

## Hepatocyte aquaporins in bile formation and cholestasis

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## 1. ABSTRACT

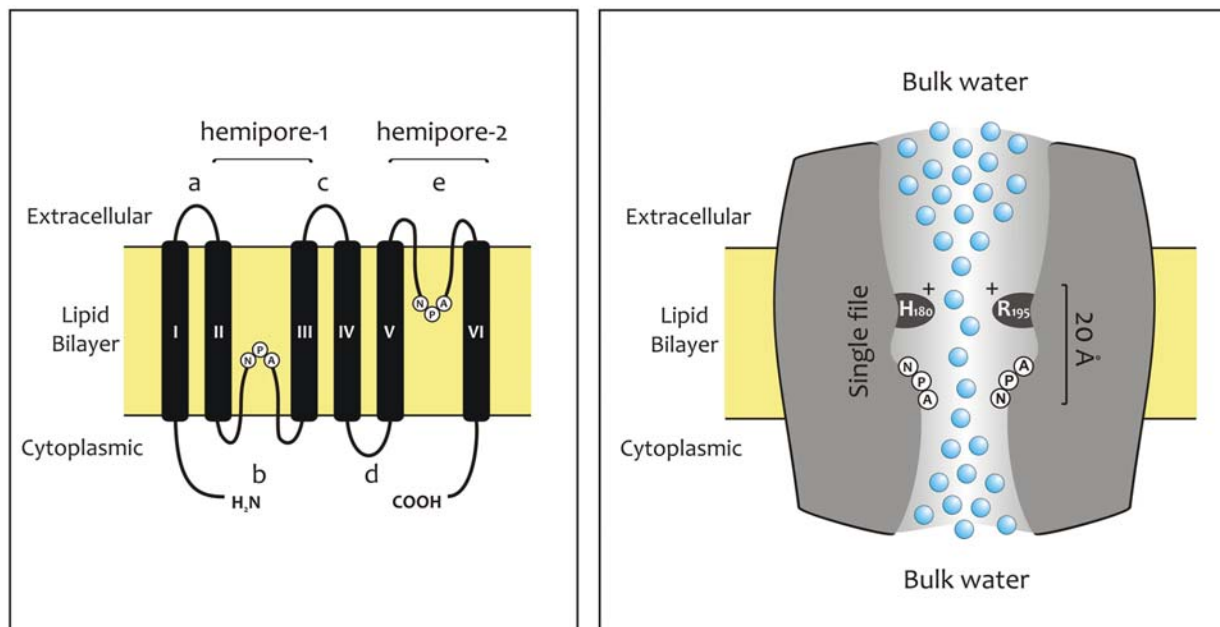
Bile formation by hepatocytes is an osmotic secretory process that is ultimately dependent on the biliary secretion of osmotically-active solutes (mainly bile salts) via specialized canalicular transporters as well as on the water permeability of the canalicular plasma membrane domain. Hepatocytes express aquaporins, a family of membrane channel proteins that facilitate the osmotically-driven movement of water molecules. Aquaporin-8 (AQP8), localized to canalicular membranes, modulates membrane water permeability providing a molecular mechanism for the osmotically-coupled transport of solute and water during bile formation. There is experimental evidence suggesting that defective hepatocyte AQP8 expression leads to alterations in normal bile physiology. Thus, AQP8 protein is downregulated (and canalicular water permeability decreased), in established rat models of cholestasis, such as sepsis-associated cholestasis, estrogen-induced cholestasis and extrahepatic obstructive cholestasis. Moreover, AQP8 gene silencing in the human hepatocyte-derived cell line HepG2 inhibits canalicular water secretion. Based on current knowledge, it is conceivable that cholestasis results from a mutual occurrence of impaired solute transport and AQP8-mediated decrease of canalicular water permeability.

## 2. MAMMALIAN AQUAPORINS

Aquaporins (AQPs) are a family of integral membrane channels which facilitate osmotically-driven water movement. Thirteen mammalian AQPs, numbered 0 to 12, have been identified thus far (1). The first AQP was identified in erythrocytes by Dr. Peter Agre (2), work for which he was awarded the 2003 Nobel Prize in Chemistry.

AQPs are homotetramers, i.e., they consist of four independent channels assembled into a functional unit. Each subunit is composed of approximately 270 amino acids with cytoplasmic carboxy- and amino-terminal ends. They consist of six bilayer-spanning domains connected by five loops. Two of these loops enclose the conserved motif asparagine-proline-alanine (NPA), which is part of the aqueous pore (3) (see Figure 1).

Several studies revealed the high-resolution structures of the AQP protein family. They confirm the so-called “hourglass model” and explain their selectivity. AQPs consist of wide extracellular and intracellular vestibules, joined at the centre by a narrow region of approximately 20 angstrom in length that shapes the filter responsible for water selectivity. Within the vestibules, water exists in bulk solution, while in the centre of the



**Figure 1.** Membrane topology and functioning of the aquaporin molecule. *Left panel:* Each monomer consists of six membrane spanning domains connected by loops A to E, with two highly conserved NPA boxes shaping the water-selective pore and the amino and carboxy termini oriented towards the cytoplasm. *Right panel:* The water channel consists of two vestibules containing water in bulk solution joined at the middle by a central constriction of 20 angstrom in length where water passes in a single file. The a/R constriction is delimited by arginine (R195) and histidine (H180) providing fixed positive charges to impede proton permeation. A subsequent constriction is delimited by two asparagine residues from the highly conserved NPA motif.

channel water transits in single file. In fact, a two-stage filter in the central region of the pore has been identified: an outer barrier termed the aromatic/arginine constriction (ar/R), and the central constriction or NPA region (Figure 1). The three key features for water channel selectivity are (3; 4):

(i) Size restriction. The pore narrows to a diameter of 2.8 angstrom (approximately the diameter of a water molecule).

(ii) Electrostatic repulsion. The ar/R constriction is composed by a highly conserved arginine residue in the 195 position and a nearby histidine residue in the 180 position. This filter provides a supplementary criteria for solute selection, as the conserved residues have a strong positive charge that repels protonated water. In the NPA region, water-water interactions are distorted so water molecules remain isolated from their solvation shell in the bulk, a process essential for the filter selectivity.

(iii) Water dipole reorientation. As water molecules approximate to the constriction, the oxygen atom rotates towards the asparagine residues from NPA motifs (asparagines 76 and 192) and creates new hydrogen bonds between the oxygen and the asparagine residues. This dipole reorientation breaks the hydrogen bonds among water molecules and avoids the passage of protonated water.

AQPs are characteristically inhibited by mercurials. The inhibitory site corresponds at the Cys-189,

proximal to the NPA motif in loop E (5). Certain AQPs (e.g., AQP4) do not have the cysteine at this site leading to the lack of inhibition by mercurials (6).

AQPs work primarily as water-transporting channels, although some of them are also permeable to certain small molecules including glycerol (7), ammonia (8), and hydrogen peroxide (9).

AQPs have been shown to be expressed in several types of epithelia. Typically, one or more specific AQPs exist in a particular water-transporting epithelial cell. They are either constitutively expressed or regulated upon physiological needs, often by agonist-induced trafficking from an intracellular vesicular compartment to the plasma membrane allowing for rapid changes in membrane permeability.

Four AQPs were demonstrated to be expressed in mammalian hepatocytes from different species, i.e. AQP8 (10; 11; 12; 13; 14), AQP9 (13; 15; 16), AQP0 (13), and AQP11 (17), which display different subcellular localizations and permeabilities (see Table 1).

## 2.1. Hepatocyte aquaporin-8 (AQP8)

AQP8 has been identified in rat, mouse, and human hepatocytes (10; 11; 12; 13; 14; 18; 19). AQP8 is a water channel that was also found to have permeability to ammonia (8) and hydrogen peroxide (9). Initial biochemical, confocal immunofluorescence, and immunoelectron microscopy studies (10; 12) showed that

**Table 1.** AQP8 expressed in hepatocytes

AQP	Permeability	Species				Subcellular Localization
		Rat	Mouse	Human	Pig	
8	Water/NH <sub>3</sub> /H <sub>2</sub> O <sub>2</sub>	+ (10; 11; 12; 13; 14)	+ (18; 83)	+ (19)	ND	ICV – CPM – Mitochondria
9	Water/glycerol/urea/certain small uncharged molecules	+ (13; 15; 16)	+ (41)	+ (37)	+ (38)	BLM
0	Water	+ (13)	ND	- (19)	ND	ICV
11	Water	+ (17)	+ (84)	ND	ND	ND

Abbreviations: ICV: intracellular vesicles; CPM: canalicular plasma membrane; BLM: basolateral plasma membrane; NH<sub>3</sub>: ammonia; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide; ND: not determined. References are given in parenthesis.

rat hepatocyte AQP8 localized to the canalicular plasma membrane domain and pericanalicular vesicles as an N-glycosylated protein of about 34 kDa. More recent studies revealed that AQP8 in hepatocytes is also present in mitochondria (20). Indeed, a non-glycosylated 28 kDa form of the AQP8 protein was found in the mitochondrial inner membrane of rat liver and several other tissues (20). AQP8 displays regulated vesicle trafficking in hepatocytes. Thus, the hormone glucagon stimulates the translocation of AQP8-containing vesicles to the canalicular plasma membrane domain (21). Canalicular AQP8 seems to be exocytically inserted in sphingolipid-enriched microdomains (i.e. rafts) (22; 23). Glucagon-stimulated AQP8 trafficking requires the activation of cAMP-PKA and PI3K as well as an intact microtubular network (21; 24). The hormone-induced increase of canalicular AQP8 levels is accompanied by an augment in membrane water permeability (12). Hence, hepatocytes are able to hormonally regulate their canalicular membrane water permeability and promote osmotically-driven water secretion via AQP8.

In addition to the described short-term regulation of canalicular AQP8 by vesicle trafficking, AQP8 can also be modulated on the long-term basis by modifying its gene expression. Accordingly, it was recently found that glucagon posttranscriptionally upregulates AQP8 protein expression in rat hepatocytes, a process that also involves the dual requirement of cAMP-PKA and PI3K signal pathways (25). Cross-talk between PKA and PI3K signaling pathways has been already suggested for the regulated translocation of Bsep to the hepatocyte canalicular membrane (26). These observations may reflect the need for a cooperative action between PKA and PI3K on a single downstream effector. Consistent with this, it has been reported that in rat hepatocytes, cAMP can activate protein kinase B/Akt, a downstream PI3K effector (26; 27). Interestingly, cAMP-PKA mediated phosphorylation of the p85 regulatory subunit of PI3K has been reported recently. This was suggested to be an important point of convergence of cAMP-PKA and PI3K signals (28).

In rodents subjected to fasting, a condition associated to increased circulating levels of glucagon, hepatic AQP8 is downregulated (18) while AQP9 is upregulated (29). Therefore, the fact that glucagon *per se* is able to increase AQP8 expression without changing that of AQP9 suggests that factors other than glucagon determine liver AQP expression during fasting.

Although the mechanisms underlying posttranscriptional stimulation of hepatocyte AQP8 by glucagon remain to be elucidated, these seem to be dependent on AQP8 protein degradation rather than on altered protein synthesis. Recent inhibitory experiments performed in rat cultured hepatocytes suggest that calpains, but not lysosomal or proteasomal degradation pathways, are implicated in the regulation of basal AQP8 protein expression (25). This suggests that the hepatocyte AQP8 upregulation induced by glucagon may result from an impaired calpain-mediated proteolysis.

Therefore, glucagon is able to regulate hepatocyte canalicular AQP8 on short and long term basis by stimulating its vesicle trafficking and gene expression, respectively. The increased amounts of canalicular AQP8 promote the osmotic water transport (30), a process that is likely to be relevant to glucagon-induced bile formation (21).

As mentioned above, AQP8 is present in hepatocyte mitochondria. Studies from Calamita and collaborators using several experimental approaches, including immunoelectron microscopy (18; 20; 31), have clearly demonstrated the expression of AQP8 in rat and mouse liver mitochondrial inner membranes. A study by Yang *et al.* (32) failed to detect mitochondrial AQP8 in mouse liver by immunoblotting. Although the reason for this discrepancy is unclear, it is likely associated to the nature of the anti-AQP8 antibody used. In agreement with Dr. Calamita's finding, we recently found that the human hepatic cell line HepG2 also expresses AQP8 at mitochondrial level (unpublished data). Surprisingly, AQP8 did not appear to have major relevance to the facilitated transport of water across mitochondrial membranes (32; 33; 34). Nevertheless, AQP8 significantly increases mitochondrial ammonia permeability (35), which suggests that ammonia diffusional transport constitutes a major role for AQP8 in mitochondria. The physiological significance of hepatic mitochondrial AQP8 in ammonia detoxification via ureagenesis is still an open question. Protein expression of mitochondrial AQP8 was also found to undergo hormonal regulation. Thus, the thyroid hormone triiodothyronine, T<sub>3</sub>, which is known to modulate metabolic activities of liver mitochondria, is able to induce the transcriptional downregulation of rat liver mitochondrial AQP8 (31). The physiological significance of this finding requires further studies.

## 2.2. Hepatocyte aquaporin-9 (AQP9)

Hepatocyte AQP9 is an approximately 32 kDa protein identified in rat, mouse, pig, and human hepatocytes (15; 29; 36; 37; 38). Immunohistochemical,

confocal immunofluorescence as well as immunoelectron microscopy studies demonstrated that AQP9 is localized on the hepatocyte surface, specifically on the sinusoidal plasma membrane domain, with no significant intracellular expression (15). AQP9 expression seems to be gender-dependent since male rats have higher levels of AQP9 protein and mRNA than female rats (16).

AQP9 is an aquaglyceroporin, i.e., a water channel membrane protein also permeable to certain small uncharged solutes such as glycerol. This AQP is thought to allow the rapid cellular uptake or exit of metabolites with minimal osmotic perturbation. AQP9 was demonstrated to play a key role in the hepatic metabolism of glycerol (39; 40). Hepatocyte AQP9 expression was found to be dependent on the nutritional status (29; 41). Accordingly, AQP9 was observed to be markedly induced in fasted rats; a state in which glycerol is actively taken up by liver for gluconeogenesis. No changes in liver AQP9 levels were observed in rats fed ketogenic or high-protein diets. Hepatocyte AQP9 expression is also affected by circulating insulin levels (29; 41). Hence, liver AQP9 levels were found to be elevated in diabetic rats and decreased after administration of insulin. The presence of a negative insulin response element in the AQP9 promoter gene (29) suggests an insulin-mediated suppression of AQP9 transcription. A recent study in the normal human liver cell line L02, confirmed that insulin treatment inhibits the expression of AQP9 protein and mRNA through a mechanism involving the PI3K signal transduction pathway (42).

AQP9 has also been implicated in the process of apoptotic cell death. Hepatocytes undergoing apoptosis decrease their volume due to changes in intracellular ion concentration and the exit of water, events that are required for the activation of proapoptotic enzymes. Thus, AQP9 is thought to be involved in hepatocyte apoptosis facilitating the water movement out of the cell (43).

AQP9 is likely to play a role in bile formation facilitating water uptake through the sinusoidal hepatocyte membranes during active biliary water secretion (30). Nevertheless, no conclusive evidence for a major role of AQP9 in bile formation has been provided thus far.

### 2.3. Hepatocyte aquaporin-0 (AQP0)

AQP0, the major intrinsic protein of lens, is an AQP with the particularity of having low water permeability (44). AQP0 has been found to be expressed in rat hepatocytes (13) and in the rat hepatoma/human fibroblast hybrid cell line WIF-B (45). AQP0 is located mainly in intracellular compartments, although it has some canalicular expression in rat hepatocytes. Rat AQP0 is not responsive to cAMP, suggesting that it does not undergo regulated vesicle trafficking (13). We found that HepG2 cells and normal human liver samples do not express any significant amount of AQP0 protein (19), which suggests that AQP0 plays no role in human canalicular bile formation.

### 2.4. Hepatocyte aquaporin-11 (AQP11)

The most recent AQP identified in rat liver is AQP11. AQP11 is likely to be expressed in hepatocytes.

However, its cellular and subcellular localization are yet to be established. Functional expression studies in *Xenopus* oocytes failed to demonstrate permeability to water, glycerol, urea or ions (17). In contrast, AQP11 reconstituted into liposomes shows high water permeability (46). The reason for this discrepancy is unknown. The presence of AQP11 in human hepatocytes as well as its physiological function remain to be studied.

## 3. ROLE OF AQPS IN CANALICULAR BILE FORMATION

Bile formation by hepatocytes is an osmotic secretory process resulting from the canalicular secretion of water in response to osmotic gradients created by the active transport of solutes. The biliary excretion of bile salts, via the bile salt transporter Bsep, glutathione, via the organic anion transporter Mrp2, and  $\text{HCO}_3^-$ , via the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2 are thought to be the major osmotic driving forces for canalicular bile formation (47; 48). Bile flow is ultimately dependent on the molecular and functional expression of these canalicular transporters as well as on the water permeability of the canalicular plasma membrane domain mainly determined by AQPs. This is assuming that water molecules flow across hepatocyte epithelial barrier largely across a transcellular route, with minimal paracellular contribution. In fact, current experimental evidence indicates that the transcellular pathway accounts for most of the water entering the bile canaliculus. Initial studies in polarized rat hepatocyte couplets showed that AQP blockers totally prevented osmotically-driven water transport into the bile canaliculus (13). Moreover, high basolateral and canalicular osmotic membrane water permeabilities were found in purified membrane preparations (30), which support a predominant transcellular route for water movement. In addition, this study demonstrated that the canalicular membrane has lower water permeability than the basolateral membrane, and thus it is rate-limiting for transcellular water transport in hepatocytes. These findings were later supported by experiments in rat hepatocyte couplets (49).

Our recent study using siRNA silencing of AQP8 expression in the human hepatocyte cell line HepG2 showed that AQP8 knockdown markedly inhibited the osmotically-driven basal and cAMP-induced canalicular water secretion (19) providing conclusive evidence not only for a predominant transcellular route but also for the key role of AQP8 in bile canalicular water transport.

AQP8 gene silencing in hepatocytes has also been accomplished by developing transgenic AQP8-null mice (50). Surprisingly, the AQP8-knockout does not seem to affect membrane water permeability of isolated mice hepatocytes. This suggests, in disagreement with the above mentioned findings in rat hepatocytes (30) and human HepG2 cells (19), that AQP8 does not play an important role in mouse canalicular water transport. Nevertheless, direct water transport studies in canalicular membrane vesicles were not performed. On the other hand, deletion of a single AQP might not significantly affect cell water transport because other known or still unidentified AQPs

**Table 2.** Molecular and functional expression of AQP8 in cholestasis

Experimental model	AQP8		AQP9		Ref
	Protein	CPM $P_f$	Protein	BLM $P_f$	
Obstructive cholestasis					
- BDL	↓↓	ND	↓↓↓	↓	(61; 63)
Intracellular cholestasis					
- EE-induced cholestasis	↓↓↓	↓	↔	ND	(66)
- LPS-induced cholestasis	↓↓↓	↓	↔	ND	(75)
- CLP-induced cholestasis	↓↓↓	ND	↔	ND	(82)

Arrows depict significant protein changes in treated rats compared with controls: ↑: increased; ↓: decreased; ↔: without change; ND: not determined;  $P_f$ : osmotic membrane water permeability; CPM: canalicular plasma membrane; BLM: basolateral plasma membrane; BDL: bile duct ligation; EE: 17- $\alpha$ -ethinylestradiol; LPS: lipopolysaccharide; CLP: cecal ligation and puncture.

could undergo compensatory upregulation. The study also showed that AQP8-null mice challenged with a high-fat diet did not show a significantly different phenotype compared to their wild-type counterparts. The lack of dietary fat misprocessing could suggest that the excretion of bile salts required for proper lipid digestion was at least preserved. However, direct studies on bile formation in the AQP8-null mice are necessary to assess if these animals develop cholestasis.

The advantages of siRNA technology for specific and effective inhibition of endogenous gene expression are becoming apparent (51), and it has been used for assessing the role of AQP8 in water transport in cholangiocytes (52; 53), colonocytes (54), astrocytes (55), and vascular endothelial cells (56). Using this technology, we provide direct experimental evidence supporting the notion that AQP8 mediates the water transport necessary for canalicular bile secretion. This strongly suggests that AQP8 is required for the efficient coupling of osmotically active solutes and water transport during canalicular bile formation in human hepatocytes.

Canalicular bile flow is known to be modulated by glucagon (57). The osmotic gradients involved in glucagon-induced choleresis are thought to be created by the facilitated transport of  $\text{HCO}_3^-$  via the canalicular AE2 (57). There is evidence to suggest that glucagon (via cAMP) is able to stimulate the microtubule-dependent vesicle trafficking of hepatocyte AE2 to the canalicular plasma membrane (57). This mechanism, together with direct activation of the exchanger, could account for the bicarbonate-rich choleresis induced by glucagon.

It has been demonstrated in rat cholangiocytes that AE2 together with AQP1 and CFTR chloride channels are packaged in the same population of vesicles possibly conforming a functional bile secretory unit (58). Interestingly, studies in the hepatoma-derived hybrid cell line WIF-B show that AQP8 and AE2 colocalize in intracellular vesicles (45). Thus, hepatocytes may also contain vesicles with functionally related proteins that can account for ion-driven water transport during bile formation. AQP8 would allow the efficient coupling of canalicular water transport to the  $\text{HCO}_3^-$  secreted by AE2 during glucagon-stimulated hepatocyte bile formation.

Therefore, AQP8 can account for the water permeability of both hepatocyte plasma membrane

domains, AQP8 modulating the canalicular, rate-limiting water transport, and AQP9 contributing to the sinusoidal uptake.

#### 4. SIGNIFICANCE OF AQPS IN CHOLESTASIS

Bile secretion is compromised in several diseases with risk of severe liver injury and systemic syndrome. It is well known that hepatocyte bile secretion results from the coordinated interaction of several solute membrane-transport systems (47). As detailed above, recent cumulative experimental evidence indicates that AQPs also play a physiological role in bile formation facilitating osmotic water transport. Biliary water plays key functions, i.e., to prevent the precipitation or adsorption of biliary constituents to the biliary epithelia so that flow is not obstructed, and to act as the liquid vehicle for bile acids and for molecules destined to excretion, such as cholesterol, bilirubin, xenobiotics, etc. Therefore, it is conceivable that a derangement of normal AQP function causes a decrease of biliary water secretion, which in turn, would contribute to bile secretory dysfunction.

Defective expression of AQPs was found to be present in experimental pathological conditions in which altered bile secretion occurs, i.e. in extrahepatic (obstructive) cholestasis and in intrahepatic (hepatocellular) cholestasis, such as estrogen-induced or sepsis-associated cholestasis. The expression of hepatocyte AQPs in experimental cholestasis is summarized in Table 2.

##### 4.1. Extrahepatic (obstructive) cholestasis

Extrahepatic cholestasis is a pathological condition caused by biliary obstruction leading to impaired canalicular bile secretion (59). Bile duct ligation in rats has been extensively used as an experimental model to assess changes in the expression of hepatocyte membrane transporters in obstructive cholestasis (60). In bile duct-ligated rats, we found defective hepatocyte AQP8 expression and impairment in AQP8 translocation to the plasma membrane (61). AQP8 expression in hepatocytes tends to quickly normalize after the bile duct ligation is released (62). The fact that AQP8 mRNA levels are increased in bile duct-ligated rats (61) suggests that posttranscriptional events are involved in AQP8 protein reduction. The two major canalicular solute transporters, Mrp2 and Bsep are also downregulated in bile duct-ligated rats (60). Nevertheless, the mechanisms involved seem to be mainly transcriptional. Thus, the impaired canalicular

expression of these solute transporters and AQP8 may cause bile secretory dysfunction in obstructive cholestasis. However, these changes in transporter expression may also be regarded as an adaptive response to the biliary obstruction leading to a reduction in the water movement into the biliary space. In addition, the functional and molecular expression of sinusoidal AQP9 was found to be decreased in bile duct-ligated rats mainly by impaired plasma membrane trafficking (63). Hence, the reduced sinusoidal water uptake might also work as another contributing factor in obstructive cholestasis.

### 4.2. Estrogen-induced cholestasis

Estrogens are known to cause intrahepatic (hepatocellular) cholestasis in susceptible women during pregnancy, administration of oral contraceptives and postmenopausal hormone replacement therapy (59). The administration of 17 $\alpha$ -ethinylestradiol (EE) to rats has been a widely accepted animal model to elucidate the molecular pathomechanisms of estrogen-induced cholestasis (64; 65). EE administration to rats causes a reduction of bile flow, and an impairment of various transport systems in both basolateral and canalicular membranes of hepatocytes (59). We recently found that in EE-induced cholestasis, the expression of hepatocyte AQP8 protein is downregulated whereas that of AQP8 mRNA is increased (66). Same results were later obtained by inducing EE cholestasis in pregnant rats (67). Cholestatic rats showed a reduction in the osmotic membrane water permeability of the hepatocyte canalicular membrane domain, which is in complete agreement with canalicular AQP8 downregulation. On the other hand, EE caused no significant changes in the expression of hepatocyte AQP9 (66).

The canalicular transport proteins, Bsep and Mrp2, are also downregulated in EE-treated rats (60; 68). Even though HCO<sub>3</sub><sup>-</sup> excretion is impaired in EE-treated rats, the functional activity of AE2 is not affected (69). Contrary to sinusoidal AQP9, expression of the solute basolateral transporters, Na<sup>+</sup> taurocholate cotransporting polypeptide Ntcp, and the Na<sup>+</sup>-independent organic anion transporting polypeptide Oatp are also reduced in EE-induced cholestasis (5; 70).

Similarly to what has been suggested for Bsep and Mrp2 (60), AQP8 protein downregulation in EE-induced cholestasis seems to mainly involve posttranscriptional mechanisms (e.g. increased protein degradation). In fact, inhibitory studies in primary cultured rat hepatocytes indicated that the EE-induced AQP8 protein downregulation was mediated by increased lysosomal degradation. EE is able to decrease plasma membrane as well as intracellular (vesicular) AQP8 in hepatocytes (66). Whether estrogens derive 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. It is however worth to mention that acute cholestasis in rats caused by the estrogen metabolite estradiol-17-D-glucuronide, in contrast to that observed for Mrp2 and Bsep (71; 72), failed to cause endocytic retrieval of canalicular AQP8 (73).

In conclusion, EE-induced intrahepatic cholestasis shows downregulation of hepatocyte AQP8 expression together with a decrease in the canalicular membrane water permeability. Both would contribute as mechanisms for bile secretory dysfunction of cholestatic hepatocytes.

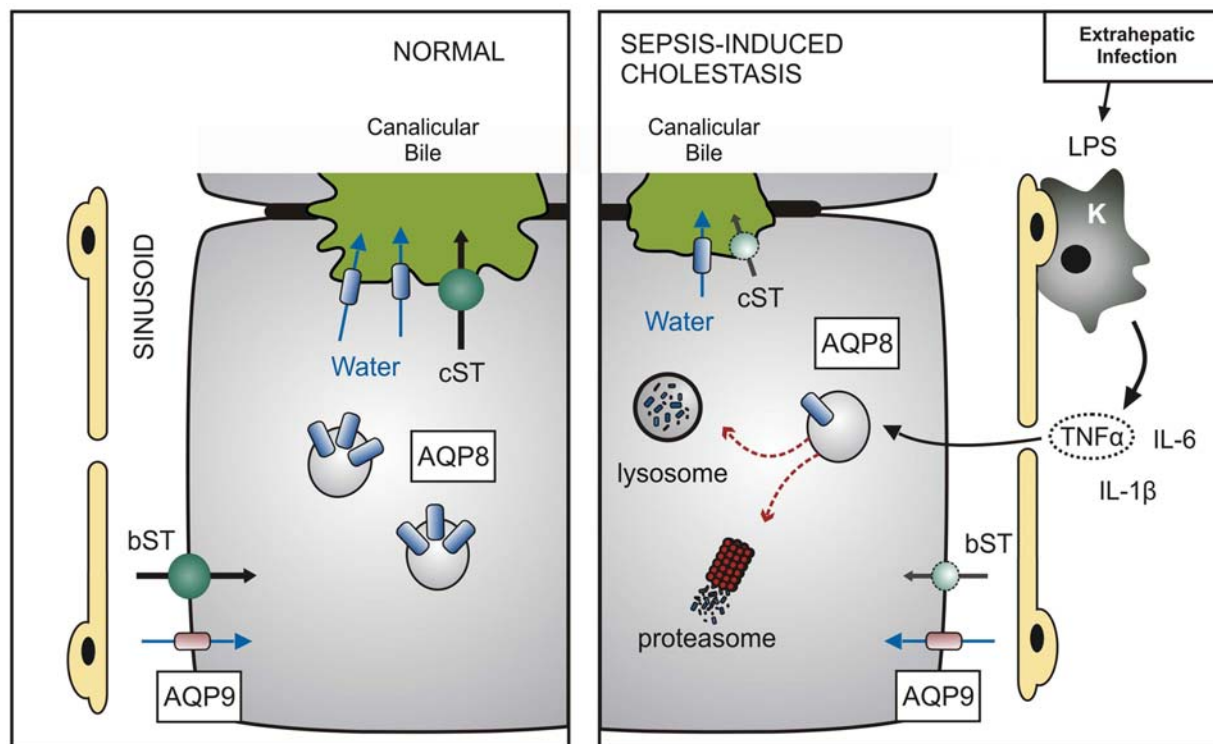
### 4.3. Sepsis-associated cholestasis

Cholestasis frequently occurs in patients with sepsis. This form of cholestasis is thought to be mediated by endotoxins, i.e., lipopolysaccharides (LPS) released into the circulation from bacterial sites of infection (74). LPS induce the secretion of proinflammatory cytokines such as tumor necrosis factor alpha (TNFalpha), interleukin-1beta and interleukin-6 mainly from Kupffer cells. Hepatocytes respond to these cytokines with a reduction in gene expression of certain solute transport proteins, which in turn would lead to bile secretory failure (74).

Treatment of rats with LPS is a widely used model for studying the pathophysiological mechanisms of sepsis-associated cholestasis. Using this experimental model, we found a significant posttranscriptional downregulation of hepatocyte AQP8 expression together with a decrease in the osmotic canalicular membrane water permeability (75). Conversely, the expression and subcellular localization of AQP9 was unaffected. Hepatocyte expression of Bsep and Mrp2 is also downregulated in LPS-treated rats (60). Both canalicular export proteins undergo a combination of transcriptional and posttranslational (i.e. canalicular retrieval) downregulation in response to LPS (76). Although HCO<sub>3</sub><sup>-</sup> excretion into bile was shown to be impaired in endotoxemic rats, the functional and molecular basis of this phenomenon remain to be clarified (77). In contrast to the lack of effect of LPS treatment on sinusoidal AQP9, the expression of some solute sinusoidal transporters, e.g. Ntcp (78) is reduced transcriptionally as well by rapid retrieval from the basolateral membranes (79). Studies using human liver slices suggest that the LPS-induced downregulation of Mrp2 and Bsep is mainly post-transcriptional (80).

The steady-state AQP8 mRNA levels are increased in LPS-induced cholestasis. Downregulation of AQP8 protein expression, concurrently with an increase in AQP8 mRNA levels were also observed in obstructive (61) and EE-induced cholestasis (66), which suggests a common compensatory mechanism. AQP8 downregulation does not seem to be related to any major modifications in the AQP8 transcript or AQP8 molecular mass, an indicator of proper protein glycosylation.

TNFalpha and other proinflammatory cytokines were demonstrated to be mediators in sepsis-induced cholestasis by altering the expression of canalicular solute transporters (81). In agreement, *in vivo* TNFalpha inactivation studies showed a complete prevention of AQP8 downregulation and bile flow reduction in LPS-treated rats. Studies in cultured rat hepatocytes using recombinant TNFalpha confirmed these results and also allowed to demonstrate that TNFalpha stimulates AQP8 protein degradation via lysosomal and proteasomal pathways (75).



**Figure 2.** Role of hepatocyte AQP8 in the mechanisms of sepsis-induced cholestasis. *Left panel:* proposed model for normal plasma-to-bile water transport in hepatocytes. Osmotically-active solutes (i.e. bile salts and endogenous and exogenous organic anions) are taken up via basolateral solute transporters (bST; i.e. the  $\text{Na}^+$  taurocholate cotransporting polypeptide Ntcp and the  $\text{Na}^+$ -independent organic anion transporting polypeptide Oatp) and secreted into the bile canaliculi via canalicular solute transporters (cST). The biliary excretion of bile salts, via the bile salt transporter Bsep, glutathione, via the organic anion transporter Mrp2, and  $\text{HCO}_3^-$ , via the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger AE2 are thought to be the major osmotic driving forces for canalicular bile formation. AQP9 is expressed at the basolateral plasma membrane, while AQP8 is expressed at the canaliculus membrane and in intracellular vesicles. Osmotically-driven water transport into the bile canaliculus occurs mainly transcellularly via AQP8. *Right panel:* model of a cholestatic hepatocyte. Circulating LPS induces the release of inflammatory cytokines from Kupffer cells, which are ultimately responsible for the transcriptional downregulation of AQP8. TNF $\alpha$  induces the posttranscriptional downregulation of canalicular AQP8 expression by increasing lysosomal and proteasomal protein degradation. The resulting defective AQP8 canalicular expression is associated with decreased osmotic water permeability. The concurrence of decreased transient osmotic gradients together with decreased water transport at the hepatocyte canaliculus membrane finally accounts for the molecular pathogenesis of sepsis-induced cholestasis.

In conclusion, canalicular AQP8 expression is downregulated by a cholestatic dose of LPS. The diminished AQP8 expression is TNF $\alpha$ -mediated and is likely caused by an increase in lysosomal and proteasomal protein degradation. AQP8 downregulation was associated with decreased canalicular membrane water permeability, a mechanism that could contribute to the molecular pathogenesis of LPS-induced cholestasis.

Because human sepsis is commonly associated with polybacterial infections, we tested our findings in a model of bacterial peritonitis induced by cecal ligation and puncture (CLP), which closely mimics the clinical situation of a perforated necrotic bowel (82). In agreement with the endotoxic model, immunoblotting and immunohistochemical studies revealed a significant decrease of AQP8 protein level in canalicular membranes, without any substantial reduction in sinusoidal AQP9 expression. These results are in entire agreement with the findings in LPS-treated rats and further support the notion that the defective expression of hepatocyte

AQP8 is involved in the pathogenesis of sepsis-associated cholestasis.

Inhibition of the basic process of canalicular fluid secretion is believed to be the primary event in the development of hepatocellular cholestasis. Thus, sepsis-induced cholestasis may ultimately be caused by impaired osmotic gradients at the hepatocyte canaliculus membrane domain generated by the downregulated expression of solute transporters (e.g., Bsep and Mrp2) together with reduced canalicular water permeability secondary to defective AQP8 expression. A schematic model for hepatocyte AQP8 contribution to the development of bile secretory failure in sepsis is depicted in Figure 2.

## 5. CONCLUSIONS

As an osmotic secretory process, hepatocyte bile formation involves the active transport of solutes, followed by the passive movement of water into the biliary space.

AQP8 water channels modulate canalicular membrane water permeability providing a molecular mechanism for the osmotically-coupled transport of solute and water during bile formation. Current experimental evidence suggests that a defective canalicular expression of AQP8 causes a decrease of biliary water secretion contributing to bile secretory dysfunction in experimental cholestasis. Further studies are needed to clarify the signaling pathways and the precise mechanisms that trigger hepatocyte AQP8 downregulation in experimental cholestasis and to evaluate its significance in clinical cholestatic disorders. This knowledge is expected to shed new light on the molecular pathogenesis of cholestasis and hopefully, to contribute to new treatment strategies.

## 6. ACKNOWLEDGEMENT

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**Abbreviations:** AQP: Aquaporins; NPA: asparagine-proline-alanine motif; ar/R: aromatic/arginine constriction; Cys: cysteine; kDa: kilodalton; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; PI3K: Phosphoinositide 3-kinase; Bsep: Bile Salt Export Pump; T3: Triiodothyronine; mRNA: Messenger RNA; Mrp2: Multidrug resistance-associated protein 2; HCO<sub>3</sub><sup>-</sup>: Bicarbonate; AE2: Anion exchanger 2; siRNA: Small interfering RNA; CFTR: Cystic fibrosis transmembrane conductance regulator; EE: 17-alpha-ethinylestradiol; Ntcp: Na<sup>+</sup>-taurocholate cotransporting polypeptide; Oatp: Organic anion-transporting polypeptide; LPS: lipopolysaccharide; TNF: tumor necrosis factor; CLP: cecal ligation and puncture.

**Key words:** Aquaporins, Water channels, Aquaporin-8, Membrane water permeability, Hepatocyte, HepG2, Bile secretion, Cholestasis, Review

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