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Recent insights into the function and regulation of the bile salt export pump BSEP (ABCB11)

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Abstract

Purpose: Generation of bile is an important function of the liver. Its impairment can be caused by inherited mutations or by acquired factors, which leads cholestasis. Bile salts are an important constituent of bile and are secreted by the bile salt export pump (also known as ABCB11) from hepatocytes.

Recent findings: Significant progress was made in the understanding of mechanisms and consequences of malfunctioning BSEP. This information was gained from extensive characterization of patients with inherited BSEP deficiency and the subsequent characterization of the identified mutations in heterologous expression systems. Furthermore and importantly, clinical evidence shows that patients with severe BSEP deficiency are at risk to develop hepatocellular carcinoma. Bile salts are now recognized to be important in the modulation of whole body energy homeostasis. Since BSEP is the rate limiting step in hepatocellular bile salt transport, it controls the spill over of bile salts into the systemic circulation. Thus, it becomes more and more likely that BSEP plays an indirect role in energy homeostasis.

Summary: In summary, knowledge on the physiological and pathophysiological roles of BSEP is rapidly progressing. It can be anticipated that the next major step in better understanding BSEP will come from information on structure-function relationships. Given the difficulty in structure determination of mammalian transporters, this will require major effort.

Introduction

In the small intestine, bile is indispensable for the absorption of lipids, which are an essential dietary energy source. Hence, ongoing and undisturbed bile flow into the gut is vital for mammals to meet their energy needs. Bile is produced by the liver and consists of phospholipids and bile salts forming mixed micelles. These mixed micelles are acceptors for poorly water soluble substances. Hence, the bile fluid not only mediates absorption of lipids from the gut, but also provides a vehicle for the disposal of poorly water soluble waste products.

Bile salts constitute a major portion of bile and are secreted by hepatocytes into the canaliculi. From there, they flow with other bile constituents into the gall bladder, except in species that are devoid of it. The gall bladder is a concentration and storage device for bile salts and other bile constituents (1). Thereafter, bile salts enter the duodenum, where they assist in the

absorption of lipids and fat soluble vitamins. In the intestine, bile salts are absorbed both passively and actively and are transported back to the liver via the portal circulation. There, bile salts are taken up from the sinusoidal blood plasma by hepatocytes, where their journey starts again. This process of bile salt circling through the gut to the liver is known as enterohepatic circulation (1-4).

Bile salt secretion by hepatocytes requires the coordinate action of distinc hepatocellular transporters located in the sinusoidal and in the canalicular membrane of hepatocytes (5-7). In the sinusoidal hepatocyte membrane, uptake of bile salts occurs predominantly in a sodium-dependent manner via the sodium-taurocholate cotransporting polypeptide and to a minor extent in a sodium-independent manner mediated by organic anion transporting polypeptides (5, 6). Bile salt secretion across the canalicular membrane is known to be the rate limiting step of hepatocellular bile salt secretion and is mediated by an ATP-binding cassette (ABC) transporter named bile salt export pump Bsep (in rodents, BSEP in humans) or Abcb11/ABCB11 (5-8). Hence, due to its strategic location, BSEP drives and maintains enterohepatic circulation of bile salts.

BSEP in liver disease

The key role of BSEP in hepatocellular bile salt handling becomes evident in patients with genes leading to defective BSEP function, who develop severe liver disease with progressive familial intrahepatic cholestasis type 2 (PFIC2) (9). A milder variant of this disease often comes with recurring episodes of cholestasis and is called benign recurrent intrahepatic cholestasis. A group of these latter patients also has mutations in the *BSEP* gene (10). Together, mutations in the *BSEP* gene represent a continuum between mild to severe, progressive forms of intrahepatic cholestasis and lead to BSEP deficiency syndrome (11, 12). Frequent mutations in the *BSEP* gene can be grouped into missense mutations, nonsense mutations, deletions, insertions and splice site mutations. A recent exhaustive study performed in 109 families of patients with severe BSEP deficiency syndrome not only analysed *BSEP* genes for mutations but also investigated BSEP expression by immunohistochemistry in biopsies. The vast majority of patients had abnormal or absent BSEP staining - the latter predominating (13). Hence, mutations in the *BSEP* gene lead frequently to absent BSEP. Even though no large studies on mRNA levels in patients with BSEP deficiency syndrome are available, quality control in the endoplasmic reticulum probably leads to degradation of truncated and wrongly folded BSEP proteins. This study (13)

also revealed that the common E297G and D482G mutant forms of BSEP varied most in their expression level between patients. This is of interest, as E297G and D482G have been shown to display residual (14) and normal (15) transport activity, respectively. In an elegant extension of the histologic characterization of patients with bile salt deficiency syndrome, the impact of mutations and single-nucleotide polymorphisms in the *BSEP* gene on the effect of pre-mRNA splicing and processing of BSEP protein was investigated (16). The authors identified 20 mutations/SNPs with reduced wild-type splicing and consequently reduced levels of normal mRNA *in vitro*. The majority of the mutants expressed in CHO-K1 cells led to protein retention in the endoplasmic reticulum and subsequent degradation. Of note, the mutant D482G, which is common in Europe displayed enhancement of aberrant splicing. This provides a possible explanation for the wide variation of BSEP expression found in patients with D482G mutations leading to clinical phenotypes with variable severity.

Missing or non-functional BSEP lead to accumulation of bile salts within hepatocytes. Clinical observations from patients with severe forms of BSEP deficiency syndrome have revealed that these patients, who are often very young, are at significant risk to develop hepatocellular carcinoma (13, 17). How elevated intracellular bile salts are transforming cells is not yet known to detail, but it is likely that bile acid induced carcinogenesis involves mitochondria as bile salts are toxic to mitochondria (18, 19). Furthermore, bile salts may interact with signalling cascades controlling cell cycle (20) and/or activate homeobox genes (21) and thereby ultimately transform cells. It is also conceivable that elevated intracellular bile salt levels may interfere with DNA repair mechanisms. And finally, bile salts have been shown to lead to a reversible induction of differentiation and cell polarization of rat hepatoma cells, again indicating pleiotropic action of intracellular bile salts (22).

Inhibition of BSEP by endogenous metabolites such as for example aberrant bile salts (23) or drugs (24-27) leads to acquired BSEP deficiency syndrome (intrahepatic cholestasis). Drug induced cholestasis constitutes a clinically important and often severe problem (28). As the risk for such events is rare (e.g. 8.5 per 100,000 in the first 45 days for first time users of flucloxacillin (29)), it is evident that patients suffering from adverse drug reactions my carry susceptibility factors. To date, c.1331T>C (p.V444A) in exon 13 and c.2029A>G (p.M677V) in exon 17 of the *BSEP* gene are two nonsynomous SNPs, which have consistently been observed with frequences of higher than 0.5 % (8, 30-32) in different cohorts. A study of hepatic protein expression levels of four canalicular ABC transporters revealed a large

interindividual variability for all the investigated transporters (BSEP, MDR1, MDR3, MRP2) (33). Interestingly, individuals carrying the p.V444A variant tended towards lower BSEP expression levels and all of the 17 % of the 110 individuals having low or very low BSEP expression levels were carrying at least one c.1331T>C allele. Functional characterization of these two BSEP variants Sf9 cells revealed no difference in their kinetic properties (34).

Individuals carrying the p.V444A variant could be at a higher risk for developing acquired BSEP deficiency syndromes. This hypothesis is supported by findings in patients with drug induced cholestasis (34) and in patients with intrahepatic cholestasis of pregnancy (35, 36), where the c.1331T>C variant of *BSEP* is significantly more frequent than in controls. These three studies therefore identified the c.1331T>C variant of *BSEP* as a susceptibility factor for acquired BSEP deficiency syndrome. It should be noted that a study in a Swedish cohort with intrahepatic cholestasis of pregnancy did not show a difference in the distribution of common BSEP haplotypes (37). This discrepancy could in part relate to the fact that the latter study investigated haplotypes, while the other studies investigated frequencies of frequent SNPs. In three case reports (2 patients with cholestasis of pregnancy and one patient with benign recurrent intrahepatic cholestasis type 2), homozygocity for 444A was observed once and heterozygocity for 444A was observed twice (38-40). Clearly, while the association of a common BSEP variant with increased risk for acquired BSEP deficiency syndromes is interesting, independent studies with large cohorts are needed to strengthens this concept.

A patient with benign recurrent BSEP deficiency syndrome associated with compound heterocygosity of two BSEP mutations with transport activity in Sf9 cells (14). The challenge will be to obtain information on minimal canalicular BSEP levels needed for proper bile salt secretion. BSEP is indirectly also important for systemic bile salt concentrations, as impaired BSEP function will lead to a spill-over of bile salts into the systemic circulation. In a study involving a large number (>5000) individuals aimed at the identification of genetic variants regulating fasting glucose levels, an SNP located in the vicinity of the *BSEP* gene was found to positively correlate with fasting glucose levels (41). It is interesting to note that the same study identified two additional SNPs in intron 19 of the *BSEP* gene, which also strongly associate with fasting glucose levels. A direct role or modulation via transcription factors of bile salts to body energy homeostasis is rapidly evolving (42-46). BSEP activity may hence have indirectly far reaching systemic effects on energy homeostasis.

Taken together, there is now overwhelming evidence from patients with liver diseases illustrating the key role of BSEP in canalicular bile formation and progress in understanding the pathophysiologic consequences of molecular defects and/or variants of BSEP is rapidly evolving.

Lessons learned from *in vitro* studies of BSEP mutations

While undoubtedly a lot has been learned from patients, such studies lack mechanistic information on the influence of changes in the BSEP protein on its biosynthesis and stability. Information pertaining to this issue can be obtained in heterologous expression systems, which provide tools resulting in "in-vitro phenotypes". This approach was initially used to characterize seven BSEP mutations leading to severe BSEP deficiency syndrome. They were introduced into the corresponding conserved positions of rat Bsep with subsequent expression of these constructs in MDCK cells (47). Five of the studied mutations prevented the Bsep protein to be targeted to the apical membrane and/or showed blunted transport activity. Interestingly, the D482G mutation was found to exhibit decreased transport activity in this study, but after being cloned into mouse Bsep displayed normal function (48). This discrepancy could be due to species differences of the Bsep backbone. In light of the recent findings (16), the divergence could also relate to different mRNA stability. Other *in vitro* phenotyping studies with different BSEP mutants demonstrated not only alterations in BSEP targeting (15, 49), a shortening of the half life of BSEP in the plasma membrane (15) (see table 1) but also differences in ubiquitin dependent BSEP turnover (50). Lately, it was observed that the severity of BSEP deficiency syndrome phenotype correlates with membrane expression and protein stability of the BSEP mutants (51, 52). Interestingly, it was demonstrated that levels of the E297G and the D482G mutants of BSEP could be increased at the plasma membrane, if cells were treated with 4-phenylbutyrate (53). This may be therapeutically relevant, as both mutants display residual transport activity. As additional factors relevant for cell surface targeting of rat Bsep, accessory proteins (54, 55) and proper glycosylation of Bsep (56) have been identified.

Regulation of BSEP expression and function

The activity of the *BSEP* gene is predominantly controlled by the nuclear farnesoid X receptor (FXR) (2, 6, 46, 57-59). Bile acids are ligands of FXR, which is the main bile salt sensor within hepatocytes. Consequently FXR regulates bile salt levels in hepatocytes by suppressing the gene encoding the sodium-taurocholate uptake system, bile salt biosynthesis and last but

not least activating the *BSEP* gene (46, 59, 60). As BSEP constitutes the rate-limiting step of transcellular transport of bile salts across hepatocytes, this later step is critical not only for bile formation but also for keeping the intracellular concentration of the potentially cytotoxic bile salts low. Hence, in many forms of liver disease, expression of BSEP is maintained, if not induced (reviewed in (57)). Therefore, FXR is a potential drug target for treatment of cholestatic liver disease (57, 61). FXR regulates the expression of target genes by binding as a heterodimer together with the retinoid X receptor RXRα. RXRα is activated by the vitamin A derivative 9-cis retinoic acid. In a recent study cholate feeding of mice with vitamin A deficiency resulted in a marked induction of Bsep expression at the mRNA and protein level (62), indicating a more complex regulation of BSEP transcription. Such a complexity is underscored by *in vitro* studies of the human promoter of *BSEP* demonstrating that vitamin D3 activated vitamin D receptor and repressed *BSEP* promoter driven reporter gene activity (63). It was also observed that liver receptor homolog 1 transcriptionally regulates the *BSEP* promoter (64). In a patient study, four heterozyogous variants of the *FXR* gene were identified in a cohort of women with intrahepatic cholestasis of pregnancy (65). *In vitro* testing of these variants revealed reduced translational activity of the variant genes leading to a reduced transactivation of a FXR-dependent promoter construct. Another clinical study observed in individuals, which were treated for three weeks with the weak FXR agonist (66) ursodeoxycholate an upregulation of BSEP protein in liver biopsies taken at the end of the treatment period (67). And finally, the canalicular transporter ATP8B1 (or FIC1), which is defective in patients with Byler's disease activates in UPS cells the activity of the promoter for BSEP in parallel with a upregulation of FXR (68). This stimulation of the BSEP promoter is absent if mutants of ATP8B1 identified in Byler's disease patients are expressed in the UPS cell system. Taken together, these findings demonstrate that regulation of BSEP expression is governed by a complex network of stimuli acting directly and indirectly on the promoter of BSEP.

Animal models of liver disease have revealed considerable evidence for posttranslational regulation of Bsep, particularly by regulating the number of Bsep transporters in the canalicular membrane (reviewed in (8)). Cholestatic liver disease is frequently treated with ursodeoxycholate (69). In model systems, ursodeoxcholate acts by stimulating canalicular bile formation, which involves, among other transporters, the insertion of Bsep from an intracellular pool into the canalicular membrane (69, 70). This anticholestatic effect is mediated by a cooperative action of protein kinases C alpha and A (71). Recent studies have

presented evidence for the presence of distinct microdomains in the canalicular plasma membrane of rat hepatocytes (72-74). These microdomains are characterized by a high cholesterol and sphingolipid content. ABC transporters including Bsep were found to partition into caveolin-1 enriched microdomains (74). Whether such microdomains are involved in regulation of the activity of Bsep and other canalicualar transporters remains to be elucidated.

Conclusion and Outlook

Evidence suggests that mouse, rat and human Bsep isoforms have similar kinetic parameters (25). This indicates that findings obtained in rodents can be extrapolated to the human situation. Studies on the inhibition of human and rodent BSEP/Bsep by drugs have indeed shown inhibition by cyclosporine and bosentan (24-27, 75). This approach should be used cautiously however, since the substrate specificity of rat and human BSEP/Bsep is not identical (76). For example, while taurolithosulfocholate is not transported by rat Bsep (24, 76), it is a substrate of human BSEP (76). Furthermore, mice with an ablated *Bsep* gene do not develop severe cholestasis on a normal diet (77), but need to be put on a high cholate diet (78). As a consequence of absent Bsep, novel, more hydrophilic bile salts have been detected in the bile of knockout animals (77) as well as an upregulation of p-glyocoprotein, which could provide a salvage pathway for bile salts (79). Nevertheless, Bsep knockout mice can be used to develop novel therapeutic approaches, such transplantation of cells to correct consequences of non-functional BSEP (80).

A first report on quantitative structure-activity relationship of inhibitors of BSEP has been published (81). Hence, it can be expected that more information on the structural requirements of BSEP inhibitors will become available. Such information undoubtedly will be highly valuable in the process of drug development for predicting potential BSEP inhibition. Certainly, knowledge on the atomic structure of BSEP will greatly enhance such predictions. Unfortunately, high resolution structural information of mammalian ABC-transporters including BSEP remains elusive (82).

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