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Aquaporin-8-facilitated mitochondrial ammonia transport

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ABSTRACT

Aquaporin-8 (AQP8) is a membrane channel permeable to water and ammonia. As AQP8 is expressed in the inner mitochondrial membrane of several mammalian tissues, we studied the effect of the AQP8 expression on the mitochondrial transport of ammonia. Recombinant rat AQP8 was expressed in the yeast *Saccharomyces cerevisiae*. The presence of AQP8 in the inner membrane of yeast mitochondria was demonstrated by subcellular fractionation and immunoblotting analysis. The ammonia transport was determined in isolated mitochondria by stopped flow light scattering using formamide as ammonia analog. We found that the presence of AQP8 increased by threefold mitochondrial formamide transport. AQP8-facilitated mitochondrial formamide transport in rat native tissue was confirmed in liver (a mitochondrial AQP8-expressing tissue) *vs*. brain (a mitochondrial AQP8 non-expressing tissue). Comparative studies indicated that the AQP8-mediated mitochondrial movement of formamide was markedly higher than that of water. Together, our data suggest that ammonia diffusional transport is a major function for mitochondrial AQP8.

Introduction

Aquaporins (AQPs) are a family of integral membrane proteins that facilitates the movement of water and certain small solutes, across cellular membranes [1]. AQP8 has been detected in many tissues and organs [2–4]. Liver AQP8 is localized as a glycosylated 34 kDa protein in the canalicular plasma membrane domain [5–8] and intracellular vesicles [5–8]. There is experimental evidence suggesting that AQP8 facilitates the canalicular water transport during hepatocyte bile formation [8–10]. A non-glycosylated 28 kDa form of the AQP8 protein was found on the mitochondrial inner membrane (IMM) of rat liver and several other tissues [11–13]. Surprisingly, in spite of its high water conductance, AQP8 did not appear to have major relevance to the facilitated transport of water across the mitochondrial membranes [14–16]. Although mitochondrial AQP8 has been speculated to be functionally relevant to many biological functions including the urea cycle [17,18], cell-to-cell signaling and generation of reactive oxygen species [19], its exact significance remains open to question and an interesting matter for consideration.

Of note, there is experimental evidence that AQP8 facilitates the ammonia transport very efficiently [17,20–22], suggesting a role of AQP8 in mammalian physiology. AQP8 was able to rescue the growth of yeast defective in ammonium uptake [20]. Expression in *Xenopus* oocytes and reconstitution in proteoliposomes showed high AQP8 permeability to ammonia analogs [17,21]. Moreover, Saparov et al. using AQP8 reconstitution into planar bilayers reported that ammonia transport by AQP8 is electrically silent, and ammonia permeability exceeds water permeability over twofold [22].

In this study, using stopped flow spectrophotometry, we assessed ammonia permeability in yeast mitochondria with heterologous expression of rat AQP8 and in mitochondria from rat native tissues. A role for AQP8 in rat liver mitochondria is suggested.

Materials and methods

Yeast strain and transformation procedure. The yeast strain BJ5457 (MAT α, *ura3-52, trp1, lys2-801, leu2Δ1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1 GAL*), lacking the *PEP4* and *PRB1* protease activities, was chosen as the basic host strain for the expression of the recombinant *rAQP8*. Yeast cells were transformed according to the standard LiCl procedure. Ura^+ transformants were selected on synthetic minimal medium (SD) containing galactose (2%) as carbon source and supplemented with the required factors unless uracil.

Abbreviations: AQP8, aquaporin-8; IMM, inner mitochondrial membrane

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The plasmid pYES2-10His-rAQP8 is carrying a full-length cDNA of rat AQP8 (rAQP8), with a His10 tag at the N-terminus, whose expression is placed under the control of the yeast GAL4 promoter and was constructed as described in a previous study [21].

Preparation of yeast mitochondria. Yeast cells containing the plasmid pYES2-10His-rAQP8 were grown until A_{600} = 2 and essentially processed as described [23] with some modifications. Briefly, yeast cells were incubated with Zymolyase-100T (1 mg/g) (MP Biomedicals, LLC) at 30 °C for 30 min in 1.2 sorbitol, 20 mM potassium phosphate, pH 7.4. Then cells were resuspended (6.5 ml/g) in the cold buffer containing 0.6 M sorbitol, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.2% BSA, added with 1 mM phenylmethylsulphonyl fluoride (PMSF) and the protease inhibitors mix (1 mg/ml pepstatin, 1 mg/ml apoprotinin, 2 mg/ml, phosphoramidon, 1 mg/ml leupeptin, 2 mg/ml antipain, and 2 mg/ml chymostatin) and lysed in an homogenizer. Cell debris was eliminated by centrifugation and the homogenate was diluted 1:1. Mitochondria were isolated by centrifugation at 12,000*g* for 15 min, washed and resuspended in 250 mM sucrose, 1 mM EDTA, and 10 mM MOPS, pH 7.2. Mitoplasts and IMM vesicles were prepared as previously described [12].

Immunoblotting analysis. Aliquots (5 μg protein) of isolated mitochondria prepared as above were heated at 90 °C and electrophoresed in an SDS, 13% acrylamide gel (Mighty Small II, Amersham Biosciences) using a low molecular weight protein ladder (Amersham Biosciences). The resolved proteins were transferred electrophoretically onto nylon membranes that were blocked in 5% (w/v) low fat milk in blocking buffer (20 mM Tris–HCl (pH 7.5), 0.15 M NaCl, and 1% Triton X-100) for 1 h, and further incubated with affinity purified antibody directed against an N-terminal peptide of rat AQP8 [11], AQP8 (Alpha Diagnostics International, San Antonio, TX), AQP9 (Alpha Diagnostics International), anti-His antibodies (Sigma and Cell Signaling Technology), or ATP11p (Santa Cruz Biotechnology) all at a final concentration of 1 μg/ml blocking solution. The blots were then washed and incubated with horseradish peroxidase-conjugated corresponding secondary antibodies, and bands were detected by enhanced chemiluminescence detection system (ECL-Plus, Amersham Biosciences). Autoradiographs were obtained by exposing nylon membranes to Kodak XAR film.

Isolation of rat liver and brain mitochondria. Mitochondria were prepared from adult male Wistar rats weighing 250–300 g (Harlan, San Pietro al Natisone, Italy). Rats were fed a standard diet and water *ad libitum*. For all experiments, rats were sacrificed and their livers and brains were quickly removed and processed depending on the specific preparation. For the isolation of mitochondria, organs were homogenized with a Potter–Elvehjem homogenizer (four strokes in 1 min at 500 rpm) in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4. The homogenate was centrifuged at 500*g* for 10 min at 4 °C and the pellet consisting of nuclei and unbroken cells was discarded; the resulting supernatant was centrifuged at 6000*g* for 10 min at 4 °C and the related pellet was washed twice before being resuspended in isolation medium to which a cocktail of protease inhibitors had been added (1 mM PMSF, 1 mM leupeptin, and 1 mM pepstatin A). All chemicals used for the preparations were from the Sigma Chemical Company (St. Louis, MO).

Stopped flow light scattering measurement of formamide and water permeabilities. The time course of mitochondrial volume change was followed from changes in intensity of scattered light intensity at 20 °C at the wavelength of 450 nm by using a BioLogic MPS-200 stopped flow reaction analyzer (BioLogic, Milan, Italy) that has a 1.6-ms dead time and 99% mixing efficiency in <1 ms.

For measurement of formamide permeability, mitochondria were subjected to a 150 mM inwardly directed gradient of formamide as previously reported by glycerol permeability measurement [24]. There was a biphasic response, a rapid shrinkage of mitochondria due to the exit of water until the osmotic equilibrium was reached, followed by a much slower swelling of mitochondria corresponding to the influx of formamide accompanied by water. Mitochondrial swelling data (i.e., formamide transport) were fitted to a single exponential function with rate constant, K_i (s^{-1}).

Mitochondria osmotic water permeability was measured by light scattering as previously described [12]. Briefly, 35 μl of a concentrated mitochondrial suspension was diluted into 2.5 ml of a hypotonic (220 mosM) isolation medium (124 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, 1 mM EDTA, and 5 mM EGTA, pH 7.4). One of the syringes of the stopped flow apparatus was filled with the specimen suspension, whereas the other was filled with the same buffer to which mannitol was added to reach a final osmolarity of 500 mosM in order to establish a hypertonic gradient (140 mosM) upon mixing. The final protein concentration after mixing was 100 μg/ml. Immediately after applying a hypertonic gradient, water outflow occurs, causing mitochondrial shrinkage and increase in scattered light intensity. Data were fitted to a single exponential function and the related rate constant (K_i, s^{-1}) of the water efflux out of the mitochondria was measured.

The K_i of swelling and shrinkage were used as indirect biophysical parameters for assessment of formamide and water mitochondrial permeabilities, respectively. The corresponding solute and water permeability coefficients were not calculated because of biophysical (i.e., presence of two membranes in series) and morphological (i.e., rod-shaped structures of heterogeneous size) constrains of mitochondria.

Statistical analysis. Data were expressed as means ± SE. Significance was determined using Student's *t*-test; $p < 0.05$ was considered statistically significant.

Results

Rat AQP8 expression in yeast mitochondria

As yeast does not express AQP8 homologs [25], the *Saccharomyces cerevisiae* BJ5457 strain was used for the heterologous expression of rAQP8. Yeast cell fractions were monitored for the presence of rAQP8 expressed from episomal copies. Fig. 1A shows that anti-poly-His antibodies identified a 28 kDa protein (i.e., the predicted rat AQP8 molecular mass) only in AQP8-expressing yeasts which was strongly enriched in the mitochondrial fraction. The same pattern was found using an antibody directed against a N-terminus AQP8 peptide (Fig. 1B). An additional 26 kDa band was also identified suggesting that targeting to yeast mitochondria is accompanied with a proteolysis at the N-terminus of the AQP8 polypeptide, which may be associated to the protein import into mitochondria. Proving the specificity, the same result was found using a polyclonal antibody directed against a C-terminus AQP8 peptide (Fig. 1C). Consistent with previous results [11,12], mitochondrial subfractionation showed AQP8 presence in the IMM subcompartment (Fig. 1D). Some AQP8 reactivity was also detected in the submitochondrial fraction enriched in outer mitochondrial membranes (not shown). Lack of AQP9 immunosignals further supports the specific expression of rat AQP8 in yeast mitochondria (Fig. 1E).

Formamide and water transport across AQP8 localized in yeast mitochondria

Whole yeast mitochondria harboring or not rat AQP8 were used to assess the mitochondrial transport of formamide and water by stopped flow light scattering. Quality and semi-quantitative normalization of yeast mitochondria were evaluated by immunoblotting with antibodies directed against the mitochondrial marker ATP11p (Fig. 2A). Fig. 2B shows the time course of mitochondrial volume

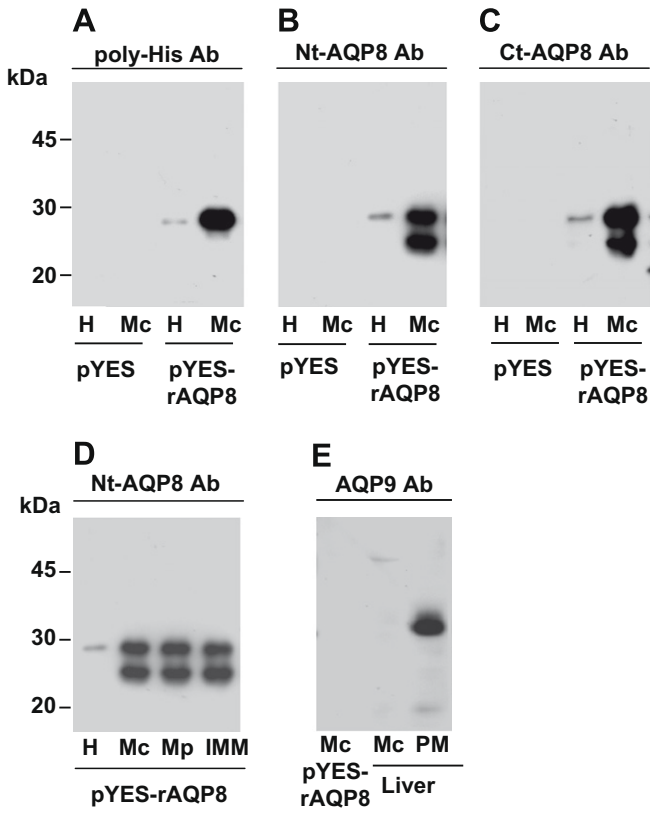

Fig. 1. Immunoblotting analysis of rat AQP8 expressed in yeast. (A) Immunoblotting using anti-poly-His antibodies. The immunoreactivity (28 kDa band) is only present in AQP8-expressing yeast (pYES-rAQP8). The mitochondrial fraction (Mc) is strongly enriched in terms of AQP8 presence respect to the whole homogenate (H). (B,C) Immunoblottings using a polyclonal antibody directed against the N- and C-terminus peptides of rat AQP8 (Nt-AQP8 Ab and Ct-AQP8 Ab, respectively). In both cases, two immunoreactive bands (28 and 26 kDa) are observed. The mitochondrial fraction (Mc) is strongly enriched in AQP8 reactivity respect to the homogenate (H). (D) Immunoblot analysis of submitochondrial compartments using anti-AQP8 antibodies. Immunoreactivity (28 and 26 kDa bands) is observed in mitoplasts (Mp) and in inner mitochondrial membranes (IMM). (E) Control immunoblotting using an antibody against AQP9. A 32 kDa band corresponding to AQP9 is seen in the rat hepatocyte plasma membrane (PM) but not in the mitochondrial fraction (Mc). No apparent immunoreactivity is observed in the mitochondrial membrane fraction of AQP8-expressing yeasts (pYES-rAQP8).

change followed by changes in scattered light intensity. Data fitted well to a single exponential curve indicating a functionally homogeneous population of mitochondria. Formamide transport resulted clearly faster in AQP8-expressing yeast mitochondria (Fig. 2B) than in control mitochondria (pYES). The calculated single exponential rate constants of mitochondrial formamide transport depicted in Fig. 2C were markedly higher in AQP8-expressing ($0.48 \pm 0.04\ s^{-1}$) than control yeasts ($0.17 \pm 0.02\ s^{-1}$).

Fig. 2D shows the time course of osmotic water movement in yeast mitochondria. The water transport data also fitted well to a single exponential curve indicating the presence of a homogeneous population of mitochondria for water transport. The calculated rate constants for mitochondrial water transport depicted in Fig. 2E were slightly higher in AQP8-expressing yeasts ($7.70 \pm 0.20\ s^{-1}$) *vs.* control yeasts ($6.55 \pm 0.33\ s^{-1}$). Thus, the expression of rat AQP8 in yeast mitochondria increased the transport of formamide by over 200%, while that of water was augmented by less than 20.0%.

Formamide and water transport across rat liver and brain mitochondria

As rat liver expresses AQP8 also in the IMMs [11,12] and rat brain lacks mitochondrial AQP8 expression [2,3], we took advantage of this feature to make a comparative transport study for formamide and water.

Formamide and water transports in rat liver and brain mitochondria were assessed by stopped flow light scattering as described in Materials and methods. Fig. 3A shows the corresponding rate constant values. The formamide transport featured by liver mitochondria was significantly faster than the one of the brain counterpart. In contrast, no significant differences were seen in terms of water transport between liver and brain mitochondria (Fig. 3B).

Discussion

Although AQP8 has been demonstrated to be an "aqua-ammoniaporin" [20–22], its functional relevance in the inner mitochondrial membranes of several tissues, including liver, remains scarcely known. In this study, using stopped flow light scattering, we found that the heterologous expression of rat AQP8 in yeast mitochondria significantly increased ammonia permeability over that of water. Also, we found that rat liver mitochondria, which express AQP8 in inner membranes, have markedly higher ammonia permeability than AQP8-lacking brain mitochondria. In contrast, osmotic water permeability in rat liver and brain mitochondria were of similar extent. These data suggest that NH_3 diffusional transport may be the main function of AQP8 in mitochondria.

Our finding that mitochondrial AQP8 exhibits preference for neutral NH_3 molecules over water is in good agreement with previous studies using purified rat AQP8 reconstituted into planar bilayers [22] in which single channel permeability for ammonia (8×10^5 ammonia molecules/s/channel) was found to be about twofold that for water with a high transport efficiency. Moreover, the fact that in our experiments the AQP8 permeability to ammonia was measured by using formamide ($HCONH_2$), an analog structurally similar to NH_3 but of larger size, might cause some underestimation of the AQP8-facilitated ammonia membrane transport. Nevertheless, our data appears not to be consistent with a cell culture study in which a relative AQP8 single-channel ammonia-to-water permeability (plasma membrane) of only 0.03 was calculated based on the fluorescence of a pH-sensing yellow fluorescent protein in presence of an ammonia (NH_3/NH_4^+) gradient [26]. This discrepancy was already explained by Saparov and coworkers [22] as, at least in part, due to compensatory transport events aimed to maintain cellular pH that may determine the kinetics of pH changes. However, while sharing the reasons of the apparent incongruity provided by the above authors, we cannot rule out the possibility that the relative ammonia-to-water permeability does also reflect the biochemistry of the lipid bilayer in which the channel is inserted (the IMM lipid composition is quite different from that of the plasma membrane). It is likely that the hydrolytic removal of the short N-terminus peptide which may be associated to the targeting of AQP8 to yeast mitochondria does not influence the NH_3-to-H_2O conductance of the channel because (1) a similar ratio was seen with rat liver mitochondria where AQP8 was not hydrolyzed, (2) the N-terminus (as also the C-terminus) of the mammalian AQPs is not known for acting on the pore peculiarities [27], and (3) many IMM transporters possess N-terminal signals that are cut away to permit the protein import into the mitochondrion without influencing the transport functions [28]. The fact that mitochondrial AQP8 is more prone in conducting NH_3 than water might also explain why its deletion does not impair the overall water permeability of mitochondria [15] and increased expression in the organelle does not raise the osmotic water permeability of the IMM [18].

The AQP8-facilitated mitochondrial ammonia transport may play important physiological roles. In liver, AQP8 may be involved in mitochondrial ammonia detoxification via ureagenesis (urea

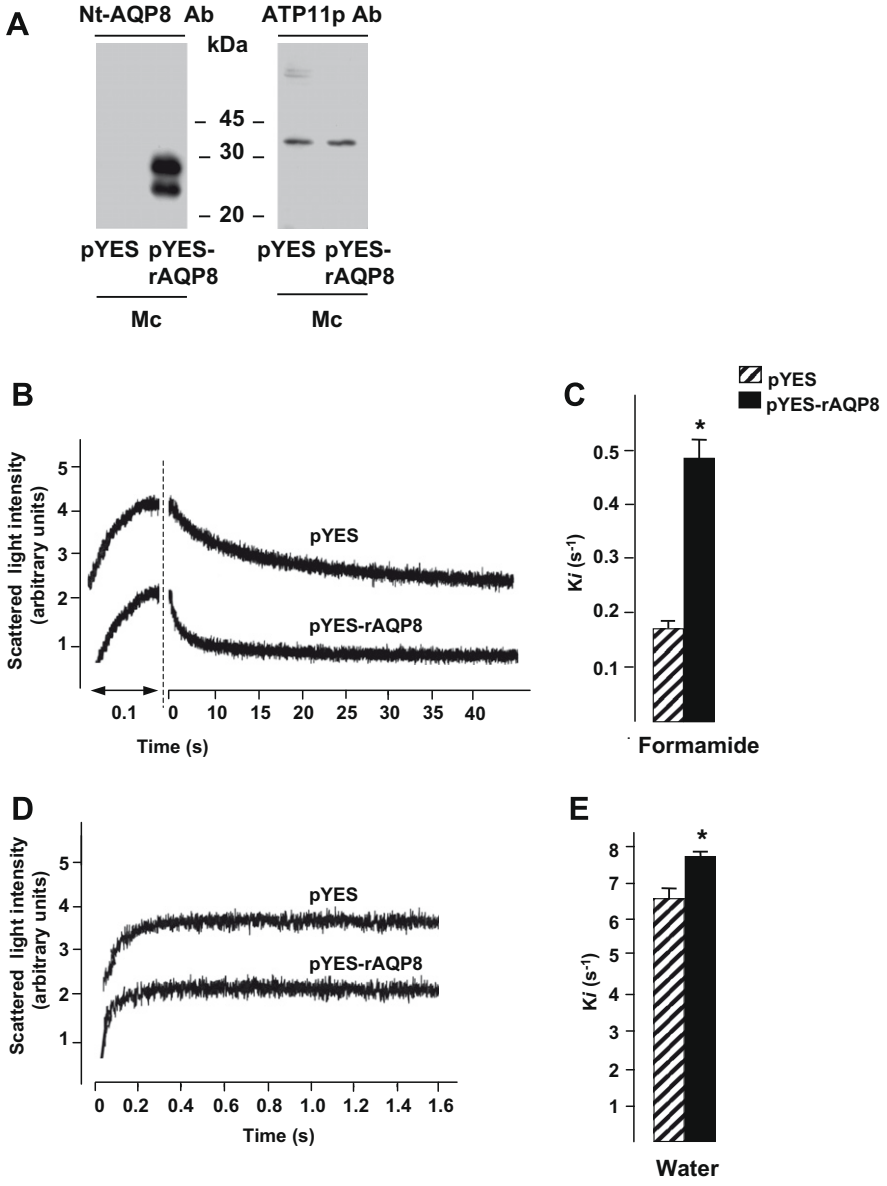

Fig. 2. Biophysical analysis of formamide and water transport across rat AQP8-expressing yeast mitochondria. (A) Immunoblot analysis of AQP8 associated with isolated mitochondria used for functional studies (left panel). Yeast ATP11p (37 kDa band) was used as a mitochondrial marker (right panel). (B) Mitochondrial transport of formamide. Representative experiments of stopped flow light scattering using mitochondria from control (pYES) and AQP8-expressing (pYES-rAQP8) yeasts in response to a 150 mM inwardly directed concentration gradient of formamide. Traces reflect a biphasic response. The initial increase in light scattering results from osmotic water efflux (mitochondrial shrinkage) is followed by a slower decrease caused by formamide diffusional entry accompanied by osmotic water influx (mitochondrial swelling). (C) Calculated rate constants (K_i, s^{-1}) of mitochondrial formamide transport from control (pYES) and AQP8-expressing (pYES-rAQP8) yeasts. Data are means ± SE; $n = 20$ $^*p < 0.001$. (D) Mitochondrial water transport. Representative experiments of stopped flow light scattering using mitochondria from control (pYES) and AQP8-expressing (pYES-rAQP8) yeasts in response to a 140 mM inwardly directed hypertonic mannitol gradient. (E) Calculated K_i values of mitochondrial water transport from control (pYES) and AQP8-expressing (pYES-rAQP8) yeasts. Data are means ± SE; $n = 20$ $^*p < 0.001$.

cycle), a way to metabolize amino acids and prevent the deleterious consequences of hyperammonemia and hepatic encephalopathy. Involvement in the urea cycle was suggested by Holm and coworkers [17] and is supported by a recent work showing that mitochondrial AQP8 is markedly augmented in hypothyroidism [18], a condition associated with increased hepatic capacity to synthesize urea as a consequence of the increased ammonia production. However, studies addressing the NH_3 transport at a plasma membrane level in AQP8-null mice reported only minor changes in hepatic ammonia handling [28] suggesting that alternative ammonia transport pathways would be of major relevance than AQP8 in plasma membranes. RhBG, a member of the Rh glycoprotein family of ammonia transporters [29], is expressed strongly in the hepatocyte basolateral plasma membrane [30]. Considering that ammonia transport might be of main significance for mitochondrial AQP8 and that, to our knowledge, no ammonia transporter other than AQP8 has been identified in mitochondria thus far, an interesting matter for further studies would be to evaluate the liver mitochondrial ammonia transport in AQP8 knockout mice. Because AQP8 features additional permeability to hydrogen peroxide, the potential relevance of mitochondrial AQP8 in generation of reactive oxygen species [19], a process underlying oxidative stress, is also an open question for consideration.

In summary, the present work shows that the AQP8-facilitated mitochondrial diffusion of the ammonia analog formamide is

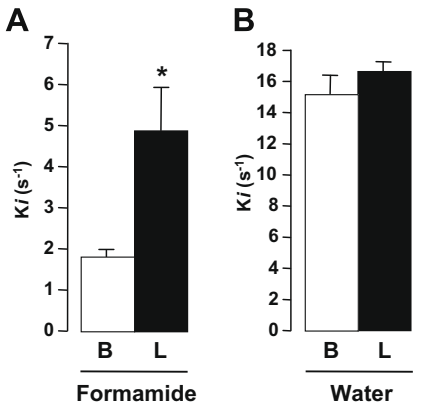

Fig. 3. Biophysical analysis of formamide and water transport across mitochondria from rat liver and brain. (A) Mitochondrial formamide transport. Calculated rate constants of formamide transport from rat liver (L) and brain (B) mitochondria. Formamide transport is significantly faster in liver, a tissue expressing AQP8 in mitochondria, than in brain, an organ lacking AQP8 in mitochondria. Data are means ± SE; $n = 5$ $^*p < 0.001$. (B) Mitochondrial water transport. Calculated rate constants of osmotic water transport from isolated rat liver (L) and brain (B) mitochondria. The K_i values (means ± SE; $n = 5$) from liver and brain are not significantly different.

markedly higher than that of water. This suggests that ammonia diffusional transport, a process avoiding the severe neurotoxicity caused by hyperammonemia, may be a major function for AQP8 in mitochondria.

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