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NOTE

Acidosis-induced downregulation of hepatocyte mitochondrial aquaporin-8 and ureagenesis from ammonia

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Abstract: It has been proposed that, during metabolic acidosis, the liver downregulates mitochondrial ammonia detoxification via ureagenesis, a bicarbonate-consuming process. Since we previously demonstrated that hepatocyte mitochondrial aquaporin-8 channels (mtAQP8) facilitate the uptake of ammonia and its metabolism into urea, we studied whether mtAQP8 is involved in the liver adaptive response to acidosis. Primary cultured rat hepatocytes were adapted to acidosis by exposing them to culture medium at pH 7.0 for 40 h. Control cells were exposed to pH 7.4. Hepatocytes exposed to acid medium showed a decrease in mtAQP8 protein expression (–30%, $p < 0.05$). Ureagenesis from ammonia was assessed by incubating the cells with ^{15}N-labeled ammonia and measuring ^{15}N-labeled urea synthesis by nuclear magnetic resonance. Reduced ureagenesis was found in acidified hepatocytes (–31%, $p < 0.05$). In vivo studies in rats subjected to 7 days acidosis also showed decreased protein expression of hepatic mtAQP8 (–50%, $p < 0.05$) and reduced liver urea content (–35%; $p < 0.05$). In conclusion, our in vitro and in vivo data suggest that hepatic mtAQP8 expression is downregulated in acidosis, a mechanism that may contribute to decreased ureagenesis from ammonia in response to acidosis.

Key words: aquaporin-8, hepatocyte, mitochondrial ammonia transport, acidosis, ureagenesis.

Résumé : Il a été proposé que durant l'acidose métabolique, le foie régule à la baisse la détoxication mitochondriale de l'ammoniac par l'uréogenèse, un processus qui consomme du bicarbonate. Parce que les auteurs avaient précédemment démontré que l'aquaporine 8 des mitochondries (mtAQP8) des hépatocytes facilite la captation de l'ammoniac et son métabolisme en urée, ils ont examiné si la mtAQP8 est impliquée dans la réponse adaptative du foie à l'acidose. Des cultures primaires d'hépatocytes de rat ont été adaptées à l'acidose en les exposant à un milieu de culture de pH 7.0 pendant 40 h. Les cellules contrôles ont été exposées à pH 7.4. Les hépatocytes exposés au milieu acide présentaient une diminution de l'expression protéique de la mtAQP8 (–30%, $p < 0.05$). L'uréogenèse à partir de l'ammoniac a été évaluée en incubant les cellules avec de l'ammoniac marqué au ^{15}N et en mesurant la synthèse d'urée marquée au N^{15} par résonance magnétique nucléaire. Une réduction de l'uréogenèse a été observée chez les hépatocytes acidifiés (–31%, $p < 0.05$). Des études in vivo réalisées chez des rats soumis à une acidose de 7 jours ont aussi montré une diminution de l'expression protéique de la mtAQP8 hépatique (–0%, $p < 0.05$) et une réduction du contenu en urée du foie (–5%, $p < 0.05$). En conclusion, les données obtenues in vitro et in vivo suggèrent que l'expression hépatique de la mtAQP8 est régulée à la baisse par l'acidose, un mécanisme qui peut contribuer à la diminution de l'uréogenèse à partir de l'ammoniac, en réponse à l'acidose. [Traduit par la Rédaction]

Mots-clés : aquaporine-8, hépatocyte, transport mitochondrial d'ammoniac, acidose, uréogenèse.

Introduction

In the liver, two ammonia detoxification systems are present: urea and glutamine synthesis. The initial and rate-limiting step in urea cycle is the synthesis of carbamoyl-phosphate from ammonia and bicarbonate in the mitochondria of periportal hepatocytes by carbamoyl-phosphate synthetase 1 (CPS1). A fraction of the ammonia used in this reaction is provided by ammonia mitochondrial uptake from portal blood. Another fraction is provided by the metabolization of amino acids, such as glutamine and alanine. Portal blood first comes into contact with periportal hepatocytes capable of urea synthesis. Ammonia that escaped periportal detoxification reaches perivenous hepatocytes, where it would be used for glutamine synthesis (Häussinger 1983, 1990; Wright et al. 2011). Glutamine is delivered to the kidney. Increased mitochondrial ammonia synthesis from glutamine in proximal tubules and its urinary excretion are key responses during metabolic acidosis to restore acid–base balance (Curthoys et al. 2007).

Some studies have demonstrated that during metabolic acidosis the liver decreases ureagenesis (a process that consumes bicarbonate) and increases glutamine synthesis (Bean and Atkinson 1984; Häussinger 1990; Nissim et al. 1992; Phromphetcharat et al. 1981). However, the mechanisms involved are not completed elucidated (Boon et al. 1994; Häussinger 1990). By contrast, some authors argue against a role of the liver in the regulation of acid–base homeostasis and propose that ureagenesis is only controlled by the need to eliminate nitrogen waste (Halperin et al. 1986;

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Hosch et al. 2004; Lardner and O'Donovan 1997). Nitrogen metabolism and acid–base balance are essential to normal health. Since impairment of urea and ammonia metabolism results from and also can lead to a variety of clinical conditions, it is worthwhile to obtain further insight into the role of liver ureagenesis in acid–base homeostasis (Weiner et al. 2014; Wright et al. 2011).

Aquaporin-8 is a member of a family of homologous membrane channel proteins demonstrated to facilitate the diffusional transport of ammonia (Holm et al. 2005; Liu et al. 2006; Saparov et al. 2007). There is evidence suggesting that mitochondrial AQP8 (mtAQP8) can facilitate ammonia transport across inner mitochondrial membranes (Molinas et al. 2012; Soria et al. 2010, 2013). Recently, using a human renal proximal tubule cell line, we reported that the *knockdown* of mtAQP8 expression decreased ammonia excretion in response to acidosis, suggesting that mtAQP8 plays an important role in the adaptive response of proximal tubule to acidosis, possibly facilitating mitochondrial ammonia transport. Moreover, acidosis upregulated mtAQP8 in renal proximal cells (Molinas et al. 2012). In addition, we demonstrated that mtAQP8 *knockdown* reduced ureagenesis from ammonia in hepatocytes, suggesting that mtAQP8 is involved in the mitochondrial uptake of ammonia and its metabolism into urea (Soria et al. 2013).

Based on the evidence that (*i*) mtAQP8 in proximal renal cells facilitates ammonia transport (Molinas et al. 2012), (*ii*) its expression is regulated during metabolic acidosis (Molinas et al. 2012), (*iii*) hepatic mtAQP8 is involved in the mitochondrial uptake of ammonia and its metabolism into urea (Soria et al. 2013), and (*iv*) there is evidence for a regulation of urea synthesis in the hepatic response to metabolic acidosis (Häussinger 1990), we think that hepatic mtAQP8 may contribute to the liver adaptive response to maintain acid–base homeostasis. In the present study, using in vivo and in vitro models, we investigated the effects of acidosis on hepatic mtAQP8 expression and ureagenesis from ammonia.

Materials and methods

Isolation and culture of hepatocytes

Hepatocytes were isolated from normal livers of male Wistar rats (Soria et al. 2013) and grown in DMEM (high glucose, with 1 mmol/L sodium pyruvate and without L-glutamine) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and 1 mmol/L $^{15}NH_4Cl$. Cultures were adapted to acidosis by incubating them at pH 7.0 medium for 40 h (Molinas et al. 2012). We used pH 7.0, based on studies that found a decrease in urea synthesis at this pH (Bean and Atkinson 1984; Boon and Meijer 1988). At lower pH values (6.9) viability was decreased after 40 h of culture (data not shown). Medium was changed every 20 h. Control cells were grown at pH 7.4 medium. Cells were harvested and mitochondrial fractions were prepared as previously described (Soria et al. 2013). Lactate dehydrogenase (LDH) activity was measured in culture medium to assess cell viability using a commercial kit (LDH-P UV AA. Wiener Lab., Argentina).

Assessment of ^{15}N-labeled urea by nuclear magnetic resonance spectroscopy (NMR)

Primary cultured hepatocytes were incubated in pH 7.4 or 7.0 medium in the presence of 1 mmol/L ^{15}N-labeled NH_4Cl (99% enriched in ^{15}N, Cambridge Isotope Laboratories, Andover, Massachusetts, USA) for 40 h in the absence of glutamine. The rate of ^{15}N-labeled urea synthesis from ammonia was measured in the medium over the last 20 h of the 40 h period by NMR, as previously described (Soria et al. 2013).

Animal model

Adult male Wistar rats were treated according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health). Protocols were approved by local Animal Welfare Committee. To induce chronic acidosis, animals were given 0.28 mol/L NH_4Cl/2% sucrose in the drinking water for 7 days (Molinas et al. 2012). Control groups received 2% sucrose. During the last 16 h, animals were housed in metabolic cages to collect urine. Rats were anesthetized with sodium thiopental (70 mg/kg b.w., i.p.) and heparinized arterial blood was collected from the femoral artery. Blood and urine acid–base status was analyzed, as previously described (Molinas et al. 2012). Livers were homogenized to isolate mitochondrial fractions (Soria et al. 2013). Urea was determined enzymatically by the urease method in liver homogenates (Soria et al. 2013).

Immunoblotting

Western blotting of isolated mitochondria from rat liver or primary cultured hepatocytes was performed as previously described by our laboratory (Soria et al. 2013), using affinity purified antibody directed against a N-terminal peptide of rat AQP8, rabbit antibody against rat CPS1 (Sigma-Aldrich), or rabbit antibody against human prohibitin (Abcam).

Statistical analysis

Data are expressed as mean ± SEM. Significance was determined using Student's *t*-test; $p < 0.05$ was considered statistically significant.

Results

Effects of acidosis on mtAQP8 expression and ureagenesis from ammonia in hepatocytes

Primary cultured rat hepatocytes were exposed to control (pH 7.4) or acidic (pH 7.0) medium for 40 h. Cell viability determined by LDH-release assay exhibited no significant differences (pH 7.4: 100 ± 15; pH 7.0: 115 ± 6%, $n = 6$). Consistent with this, examination by light microscopy demonstrated that hepatocytes exposed to pH 7.0 remained adherent and morphologically similar to control.

In acidified hepatocytes, mtAQP8 protein expression was significantly decreased while CPS1was unaltered (Fig. 1A).

Ureagenesis from ammonia was measured by NMR in hepatocytes incubated in medium without glutamine (an intramitochondrial ammonia source) and supplemented with ^{15}N-labeled ammonia (Soria et al. 2013). As shown in Figs. 1B and 1C, ^{15}N-labeled urea synthesis was significantly reduced in hepatocytes exposed to pH 7.0.

Hepatic mtAQP8 expression and urea content in a rat model of chronic metabolic acidosis

Chronic metabolic acidosis was induced by NH_4Cl loading, an established rodent model (Molinas et al. 2012; Nissim et al. 1992; Phromphetcharat et al. 1981). As shown in Fig. 2A, metabolic acidosis was confirmed by the decrease in plasma pH and bicarbonate. Acidotic rats presented increased urinary but normal blood ammonia.

As shown in Fig. 2B, mtAQP8 protein levels were significantly decreased after 7 days of acidosis. CPS1was not affected (Fig. 2B). Liver urea content in acidotic rats was significantly decreased (Fig. 2C), suggesting reduced urea synthesis.

Discussion

While the role of the kidney in the adaptive response to acidosis is well characterized, there is still controversy about the role of the liver. The main purpose of the present study was to gain further insight into the role of mtAQP8 in the hepatic adaptive response to maintain acid–base homeostasis. Our major finding was that hepatic mtAQP8 expression was downregulated in in vitro and in vivo models of acidosis. Moreover, we provided additional evidence in favor of decreased ureagenesis from ammonia in response to acidosis.

Some studies have shown that urea synthesis from glutamine and other amino acids is reduced in response to acidosis by mechanisms that include inhibition of liver amino acid uptake and

Fig. 1. Effect of acidosis on mtAQP8 expression and ureagenesis from ammonia in primary cultured rat hepatocytes. Primary cultured rat hepatocytes were exposed to control (pH 7.4) or acidic (pH 7.0) medium for 40 h. Hepatocytes were incubated with ^{15}N-labeled ammonium chloride (1 mmol/L) as source of nitrogen. (A) mtAQP8 and CPS1 protein abundance evaluated by immunoblotting in hepatocyte mitochondria. Densitometric analysis for 3 separate experiments are shown. Prohibitin (Pro), an inner mitochondrial membrane protein, is shown as control for equal protein loading. Data (means ± SEM) are expressed as a percentage of controls. *$p < 0.05$ vs. pH 7.4. (B) ^{15}N-urea synthesis was measured by NMR as described in Materials and methods. Representative ^{15}N-NMR spectra for urea in pH 7.4 or 7.0 culture medium are shown. The peaks corresponding to ^{15}N-urea are shown in the enlarged insets on the right. (C) Quantitation of total ^{15}N-labeled urea normalized to the internal standard, corresponding to 3 independent ^{15}N-NMR spectra. Data are expressed as the percentage of control and represent the mean ± SEM. *$p < 0.05$ *vs.* pH 7.4.

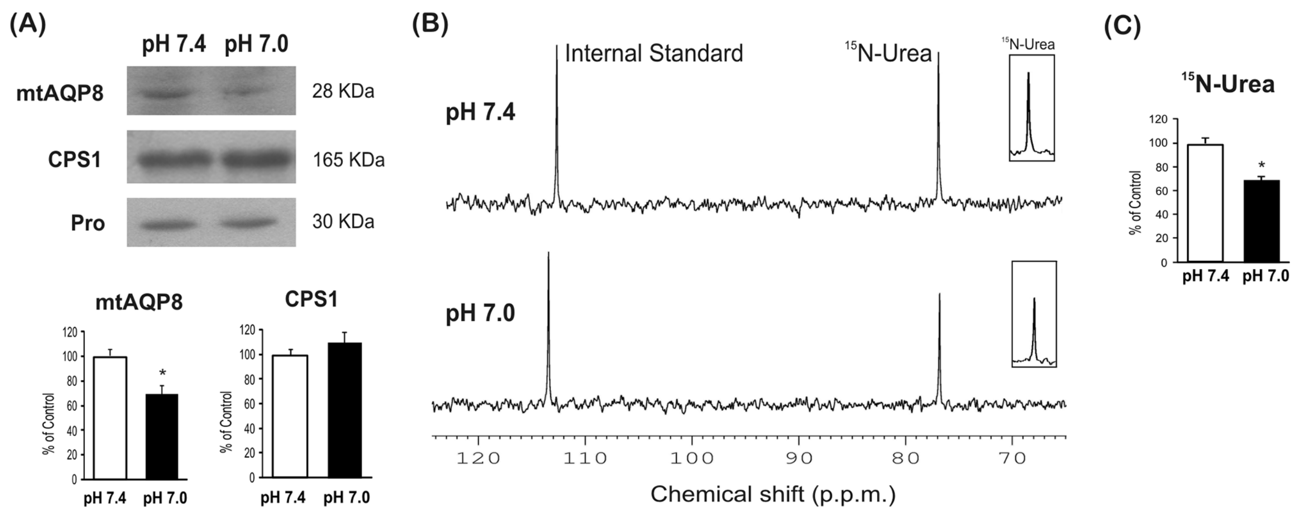
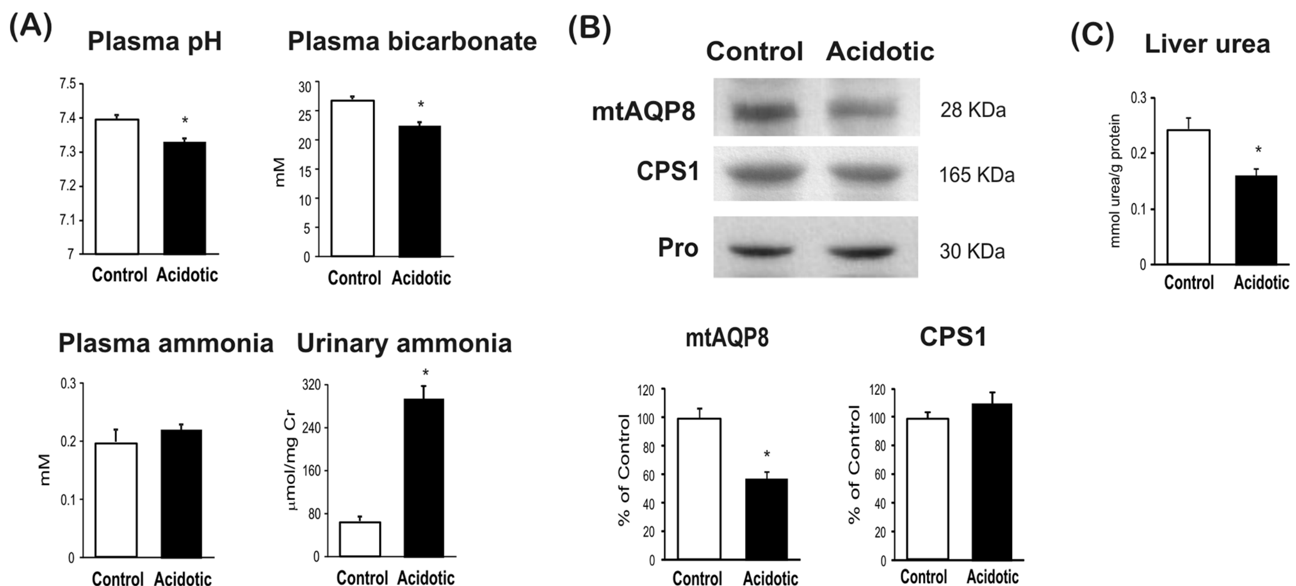


Fig. 2. Blood and urine analysis, hepatic mtAQP8 expression, and urea content in rats with metabolic acidosis. Metabolic acidosis was induced as described in Material and methods. (A) pH, bicarbonate, and ammonia concentration in blood, and urinary ammonia concentration measured in control and acidotic rats. Urine and arterial blood samples were collected as described in Materials and methods. Results are expressed as means ± SEM. $n = 6$. *$p < 0.05$ *vs.* control. Cr, creatinine. (B) mtAQP8 and CPS1 protein abundance evaluated by immunobloting in mitochondria isolated from liver of control and acidotic rats. Densitometric analysis for mtAQP8 and CPS1 protein abundance of 6 rats per experimental group are shown. Prohibitin (Pro), an inner mitochondrial membrane protein, is shown as control for equal protein loading. Data (means ± SEM) are expressed as a percentage of controls. *$p < 0.05$ vs. control. (C) Urea content in liver homogenates from control and acidotic rats. Results are expressed as means ± SEM. $n = 6$. *$p < 0.05$ vs. control. Results are expressed as mmol urea per g of liver protein.



consumption (Boon and Meijer 1988; Boon et al. 1994, 1996). Consistent with our results, reduced urea synthesis from ammonia was observed in experiments in which isolated liver (Häussinger and Gerok 1985) or hepatocytes (Bean and Atkinson 1984; Boon and Meijer 1988) were perfused or incubated with NH_4Cl as nitrogen source. Several mechanisms have been proposed, including inhibition of hepatic mitochondrial carbonic anhydrase (Häussinger and Gerok 1985), the degree of ammonia protonation (Rémésy et al. 1986), and decreased synthesis of the CPS1 activator, N-acetylglutamate (Kamemoto and Atkinson 1985). In the present study we found that acidosis in vitro and in vivo did not change CPS1 protein expression. Consistent with our

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findings, different studies reported no decrease in CPS1 activity in response to metabolic acidosis in rat (Lardner and O'Donovan 1997, 1998; Welbourne and Joshi 1986). Although we could not disregard that some of the mechanisms mentioned above could be responsible for the reduced urea synthesis found in the present study, we propose that mtAQP8 downregulation, by reducing mitochondrial uptake of ammonia, could be an additional mechanism that contributes to reduce ureagenesis at low pH.

Although the administration of NH_4Cl in vivo constitutes a nitrogen load per se and a potential ureagenic stimulus (Phromphetcharat et al. 1981), our present study and previous results of other researchers (Nissim et al. 1992; Phromphetcharat et al. 1981) showed that after chronic NH_4Cl^- loading, urea synthesis was reduced in rats. In this chronic model of acidosis, reduced ureagenesis could be a compensatory mechanism of the liver to spare bicarbonate consumption.

During metabolic acidosis, the kidney displays a wide change of gene expression, which is spatially and temporally regulated (Curthoys et al. 2007; Nowik et al. 2008). One of the complex adaptive responses that the kidney exhibits in response to metabolic acidosis is the pronounced increase in ammonia production and excretion. Adaptive response of proximal tubule to partially restore plasma bicarbonate and pH involves the regulation of relevant transporters and enzymes (Curthoys et al. 2007; Nowik et al. 2008), including key mitochondrial ammoniagenic enzymes. In the liver, although not as deeply studied as in kidney, some genes change their expression in response to acidosis (López-Flores et al. 2006; Masuda et al. 2003). mtAQP8 downregulation by acidosis could represent a homeostatic response of the liver to regulate acid–base balance. Thus, in response to metabolic acidosis, mtAQP8 downregulation in periportal hepatocytes would contribute to reduce ammonia availability to supply urea cycle (decreasing bicarbonate consumption) and increase ammonia availability in perivenous hepatocytes to stimulate glutamine synthesis.

Based on our previous results in renal proximal cells that showed that mtAQP8 was upregulated in response to metabolic acidosis (Molinas et al. 2012), we propose there could exist a tissue-specific regulation as a consequence of different ammonia transport physiological needs. Accordingly, a differential regulation in liver and kidney in response to metabolic acidosis has been also reported for genes involved in gluconeogenesis (López-Flores et al. 2006; Masuda et al. 2003).

In summary, using in vitro and in vivo models, we have demonstrated that hepatic mtAQP8 expression and ureagenesis from ammonia were reduced in response to acidosis. Our data suggest that mtAQP8 may play a contributing role in the adaptive response of hepatocyte to acidosis.

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