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Ammonia Detoxification via Ureagenesis in Rat Hepatocytes Involves Mitochondrial Aquaporin-8 Channels

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Hepatocyte mitochondrial ammonia detoxification via ureagenesis is critical for the prevention of hyperammonemia and hepatic encephalopathy. Aquaporin-8 (AQP8) channels facilitate the membrane transport of ammonia. Because AQP8 is expressed in hepatocyte inner mitochondrial membranes (IMMs), we studied whether mitochondrial AQP8 (mtAQP8) plays a role in ureagenesis from ammonia. Primary cultured rat hepatocytes were transfected with small interfering RNAs (siRNAs) targeting two different regions of the rat AQP8 molecule or with scrambled control siRNA. After 48 hours, the levels of mtAQP8 protein decreased by approximately 80% ($P < 0.05$) without affecting cell viability. mtAQP8 knockdown cells in the presence of ammonium chloride showed a decrease in ureagenesis of approximately 30% ($P < 0.05$). Glucagon strongly stimulated ureagenesis in control hepatocytes (+120%, $P < 0.05$) but induced no significant stimulation in mtAQP8 knockdown cells. Contrarily, mtAQP8 silencing induced no significant change in basal and glucagon-induced ureagenesis when glutamine or alanine was used as a source of nitrogen. Nuclear magnetic resonance studies using 15N-labeled ammonia confirmed that glucagon-induced 15N-labeled urea synthesis was markedly reduced in mtAQP8 knockdown hepatocytes (−90%, $P < 0.05$). *In vivo* studies in rats showed that under glucagon-induced ureagenesis, hepatic mtAQP8 protein expression was markedly up-regulated (+160%, $P < 0.05$). Moreover, transport studies in liver IMM vesicles showed that glucagon increased the diffusional permeability to the ammonia analog [^{14}C]methylamine (+80%, $P < 0.05$). *Conclusion:* Hepatocyte mtAQP8 channels facilitate the mitochondrial uptake of ammonia and its metabolism into urea, mainly under glucagon stimulation. This mechanism may be relevant to hepatic ammonia detoxification and in turn, avoid the deleterious effects of hyperammonemia. (HEPATOLOGY 2013;57:2061-2071)

By means of the urea cycle, hepatocytes metabolize waste nitrogen and prevent the deleterious consequences of hyperammonemia and hepatic encephalopathy. The initial and rate-controlling step in the urea cycle is the mitochondrial synthesis of carbamoyl-phosphate from ammonia by carbamyl phosphate synthetase 1 (CPS1),[1] a process that involves the mitochondrial uptake of ammonia and the metabolization of ammonia from amino acids such as glutamine and alanine.[2] However, the mechanisms involved in the mitochondrial entry of ammonia are still unknown (Fig. 1). Aquaporin-8 (AQP8) is a member of a family of homologous membrane channel proteins that facilitates the movement of water and certain small solutes across biological membranes.[3] Hepatocyte AQP8 is localized as a glycosylated 34-kDa protein in the canalicular plasma membrane domain and intracellular vesicles.[4–7] Canalicular AQP8 works as a water channel facilitating the movement of water molecules coupled to osmotic gradients,[8,9] a process that may play a role in hepatocyte bile formation and cholestasis.[7,10–13] A nonglycosylated 28-kDa form of the AQP8 protein

Abbreviations: AQP8, aquaporin-8; ATP, adenosine triphosphate; CPS1, carbamyl phosphate synthetase 1; IMM, inner mitochondrial membrane; mtAQP8, mitochondrial aquaporin-8; NMR, nuclear magnetic resonance; OTC, ornithine transcarbamylase; SCR, scramble; siRNA, small interfering RNA.

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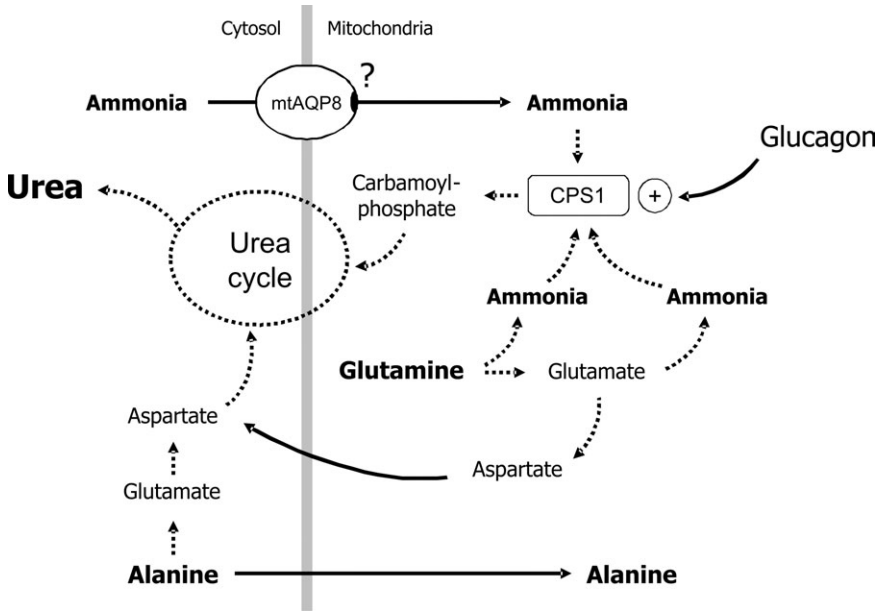

Fig. 1. Schematic representation of ureagenesis from ammonia, glutamine, and alanine. After entering mitochondria, ammonia is incorporated into carbamoyl-phosphate by CPS1. Carbamoyl-phosphate is then converted into urea via the urea cycle. In mitochondria, glutamine is converted into glutamate by phosphate-dependent glutaminase releasing one molecule of ammonia. Mitochondrial glutamate is then used to form ammonia via glutamate dehydrogenase. Alanine can provide glutamate in either the cytoplasmic or mithochondrial compartment of hepatocytes. The mitochondrial glutamate may be used to form aspartate via the mitochondrial transaminase reaction. This mitochondrial aspartate is transported to the cytosol and then converted into urea. Some of the aspartate may be formed in the cytosol via the cytosolic transaminase reaction. The initial and rate-controlling step in the urea cycle is the mitochondrial synthesis of carbamoyl-phosphate from ammonia by CPS1. Glucagon stimulates ureagenesis via an *N*-acetylglutamate-dependent CSP1 activation, which in turn, induces mitochondrial uptake of ammonia to sustain urea cycle. Because the mechanisms of ammonia transport from cytoplasm to mitochondria are still unknown, we hypothesized that mtAQP8 is involved in this process.

was found on the inner mitochondrial membrane (IMM) of hepatocytes[14,15] and kidney proximal cells.[16,17] Mitochondrial AQP8 (mtAQP8) did not appear to have major relevance in mediating the transport of water across mitochondrial membranes.[18–20] Studies in heterologous expression and reconstituted systems[21–24] and in testis plasma membrane vesicles[25] demonstrated that rat, mouse, and human AQP8 are able to efficiently facilitate the transport of ammonia. We recently reported that recombinant rat AQP8 expressed in inner membranes of yeast mitochondria significantly increased the ammonia permeability.[26] Moreover, comparative studies with mitochondria from liver versus brain (an organ where AQP8 is not expressed) suggested that facilitation of ammonia membrane transport may be an important function for hepatic mtAQP8.[26] Thus, it is reasonable to suppose that the mtAQP8-facilitated ammonia transport may play a role in the hepatic mitochondrial ammonia detoxification and ureagenesis. Glucagon is well known to up-regulate hepatic ureagenesis on short- and long-term basis.[27] In this study, we have combined data obtained from mtAQP8 knockdown hepatocytes and whole animal in an effort to elucidate the role that mtAQP8 plays in the ureagenesis from ammonia, particularly under glucagon-stimulation.

Materials and Methods

Additional details are provided in the Supporting Methods.

Isolation and Culture of Hepatocytes. Hepatocytes were isolated from normal livers of male Wistar rats by collagenase perfusion and mechanical disruption.[6] Cell viability (assessed by Trypan blue exclusion) was >85%. Hepatocytes were plated onto collagen-coated glass plates at 1.9×10^4 cells/cm^2, containing

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Dulbecco's modified Eagle's medium (high-glucose, with 1 mM sodium pyruvate, and without L-glutamine) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen, San Diego, CA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. At the end of the experiments, cells were washed and sonicated in 0.3 M sucrose (Merck Chemicals, Darmstadt, Germany).

Animals and Treatments. Adult male Wistar rats were maintained on a standard diet and water *ad libitum*, and housed in a temperature- and humidity-controlled environment under a constant 12-hour light/dark cycle according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD). Protocols were approved by the local Animal Welfare Committee. Highly purified recombinant glucagon (Eli Lilly, Indianapolis, IN) was administered at a final dose of 0.2 mg/100 g intraperitoneally in 2, 3, or 6 doses for 8, 16, and 36 hours, respectively. Because of the short half-life of glucagon in plasma,[28] repetitive administrations are required for long-term studies. Control rats received saline (i.e., glucagon vehicle). Animals were euthanized, and livers were harvested for evaluation.

Synthesis of Small Interfering RNA and Knockdown of mtAQP8 Expression. The 21-nucleotide RNA duplexes were synthesized using the Silencer small interfering RNA (siRNA) kit (Ambion, Austin, TX) as described.[10] siRNA1 was specifically targeted to nucleotides 186-206 (AACATGGCTGACAGTTACCAT) and siRNA2 to nucleotides 236-256 (AACCGTGTGTGGTGGAACTTT) of rat AQP8. The control siRNA (siRNA scramble [SCR]) was designed by randomly scrambling the nucleotides of siRNA1 (AATATCGCGATGTGCACTACA). After 18 hours of culture, hepatocytes were transfected with AQP8 siRNAs by use of Lipofectamine 2000 transfection reagent (Invitrogen).

Hepatocyte Urea Production. Hepatocytes transfected with corresponding siRNAs were incubated in the presence of 1 mM ammonium chloride, 4 mM L-glutamine, or 4 mM L-alanine. In some experiments, hepatocytes were also incubated for 4 hours with 1 μM glucagon or with the mitochondrial urea cycle intermediate carbamoyl-phosphate (15 mM). All the experiments were done in the presence of L-ornithine (4 mM) (Sigma, St. Louis, MO). In addition, because urea has two nitrogens, one coming from ammonia and one from aspartate,[2] it is worth mentioning that culture media contain pyruvate as a source of aspartate.[29] At the end of the experiments, the culture medium was aspirated and centrifuged at 500*g* for 5 minutes to obtain a cell-free supernatant for determination of urea via the urease method[30] and ammonia by using a commercial kit (Randox Laboratories, Crumlim, UK).

Assessment of ^{15}N-labeled Urea by NMR Spectroscopy. Control and mtAQP8 knockdown hepatocytes were incubated with 1 mM ^{15}N-labeled ammonium chloride (99% enriched in ^{15}N; Cambridge Isotope Laboratories, Andover, MA) at 37°C for 4 hours in the absence or presence of 1 μM glucagon. The culture medium was then aspirated, centrifuged at 500*g* for 5 minutes at 4°C and lyophilized. The lyophilizate was resuspended in 0.5 mL of dimethyl sulfoxide-d_6 (99.9 atom% D; Cambridge Isotope Laboratories) containing 0.02 mL of formamide (natural abundance [0.365%] in ^{15}N) (Promega), that was used as an internal standard. ^{15}N-labeled urea synthesis was determined by nuclear magnetic resonance (NMR) spectroscopy[31] using a Bruker Avance 300 MHz NMR Ultrashield (Bruker AXS Inc., Madison, WI) operating at 30.41 MHz. The instrument is equipped with a 5-mm broadband multinuclear probe. The operating conditions for ^{15}N employed a 21-μs (90° flip angle) pulse width, a relaxation delay time of 5 seconds, acquisition time of 2.7 seconds, spectral width of 200 ppm, and an INEPT (insensitive nuclei enhanced by polarization transfer) pulse sequence. The sample was measured at 25°C. The total measurement time was about 29.75 minutes. Biological ^{15}N-labeled urea and the internal standards were identified by comparison of their chemical shifts with those of the standard compounds (NMR standard samples from Bruker). The signals from ^{15}N-labeled urea and from formamide were observed at 76.75 ppm and 113.33 ppm, respectively. Biological ^{15}N-labeled urea in each NMR sample was quantified from the observed ^{15}N peak intensities (in integrated areas) compared with that of the internal standard.

Statistical Analysis. Data are expressed as the means ± SE. Significance was determined using a Student *t* test or one-way analysis of variance (Tukey test). $P < 0.05$ was considered statistically significant.

Results

Mitochondrial AQP8 Expression and Ureagenesis in Primary Cultured Rat Hepatocytes. As a first step, we confirmed the expression and subcellular localization of AQP8 in hepatocyte mitochondria as reported.[14,15] This was done by immunogold electron microscopy, confocal immunofluorescence microscopy and inmunoblot analysis. Fig. 2A shows immunogold particles at the IMM from rat hepatocytes. Confocal

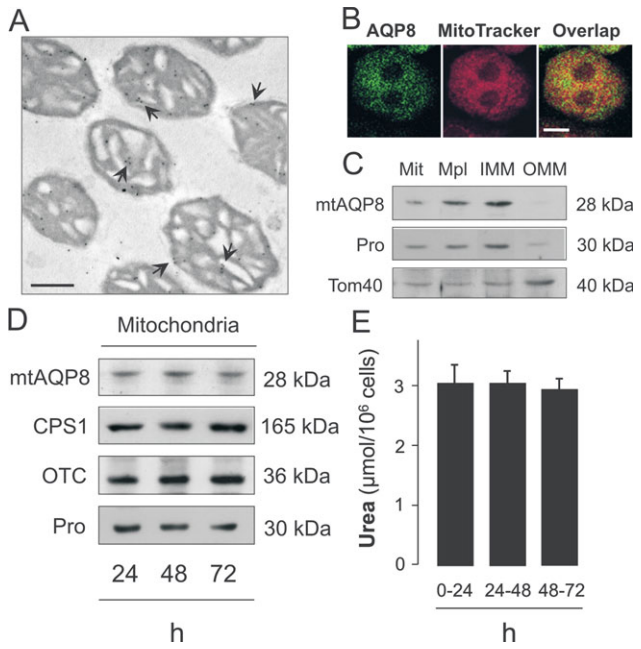

Fig. 2. Mitochondrial AQP8 expression and ureagenesis in primary cultured rat hepatocytes. (A) Immunogold electron microscopy for AQP8 in purified rat hepatocyte mitochondria. Immunogold particles are seen in IMMs (cristae and border; arrows) as reported.[14,15] Scale bar = 300 nm. (B) Confocal immunofluorescence microscopy for AQP8 (green) and mitochondrial dye MitoTracker (red) in primary cultured rat hepatocytes. AQP8 is largely localized to cytoplasmic structures with partial overlapping with red MitoTracker (yellow). Scale bar = 10 μm. (C) Inmunoblot analysis for mtAQP8 in hepatic submitochondrial fractions (20 μg of protein/lane). Immunoreactivity (28 kDa) is observed in mitochondria (Mit), mitoplasts (Mpl), and IMMs. Note the AQP8 enrichment among these fractions. Immnoblottings were reprobed for prohibitin (IMM marker) and Tom40 (marker of outer mitochondrial membrane [OMM]) as controls. (D) Inmunoblot analysis for mtAQP8 and the mitochondrial urea cycle enzymes. CPS1 and OTC after 24, 48, and 72 hours of primary hepatocyte culture. Expression levels of these proteins showed no significant change over time. Each lane was loaded with 20 μg of protein. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading. (E) Urea was determined enzymatically in the cultured media after 24, 48, and 72 hours of culture. Primary cultured rat hepatocytes showed stable urea production during the time of the experiments. Data represent the mean ± SE of three independent experiments.

immunofluorescence studies showed that hepatocyte AQP8 labeling is largely localized to cytoplasmic structures as we first reported,[6] with partial overlapping with mitochondrial dye MitoTracker (Fig. 2B). For the immunoblotting experiments, using an antibody directed against an N-terminus AQP8 peptide,[14,15] mitochondrial subfractionation showed a band of expected size (28 kDa) that was strongly detected in the IMM subcompartment (Fig. 2C). Same results were obtained in immunoblotting studies by using commercially available N-terminal affinity-purified antibody against AQP8 (E-20, Santa Cruz Biotechnology, Santa Cruz, CA) (data not shown). Fig. 2D,E shows that after 24, 48, and 72 hours of primary hepatocyte culture, the expression levels of mtAQP8 and the mitochondrial urea cycle enzymes; CPS1 and ornithine transcarbamylase (OTC) as well as urea production showed no significant change over time. In contrast, the expression of plasma membrane 34 kDa AQP8 declined in primary hepatocytes after 24 hours of culture being almost undetectable at 72 hours (not shown). This seems to be a common feature of hepatocyte plasma membrane transporters, which are down-regulated in primary culture.[32]

mtAQP8 Knockdown in Primary Cultured Rat Hepatocytes. To induce a decrease in mtAQP8 expression, primary rat hepatocytes were transfected with siRNAs containing sequences specific for rat AQP8. After 24 hours of transfection, mtAQP8 expression was unaffected (Fig. 3A). Nevertheless, after 48 hours, mtAQP8 expression decreased 80% and 75% in siRNA1- and siRNA2-treated cells, respectively (Fig. 3A,B). No significant changes in CPS1 and OTC expression were observed in hepatocytes with mtAQP8 silencing. (Fig. 3A,B). Neither the other members of the aquaporin family of proteins in hepatocytes (i.e., aquaporins 0, 9, and 11) showed any change of expression (Supporting Fig. 1). Viability of mtAQP8 knockdown hepatocytes assessed either by lactate dehydrogenase leakage (Fig. 3C) or the activity of the mitochondrial complex II/succinate dehydrogenase (i.e., MTT assay) (Fig. 3D) was unaffected. It is well known that ATP plays an important role in the urea cycle.[33] Figure 3E shows that no significant changes in ATP levels were observed in hepatocytes with mtAQP8 silencing. Accordingly, the expression of mitochondrial F_1F_0-ATP synthase (assessed by immunoblotting using antibodies against the alpha-subunit of the F_1-ATPase complex from Sigma Co.) was also found to be unaltered (data not shown).

Effect of Rat Hepatocyte mtAQP8 Knockdown on the Basal and Glucagon-Stimulated Ureagenesis. Ureagenesis analysis indicated that there is not a cell phenotype associated with transfection of siRNA1 or siRNA2 at 24 hours; nevertheless, at 48 hours, when mtAQP8 silencing is evident, basal ureagenesis decreased 38% and 29% ($P < 0.05$) in siRNA1- and siRNA2-treated cells, respectively. Consequently, ammonia levels in culture were found to be increased (Fig. 4A). It is worth mentioning that the glutaminase activity in hepatocytes did not change among experimental groups (nmol/minute/mg protein, n = 3): 22.1 ± 1.1 (siRNA SCR) versus 22.4 ± 0.5 (siRNA 1) and 21.5 ± 0.8 (siRNA 2).

As glucagon is a known inducer of ureagenesis,[27] its action on urea production was directly explored in mtAQP8 knockdown hepatocytes cultured in media

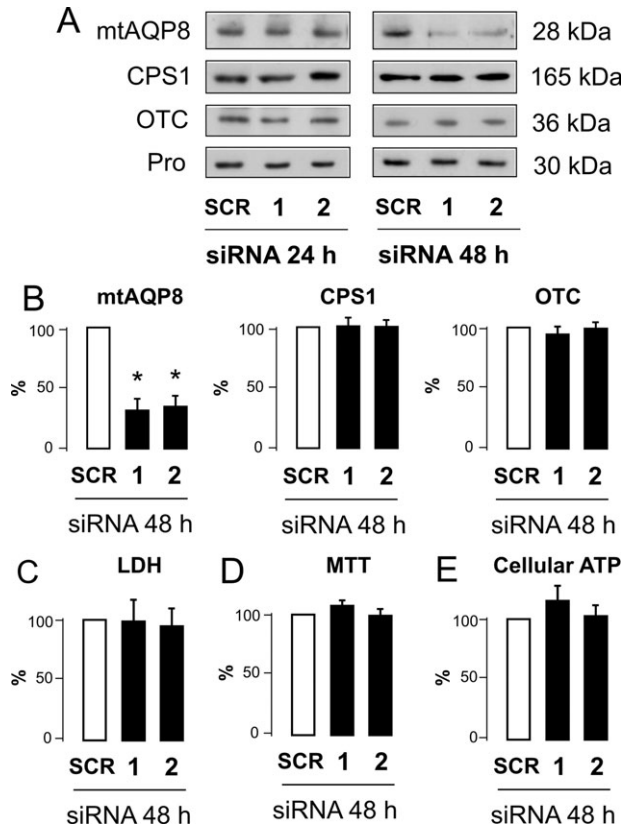

Fig. 3. mtAQP8 knockdown in primary cultured rat hepatocytes. Hepatocytes were transfected with siRNA duplexes targeting specific sequences of rat AQP8 mRNA (siRNA1 and siRNA2 [1 and 2, respectively]) or a scrambled siRNA1 sequence (SCR). (A) Protein expression of mtAQP8 and the urea cycle enzymes CPS1 and OTC were analyzed by immunoblotting in total mitochondrial fractions 24 and 48 hours after transfection. siRNA1 and siRNA2 induced a specific decrease in mtAQP8 expression 48 hours after transfection. Each lane was loaded with 20 μg of protein. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading. (B) Densitometric analysis of mtAQP8, CPS1, and OTC expression 48 hours after transfection. (C-E) The viability of mtAQP8-knockdown hepatocytes was determined by the leakage of cytosolic lactate dehydrogenase to the culture media (C), the activity of mitochondrial succinate dehydrogenase (respiratory chain complex II), assayed by MTT (D), and the levels of hepatocyte ATP (E) 48 hours after transfection. No significant changes in cell viability were observed in hepatocytes with mtAQP8 silencing. Data are expressed as the percentage of SCR controls and represent the mean $\pm$ SE of three independent experiments. *$P < 0.05$ from SCR.

containing 1 mM ammonia. Glucagon significantly increased urea synthesis by about 120% ($P < 0.05$) in hepatocytes transfected with siRNA SCR (Fig. 5A). However, glucagon-stimulated ureagenesis was significantly reduced in AQP8 knockdown hepatocytes by 85% and 90% in siRNA1- and siRNA2-treated cells, respectively. Similar results were obtained in the presence of lower ammonia concentration (i.e., 0.3 mM). The data were as follow: glucagon-induced ureagenesis (μmol urea/10^6 cells/4 hours): 0.52 $\pm$ 0.10 (siRNA scramble) versus 0.16 $\pm$ 0.08 (siRNA1) and 0.12 $\pm$ 0.10 (siRNA2).

Carbamoyl phosphate is synthesized from ammonia by mitochondrial CPS1. This is the first step in the urea cycle, which is necessary to get rid of ammonia. Thus, to assess the integrity of urea cycle in mtAQP8 knockdown hepatocytes, we made use of carbamoyl phosphate to bypass ammonia as substrate for urea synthesis.[33] No significant changes in urea production were observed in hepatocytes with mtAQP8 silencing under carbamoyl-phosphate treatment (μmol urea/10^6 cells/4 hours, n = 3): 1.20 $\pm$ 0.30 (siRNA scramble) versus 1.25 $\pm$ 0.15 (siRNA1) and 1.00 $\pm$ 0.10 (siRNA2).

According to our hypothesis, the ureagenesis from glutamine and alanine would not require mtAQP8 as mitochondrial facilitator of ammonia. Thus, we also tested the synthesis of urea from such amino acids. Basal and glucagon-induced ureagenesis from glutamine (Figs. 4B and 5B, respectively) and from alanine (Figs. 4C and 5C, respectively) were found not to be affected in AQP8 knockdown hepatocytes.

To directly test the relevance of AQP8-facilitated mitochondrial ammonia transport in glucagon-induced ureagenesis, we made use of ^{15}N-labeled ammonium chloride. ^{15}N-labeled urea synthesis was measured via NMR as described in Materials and Methods and Supporting Fig. 2. Fig. 6A shows representative experiments of NMR studies of ureagenesis under the glucagon-stimulated condition. The ^{15}N-labeled urea synthesis was significantly reduced in mtAQP8 knockdown hepatocytes (Fig. 6A,B). As shown above for enzymatically assessed urea (see Fig. 5A, white bars), 4-hour unstimulated production of ^{15}N-urea (i.e., in the absence of glucagon) was not significantly reduced by mtAQP8 knockdown (data not shown). Thus, we estimated that the glucagon-induced ^{15}N-urea synthesis (i.e., ^{15}N-urea over unstimulated values) was significantly inhibited by approximately 90% in mtAQP8 knockdown cells (Fig. 6C).

Taken together, these results suggest that mtAQP8 plays a role in hepatocyte ureagenesis from ammonia, particularly under glucagon-stimulation, possibly facilitating mitochondrial ammonia uptake.

Hepatic mtAQP8 Expression and Mitochondrial Ammonia Transport During* In Vivo *Glucagon-Induced Ureagenesis. It has been well described that glucagon is able to stimulate ureagenesis by up-regulating the expression of urea cycle enzymes.[34–36] Thus, we decided to test if glucagon was able to induce hepatocyte mtAQP8 expression *in vivo* in the rat. Our studies indicate that after 36 hours of treatment,

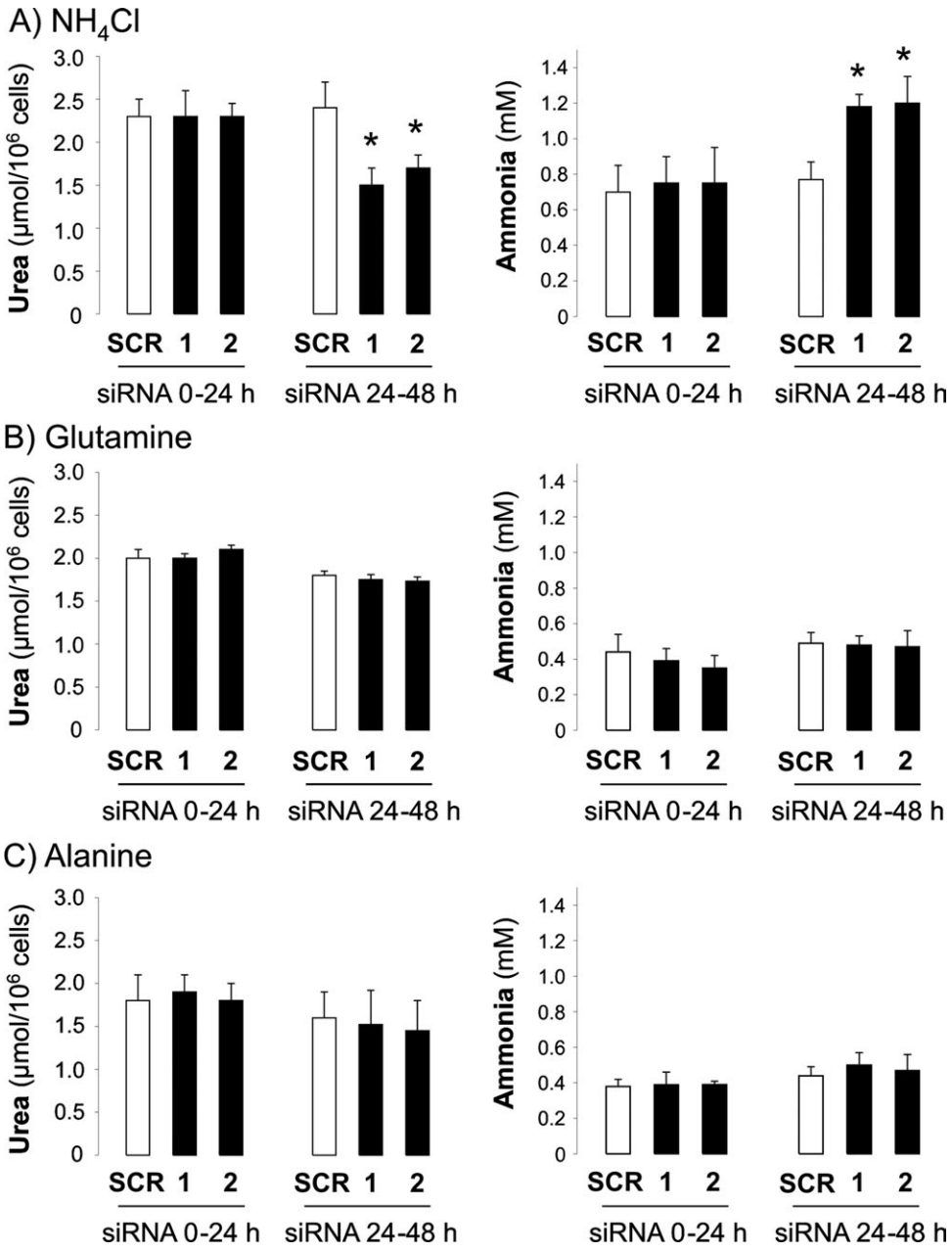

Fig. 4. Effect of rat hepatocyte mtAQP8 knockdown on ureagenesis from ammonia and amino acids. Hepatocytes were transfected with siRNA duplexes targeting specific sequences of rat AQP8 mRNA (siRNA1 and siRNA2 [1 and 2, respectively]) or a scrambled siRNA1 sequence (SCR) and were incubated in the presence of 1 mM ammonium chloride (A), 4 mM glutamine (B), or 4 mM alanine (C). Urea and ammonia were determined enzymatically in the culture media 24 and 48 hours after transfection. Only when ammonia was used as a source of nitrogen did the siRNAs induce a specific decrease in urea production and a concomitant increase in ammonia levels. This was observed 48 hours after transfection, when mtAQP8 expression was knocked down. Data represent the mean ± SE of four independent experiments. *$P < 0.05$ from SCR.

glucagon was able to increase protein expression of the urea cycle enzyme OTC by 140% ($P < 0.05$) (Fig. 7A,B) as well as the liver urea content by approximately 100% (1.1 ± 0.3 versus 2.3 ± 0.3 mg urea/g liver; $P < 0.05$; n = 4). The mtAQP8 protein expression was also found to be induced by glucagon at this time point (+160%, $P < 0.05$) (Fig. 7A,B). In agreement with the *in vivo* data, exposure of cultured hepatocytes to glucagon (1 μM) for 12 hours also led to an increase in mtAQP8 protein expression (+80%; $P < 0.05$; n = 3) (data not shown).

We then investigated mitochondrial ammonia transport in liver IMM vesicles using the ammonia analog, [^{14}C]methylamine. IMM vesicles were of good quality, as indicated by the over 10-fold enrichment of mtAQP8 from crude mitochondrial fraction (Fig. 2C). Fig. 8A shows that the uptake of [^{14}C]methylamine in liver IMM vesicles from control rats was linear up to 10 minutes at pH 7.4 and 8.2. The extent of [^{14}C]methylamine uptake in IMM vesicles increased with pH, as reported from transport studies in AQP-expressing oocytes.[37] Methylamine is a weak base (pKa 10.7) largely protonated at pH 7.4. As a result of the increasing pH, there is an increase of the unprotonated (neutral) form of the molecule, which is expected to favor diffusion across AQPs. In fact, AQP8 was found to allow the exclusive transport of the neutral ammonia molecules.[24] In line with the above results,

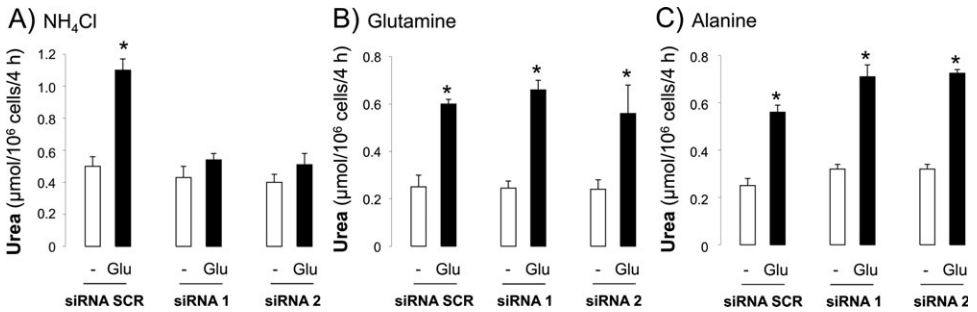

Fig. 5. Effect of rat hepatocyte mtAQP8 knockdown on the glucagon-stimulated ureagenesis from ammonia and amino acids. Control and mtAQP8 knockdown hepatocytes were incubated in presence of 1 mM ammonium chloride (A), 4 mM glutamine (B), or 4 mM alanine (C) at 37°C for 4 hours in the absence or presence of 1 μM glucagon (Glu). Urea was determined enzymatically in the culture media. Glucagon strongly stimulated ureagenesis in control (SCR) hepatocytes regardless of the source of nitrogen. In mtAQP8-knockdown cells, glucagon induced no significant stimulation only when ammonia was used as a source of nitrogen for ureagenesis. In contrast to Fig. 4A, in which ureagenesis (24-hour period) was shown to be slightly but significantly decreased, Fig. 5A (white bars) shows that the urea production in a shorter period of time (4 hours) is not found to be significantly reduced by mtAQP8 knockdown. Data represent the mean ± SE of three independent experiments. *$P < 0.05$ from corresponding non-Glu treated cells.

glucagon increased the uptake of [^{14}C]methylamine by about 80% ($P < 0.05$) in the 36-hour-treated rats, whereas no changes were seen in 8-hour-treated rats (Fig. 8B). Figure 8C shows the increase in mAQP8 expression at 36 hours (but not at 8 hours) after glucagon treatment directly assessed in the IMM vesicles used for the transport studies. Together, these observations indicate that under *in vivo* glucagon-induced ureagenesis, there is an up-regulation of hepatocyte mtAQP8 protein expression along with an increase in mitochondrial ammonia diffusional permeability. These *in vivo* data support a role for mtAQP8 in glucagon-induced ureagenesis from ammonia.

Discussion

The major finding in this study relates to the functional significance of AQP8, an aqua-ammoniaporin, in rat hepatocyte mitochondria. We provide experimental evidence that (1) the knockdown of mtAQP8 significantly reduced ureagenesis in primary cultured rat hepatocytes under basal and glucagon-stimulated

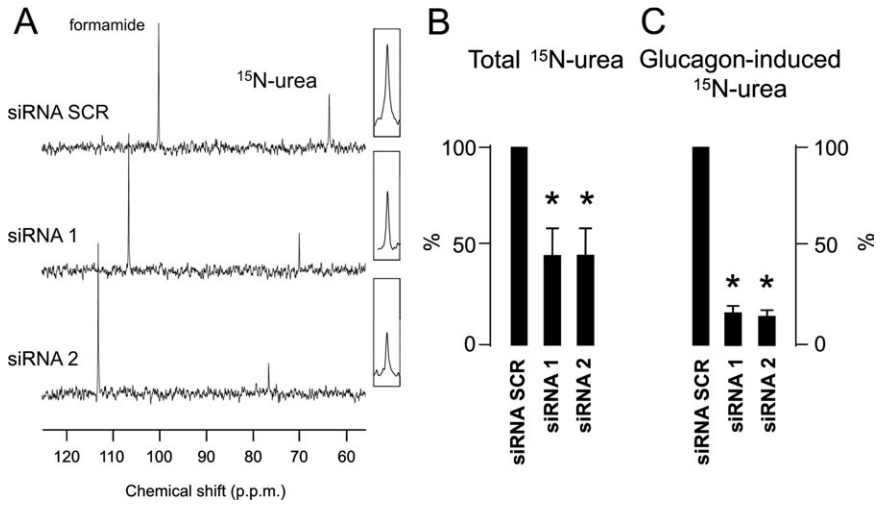

Fig. 6. Effect of rat hepatocyte mtAQP8 knockdown on glucagon-stimulated ^{15}N-labeled urea synthesis from ^{15}N-labeled ammonia. Primary cultured hepatocytes were incubated with ^{15}N-labeled ammonium chloride (1 mM) to assess the role of mtAQP8 in the glucagon-induced ureagenesis. ^{15}N-labeled urea synthesis was measured by NMR as described in Materials and Methods. (A) Representative ^{15}N-NMR spectra for urea under glucagon stimulation. (B) Quantitation of total ^{15}N-labeled urea (i.e., unstimulated + glucagon-induced) normalized to the internal standard, corresponding to four independent ^{15}N-NMR spectra. (C) Glucagon-induced ^{15}N-urea synthesis (i.e., ^{15}N-urea over unstimulated values). As shown in Fig. 5A (white bars), 4-hour unstimulated production of ^{15}N-urea (i.e., in the absence of glucagon) was not significantly reduced by mtAQP8 knockdown (data not shown). Data are expressed as the percentage of SCR controls and represent the mean ± SE of four independent experiments. *$P < 0.05$ from SCR.

conditions and (2) the glucagon-induced ureagenesis *in vivo* in rats is associated with up-regulation of both hepatic mtAQP8 protein expression and IMM ammonia permeability. An important role for mtAQP8 in facilitating mitochondrial ammonia import in ureagenesis is suggested.

Hepatocyte mitochondrial ammonia detoxification via ureagenesis is critical for prevention of hyperammonemia and hepatic encephalopathy[38,39] and mtAQP8-facilitated ammonia transport may have an important functional implication in this process. The AQP8-mediated transport of ammonia into mitochondrial matrix to sustain the urea cycle was initially speculated in works characterizing the conductance properties of the AQP8 channel.[21,23] While supported by studies showing liver mtAQP8 up-regulation in rat hypothyroidism, a condition associated with increased hepatocyte urea synthesis,[40] this possibility appeared to be weakened by a work using AQP8 null mice, where defects in ammonia metabolism (e.g., high blood ammonia) were only observed in response to high ammonia loading.[25] In our study, small but significant defects in ammonia metabolism (Fig. 4A) were also seen in mtAQP8-deficient hepatocytes exposed to a supraphysiologic concentration of ammonia (i.e., 1 mM) when considering that the ammonia levels in rat portal blood is 0.3 mM.[41]

The relevance of AQP8 ammonia-based hepatocyte ureagenesis seems to occur mainly in the presence of glucagon. This is indicated by the marked reduction of the glucagon-induced ureagenesis in hepatocytes cultured with media containing 1 mM ammonia, where

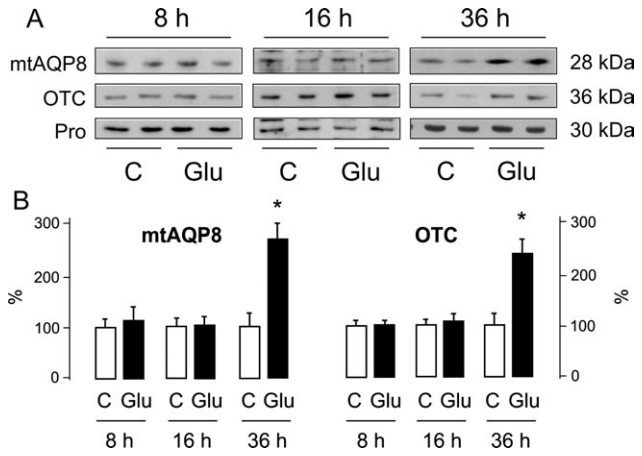

Fig. 7. Hepatic mtAQP8 protein expression during *in vivo* glucagon-induced ureagenesis. Glucagon (Glu) or vehicle (C) were administered to rats at the final dose of 0.2 mg/100 g intraperitoneally for 8, 16, and 36 hours. Three or four rats were used per experimental group. Total mitochondrial membranes were prepared as described in Materials and Methods. (A) Immunoblots for mtAQP8 and the urea cycle enzyme OTC. (B) Corresponding densitometric analysis. Data are expressed as a percentage of controls and represent the mean ± SE. *$P < 0.05$ respect to C 36 hours. Each lane was loaded with 20 μg of protein. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading.

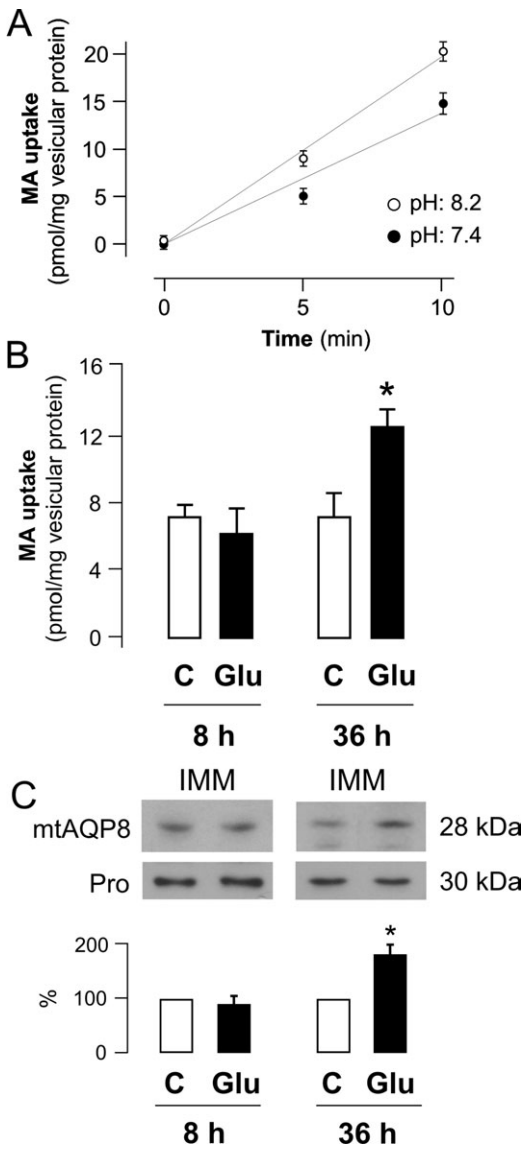

Fig. 8. Hepatic mitochondrial ammonia transport during *in vivo* glucagon-induced ureagenesis. Transport studies were performed in liver IMM vesicles using the ammonia analog [^{14}C]methylamine (MA) and the rapid filtration technique (see Materials and Methods). (A) [^{14}C]methylamine uptake in liver IMM vesicles from control rats at pH 7.4 and 8.2. The [^{14}C]methylamine uptake was linear up to 10 minutes and increased with pH due to the increase of the unprotonated form of the molecule. (B) Ammonia transport studies in liver IMM vesicles prepared from 8- and 36-hour glucagon-treated rats. The experiments were performed at 5 minutes at pH 8.2. The diffusional permeability of [^{14}C]methylamine increased by about 80% ($P < 0.05$) only in the 36-hour glucagon-treated rats. (C) Representative immunoblotting for mtAQP8 protein with the corresponding densitometric analysis in the IMM vesicles used for the transport studies of panel B. In agreement with the data presented in Fig. 7, mtAQP8 protein expression was increased only at 36-hour glucagon treatment. Data represent the mean ± SE of three independent experiments. *$P < 0.05$ respect to C 36 hours. Prohibitin (Pro), an IMM marker, is shown as a control for equal protein loading. Each lane was loaded with 20 μg of protein.

mtAQP8 had been silenced (Fig. 5A). Moreover, similar results were observed in the presence of the physiologic ammonia concentration (see Results). Strong support to the hypothetic role exerted by mtAQP8 in glucagon-induced ureagenesis also comes from the experiments using ^{15}N-labeled ammonia (Fig. 6). After being taken up into mitochondria via mtAQP8, the ammonia is expected to be incorporated to urea via mitochondrial CPS1[2] or as ^{15}N-labeled aspartate via mitochondrial glutamate dehydrogenase.[42]

Ammonia, along with bicarbonate and ATP, are substrates for the synthesis of carbamoyl phosphate by mitochondrial CPS1. This is the first step in the urea cycle, which is necessary for ammonia detoxification. The lack of effect of mtAQP8 silencing on the urea synthesis from carbamoyl phosphate (see Results), together with the unaltered hepatocyte ATP content (Fig. 3D and Results), constitutes important experimental evidence supporting the notion of an mtAQP8-mediated ammonia transport to supply urea cycle. Additional support comes from the lack of effect of mtAQP8-knockdown on ureagenesis from the amino acids glutamine and alanine (Figs. 4 and 5). This is consistent with the fact that glutamine is an intramitochondrial source of urea precursors. Glutamine is metabolized to ammonia and glutamate via glutaminase, and then glutamate forms aspartate via mitochondrial aspartate aminotransferase.[42,43] Alanine is a urea precursor mainly via mitochondrially generated aspartate.[44]

Glucagon is known to regulate hepatocyte urea synthesis via short and long-term actions. The synthesis of carbamoyl phosphate by CPS1 is considered a key step for short-term regulation of ureagenesis.[1] CPS1, with an ammonium *K*m of 1 to 2 mM, is the rate-controlling enzyme for the urea cycle. CPS1 is activated by its natural activator *N*-acetylglutamate, which is synthesized from acetyl coenzyme A and glutamate by *N*-acetylglutamate synthetase. Glucagon activates the synthesis of *N*-acetylglutamate[29,45] by mechanisms that may involve the increased provision of glutamate via glucagon-induced glutaminase activation.[46] Glucagon-induced *N*-acetylglutamate synthesis has been demonstrated to take place in isolated rat hepatocytes.[29] Thus, in our studies, it conceivable to suppose that glucagon stimulated ureagenesis via an *N*-acetylglutamate-dependent CSP1 activation, which in turn induced mitochondrial uptake of ammonia to sustain urea cycle.

In the longer term, glucagon is able to induce the hepatic urea production by up-regulating urea cycle enzyme expression.[34–36] This is consistent with what we observed in our *in vivo* studies in rats with glucagon administration (Fig. 7). Accordingly, the expression of the mitochondrial urea cycle enzyme OTC was increased in our *in vivo* studies in rats with glucagon administration (Fig. 7). The protein mtAQP8 expression was also found to be up-regulated (Fig. 7), suggesting its involvement in the *in vivo* hormone-modulated ureagenesis.

Glucagon has been described to modulate hepatic urea cycle enzyme expression at multiple levels by transcriptional, posttranscriptional, and posttranslational mechanisms.[34–36] Our previous *in vivo* experiments showed that glucagon up-regulates the glycosylated 34-kDa canalicular AQP8 protein without changing the related AQP8 mRNA levels.[47] In fact, posttranslational mechanisms seem to account for the hormone-induced plasma membrane AQP8 protein expression.[47] However, further studies are needed to determine whether these mechanisms are implicated in the *in vivo* glucagon-induced up-regulation of mtAQP8.

The fact that the mitochondrial ammonia permeability parallels the increase in mtAQP8 protein seen after glucagon treatment (Fig. 8), besides suggesting an involvement of mtAQP8 in ammonia ureagenesis, strongly supports the idea of a major involvement of AQP8 in mitochondrial ammonia uptake. In our biophysical measurements, the extent of the IMM ammonia permeability was likely underestimated due to the use of methylamine, an analog structurally similar to ammonia but of larger size. Although glucagon-induced mitochondrial ammonia permeability appears to be mainly attributable to mtAQP8 (Figs. 7 and 8), the potential involvement of additional ammonia pathways cannot be ruled out completely. Currently, the lack of specific AQP8 inhibitors precludes conclusive experimental evidence on this issue.

Following ureagenesis, the urea efflux from hepatocytes could be mediated by phloretin-inhibitable urea transporters, such as UT-A and AQP9.[48,49] No accumulation of ^{15}N-labeled urea in cytosol of mtAQP8 knockdown hepatocytes was observed (data not shown), indicating no significant alteration in terms of hepatocyte urea efflux. Accordingly, AQP9 expression was unchanged in AQP8-knockdown hepatocytes (Supporting Fig. 1).

In conclusion, our data in rat hepatocytes suggest that mtAQP8 facilitates mitochondrial ammonia transport and its metabolism into urea, mainly under glucagon stimulation. This mechanism may be relevant to hepatic ammonia detoxification and in turn, avoid the deleterious effects of hyperammonemia.

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