium-dependent chloride bicarbonate transporters, and sodium-independent chloride bicarbonate transporters (*SLC4A1*-3; Alper, 2006). The genes have similar structures, and their encoded proteins share common functional features. The expression of these genes has been demonstrated previously in HEK293 cells, in which the efflux and influx of bicarbonate were measured during changes in chloride gradients (Sterling and Casey, 1999). HEK293 cells were chosen in this study because they have been widely used in cell biology research for many years and are a convenient model for toxicity and induced gene expression testing.

To determine the toxicity of boric acid on HEK293 cell, we applied the MTT test and evaluated cell viability after treatment with boric acid. The viability of HEK cells treated with boric acid (0-1000 μM) for 6, 12, 24, and 36 h was measured. Cells survived after treatment with concentrations of <250 μM boric acid. Treatment with concentrations of >500 μM boric acid appeared to cause cytotoxicity, an observation consistent with previous studies (Landolph, 1985). Longer exposure to boric acid reportedly results in toxic effects because of an accumulation of intracellular boron (Tilly et al., 1996). Thirty-six hours of incubation was sufficient to find cytotoxicity activity. The increased incubation time caused boron to accumulate in intracellular spaces and induced gene expression to maintain cellular homeostasis accordingly; up-regulation of anion exchanger genes is also expected to play a role in this cellular response.

We tested HEK293 cells with 250 μM boric acid for various times (6, 12, 24, and 36 h). qRT-PCR results showed a gradual increase in *SLC4A2* gene expression, which indicates that boric acid causes up-regulation of this gene as high as 8.56-fold. Under the same conditions, the *SLC4A3* gene expression level slightly increased up to 2.6-fold. These results are the first to show the regulatory effect of boric acid on *SLC4A1*, *SLC4A2*, and *SLC4A3* mRNA levels. However, the underlying molecular mechanism remains unclear and should be examined. Owing to changes in ion balance with increased in time during cell culture, up-regulation of *SLC4A2* and *SLC4A3* mRNAs may be considered a consequence of the direct or indirect effects of boric acid.

Understanding the molecular mechanisms of the membrane transportation of boron in humans may lead to a more comprehensive understanding of the pathophysiology and genetics of anion exchangers. This gene family also has a role in the deficiency diseases of hereditary spherocytosis, distal renal tubular acidosis, and hereditary stomatocytosis (Alper, 2009). The functional analyses of *SLC4A1-3* genes and their pathophysiology should be tested using knockout animal models to obtain more detailed molecular information.

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