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Role of AQP9 in transport of monomethyselenic acid and selenite

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Abstract

AQP9 is an aquaglyceroporin with a very broad substrate spectrum. In addition to its orthodox nutrient substrates, AQP9 also transports multiple neutral and ionic arsenic species including arsenic trioxide, monomethylarsenous acid (MAs^{III}) and dimethylarsenic acid (DMA^{V}). Here we discovered a new group of AQP9 substrates which include two clinical relevant selenium species. We showed that AQP9 efficiently transports monomethylselenic acid (MSeA) with a preference for acidic pH, which has been demonstrated in *X. laevis* oocyte following the overexpression of human AQP9. Specific inhibitors that dissipate transmembrane proton potential or change the transmembrane pH gradient, such as FCCP, valinomycin, and nigericin did not significantly inhibit MSeA uptake, suggesting MSeA transport is not proton coupled. AQP9 was also found to transport ionic selenite and lactate, with much less efficiency compared with MSeA transport. Selenite and lactate uptake via AQP9 is pH gradient dependent and inhibited by FCCP and nigericin, but not valinomycin. The selenite and lactate uptake via AQP9 can be inhibited by different lactate analogs, indicating that their translocation share similar mechanisms. AQP9 transport of MSeA, selenite and lactate is all inhibited by a previously identified AQP9 inhibitor, phloretin, and the AQP9 substrate As^{III}. These newly identified AQP9 selenium substrates imply that AQP9 could play a significant role in MSeA uptake and possibly selenite uptake involved with cancer therapy under specific microenvironments.

Keywords

AQP9; liver; water; glycerol; arsenic trioxide (As^{III}); lactate; selenite; monomethyselenic acid (MSeA)

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Conflict of interest

There is no conflict of interest involved in this paper.

Introduction

Selenium is an essential micro-nutrient for all mammals. Dietary selenium can be metabolized into different organic species and is dominantly present as a methylated species. Selenite (Se^{IV}) and selenate (Se^{VI}) are two common inorganic selenium species, and selenite has been approved by the USDA as a food additive for farm animals. Organic dietary selenium includes selenocysteine and selenomethione, in which elemental selenium replaces sulfur in the corresponding amino acids cysteine and methionine. All of the selenium containing amino acids can be metabolized intracellularly for the synthesis of essential selenoproteins.

Inorganic selenite has been applied in many clinically relevant studies involving cell culture and animals, mainly for cancer prevention and treatment (Brodin et al. 2015; Ganther 1999; Jackson and Combs 2008). Selenite regulates a wide range of downstream cell signals. For example, selenite affects the phosphoinositide 3-kinase(PI3K)- serine-threonine kinase Akt pathway and three major mammalian mitogen-activated protein kinase(MAPK) pathways: exctracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase(JNK), and p38(Jiang et al. 2002; Zou et al. 2008). Membrane transporters that facilitate the cellular permeation of selenite have been recently reported. In yeast, the major selenite transporter is characterized as the lactate transporter, Jen1p(McDermott et al. 2010b). It is predicted that selenite resembles the structure of lactate and is recognized by Jen1p by molecular mimicry. However, functional homologues of Jen1p, mammalian monocarboxylate transporters (MCT), have no detected selenite transport activity (unpublished data, our lab). In 2016, the first direct transporter for selenite permeation was identified as ZIP8, which is a member of the Zinc import family (ZIP)(McDermott et al. 2016). ZIP8 transports selenite in a zinc and bicarbonate dependent manner. Since ZIP8 is found upregulated during inflammation, its role is predicted to recruit circular anti-inflammatory selenite and zinc to combat inflammation. This is consistent with the observation of selenite application in inflammatory diseases such as severe sepsis(Yang et al. 2016). While ZIP8 may play a critical role to facilitate selenite transport into inflammatory tissues, here we studied selenite uptake via AQP9. Our results showed AQP9 transports selenite under acidic conditions, but not under physiological pH, which indicates that AQP9 may serve as a secondary transporter under specific conditions, such as in an acidic microenvironment. The AQP9 mediated transportation of lactate has been determined to be comparable with that of selenite uptake, and results showed their transportation shares a similar translocation mechanism as we observed in yeast.

Monomethylselilinic acid (MSeA) is an intracellular metabolite derived from inorganic selenium methylation. Recently, MSeA was found to exhibit promising effects in the prevention and treatment for multiple cancer types, such as pancreatic cancer (Wang et al. 2014), lung cancer (Swede et al. 2003), breast cancer (Qi et al. 2012a), prostate cancer (Jiang et al. 2002), ovarian cancer (Swede et al. 2003; Zhang and Azrak 2009), and primary effusion lymphoma (PEL) (Wang et al. 2016), when MSeA was used at lower micro-molar range. In addition, MSeA was used as an adjuvant to synergistically enhance growth-inhibitory effect of the chemotherapeutic drugs doxorubicin and paclitaxel in breast cancer

cells (Hu et al. 2008; Li et al. 2007). At *in vivo* levels, MSeA showed a dose-dependent restriction of xenograft tumor growth (Li et al. 2008; Wu et al. 2012).

Mechanisms of MSeA function includes inhibition of specific cell signaling pathways, some growth factors or extracellular matrix proteins, as well as inducing G1 arrest, DNA fragmentation, and caspase-mediated apoptosis. For example, treatment of primary effusion lymphoma (PEL) with MSeA was found induce an anti-proliferative effect by causing endoplasmic reticulum (ER) stress and subsequent apoptosis (Shigemi et al. 2017). MSeA induces apoptosis and G1 cell cycle arrest by perturbing PI3K through Akt kinase and forkhead box O protein (FOXO) dephosphorylation (Tarrado-Castellarnau et al. 2015). In human umbilical vein endothelial cells (HUVECs), MMP2 and VEGF expression was decreased upon short-term exposure to MSeA (Jiang et al. 2000). MSeA has a higher reactivity and displays superior efficacy against human cancer than other selenium species such as selenite. It is discovered that MSeA is readily metabolized to methylselenol, a bioactive selenium metabolite for cancer chemoprevention(Ip et al. 2000; Li et al. 2008).

However, despite its high toxicity for cells and therapeutic effects, mechanisms of MSeA permeation into cell membranes have not been studied. Given the higher toxicity and efficient cellular effect, one or more transporters for MSeA is predicted to universally exist. Here for the first time, we report that AQP9 transports MSeA effectively in a wide pH range and suggest it may serve as a major transporter for MSeA cell permeation. We demonstrated that the uptake is in favor of anacidic pH. Inhibitory studies have supported a hypothesis that MSeA transport does not require a transmembrane proton gradient. Since membrane permeation of MSeA is the rate limiting step for intracellular concentration and determines its potency, identification of a MSeA transporter can aid future studies of MSeA pharmacokinetics. In addition, the selective toxicity of MSeA for cancer cells implies that the expression of an AQP9 membrane transporter may play a role in the outcome of MSeA treatment.

Material and Methods

Expression of AQP9 in Xenopus oocytes

The human AQP9 were cloned into pXβG-ev1, as described previously (Liu et al. 2004; Qi et al. 2012b). Capped cRNAs were synthesized in an *in vitro* reaction using mMessage mMachine T3 ultra kit (Applied Biosystem) with pXβG-ev1 plasmids linearized with *NotI* (Liu et al. 2006a). Oocytes from *Xenopus laevis* were defolliculated and injected with 25 ng of cRNA or with 50 nl of water. They were then incubated in complete ND96 buffer for 3 days at 16 °C and used for uptake assays.

Transport Assays of MSeA and selenite

For the assay of selenite and MSeA accumulation in AQP9 expressed oocytes, oocytes with either AQP9 cRNA or water injected were incubated in 1 mM of sodium selenite (Sigma), 100 μM monomethylselilinic acid (Sigma), respectively, at room temperature for 60 min or indicated time. When necessary, oocytes were pretreated by 20 μM carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP, Sigma), 10 μM phloretin (Sigma), 100 μM

valinomycin (Sigma) or 100 μM nigericin (Sigma) for 30 min. When organic acid competitors, including formate, acetate, pyruvate, benzoate and succinate, were used, oocytes were pretreated with these substrates at 1mM of each for 5 minutes prior to adding the tested selenium substrates. Sodium arsenite (As^{III}) is added at final concentration of 1mM (Sigma) to study the inhibitory effect. All inhibitory experiments were performed under pH 5.5. The oocytes were then collected and washed in ND96 buffer three times. Oocytes were completely digested using 70% (vol/vol) HNO_3 for at least 2 hrs. The samples were then diluted with HPLC grade water for selenium quantification.

Transport Assays of lactate

For assay of lactate accumulation in oocytes, oocytes were incubated in 1 mM of sodium lactate mixed with appropriate ^{14}C labeled lactate at room temperature for 60 min. When necessary, oocytes were pretreated by FCCP, phloretin, valinomycin, or nigericin for 30 min before transport assay, as described above. When organic acid competitors, including formate, acetate, pyruvate, benzoate and succinate, were used, oocytes were pretreated with these substrates at 1mM concentration for 5 minutes at indicated concentrations prior to adding the tested substrates, as described above. All inhibitory experiments were performed under pH 5.5. After transport, the oocytes were collected and washed in ND96 buffer three times. Oocytes were completely dissolved in 10% SDS and cocktail was added. Radioactivity (CPM) is determined by a scintillation counter.

Selenium quantification

Total elemental selenium in each sample was determined by inductively coupled plasma mass spectroscopy (ICP-MS) (Nexion 300, PerkinElmer, Norwalk, CT).

Statistical Analysis

All experiments contain at least two batches of oocytes from two animals; at least 3 replicates are used each time. One batch of experiments is used to present in this paper. Quantitative results are shown as means ± standard deviations. The statistical analysis was performed by Student's t test for paired data between control and treated groups. P values <0.05 were considered significant.

Results

MSeA transport by AQP9 in Xenopus oocytes

The ability of human AQP9 to conduct MSeA in oocytes was examined in a time course. The transport is time-dependent, which does not saturate within 60 minutes (Fig. 1A). AQP9 transport of MSeA was examined under different pH conditions (pH 4.5, 5.5, 6.5 and 7.5, respectively) (Fig. 1B). Results showed AQP9 has a high efficiency to facilitate MSeA with a preference of lower pH, but significant MSeA uptake is still observed at physiological pH. In order to know whether other organic acids can affect AQP9-mediated MSeA transport, we performed the transport assay in the presence of different organic acid substrates. Our results showed that these organic acids have no significant inhibition for MSeA uptake. However, the inorganic As^{III}, one well characterized substrate for AQP9, can effectively inhibit MSeA transport (Fig. 1C). In order to investigate whether AQP9 mediated transport depends on

transmembrane proton potential or pH gradient. Four different inhibitors including FCCP, valinomycin, pholoretin, and nigericin were applied in the transport assay., As is shown in Fig. 1D, these inhibitors has no effect on MSeA uptake via AQP9, indicating transport is independent on transmembrane potential and pH gradient. However, the AQP9 specific inhibitor phloretin exhibits substantial inhibition of MSeA uptake (Fig. 1D).

Lactate transport by AQP9 in Xenopus oocytes

Human AQP9 transport of lactate in oocytes was investigated by transport assay. AQP9 transportation of lactate is time dependent and stays linear within 60 minutes, indicating human AQP9 facilitates the uptake of lactate in a time-dependent manner. (Fig. 2A). The ability of human AQP9 to increase lactate permeability at different pH levels from 4.5 to 7.5 was examined in oocytes. The results show that lactate exhibits much lower transport at lower pH, indicating the neutral forms of these compounds are substrates for AQP9 (Fig. 2B). Moreover, lactate analogs including the weak organic acids formate, acetate, pyruvate, benzoate, and succinate showed universal inhibition of lactate uptake, indicating they are competing with lactate in membrane transport via AQP9 (Fig. 2B). In addition, consistent with the result As^{III} blocks MseA uptake, As^{III} can effectively inhibit lactate permeation as well (Fig. 1C). In contrast, the inhibitors FCCP and nigericin showed effective inhibition of lactate permeation, which supports that AQP9 transports lactate requires a transmembrane proton and pH gradient. Phloretin expectedly exhibits substantial inhibition of lactate uptake (Fig. 2D).

Selenite transport by AQP9 in Xenopus oocytes

AQP9 transport of inorganic selenite has been shown to have a much lower efficiency compared with MSeA (approximately 1000-times less). The selenite transport resembles the pattern of lactate uptake, demonstrating that selenite and lactate are transported similarly via AQP9. Selenite transport is linear within 60 minutes (Fig. 3A). The selenite transport is in favor of a lower pH, indicating the neutral form selenite is the transported species. There is no visible selenite uptake at physiological pH (pH 7.5) (Fig 3B). Organic acids including formate, acetate, pyruvate, benzoate, and succinate showed inhibition of selenite uptake similar to lactate. As^{III} also inhibits selenite permeation (Fig. 3C). Inhibitors of FCCP and nigericin showed effective inhibition of selenite permeation, which supports that selenite transport also requires a transmembrane proton and pH gradient. AQP9 inhibition by phloretin effectively inhibits MSeA, lactate, and selenite uptake in a similar pattern (Fig. 3D).

Discussion

Inorganic selenite (Se) and organic methylseleninic acid (MSeA) have clinical importance in cancer prevention and treatment. They share different valence, structures, and charges and therefore have distinct mechanisms in inducing cell responses and regulating downstream targets once entering cells.

MSeA transported by AQP9 can be reasonably explained. MSeA was reported to have a pKa of 8.5, which means it exists mostly as a non-charged neutral molecule at physiological pH.

Our results showed that AQP9 serves as an effective transporter to permeate MSeA in a pH range of 4.5–7.5, with a preference for acidic pH. The fully protonated MSeA species is predicted as the substrate for translocation, similarly to that of AQP9 transport of inorganic arsenite (As^{III}), which is also a neutral form at physiological pH. The As^{III} substrate has significant inhibition of MSeA uptake (Fig. 1C), which supports this prediction. This hypothesis is further supported by the observation that the inhibitors of the transmembrane electron potential and proton gradient, including FCCP, valinomycin, and nigericin, do not inhibit MSeA uptake. The result of the pH preference of MSeA transportation could explain the selective toxicity of MSeA to cancer tissue. Cancer tissues have been known to have a lower pH and therefore they can take up more MSeA than normal tissues. It is a plausible prediction that expression of AQP9 controls the MSeA availability and determines MSeA toxicity in cancers, which requires more studies. AQP9 expression was detected in multiple tumor cells. For example, in human hepatocellular carcinoma, AQP9 expression was decreased compared with non-tumourigenic liver tissue (Jablonski et al. 2007). AQP9 levels in human astrocytic tumors were positively related to physiological grade(Tan et al. 2008). In addition, microarray analysis found that AQP9 mRNA was lower in the adjuvant chemotherapy nonresponse colorectal cancer patients (Dou et al. 2013). Therefore, manipulation of AQP9 expression, as well as AQP9-mediated drug sensitivity would be a promising anti-cancer therapy. This strategy has been investigated in leukemia treatment by using As^{III}. Overexpression of human AQP9 in K562 leukemia cells was applied to increase As^{III} sensitivity (Bhattacharjee et al. 2004).

Lactate is a weak acid with a pKa of 3.85 and exists as a monovalent anion under physiological pH. Lactate permeation by AQP9 has been previously reported (Tsukaguchi et al. 1998; Tsukaguchi et al. 1999a). Mechanisms of AQP9 anion transport have been discussed, and AQP9 is proposed to act as a channel for the protonated lactic acid form (Rambow et al. 2014). Since humans have a large family of monocarboxylate transporters (MCTs) expressed in all tissues(Halestrap 2013), the physiological role of AQP9 in lactate transport is questionable.

In our study, we compared AQP9 transportation of lactate and selenite since selenite is an analog of lactate and transported by the lactate transporter Jen1p in yeast. Our results showed that selenite is a weak substrate for AQP9 with a preference of acidic pH, with a low efficiency comparable to lactate uptake. In addition, the transport is not observed at physiologic pH, it is therefore predicted that AQP9 selenite transport does not represent a major pathway under normal physiological conditions. However, at some acidic microenvironments, such as the stomach or cancerous environments, selenite could have an improved permeation rate by AQP9. Under most situations, the direct selenite transport is believed to be mediated by ZIP8, a ZIP family member which has been identified to transport selenite under neutral conditions with cofactors Zn and bicarbonate. Roles of these two different selenite transporter systems under different conditions are illustrated in Fig. 4.

Results from inhibitory experiments by different lactate analogs showed that they inhibit both lactate and selenite permeation with a similar pattern. Organic acids including formate, acetate, pyruvate, benzoate, and succinate can compete with lactate and selenite transport via AQP9, indicating these organic acids are likely to be substrates of AQP9 as well. Lactate and

selenite inhibit each other reciprocally, which supports the hypothesis that they share similar transport mechanisms and compete with each other for AQP9 transport. On the contrary, these organic acids had no effect on MSeA uptake via AQP9, indicating AQP9 transports MSeA in a different mechanism. One well characterized AQP9 substrate, $As^{III,}$ can inhibit both selenium species and lactate permeation. Likely transportation of these substrates share partial similarity in substrate translocation.

Both lactate and selenite were transported under lower pH conditions. The lower pH would alter the transmembrane gradient and membrane potential. Therefore, the dissipation of these gradients by the inhibitor FCCP significantly decreases lactate and selenite transport via AQP9. Nigericin, which disrupts pH concentrations across membranes, also blocks AQP9-mediated lactate and selenite transport, indicating the transmembrane ΔpH is a critical factor for effective transport. Phloretin, another inhibitor of AQP9 that inhibits both water and glycerol permeation (Ishibashi and Sasaki 1998; Tsukaguchi et al. 1999b), completely inhibited transport of MSeA (Fig. 1D). Instead, valinomycinhas no effect on lactate, selenite, and MSeA uptake, which indicates the electron potential is not involved in driving substrate movement. These results suggest selenite and lactate share the same translocation pathway, while MSeA, a neutral molecule, may transport similarly to that of As^{III} and MAs^{III}.

MSeA was transported at much higher rate than that of selenite. Considering different concentration levels used in the assays (100μM versus 1mM), the uptake of MSeA is approximately 1000 times higher than that of selenite at pH 4.5–6.5. Consistent with this, MSeA is also a more toxic selenium form for liver cells, while liver cells are more resistant to selenite (Li et al. 2013; Zhao et al. 2004). The selective uptake for methylated species was also observed in arsenic substrates, which monomethylated arsenic has 5-time higher efficiency than the inorganic arsenite with same valence(Liu et al. 2006b). This shows that molecular polarity and hydrophobicity are critical factors involved in AQP9 permeation.

AQP9 transport many neutral nutrient molecules and multiple arsenic species(McDermott et al. 2010a). The current new findings additionally address the multiple roles of AQP9 in the uptake of therapeutic selenium compounds, particularly MSeA. The dependence of AQP9 in MSeA transport renders AQP9 as an important consideration for future MSeA application. More work in cultured cell lines and experimental animals with altered AQP9 expression are required to investigate the physiological and pharmacological relevance of this transport.

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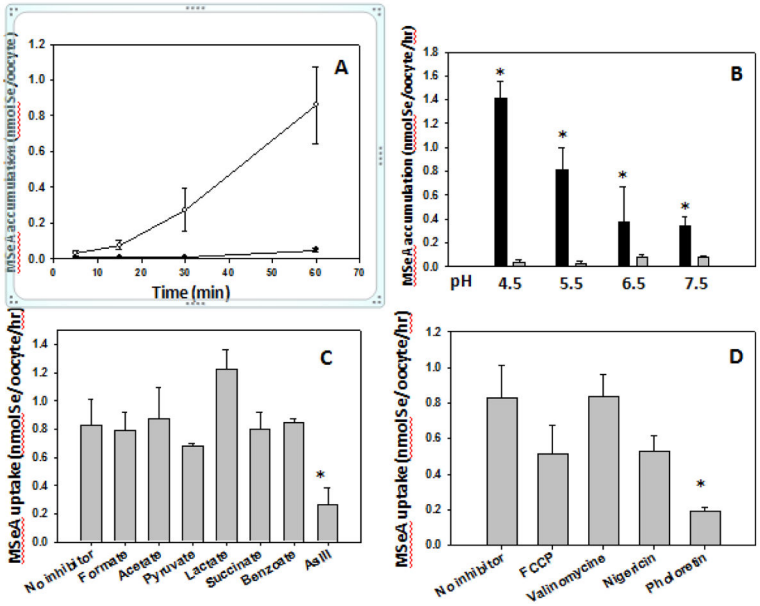

Fig. 1. Monomethyselenic acid (MSeA) transport by AQP9

Transport of MSeA by human AQP9 was examined under different pH conditions. **A:** Time course of MSeA uptake via AQP9. MSeA was added in transport buffer at a final concentration of 100μM. **B.** Transport of MSeA under different pH. MSeA was added in transport buffer at a final concentration of 100μM for 60 minutes. **C:** Inhibition of MSA transport by different organic acids and AQP9 substrate As^{III} Transport assay was performed under pH 5.5 for 60 minutes. **D**. Effect of FCCP, valinomycin and nigericin on MSeA transport by hAQP9. Oocytes were pretreated with FCCP (20 μM), valinomycin (100 μM), or nigericin (10 μM) for 60 min at pH 5.5. The open bars represent arsenic uptake via AQP9, and the solid bars are the water injected controls.

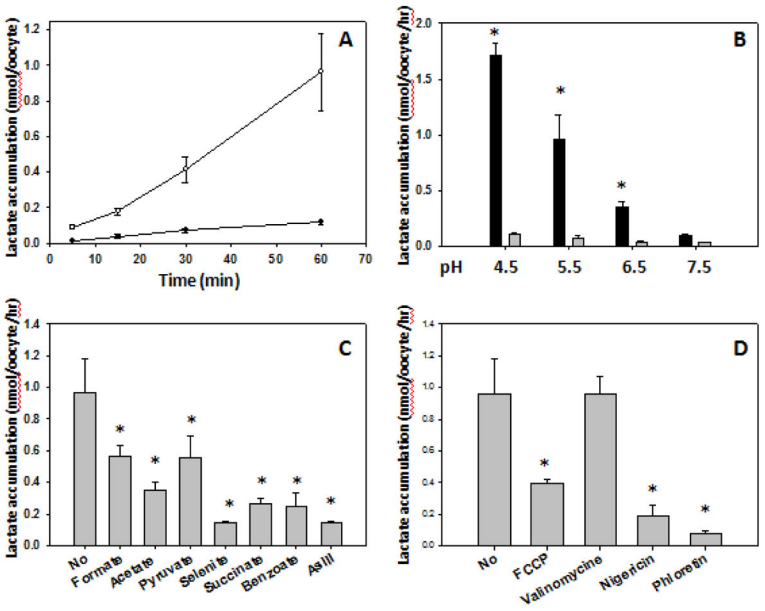

Fig. 2. Lactate transport by AQP9

Transport of Lactate species by AQP9 was examined under different pH conditions. **A:** Time course of lactate uptake via AQP9. Lacate was added at a final concentration of 1mM. **B.** Transport of lactate under different pH. Lactate was added in transport buffer at a final concentration of 1mM for 60 minutes. **C:** Inhibition of lactate transport by different AQP9 substrates. Transport assay was performed under pH 5.5 for 60 minutes. **D**. Effect of FCCP, valinomycin and nigericin on lactate transport by AQP9. Oocytes were pretreated with FCCP (20 μM), valinomycin (100 μM), or nigericin (10 μM) for 60 min at pH 5.5. The open bars represent arsenic uptake via AQP9, and the solid bars are the water injected controls.

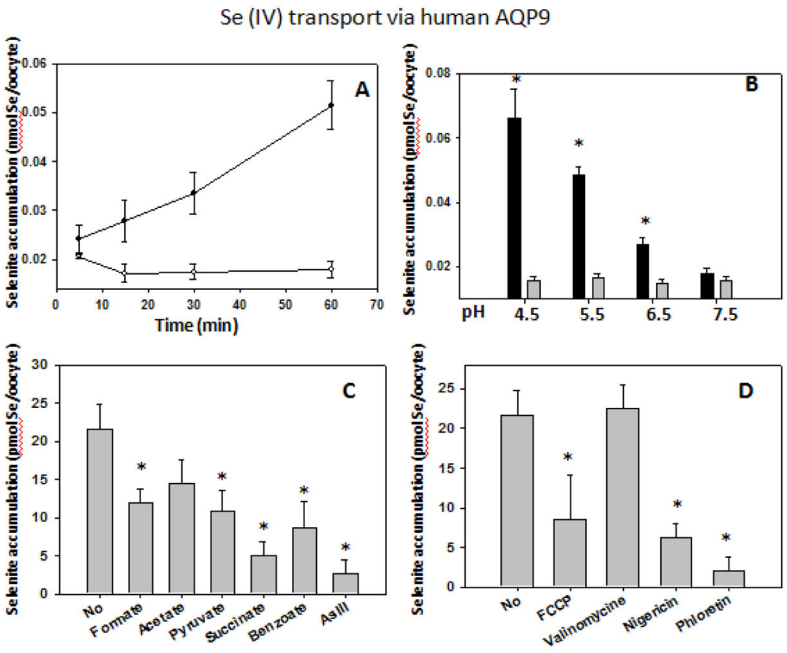

Fig. 3. Selenite transport by AQP9

Transport of selenite by human AQP9 was examined under different pH conditions. **A:** Time course of lactate uptake via human AQP9. Selenite was added in transport buffer at a final concentration of 1mM for 60 minutes. **B.** Transport of lactate under different pH. Selenite was added in transport buffer at a final concentration of 1mM for 60 minutes. **C:** Inhibition of lactate transport by different AQP9 substrates. Transport assay was performed under pH 5.5 for 60 minutes. **D**. Effect of FCCP, valinomycin and nigericin on selenite transport by AQP9. Oocytes were pretreated with FCCP (20 μM), valinomycin (100 μM), or nigericin (10 μM) for 60 min at pH 5.5. The open bars represent arsenic uptake via AQP9, and the solid bars are the water injected controls.

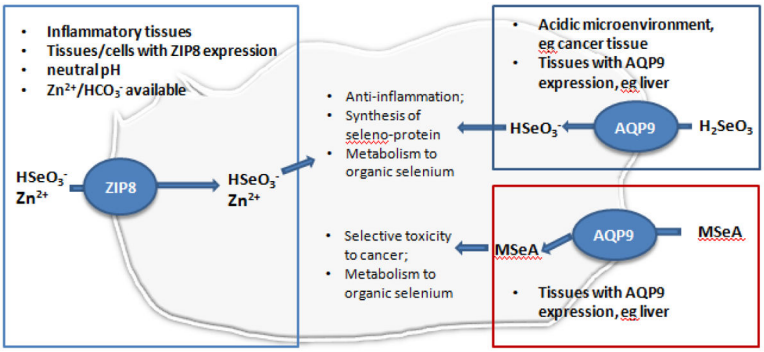

Fig. 4. Hypothetical pathway of MSeA and selenite uptake and cellular metabolism in cells

Inorganic selenite has two uptake pathways: under physiological pH, it is facilitated via ZIP8 with Zn and bicarbonate, which is predicted a major pathway under these conditions; when under acidic microenvironment while ZIP8 is not expressed, uptake of Se could be by AQP9. MSeA cellular uptake is mainly dependent on AQP9 expression, such as in liver tissue.