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Hepatic Bile Formation: Canalicular osmolarity and paracellular and transcellular water flow

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Running title page

Osmolarity and paracellular and transcellular water flow

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Abbreviations: $E_2$17G, Estradiol-17β-D-glucuronide; $E_2$3G, estradiol-3-glucuronide

Abstract

The purpose of this minireview is to indicate that a new paradigm is developing regarding hepatic bile flow. The focus thus far has been on the carrier-mediated transport of bile acids and other solutes, such as glutathione, which create an osmotic gradient for the transcellular and paracellular flow of water into canaliculi. In addition to the physico-chemical properties of the bile acids, which govern the osmotic gradient, data now exist that the tight junctions, governing paracellular water flow, and Aquaporin-8 water channels, governing transcellular water flow, are regulated independently. Thus, the rate of water flow into the canaliculus in response to bile acid transport is variable and determines canalicular bile acid concentration, which affects the production and solubilization of cholesterol-lecithin vesicles. These new considerations modify thinking regarding the occurrence of cholestasis and its progression and re-orient the design of experimental studies that can distinguish the different determinants of bile flow.

Significance

The paradigm that water flow into the canaliculus is determined only by the rate of carrier-mediated transport has been challenged recently by the changes that occur in hepatic bile composition in the Claudin-2 knockout mouse and with the cholestatic effect of estradiol 17β-D-glucuronide. Thus, respectively, a reduction in paracellular or transcellular canalicular water flow, probably via Aquaporin 8, has no significant effect on bile acid excretion.

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Introduction

Claudin-2 is one of the proteins comprising the tight junctional apparatus which regulates paracellular water flow (Tanaka et al., 2017). Therefore, we can pinpoint the reduction in hepatic bile flow found in the Claudin-2 null mouse (Matsumoto et al, 2014) to events occurring in the canaliculus.

A reduction in transcellular canalicular water flow by estradiol-17β-D-glucuronide ($E_2$17G) can also be pinpointed to the canaliculus because of a related increase in bile acid concentration. The focus on transcellular water flow relates to the effect of $E_2$17G on the expression of Aquaporin-8 identified in the canalicular membrane (Huebert et al., 2002). The increase in the concentration of bile acids in the canaliculus because of decreased paracellular and/or transcellular water flow, affects their osmolarity and the solubilization of cholesterol and lecithin.

Accepting that water flow into the canaliculus is osmotically determined, proposed in the 1950's (Sperber 1959), we need to integrate this concept with these determinants of the rate of paracellular and transcellular water entry which appear to be independently regulated by the expression of the claudin-2 and aquaporin-8 proteins.. The concept that paracellular and transcellular water flow, although governed by osmotic gradients, are nevertheless independently regulated, broadens our approach to understanding the pathogenesis of cholestasis, particularly regarding the effects of xenobiotics.

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Determinants of canalicular water flow (Figure 1)

Claudin-2

Claudin-2 null [Cldn2−/−] mice are born in the expected Mendelian ratios. Their growth rate, appearance, activity, and behavior are normal (Matsumoto et al., 2014) in contrast to other claudin null models which cause early lethality (Muto et al., 2010; Tsukita et al., 2019).Therefore, this model is amenable to many long-term studies. Hepatic bile flow in the claudin-2 null mouse is 50 % less than the wild-type animal, but bile acid, bilirubin, lecithin and cholesterol output are not significantly different from the normal, each having an approximately 2-fold increase in concentration (Matsumoto et al., 2014). There is no increase in hepatic or plasma bile acid concentration. The challenge is to understand why water flow can vary in response to a solute load, generated mostly by the bile acids.

B. Aquaporin-8

Aquaporin-8 belongs to a family of transmembrane proteins with the primary role of conducting osmotically driven water molecules across cell membranes (Calamita et al., 2018). Aquaporin-8 pore structure and substrate specificity differs substantially from other human aquaporin water channels (Horner and Pohl, 2018). The single water permeability (p_f) of Aquaporin-8 expressed in hepatocyte canalicular membranes is approximately 100-fold less than Aquaporin-1 found in the nephron and ductular regions of the biliary tree and is much closer to Aquaporin-0 found in the lens of the eye, which

has the lowest permeability coefficient (Horner and Pohl, 2018). The biologic significance of these differences is an area of active investigation. The low Aquaporin-8 intrinsic water permeability likely contributes to the fact that the canalicular membrane has lower osmotic water permeability than the basolateral membrane, and thus it is rate-limiting for the osmotically driven transcellular water transport in hepatocytes (Marinelli et al., 2003). Also, it is reasonable to think that low permeability of Aquaporin-8 favors relatively more rapid bile acid transport than water flow thus accounting for micellar concentrations of bile acids in the canaliculus which are necessary for the solubilization of the water-insoluble cholesterol-lecithin vesicles (Crawford et al., 1995).

No published studies exist to demonstrate the effect of deleting Aquaporin-8 on bile flow. Studies not yet published (Ma T, College of Basic Medical Sciences, Dalian Medical University, Dalian, China. Personal Communication to R.A. Marinelli) report a severe reduction in bile flow. A published study of the physiologic effects in an Aquaporin-8 null mouse focused on fat absorption which was minimally affected (Yang et al., 2005) and therefore concluded there was little effect on bile acid excretion. Although the conclusion is reasonable, it does not relate to an effect on canalicular water flow. The findings in the Claudin-2 null mouse indicate that a 50% reduction in water flow can occur with no change in bile acid output. Thus monitoring only bile acid output in the Aquaporin-8 null mouse does not provide information concerning changes that may have occurred in bile flow.

Canalicular Aquaporin-8 expression has been found to be increased in the choleresis induced by glucagon (Gradilone et al., 2003; Soria et al., 2009) and endothelin 1 and 3

(Rodriguez et al., 2013) via mechanisms involving trafficking and insertion of Aquaporin-8-containing vesicles as well as by modifying its gene expression. Decreased canalicular Aquaporin-8 expression has been observed in cholestatic liver disease associated not only to estrogen, as mentioned above, but also to endotoxin (Lehmann et al., 2008), sepsis (Lehmann et al., 2009) and extrahepatic biliary obstruction (Carreras et al., 2003). An increase in Aquaporin-8 expression has also been reported to be the physiologic basis for the 35% increase in bile flow that occurs in the Hypoxia-inducible factor knockout mouse (Asai et al., 2017). Thus, all these findings together with the sustained effects of $E_2$17G imply an important regulatory role for Aquaporin-8 expression in the pathogenesis of naturally occurring and xenobiotic-related cholestatic events.

In normal or cholestatic hepatocytes, it is unknown whether Aquaporin-8 single water permeability can be modified. Nevertheless, recent evidence in non-hepatic cells indicates that the Aquaporin-8-dependent water transport can be reduced by reversible inhibition of the water channel conductance (Medrano-Fernandez et al., 2016). This may be important in $E_2$17G cholestasis where there is clear evidence for an osmotic gradient-independent inhibition of the biliary water secretion (see Figure 2). In $E_2$17G-induced cholestasis the activation of Ca(2+)-dependent protein kinase C plays a critical role (Crocenzi et al., 2008). Protein kinase C can phosphorylate BSEP/ABCB11 which would alter its canalicular localization and likely its intrinsic activity (Lam et al., 2010). Diverse Aquaporins, including Aquaporin-8, may undergo Protein kinase C phosphorylation (Nesverova et al., 2019) which in some cases causes the decrease of water channel permeability. Thus, the possibility exists that in $E_2$17G-induced

cholestasis, protein kinase C activation may directly or indirectly impair Aquaporin-8 single water permeability.

C. Estradiol-17β-D-glucuronide ($E_2$17G)

This naturally occurring hormone metabolite is utilized to induce cholestasis in animals because it is known that some women will develop cholestasis when taking ethynyl estradiol and, when not taking an oral contraceptive, will develop cholestasis of pregnancy, usually in the third trimester. However, the latter is an uncommon event, not known to be related to excessive hormone production, and therefore attributed to host-related environmental and/or genetic determinants. A variety of experimental designs have been used to characterize the cholestatic effects of E217G, including a range of single, acute doses and infusion of high doses. However, it remains difficult to distinguish physiologic effects of the hormone from pathophysiologic or secondary effects.

The lowest acute dose of $E_2$17G given to a rat with bile duct drainage and not receiving a bile acid infusion caused an immediate transient 63 % reduction in bile flow that returned to the pre-injection level within 90 minutes (Meyers et al.,1980). No change in bile acid concentration occurred in the fluid collected as bile flow decreased, however a significant increase in bile acid concentration occurred during the recovery of bile flow (Figure 2).

If the effect of $E_2$17G was to cause water reabsorption from the biliary tree distal to the canaliculi, i.e., in the bile ducts, then the increase in bile acid concentration should have

occurred initially as bile flow diminished. The significant increase in bile acid concentration in the fluid collected during the recovery phase indicates that $E_2$17G lowered canalicular water flow to a greater extent than carrier-mediated bile acid transport.

Utilizing a different experimental design, the perfused rat liver with a constant infusion of sodium taurocholate, the lowest dose of $E_2$17G again caused an immediate transient reduction in bile flow (Adinolfi et al., 1984). Neither an increase nor decrease in bile acid concentration was found in collected bile samples, perhaps related to the accumulation of taurocholate in hepatocytes, since the infusion rate exceeded the excretion rate of this bile acid. Nevertheless, both models focus on reduced water flow independent of bile acid concentration. These data are consonant with the experimental model in which the intact rat is treated with ethynyl estradiol daily for five days, and where basal bile flow is significantly less (Harkavy and Javitt, 1969).

Figure 2, taken from the initial study (Meyers et al.,1980) summarizes the total volume of hepatic bile and total bile acid excretion during the 120-minute period following a low dose of $E_2$17G (11 μmol/kg, iv). Compared to the control, a 26.7% reduction in bile flow occurred and a 13% reduction in bile acid output, accounting for a significant increase in bile acid concentration.

Studies in hepatocytes and in polarized hepatocyte-derived WIF-B cells (Tietz et al., 2005; Gradilone et al., 2005) suggest that Aquaporin-8, the organic anion transporter MRP2/ABCC2, the bile salt transporter BSEP/ABCB11 along with other transporters are co-localized in the canalicular membrane. Other studies indicate that $E_2$17G binds to

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Mrp2 and then is rapidly transported into the canaliculus (Gerk et al., 2004). Although the number of Aquaporin-8 channels is not altered in acute studies (Mottino et al., 2006), transient changes in the topography of the membrane as $E_2$17G is transported via Mrp2 (Mottino et al., 2002) into the canaliculus could alter rates of both water flow and solute transport via their respective proteins.

Thus, the data support the view that the acute effect of $E_2$17G is localized to the canalicular membrane where it binds to and is transported by MRP2/ABCC2. It is quite possible that MRP2/ABCC2-mediated transport of $E_2$17G can change MRP2/ABCC2 topography in a manner that decreases Aquaporin-8 function. It may now be possible to determine whether E217G binds directly to Aquaporin-8 expressed in a lipid membrane and alters its function. Thus, measurement of osmotic membrane water permeability could be assessed following reconstitution of purified Aquaporin-8 in lipid vesicles pretreated with $E_2$17G or $E_2$3G. Similarly, the requirement for MRP2/ABCC2 could be assessed by expression of MRP2/ABCC2 together with Aquaporin-8 in lipid vesicles pretreated with $E_2$17G or $E_2$3G. Characterization of the effects of estradiol-3-glucuronide ($E_2$3G), which does not induce cholestasis (Meyers et al., 1980) but is transported by Mrp2 (Gerk et al., 2004), on Aquaporin-8 function is needed as a critical control. An effect of $E_2$17G on the Claudin-2 protein has not been studied. The elegant studies of Matsumoto et al. (Matsumoto et al., 2014) demonstrating the normal role of Claudin-2 in increasing paracellular water inflow could be adapted to assess directly the effect of $E_2$17G on tight junction permeability. These authors used isolated mouse intrahepatic bile duct units (IBDUs) to measure transepithelial water permeability in the presence of hypo- or hyper-osmotic buffer. Comparable studies in IBDUs from wild-type mice could

thus determine if transepithelial water permeability is altered in the presence of $E_2$17G vs. $E_2$3G. However, the possibility remains that changes in Claudin-2 water permeability might function to diminish the effect on Aquaporin-8-mediated transmembrane water flow. $E_2$17G is able to induce the post-translational down-regulation of hepatocyte Aquaporin-8 (Carreras et al., 2007). Many other biologic effects of $E_2$17G have been found, such as the increase in permeability of the tight junctions (Mottino et al., 2007), the transient effect on the biliary excretion of taurocholate (Vore et al., 1991) and endocytic internalization of BSEP/ABCB11 (Crocenzi et al., 2003). The molecular basis for the transition from the acute to sustained effects of $E_2$17G merit further scrutiny with respect to the roles of Aquaporin-8 and Claudin-2 in their occurrence.

D. Bile acids and osmotically determined flow

Two models now exist in which reductions in canalicular water flow lead to an increase in bile acid concentration in the canaliculi, at least initially, which implies that their carrier-mediated transporter is little affected. The increase in bile acid concentration is at variance with the linear relationship between flow and bile acid excretion that occurred with infusion of sodium taurocholate (Wheeler et al., 1960). Later, it was found that the aggregation number for this bile acid remains relatively constant under varying conditions compared to other bile acids (Small, 1968). Figure 3, adapted from the studies of Carpenter and Lindenbaum (Carpenter and Lindenbaum, 1979) illustrate the fall in the osmotic coefficient of sodium glycochenodeoxycholate attributable to aggregate formation, which occurs as the concentration of bile acids increases. Thus, the osmotic gradient is proportionally less than the increase in the molar concentration

of bile acids. Relatively less water is obligated to flow into the canaliculus.

Adding to the complexity of changes that occur when canalicular bile acid concentration increases is the relationship to carrier-mediated lecithin and cholesterol transport. Ultrastructural studies demonstrate (Crawford et al., 1995) that they appear in the canaliculus as water-insoluble vesicles, which are incorporated into bile acid aggregates to form mixed micelles. Their rate of formation is thought to be dependent on the rate of bile acid secretion (Crawford et al., 1995). The transport of lecithin from the canalicular membrane is not sufficient to cause hepatocellular injury. Whether larger doses of $E_2$17G, which lower canalicular water flow to 90% or more, will cause further increases in bile acid concentrations that extract sufficient amounts of lecithin from the membrane to cause hepatocellular injury has not been studied.

Conclusion and perspectives

Cholestasis remains a major clinical problem because of its role in progression to cirrhosis and also because it is a hindrance to the development of medications that otherwise would have major beneficial effects. This review focuses on new and old studies which bear on canalicular water flow, the initial site for hepatic bile formation. Although dependent on osmotic gradients, it nevertheless is independently regulated by determinants of Aquaporin-8 expression and/or water channel activity, Claudin-2 expression, and probably other determinants that need to be identified. This minireview provides a rationale for evaluating the effects of xenobiotics on hepatic bile formation using acute and chronic administration to wild-type and null animals as claudin-2 and aquaportin-8. By differentiating the events occurring in the canaliculus from the more

distal regions of the biliary tree a more precise evaluation is obtained. Knowledge of what happens to the canalicular fluid distal to the canaliculus is also essential to our understanding of hepatic bile formation but in this minireview we begin at the beginning.

Authorship contributions

Wrote or contributed to the writing of the manuscript: Marinelli, Vore, Javitt

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Footnotes

Conflict of interest

The authors declare no conflict of interest.

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Legends to Figures

Figure 1. Hepatocyte canalicular water transport. The output of bile salts via BSEP/ABCB11 generates an osmotic gradient for water movement transcellularly via Aquaporin-9 (AQP9) at the basolateral surface and Aquapotin-8 (AQP8) in the canalicular membrane and paracellularly through claudin-based tight junctions. $E_2$17G binds to MRP2/ABCC2 and is transported into the canaliculus. Following low dose intravenous injection, canalicular water flow rapidly decreases and bile acid concentration increases (see Figure 2). Canalicular water permeability is modulated by changes in the number of AQP8 molecules by trafficking and insertion of AQP8-containing vesicles. $E_2$17G does not decrease the number of AQP8 molecules in the canalicular membrane in acute low dose studies in contrast to the reduced numbers that occur with chronic administration. Nevertheless, the possibility exists that the intrinsic water conductance of AQP8 may be modulated or impaired by perturbations in the canalicular membrane related to the binding of $E_2$17G to MRP2/ABCC2. OA = organic anions.

Figure 2. Effect of single low dose Estradiol-17β D-glucuronide on bile flow and bile acid excretion. As shown in bottom panel D, bile flow fell to a nadir by 15 minutes after injection of 11 μmole/kg of [^{3}H]-$E_2$17G intravenously. However, as shown in Panel C, no significant change in bile acid concentration occurred until the recovery phase. As bile flow returned toward the pre-injection level, a significant increase in bile acid concentration was found in 3 successive samples (15, 30 and 45 min). Percent recovery of the injected [^{3}H] is shown in Panel A (top) and bile acid excretion rates (μmole/min/kg

body wt) in Panel B. Data from Meyers et al. 1980.

Figure 3. Osmotic coefficient of sodium glycochenodeoxycholate. The osmotic coefficient of sodium glycochenodeoxycholate at different molar concentrations was determined by vapor pressure osmometry (Carpenter and Lindenbaum, 1979). Common to all micelle forming bile acids, the osmotic coefficient decreases with increasing bile acid concentration. In the Claudin-2 null mouse bile acid concentration in bile was 35 mM compared to 18 mM in the wild-type mouse. Mouse bile is mostly the taurine conjugates of cholic and muricholic acids and would probably have similar changes in their osmotic coefficients.

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FIGURE 1

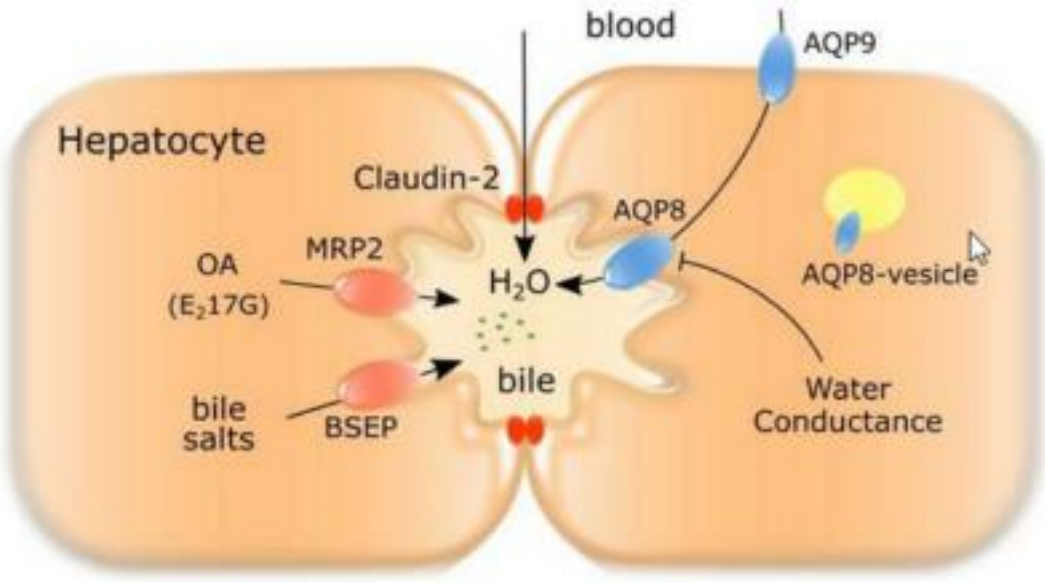

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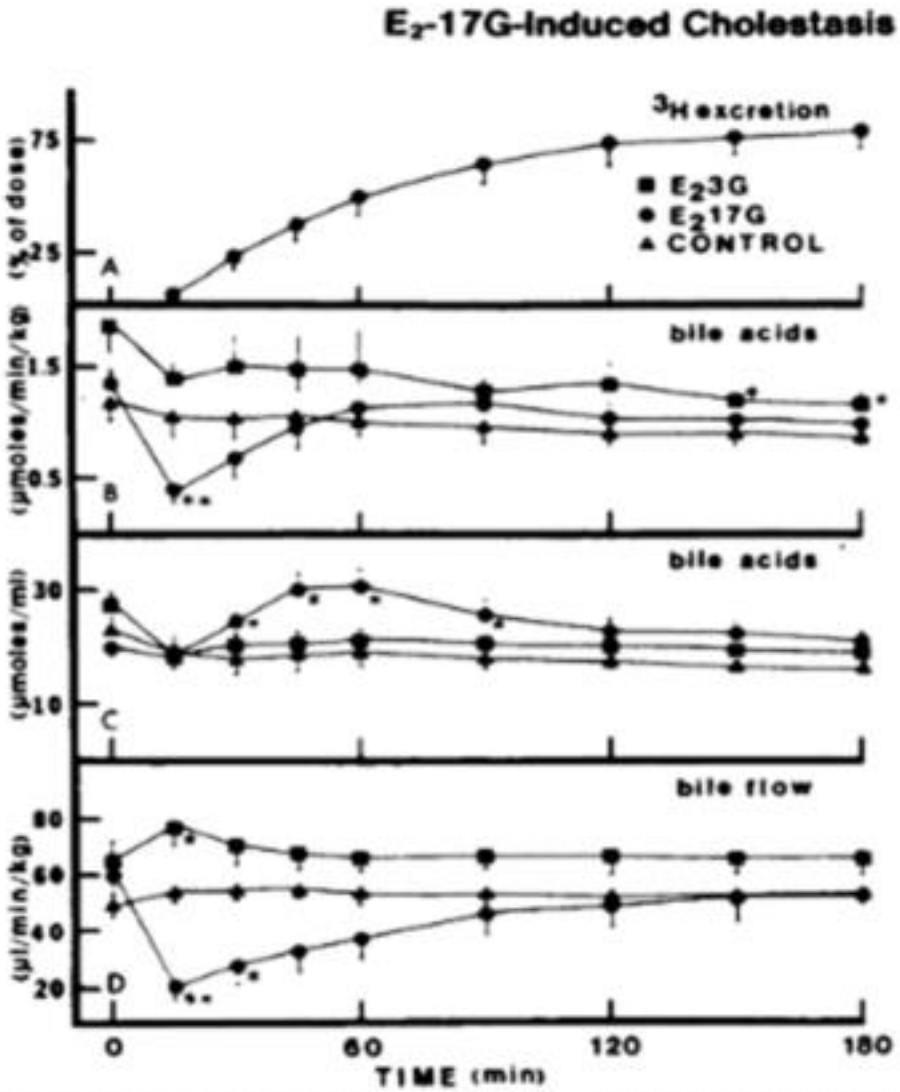
FIGURE 2



Bile acid output	minutes	0-15	15-30	30-45	45-60	60-90	90-120	TOTAL
Control	μmole	16.5	15	15	15	30	30	121.5
E2 17G	μmole	6	10.5	14.3	16.5	36	36	105.8
Volume								ml
Control	μl	750	780	780	780	1560	1560	6,21
E2 17G	μl	300	345	450	525	1350	1470	4.55

Flow reduction = 26.7% Reduction Bile acid output = 13 %

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FIGURE 3