AQP9 expression and phosphorylation affect arsenic uptake and tolerance in human liver hepatocellular cells

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Abstract

Background

Little is known about the signaling mechanisms involved in arsenic tolerance or detoxification, although the water channel protein aquaporin-9 (AQP9) and components of the mitogen-activated protein kinase (MAPK) pathway have putative roles. Elucidation of the mechanisms of resistance in mammalian cells would be helpful in developing effective, arsenic-based therapeutic strategies.

Methods

The association between AQP9 and arsenic accumulation and tolerance was investigated in arsenic-sensitive human liver hepatocellular cells (HepG2) and arsenic-resistant HepG2 (AsHepG2) cells.

Results

The IC_{50} value for arsenic of AsHepG2 cells (15.59 μ M) was significantly higher than that of HepG2 cells (7.33 μ M; $P < 0.05$), and AsHepG2 cells accumulated significantly low levels of arsenic after treatment with sodium arsenite ($NaAsO_2$; $P < 0.01$). Arsenic accumulation in AsHepG2 cells reached a plateau 6 h after treatment, while in HepG2 cells it continued to increase throughout the experimental period. Additionally, intracellular arsenic accumulation in AQP9-overexpressing AsHepG2 cells significantly increased within 10 h of treatment, whereas in HepG2 cells it increased throughout the experimental period. The level of AQP9 protein in AsHepG2 cells decreased in a concentration-dependent manner, but was not markedly different in HepG2 cells. Furthermore, after $NaAsO_2$ treatment the level of phosphorylated AQP9 and p38 was significantly increased with time in the two cell lines. Partial inhibition of p38 activity by the specific inhibitor SB203580 did not significantly affect AQP9 protein expression or phosphorylation.

Conclusion

AQP9 expression and its state of phosphorylation are closely related to arsenic uptake and may regulate cellular arsenic tolerance by reducing its uptake rate. p38 may have a limited role in the regulation of AQP9 phosphorylation in AsHepG2 cells.

1. Background

Some arsenic compounds, such as arsenic trioxide, are widely used as chemotherapeutic agents to treat certain types of cancers [1–3], especially acute promyelocytic leukemia [4; 5]. Despite the effectiveness of arsenic chemotherapy, the development of resistance has been observed in many cells including

leukemia [6], lung cancer [7; 8] and Chinese hamster ovary cells [9]. Such tolerance can become an important limitation in the clinical application of arsenic. Elucidation of the mechanisms of resistance in mammalian cells would be helpful in developing effective, arsenic-based therapeutic strategies.

It has been suggested that acquired tolerance to arsenic is mainly due to changes in cellular uptake and efflux pathways. Aquaporin (AQP) water channels, members of the aquaglyceroporin family are considered the principal entry routes for As(III) in bacteria [10; 11], *Leishmania* protozoa [12], yeast [13], and vertebrate [14–16]. Among these, AQP9 plays a particularly important role in arsenic uptake [14; 17–19]. In the yeast *Saccharomyces cerevisiae*, the AQP9 homolog Fps1p contributes to show highly sensitivity to both As(III) and trivalent antimony Sb(III) [20]. Moreover, the mammalian-derived AQP9 restored arsenic sensitivity in a *S. cerevisiae* strain in which the gene encoding FPS1 was deleted [14]. Overexpression of AQP9 in the leukemia cell line K562 led to intracellular arsenic accumulation and arsenic hypersensitivity [21]. More recently, other studies have supported the hypothesis that AQP9 mediates arsenic uptake in human lung cancer cells [22] and primary mouse hepatocytes [23]. We found that AQP9 expression and phosphorylation levels may play an important role in regulating arsenic influx in previous study [24–25]. Tang Jie also found the expression of AQP9 affected biological behaviors of HepG2 cells and their sensitivity to arsenic [26].

Little is known about the signaling proteins and transcriptional molecules that regulate expression and activity of aquaglyceroporin channels and, consequently, arsenic uptake in eukaryotic cells. In mammals, As(III) activates p38 mitogen-activated protein kinase (MAPK), which then activates several stress response genes [27–29]. By contrast, inhibition of p38 suppressed the expression of AQP4 and AQP9 in cultured rat astrocytes [30], and enhanced arsenic trioxide (As₂O₃)-induced cytotoxicity in myeloma cells [31]. Throsen et al. [13] found a direct link between MAPK-mediated regulation of an AQP9 analogue and arsenic transport in *S. cerevisiae*, which suggested that downregulation of MAPK may sensitize cells to arsenic by increasing uptake. We found that p38 kinase attends partly AQP9 phosphorylation in liver normal cells L-02 [32]. However, it is not known whether or not p38 modulates expression and phosphorylation of AQP9, arsenic uptake, or arsenic sensitivity in mammalian cells.

In the present study, the association between AQP9 and arsenic tolerance and a potential role for p38 MAPK in regulation of AQP9 activity were investigated in mammalian cells. The results suggest that AQP9 plays an important role in arsenic uptake and that AQP9 phosphorylation is partially regulated by p38 MAPK.

2. Methods

2.1. Cell lines and cell culture

The human liver hepatocellular carcinoma cell line HepG2 was obtained from the cell bank of the Chinese Academy of Sciences in Shanghai, China. Arsenic-resistant HepG2 (AsHepG2) cells were generated by treating the HepG2 cells with a low concentration of arsenic [33]. Both HepG2 and AsHepG2 cells were

cultured in RPMI-1640 medium (Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) and maintained at 37°C in a humidified incubator with 5% CO₂.

2.2. Assay of cell viability

Cell viability was measured with Cell Counting Kit-8 (CCK8, Beyotime, Haimeng, China). Briefly, cells seeded on a 96-well plate at a concentration of 10⁴ cells/mL were treated with various concentrations of sodium arsenite (NaAsO₂) for 24 h. The CCK8 reagent was then added and incubation was continued for 1 h. Absorbance was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.3. Measurement of arsenic accumulation

Cells treated with various concentrations of NaAsO₂ for different times were washed three times with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) and harvested in 0.5 mL nitrosonitric acid (98%). Arsenic accumulation was measured using an inductively coupled plasma mass spectrometer (ICP-MS, HP4500; Yokogawa Analytical Systems, Japan).

2.4. Plasmids and transfections

The *AQP9* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR). The PCR product was purified and cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Shanghai, China), thereby generating the pcDNA3.1-AQP9 plasmid.

AsHepG2 cells were transfected with pcDNA3.1-AQP9 and pcDNA3.1 (as a control) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions.

2.5. Quantitative real-time quantitative PCR (Q-PCR)

Total RNA was extracted from NaAsO₂-treated cells using the one-step Trizol-chloroform method, and the first strand cDNA was synthesized using a PrimeScript® RT reagent Kit (Takara, Osaka, Japan). The following primers were used for the Q-PCR reaction: *AQP9* forward 5'-ACT CAG TGT CAT CAT GTA GTG G-3' and reverse 5'-CAC CTC AGG CTT ACA AGA ACA-3'; β -actin forward 5'-TGG CAC CCA GCA ATG AA-3' and reverse 5'-CTA AGT CAT AGT CCA CCT AGA AGC A-3'. PCR was performed on a GeneAmp PCR® system 7300 (Applied Biosystems, Carlsbad, CA, USA) using a SYBR® *Premix Ex Taq* kit (Takara). Expression levels of AQP9 were normalized to β -actin. Measurement of each sample was independently repeated three times, the relative mRNA expression was quantified using the comparative Ct (Δ Ct) method, and data was expressed as $2^{-\Delta\Delta C_t}$, where Ct values were analyzed according to a previously reported method [34].

2.6. Western blot analysis

Cells treated with or without NaAsO₂ were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% [w/v] SDS, 0.5% [w/v] deoxycholate, and 1% [v/v] protease inhibitor cocktail). 20 μ g of protein extract was separated by SDS-PAGE and transferred to a

polyvinylidene fluoride membrane with a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked with 5% bovine serum albumin (BSA; Invitrogen) and then incubated sequentially with mouse monoclonal antibodies to AQP9 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p38 (1:500, Abcam, London, UK), or a rabbit polyclonal antibody to phospho-p38 MAPK (Thr180/Tyr182; 1:500, Abcam). Western blot for β -actin (1:500, Santa Cruz Biotechnology) served as a loading control. Membranes were then incubated with either a secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2000, Santa Cruz Biotechnology). The signals were visualized with an enhanced chemiluminescence kit (Beyotime) using a gel imaging system (Bio-Rad). Quantitative analysis was performed with the digital Kodak Gel Logic 200 (Carestream Molecular Imaging, Woodbridge, CT, USA).

2.7. Immunoprecipitation

Cells were lysed in RIPA buffer. The cellular lysates were pre-cleared with protein A + G agarose beads for 1 h and then immunoprecipitated overnight with anti-AQP9 antibody at 4°C on a rotator followed by incubation with protein A + G agarose beads for 1 h at 4°C. The pellets were collected by centrifugation and washed three times with PBS buffer. The precipitates were analyzed by SDS-PAGE and Western blot as described above. Phosphorylated (phospho)-AQP9 was detected using a rabbit polyclonal antibody against phosphoserine (1:500, Abcam).

2.8. p38 inhibition assay

To evaluate the effects of p38 activation on the level of AQP9 phosphorylation, cells were treated with or without the p38 inhibitor SB203580 (18 μ M; Beyotime) for 30 min and then treated with 12 μ M NaAsO₂ for 2 h. Cell lysates were extracted using RIPA buffer and then analyzed by SDS-PAGE and Western blot as described above.

2.9. Statistical analyses

Data were expressed as the mean $\pm$ SE. One-way analysis of variance (ANOVA) was used with Tukey's multiple comparison test to compare differences in means among treatment groups. SPSS 16.0 software (SPSS, Chicago, IL, USA) was used for the statistical analysis. Differences with probability (*P*)-values < 0.05 were considered significant.

3. Results

3.1. AsHepG2 cells accumulate less arsenic than HepG2 cells

The effect of arsenic treatment on the viability of HepG2 cells and their arsenic-resistant (AsHepG2) counterparts was measured in terms of the half-maximal inhibitory concentration of NaAsO₂ (IC₅₀). As shown in Fig. 1A, the IC₅₀ of NaAsO₂ for AsHepG2 cells (15.59 μ M) was significantly higher than that for HepG2 cells (7.33 μ M) (*P* < 0.05).

The rates of arsenic accumulation in the two cell lines were then determined by ICP-MS. After treatment with 12 μM NaAsO_2 , arsenic accumulation in AsHepG2 cells was significantly lower than that in HepG2 cells ($P < 0.01$; Fig. 1B), suggesting that a lower intracellular accumulation of arsenic may be responsible for the arsenic tolerance of AsHepG2 cells.

3.2. Overexpression of AQP9 protein increases intracellular arsenic accumulation

AQP9 overexpression was shown to increase uptake and toxicity of arsenic in lung cancer and leukemia cells [21; 22]. Similarly, overexpression of AQP9 in both AsHepG2 and HepG2 cells by transfection with an AQP9 construct (pcDNA3.1-AQP9) resulted in higher intracellular arsenic levels as compared with cells transfected with a control vector (Fig. 2A, B).

Furthermore, AsHepG2 and HepG2 cells showed a different time-dependent pattern of accumulation. In AQP9 overexpressed AsHepG2 cells, a linear increase of intracellular arsenic levels was observed only during the first 10 hrs of arsenic treatment, and the rate of accumulation significantly showed down during 10–20 hrs after the treatment (Fig. 2A). In contrast, intracellular levels of arsenic gradually increased throughout the course of the experiment in AQP9 overexpressed HepG2 cells (Fig. 2B). These results suggest that arsenic tolerance in AsHepG2 cells may be due to reduced arsenic uptake.

3.3. *NaAsO₂* downregulates AQP9 mRNA levels in AsHepG2 cells

AQP9 is one of the main channels involved in arsenic uptake in mammalian cells [14; 19]. Because overexpression of AQP9 resulted in increased arsenic accumulation, we examined whether exogenous arsenic treatment affects expression of the endogenous *AQP9* gene. We used Q-PCR to compare *AQP9* mRNA levels in AsHepG2 and HepG2 cells treated with NaAsO_2 . Results showed that NaAsO_2 treatment significantly decreased AQP9 mRNA levels in a dose- and time-dependent manner in AsHepG2 cells ($P < 0.05$; Fig. 3A, 3B). Whereas in HepG2 cells, AQP9 mRNA levels were not affected by arsenic treatment (Fig. 3A, 3B). These results indicated that under conditions of acute toxicity, reduced endogenous *AQP9* mRNA levels may attribute to the tolerance in arsenic-resistance cells.

3.4. *NaAsO₂*AQP9 phosphorylation levels in arsenic-treated cells

To further explore the relationship between AQP9 and arsenic tolerance, we examined the effect of NaAsO_2 treatment on AQP9 protein levels and the phosphorylation state of the protein in both cell lines. NaAsO_2 treatment significantly decreased AQP9 protein levels in a concentration-dependent manner in AsHepG2 cells, While the protein levels were increased by NaAsO_2 treatment in HepG2 cells (Fig. 4A, 4B), which was almost consistent with the changes of AQP9 mRNA expression.

Phosphorylation levels of AQP9 did not significantly affected by NaAsO₂ treatment in AsHepG2 cells; however, in HepG2, NaAsO₂ treatment significantly increased AQP9 phosphorylation(Fig. 4A, 4B). These results suggested that phosphorylation of AQP9 protein may contribute to cellular arsenic tolerance in mammalian cells.

3.5. p38 may be partially involved in phosphorylation of AQP9

It has been reported that in yeast cells, phosphorylation of the AQP9 homolog Fps1p is regulated by the p38 homolog Hog1p, which results in the decreased cellular uptake of arsenic [13]. Therefore, the levels of p38 protein and its phosphorylation status were analyzed in both cell lines. As shown in Fig. 4A, p38 protein levels did not significantly change after NaAsO₂ treatment in HepG2 cells, while in AsHepG2 cells p38 protein levels were significantly increased by the treatment. However, phosphorylated p38 were slightly increased in dose- and time dependent manner by NaAsO₂ treatment in both cells (Fig. 4A and 4B), suggesting that p38 may partially regulate phosphorylation of AQP9 in mammalian cells.

The role of p38 in the regulation of AQP9 phosphorylation was further investigated by use of SB203580, a selective inhibitor of p38 MAPK. Treatment with SB203580 and NaAsO₂ resulted in only slight decreases in AQP9 protein levels in both cells, but AQP9 phosphorylation levels were not affected (Fig. 5), indicating that p38 may only partially regulate AQP9 phosphorylation.

4. Discussion

In the present study, we showed AsHepG2 cells had a higher survival rate and a lower arsenic accumulation level than HepG2 cells after arsenic treatment, which agrees with previous observations that arsenic uptake in arsenic-resistant R15 cells was significantly lower than in the parent CL3 cells generated from a human lung-derived adenocarcinoma cell line [35]. Previous studies have also shown that the accumulation of arsenic in AsRE cells (arsenic-resistant cells from a human umbilical vein endothelial cells ECV304) was lower than that in ECV-304 cells [25]. These findings suggest that reduced arsenic accumulation through restriction of arsenic uptake is one of the mechanisms that contribute to arsenic tolerance in mammalian cells.

AQP9 is a water-glycerol channel that allows extracellular arsenic to enter into cells, thereby leading to intracellular arsenic accumulation and cytotoxicity. As shown in Fig. 2, overexpression of AQP9 increased arsenic accumulation in both cell lines. However, arsenic accumulation in the AsHepG2 cells slowly increased to a plateau after 10–20 h of treatment, whereas such a plateau was not observed in the HepG2 cells, further suggesting that AQP9 is one of the primary facilitators of arsenic uptake and that arsenic tolerance may be due to reduced rates of such uptake.

Several studies have shown that *AQP9* mRNA levels are correlated with cellular sensitivity to arsenic. For example, the acute promyelocytic leukemia cell line NB4 expresses the highest levels of AQP9 and is the

most sensitive to As_2O_3 , with the lowest IC_{50} value for arsenic among leukemia cell lines. In contrast, the chronic myeloid leukemia cell line K562 has very low levels of endogenous AQP9 expression and has the least sensitivity to As_2O_3 among a variety of leukemia cell lines [21]. In addition, overexpression of AQP9 in K562 cells dramatically sensitizes the cells to As_2O_3 [8]. Moreover, this sensitivity was associated with high levels of arsenic uptake [36]. Recently, it has been found that expression level and sensitivity of primary culture chorion cells to arsenic was higher than amnion cells [37]. In this study, we found that AsHepG2 cells, which have significantly lower AQP9 mRNA levels, had a higher tolerance to arsenic (Fig. 1). HepG2 cells were more sensitive to arsenic, and the AQP9 mRNA levels of HepG2 cells were slightly but significantly increased by NaAsO_2 treatment (Fig. 4). The changes of AQP9 protein level in both cells at different concentration and time points after NaAsO_2 treated were consistent with the changes of AQP9 mRNA levels. Overall, AsHepG2 cells seem to have a saturation threshold for arsenic accumulation; once arsenic accumulation reaches such the threshold, the cells can reduce the uptake by down-regulation of AQP9 expression.

In the present study, phosphorylation of AQP9 in AsHepG2 cells was maintained at high levels after arsenic treatment (Fig. 4). Nevertheless, the phosphorylation level of AQP9 in HepG2 cells was increased in the two situations. These changes in the phosphorylation state of AQP9 may lead to an increased ability to inhibit arsenic uptake. We have previously found that AQP9 expression and phosphorylation levels may be related to regulation of arsenic influx [24, 25]. However, other mechanisms underlying the differential arsenic uptake between these cell lines cannot be ruled out at this time.

Little is known about how AQP9 expression is regulated. In 2003, it was reported that p38 signaling plays a role in regulating AQP protein expression [27]. The most direct correlation between MAPK activation and aquaglyceroporin-regulated arsenic transport was shown in *S. cerevisiae* [2]. In the study, the levels of p38 protein were increased with the concentration and time except those in HepG2 at different concentration of arsenic. The phosphorylation levels of p38 increased significantly with the time and concentration of the treatment, while maintained a same levels in AsHepG2 over different concentrations. These results suggest that increased levels of p38 phosphorylation may, at least in part, increase the phosphorylation level of AQP9. Furthermore, when p38 was inhibited by SB203580, phosphorylation levels of AQP9 exhibited a slight increase in AsHepG2 cells (Fig. 5), further suggesting that p38 can only partially affect AQP9 phosphorylation.

Taking all the results of this study into consideration, we propose that in addition to p38, other kinases or proteins contribute to the regulation of AQP9 phosphorylation. This conclusion is consistent with previous findings demonstrating that the p38 homolog, Hog1p, is not the only kinase that phosphorylates the AQP9 homolog in yeast [2]. Identification of the kinase(s) that phosphorylate AQP9 will be our next challenge, which will allow us to explore the mechanisms that regulate AQP9 phosphorylation and arsenic tolerance in mammalian cells.

5. Conclusion

In this study, the expression of AQP9 and roles of p38 involved in the MAPK pathway was compared between arsenic-sensitive HepG2 cells and arsenic-resistant HepG2 (AsHepG2) cells under arsenic-treatment. AsHepG2 cells had a higher survival rate and a lower arsenic accumulation level than HepG2 cells after arsenic treatment. Overexpression of AQP9 increased intracellular arsenic accumulation in both cells, extremely AsHepG2 cells significantly increased within 10 h of treatment. Both the expression and phosphorylation of AQP9 are closely related to arsenic uptake and may regulate cellular arsenic tolerance by reducing its uptake rate. p38 may only play a limited role in the regulation of AQP9 phosphorylation in AsHepG2 cells. The results provide signaling mechanisms involved in arsenic tolerance or detoxification.

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

No conflicts of interest to disclose.

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Authors` contributions

RC, YFC, FZL designed the experiment, presided over the expert seminar and analyzed the data. RC, QQW, XZW performed functional analysis and WB analysis. RC was the first writing author of the manuscript. YH, XRX, JY participate in literature review, consensus discussion, discussion and suggestions, recommendations. All authors read and approved the final manuscript.

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Figures

Figure 1

Cell viability and arsenic accumulation in AsHepG2 and HepG2 cells after NaAsO₂ treatment. (A) Cells grown in 96-well plate were treated with different concentrations of NaAsO₂ for 4, 8, 16, 32 or 64h and CCK8 assay was performed. Data presented are the mean $\pm$ SE (n=5). (B) Cells were treated with 12 μ mol/l NaAsO₂ for 2 h and then arsenic accumulation was determined by ICP-MS. Data presented are the mean $\pm$ SE (n=3).

Figure 2

Effect of overexpression of AQP9 on As accumulation in AsHepG2 and HepG2 cells. (A) AQP9- or empty vector-transfected AsHepG2 cells were exposed to NaAsO₂ (12 μ mol/l) for 2, 4, 6, 8, 10 and 20 h; (B) AQP9- or empty vector-transfected HepG2 cells were exposed to NaAsO₂ (12 μ mol/l) for 2, 4, 6, 8, 10 and 20 h, arsenic accumulation was determined by ICP-MS. Data presented are the mean $\pm$ SE (n=3).

Figure 3

Effect of NaAsO₂ on AQP9 mRNA levels in AsHepG2 and HepG2 cells. (A) Cells were treated with 2, 4, 8 or 16 μmol/l NaAsO₂ for 2h; (B) Cells were treated with 12 μmol/l NaAsO₂ for 2, 4, 8h. Total RNA was isolated by Trizol and real-time quantitative RT-PCR was performed. Data presented are the mean ± SE (n=3). Single asterisks above the columns represent significant difference at p<0.05 level.

A

B

Figure 4

Effect of NaAsO₂ on protein and phosphorylation levels of AQP9 and p38 in AsRE and ECV-304 cells. (A) Cells were treated with 2, 4, 8 or 16 μmol/l NaAsO₂ for 2 h and Western blotting was performed with 20 μg of cell lysates. The bar graph below the western blot represents the densitometry measurement of the AQP9 (A1), p-AQP9 (A2), p38 (A3) and p-p38 (A4) to β-actin ratio. Data presented are the mean ± SE (n=3). Single and double asterisks above the columns represent significant difference at p<0.05 and p<0.01 levels, respectively. (B) Cells were treated with 12 μmol/l NaAsO₂ for 2, 4 and 8 h. The graphs below the western blot represents the densitometry measurement of the AQP9(B1), p-AQP9 (B2), p38 (B3) and p-p38 (B4) to β-actin ratio. Data presented are the mean ± SE (n=3). Single and double asterisks above the columns represent significant difference at p<0.05 and p<0.01 levels, respectively.

A

B

Figure 5

Effect of NaAsO₂ and p38 inhibitor (SB203580) on protein and phosphorylation levels of AQP9 and p38 in AsHepG2 and HepG2 cells. (A) Cells were treated with 12 μ mol/l NaAsO₂ for 2 h after incubation with or without SB203580 (18. μ mol/l) for 30 min and Western blotting was performed using 20 μ g of cell lysates. Protein and phosphorylation levels of AQP9 and p38 were detected with anti-AQP9, anti-p38 and anti-p-p38 antibody, respectively. Phosphorylated AQP9 (p-p38) was determined by immunoprecipitation using anti-phosphoserine antibody. β -actin was detected with anti- β -actin antibody as an internal control. (B) Densitometry measurement of the phosphorylated AQP9 to AQP9 ratio. Data presented are the mean $\pm$ SE (n=3).