Defective hepatocyte aquaporin-8 expression and reduced canalicular membrane water permeability in estrogen-induced cholestasis

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1Instituto de Fisiología Experimental and 2Instituto de Biología Molecular y Celular de Rosario, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina; 2Dipartimento di Zoologia and 3Dipartimento di Fisiologia Generale ed Ambientale, Università degli Studi di Bari, Bari, Italy

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Carreras FI, Lehmann GL, Ferri D, Tioni MF, Calamita G, Marinelli RA. Defective hepatocyte aquaporin-8 expression and reduced canalicular membrane water permeability in estrogen-induced cholestasis. Am J Physiol Gastrointest Liver Physiol 292: G905–G912, 2007. First published November 16, 2006; doi:10.1152/ajpgi.00386.2006.—Our previous work supports a role for aquaporin-8 (AQP8) water channels in rat hepatocyte bile formation mainly by facilitating the osmotically driven canalicular secretion of water. In this study, we tested whether a condition with compromised canalicular bile secretion, i.e., the estrogen-induced intrahepatic cholestasis, displays defective hepatocyte AQP8 functional expression. After 17α-ethinylestradiol administration (5 mg·kg body wt⁻¹·day⁻¹ for 5 days) to rats, the bile flow was reduced by 58% (P < 0.05). By subcellular fractionation and immunoblotting analysis, we found that 34 kDa AQP8 was significantly decreased by ~70% in plasma (canalicular) and intracellular (vesicular) liver membranes. However, 17α-ethinylestradiol-induced cholestasis did not significantly affect the protein level or the subcellular localization of sinusoidal AQP9. Immunohistochemistry for liver AQPs confirmed these observations. Osmotic water permeability (P) of canalicular membranes, measured by stopped-flow spectrophotometry, was significantly reduced (73 ± 1 vs. 57 ± 2 μm/s) in cholestasis, consistent with defective canalicular AQP8 functional expression. By Northern blotting, we found that AQP8 mRNA expression was increased by 115% in cholestasis, suggesting a posttranscriptional mechanism of protein level reduction. Accordingly, studies in primary cultured rat hepatocytes indicated that the lysosomal protease inhibitor leupeptin prevented the estrogen-induced AQP8 downregulation. In conclusion, hepatocyte AQP8 protein expression is downregulated in estrogen-induced intrahepatic cholestasis, presumably by lysosomal-mediated degradation. Reduced canalicular membrane AQP8 expression is associated with impaired osmotic membrane water permeability. Our data support the novel notion that a defective expression of canalicular AQP8 contributes as a mechanism for bile secretory dysfunction of cholestatic hepatocytes.

AQUAPORIN (AQP) water channels are a family of integral membrane proteins known to facilitate the osmotic water movement across the cellular membranes (3). Three members of the AQP family are expressed in rat hepatocytes: AQP8 (7, 13, 16, 21), AQP9 (14, 21, 40), and AQP0 (21). AQP8 is localized in the canalicular plasma membrane domain (7, 13, 16, 21) as well as in intracellular vesicles (7, 13, 16, 21) and mitochondria (6). Its trafficking from a vesicular compartment to the canalicular membrane can be induced by a choleretic stimulus, such as dibutylryl-cAMP (16, 21) or the hormone glucagon (18, 19, 30). AQP9 resides exclusively on the sinusoidal plasma membranes of hepatocytes and thereby may facilitate the movement of water and certain small solutes (14, 21). AQP0 is localized intracellularly, and its significance is currently unclear (21).

Bile secretion results from the coordinated interactions of several solute membrane-transport systems and involves the movement of water into the biliary space in response to transient osmotic gradients generated by active solute transport (31). There is recent evidence suggesting that AQP8 facilitates the osmotically induced canalicular membrane water transport (28) and bile formation by rat hepatocytes (21). Thus it is conceivable that defective AQP membrane expression may lead to alterations of normal bile physiology. Currently, there is no conclusive evidence indicating that derangements of normal AQP function are causative of bile secretory dysfunction. Nevertheless, we found a downregulated expression of rat hepatocyte AQP8 in obstructive extrahepatic cholestasis, a pathological condition in which altered bile secretion occurs (8).

Estrogens are well known to cause intrahepatic cholestasis, whose clinical manifestations are oral contraceptive-induced cholestasis and cholestasis associated with pregnancy or post-menopausal replacement therapy (22). Experimental cholestasis induced by estrogen administration to rodents, mainly 17α-ethinylestradiol (EE), is an established experimental model to assess the mechanisms of estrogen-induced cholestasis (22). This model has been widely used to investigate alterations in the expression of hepatocyte membrane transporters associated with intrahepatic cholestasis (23, 37, 39). The aim of this study was to test whether a condition with compromised canalicular bile secretion, such as the estrogen-induced intrahepatic cholestasis, displays a defective hepatocyte AQP8 functional expression.

MATERIALS AND METHODS

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-h light-dark cycle, according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), EE (Sigma-Aldrich, St.

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glucose-6-phosphatase), mmol product formed

plated onto collagen-coated glass plates at 3.8

exclusion) was greater than 86%. Freshly isolated hepatocytes were

mechanical disruption (16). Cell viability (assessed by Trypan blue

from normal livers of male Wistar rats by collagenase perfusion and

microsomal membranes; ND, not detected.

Table 1. Assessment of plasma and microsomal membrane purity

<table>
<thead>
<tr>
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<th>Control</th>
<th>EE</th>
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<tbody>
<tr>
<td></td>
<td>H</td>
<td>PM</td>
</tr>
<tr>
<td>Plasma membrane 5'-nucleotidase</td>
<td>0.8±0.0</td>
<td>11.3±1.8</td>
</tr>
<tr>
<td>Microsomal glucose-6-phosphatase</td>
<td>3.6±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>Lysosomal acid phosphatase</td>
<td>0.1±0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Mitochondrial aspartate aminotransferase</td>
<td>0.4±0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Protein yield</td>
<td>173.3±6.7</td>
<td>4.2±0.1</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 3). Specific activities are expressed as μmol product formed·mg protein⁻¹·h⁻¹ (plasma membrane 5'-nucleotidase and microsomal glucose-6-phosphatase), mmol product formed·mg protein⁻¹·h⁻¹ (mitochondrial aspartate aminotransferase), and μmol product formed·μg protein⁻¹·min⁻¹ (lysosomal acid phosphatase). Protein yield is expressed as mg protein/g liver. EE, ethinylestradiol; H, homogenate; PM, plasma membrane; IM, intracellular microsomal membranes; ND, not detected.
Before staining, semithin sections were incubated for 5 min at 37°C in resin Technovit 8100 (Heraeus-Kulzer, Wehrheim, Germany) at 4°C with 6.8% sucrose, dehydrated with acetone, and embedded in the resin. The samples were incubated overnight in phosphate-buffered saline and then sliced and fixed by immersion with 4% paraformaldehyde. The livers from control and EE-treated rats were perfused with phosphate-buffered saline to eliminate the blood flowing in the liver vessels. The sections were cut, washed, and blocked with serum in PBS (pH 7.4) before incubation with the primary antibodies. The sections were then incubated with a biotinylated secondary antibody and developed with an ABCElite kit (Vector). The sections were then washed and incubated with peroxidase-antiperoxidase at a dilution of 1:100 for 1 h at 37°C. Finally, the immunolabeling was visualized by incubation with 3,3'-diaminobenzidine-H2O2 medium for 10 min at room temperature. Controls were performed by omitting the primary antibodies. Images were captured with an E 600 photomicroscope equipped with a DMX 1200 digital camera (Nikon, Kawasaki, Japan).

Northern blotting. Total RNA from livers of control and EE-treated rats was isolated by the TRIzol reagent (Invitrogen) following the manufacturer’s protocol. RNA samples (20 μg per lane) were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Hybond-N+, Amersham Biosciences). Hybridization was performed in 50% formamide, 5× saline-sodium citrate, 5× Denhardt’s solution, and 0.1% SDS at 42°C for 20 h, with a specific rat AQPh complementary DNA probe labeled with [α-32P]deoxyctydine 5’-triphosphate. The membranes were washed and autoradiographed with intensifying screens for 5 days. Expression of the AQPh mRNA was normalized against the expression of the 28S rRNA which was not altered with the treatment.

Statistical analysis. Data are expressed as means ± SE. Significance was determined by Student’s t-test or the 1-way ANOVA, Tukey’s test; P < 0.05 was considered statistically significant.

RESULTS

Administration of EE to rats daily during 5 days resulted in intrahepatic cholestasis as indicated by a decrease in bile flow and both a significant decrease in body weight and increase in liver weight, in agreement with results reported elsewhere (11). Bile flow was reduced by 58% (P < 0.05) from 1.9 ± 0.3 μl·min⁻¹·g liver⁻¹ in control rats to 0.8 ± 0.2 μl·min⁻¹·g liver⁻¹ in EE-treated rats.

Expression of AQPh proteins in EE-induced cholestatic liver. To study the AQPh protein expression and subcellular localization in EE-induced cholestasis, we initially performed immunoblotting on liver membrane fractions. Compared with controls, AQPh protein levels were significantly reduced in all membrane fractions, i.e., 81% in total membranes, 87% in plasma membranes, and 76% in intracellular membranes (Fig. IA). EE treatment did not significantly affect AQPh protein...
levels in total or plasma membranes (Fig. 1B). Consistent with the predominant hepatocyte surface localization of AQP9 (21), intracellular membranes showed no detectable signal (data not shown).

Immunohistochemical studies in serial liver sections were performed to confirm the expression and subcellular localization of hepatocyte AQP8s 8 and 9 in EE-induced cholestasis (Fig. 2). Immunohistochemical data were in agreement with the quantitative immunoblotting analysis. As previously observed (7, 8, 21), AQP8 in control rat livers was found to be predominantly intracellular in hepatocytes, although some immunoreactivity was observed on the plasma membrane (Fig. 2A). After EE administration, AQP8 staining was markedly decreased (Fig. 2B). The staining pattern for AQP9 in control livers was in agreement with previous results (21), i.e., AQP9 was mainly observed on the hepatocyte sinusoidal plasma membranes (Fig. 2D). AQP9 staining remained unaltered in EE-treated rat livers (Fig. 2E). Immunohistochemical controls showed no labeling when the corresponding primary antibody was omitted (Fig. 2, C and F). Similar immunostaining patterns were observed in three separate control and EE-treated rats.

The pair-feeding experiments indicated that EE reduced AQP8 protein expression in total liver membranes (assessed by immunoblotting) by 78% \((P < 0.001)\) and bile flow by 58% \((P < 0.05)\). AQP9 protein expression was found not to be altered. These data did not differ from those obtained with rats allowed to have free access to food.

**Water permeability of canalicular membranes from EE-induced cholestatic liver.** To study whether EE-induced AQP8 downregulation caused a reduction in canalicular water permeability, we assessed the osmotic water permeability by a stopped-flow method in which vesicles were subjected rapidly (1 ms) to a hypertonic osmotic gradient. The time course of vesicle volume was followed from the change in scattered light. Figure 3A shows the typical tracings of a time course of scattered light intensity (water transport) in canalicular plasma membrane vesicles from normal and EE-cholestatic livers in response to a 300 mosM hypertonic sucrose gradient. No change in scattered light was observed when vesicles were mixed with isosmotic buffer, showing an absence of mixing artifacts. Data fit well to a single exponential function indicating the presence of functional homogenous populations of canalicular vesicles. The corresponding calculated \(P_f\) values are shown in Fig. 3B. The canalicular \(P_f\) value for control rats was 73 ± 1 \(\mu m/s\), comparable to that reported previously (28). Canalicular \(P_f\) for EE-cholestatic rats was significantly reduced \((57 ± 2 \mu m/s, P < 0.05)\). Figure 3C shows, consistent with data from Fig. 1A, a significant reduction of canalicular AQP8 protein levels in EE cholestasis.

The size of control and EE canalicular plasma membrane vesicles was not significantly different. Mean canalicular vesicle diameter from control liver was 176 ± 7 nm \((n = 1,102)\), and mean canalicular vesicle diameter from EE-treated liver was 160 ± 9 nm \((n = 1,018)\). Thus the decreased canalicular \(P_f\) in EE-treated rats cannot be due to a difference in either the initial vesicle size or the purification of the membranes (see MATERIALS AND METHODS).

Expression of AQP8 mRNA in EE-induced cholestatic liver.

To begin to explore the mechanisms of EE-induced AQP8 protein downregulation, we assessed the steady-state mRNA levels by Northern blot analysis. AQP8 mRNA expression was found not to be reduced, actually increased by 115% with the treatment (Fig. 4), suggesting the involvement of posttranscriptional mechanisms.

**Role of lysosomal and proteasomal proteolytic pathways in estrogen-induced AQP8 downregulation.** An in vitro model was established to study whether a posttranscriptional mechanism, such as an increased proteolysis, is involved in the AQP8 protein downregulation observed in estrogen cholestasis. As shown in Fig. 5A, the hepatocyte protein expression of AQP8s 8 and 9 (but not that of the organic anion transporter Mrp2) was stably maintained under our culture conditions. This seems to be a particular feature of hepatocyte AQP8s, since most of the solute transporters (including Mrp2) are quickly downregulated in primary hepatocytes (35). In agreement with the in vivo data (see Fig. 1), we found that the cholestatic estrogen E17G decreased hepatocyte AQP8 protein expression, whereas that of AQP9 was unaltered (Fig. 5B). The lysosomal protease inhibitor leupeptin, but not the proteasome inhibitor MG132, prevented the estrogen-induced AQP8 downregulation (Fig. 5B). These data suggest a lysosomal-mediated degradation of AQP8 protein in estrogen-induced cholestasis.
DISCUSSION

Our results demonstrate for the first time that the expression of hepatocyte AQP8 water channels as well as the canalicular membrane water permeability is downregulated in estrogen-induced intrahepatic cholestasis. This supports the novel concept that a defective AQP canalicular expression contributes as a mechanism for bile secretory dysfunction of cholestatic hepatocytes.

Bile formation is an osmotic secretory process that involves the secretion of osmotically active solutes, followed by the passive movement of water into the biliary space. Water may move across the hepatic epithelial cells by either of two pathways: a paracellular pathway between the tight junctions of adjacent cells or a transcellular pathway across the cells. Although the quantitative contribution of these two pathways of water transport is still unclear, current experimental evidence appears to favor the transcellular pathway (5, 9, 27–29). AQPs partially account for the water permeability of both hepatocyte plasma membrane domains, AQP9 facilitating the basolateral movement of water and AQP8 modulating its canalicular, rate-limiting, transport (28). Thus the generation of bile flow would be ultimately dependent on the molecular and functional expression of solute transporters in the canalicular plasma membrane domain as well as on the canalicular membrane water permeability determined by the level of AQP expression and the lipid composition. The biliary excretion of bile salts, via the bile salt transporter Bsep, glutathione, via the organic anion transporter Mrp2, and HCO₃⁻/H₂CO₃ exchanger AE2, are thought to be the major osmotic driving forces for canalicular bile flow (31). Canalicular AQPs would allow the efficient coupling of osmotic solutes and water transport during bile formation.

As observed in this and previous studies (6, 7, 13, 16, 21), rat hepatocyte AQP8 is localized in intracellular compartments as well as on the plasma (canalicular) membrane. Nevertheless, there are conflicting data about AQP8 subcellular localization. Thus, whereas immunohistochemical and immunogold electron microscopy studies showed similar AQP8 localization (i.e., in intracellular compartments and on canalicular membrane) between mouse and rat hepatocytes (15), studies from other investigators showed a predominant plasma membrane (basolateral) localization of mouse AQP8 with no major con-
Fig. 5. Effect of lysosomal and proteasome inhibitors on estrogen-induced AQP8 downregulation in cultured hepatocytes. Rat hepatocytes were cultured and subjected to subcellular fractionation as described in MATERIALS AND METHODS. A: expression of AQP8, AQP9, and Mrp2 after 3 h of cell attachment (time 0) and after additional 20 h of culture. Representative immunoblots of 3 separate experiments in total hepatocyte membrane fraction (20 μg protein/lane). Expression of AQP8 8 and 9 were not significantly changed, whereas that of Mrp2 decreased by 80% (P < 0.05). B: primary cultured rat hepatocytes were preincubated for 30 min in the presence of the lysosomal protease inhibitor leupeptin (250 μM) or the proteasome inhibitor MG132 (10 μM), and then for additional 20 h in the absence (−) or presence (+) of the estrogen (10 μM; E, estradiol-17β-glucuronide). Anti-AQP8 and anti-AQP9 immunoblots of total hepatocyte membranes (20 μg protein/lane) and corresponding densitometric analysis (n = 4) are shown. Data (means ± SE) are expressed as percentage of controls. *P < 0.05 compared with controls.

Water transport through the cell membranes may either occur through the lipid bilayer or be channel mediated. We previously provided evidence for the presence of both lipid- and AQP-mediated pathways for water movement across hepatocyte canalicular plasma membranes (28). The lipid membrane pathway can be disturbed by increasing cholesterol content, which is known to reduce water permeability (12). Nevertheless, because canalicular lipid composition (including cholesterol level) is known to be unaltered in EE cholestasis...
(4), the data strongly suggest a decrease in the fraction of water that moves through AQPs instead of through the lipid bilayer. The AQP-mediated water pathway was roughly estimated to contribute by \( \sim 30\% \) to total canalicular water transport under nonstimulated (basal) conditions (28). In agreement with this, present data showed that a decrease of 60% in canalicular AQP8 expression in cholestasis (see Fig. 3C) is associated with a 22% reduction in membrane water permeability. This alteration in canalicular water permeability may be sufficient to impair the efficient coupling between osmotic solutes and water transport during bile formation.

Our data show that steady-state AQP8 mRNA levels are increased in EE-treated rat livers. Similar observations (i.e., decrease protein and increased AQP8 mRNA) were previously observed by us in obstructive extrahepatic cholestasis (8), suggesting a common compensatory mechanism for AQP protein reduction in cholestasis. Besides, as we observed in extrahepatic cholestasis (8), AQP8 downregulation does not seem to be associated with any major alteration of AQP8 mRNA structure, as judged by the Northern blotting studies, or AQP8 molecular mass (34 kDa), which indicates that its glycosylation process properly. Our data suggest, as previously for both Bsep and Mrp2 (23), the involvement of some posttranscriptional mechanism (e.g., increased protein degradation) for AQP8 protein downregulation in estrogen-induced cholestasis. Other members of the AQP family of proteins (i.e., AQPs 1, 2, 4, and 5), which are not expressed in hepatocytes, have been described to be targeted for proteolysis through the lysosomal and/or the proteasome system (20, 24, 26, 36). Our studies in primary cultured hepatocytes support the notion of an estrogen-driven lysosomal degradation of AQP8, since the lysosomal protease inhibitor leupeptin, but not the proteasome inhibitor MG132, specifically prevented AQP8 downregulation. An increased lysosomal proteolysis has been implicated in the Mrp2 downregulation in obstructive cholestasis (34), but, to our knowledge, degradative pathways have not been explored for the known downregulation of solute transporters in estrogen-induced cholestasis. Our data indicate that estrogen treatment was able to decrease plasma membrane as well as intracellular (vesicular) AQP8 (see Fig. 1A). Whether estrogens drive AQP8 to the lysosomal degradation pathway by causing either mistrafficking of canalicularly targeted AQP8-containing vesicles or endocytic retrieval of canalicular AQP8 needs further investigation. Nevertheless, it is worth mentioning that after short-term treatment, E17G, in contrast to that observed for Mrp2 and Bsep (10, 32), failed to cause endocytic retrieval of canalicular AQP8 (33).

In conclusion, hepatocyte canalicular AQP8 (but not sinusoidal AQP9) protein expression is downregulated in estrogen-induced cholestasis, presumably by increased lysosomal-mediated degradation. Reduced AQP8 expression is associated with impaired canalicular membrane water permeability. Our data support the novel notion that a defective expression of canalicular AQP water channels contributes as a mechanism for bile secretory dysfunction of cholestatic hepatocytes.

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