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Genetic determinants of Drug Induced Cholestasis and Intrahepatic Cholestasis of Pregnancy

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Key Words

Pharmacogenetics, cholestasis, drug-induced, pregnancy

ABSTRACT

Intrahepatic cholestasis of pregnancy and drug induced cholestasis are two clinically important forms of acquired cholestatic liver disease. The understanding of the underlying mechanisms of acquired cholestasis has recently made considerable progress by the identification of canalicular ATP-binding cassette (ABC) transporters as likely targets for these forms of cholestasis. Cholestasis of pregnancy is linked to estrogen and progesterone metabolites. These metabolites have been shown to impair the bile salt export pump BSEP function by an indirect mechanism. In addition, genetic variants (as well as mutants) of the genes coding for the phosphatidylcholine translocator MDR3 and BSEP and for the farnesoid X receptor, which is critical in the transcriptional activation of *MDR3* and *BSEP* have been associated with intrahepatic cholestasis of pregnancy. The pathogenesis of drug induced liver injury encompasses a wide spectrum of mechanisms, some of which are still poorly understood. BSEP is now known to be subject to drug inhibition in susceptible patients. Information on genetic factors rendering individuals susceptible to inhibition of BSEP by drugs or their metabolites is still scarce. Besides rare mutations that have been linked to drug induced cholestasis, the common p.V444A polymorphism of BSEP has been identified as a potential risk factor. This review summarizes key concepts of physiology of bile formation, diagnostic principles to indentify these forms of acquired cholestasis as well as pathogenetic mechanisms leading to intrahepatic cholestasis of pregnancy or drug induced cholestasis. Furthermore, it summarizes the current knowledge on genetic susceptibility factors for these two forms of cholestasis.

PHYSIOLOGY OF BILE FORMATION

The liver is instrumental for maintaining enterohepatic circulation of bile salts. Bile salts are synthesized in a multistep cascade consisting of 16 enzymes catalyzing 17 reactions in hepatocytes [1] and secreted into the canaliculi, from were they enter the biliary tree [2-4]. In the biliary tree, the composition of bile with bile salts is modified and drained to the gall bladder, from where it enters the duodenum. In the duodenum, bile salts promote the digestion of fat and absorption of lipids and fat soluble vitamins [5,6]. Bile salts are reclaimed to more than 90 % in the small intestine and transported back to the liver via the portal circulation. In the liver, bile salts are taken up again from the sinusoidal blood plasma and their journey to the intestine restarts [7]. For efficient transport of bile salts from the sinusoids into the canaliculi as well as for controlling this process, hepatocytes are equipped with an elaborate array of transporters and regulatory mechanisms. Regulation of bile salt flow across hepatocytes is crucial, as bile salts are amphipathic molecules and display detergent properties. Hence, any surplus of bile salts within hepatocyte can become cytotoxic or even lethal to the cells.

In the basolateral plasma membrane, bile salts are taken up predominantly in a sodium dependent manner and to a minor portion via sodium independent processes. The sodium dependent uptake of bile salts is mediated by the sodium-taurocholate cotransporting polypeptide NTCP (*SLC10A1*) and shows a preference for conjugated bile salts [7,8]. Sodium independent uptake of bile salts is fostered by organic anion transporting polypeptides or OATPs (*SLCOs*), namely OATP1B1 and OATP1B3 [2,9]. A third OATP, OATP2B1 is also expressed in hepatocytes but does not mediate transport of conjugated bile salts [2,10]. OATPs are also mediators of hepatocellular drug and xenobiotic uptake whereby OATP1B1 and OATP1B3 exhibit considerable overlap in their substrate specificity. Both, NTCP and OATPs are subject to considerable interindividual differences in their hepatocellular expression levels. Knowledge on intracellular transport of bile

salts from the basolateral to the apical plasma membrane is still scarce, but it is assumed that binding proteins are involved. Canalicular export occurs against a steep concentration gradient and is mediated by a member of the ATP-binding cassette (ABC) transporter family: the bile salt export pump BSEP (ABCB11) [7,11,12]. The rate limiting step in the overall transport from the portal blood into bile is located to the canalicular membrane of hepatocytes [2,13]. Hence, proper functioning of BSEP is essential for keeping the potentially cytotoxic bile salts at a low intracellular level in hepatocytes. Consequently, mutations leading to a non-functional BSEP protein were associated with familial cholestatic syndromes, the so called progressive familial intrahepatic cholestasis type 2 [4]. Furthermore, as bile formation is an isoosmotic process, bile salts are a major driving force for the generation of canalicular bile flow. In addition to bile salts, canalicular bile contains lipids. Phosphatidylcholine is the major lipid constituent and its release from the extracellular leaflet of the canalicular membrane into bile is mediated by MDR3 or ABCB4. This ABC transporter acts as a phosphatidylcholine translocator supplying phosphatidylcholine to the outer hemileaflet of the canalicular membrane [14]. From there, phosphatidylcholine is released into bile by the detergent action of bile salts [15]. Mutations in the gene coding for MDR3 lead to progressive familial intrahepatic cholestasis type 3 [16]. In primary bile, phosphatidylcholine and bile salts form mixed micelles, which act as acceptors for poorly water soluble substances, such as cholesterol [15]. The release of cholesterol from the canalicular membrane into bile is facilitated by the heterodimeric transporter ABCG5/ABCG8 [17].

A reduction of bile flow represents a pathophysiologic situation and is called cholestasis. Metabolism of bile salts within hepatocytes leads to sulfated and glucuronidated bile salts, particularly in cholestatic conditions [18,19]. Such bile salt derivatives are excreted into bile via the multidrug resistance protein MRP2 (ABCC2) [20], or back into the sinusoids by MRP3 and MRP4 [21], two salvage systems which help to reduce the concentration of potentially cytotoxic

intracellular bile salts in hepatocytes. In addition, the heterodimeric organic solute transporter OSTα-OSTβ is also expressed in the basolateral membrane and might act as an additional salvage system [22]. The relative contribution of these three adaptive efflux systems is at the moment not fully understood and needs to be worked out in detail.

NTCP has a rather restricted substrate specificity and transports in addition to bile salts sulfated compounds such as bromosulfophthalein and sulfated steroid metabolites [7,8,23,24]. Furthermore, in heterologoues expression systems NTCP transports bile salt-drug conjugates and sulfated thyroxin. Taken together, NTCP acts as the key hepatocellular bile salt uptake system, but may also contribute to hepatocellular handling of additional compounds and even drugs (see below). NTCP transports one bile salt molecule together with two sodium ions and is therefore electrogenic [25]. Consequently, it can take up bile salts against a concentration gradient into hepatocytes.

OATPs transport a large variety of endogenous substrates, metabolic end products as well as xenobiotics, such as for example bile salts, estrogen metabolites, drugs and toxins [10,26,27]. In hepatocytes, OATP1B1 and OATP1B3 are the two key uptake transporters for unconjugated and conjugated bile salts and for hydrophobic, anionic xenobiotics, while OATP2B1 is so far considered to be mainly a transporter for bromosulfophthalein and steroid sulfates. OATP1B1 and OATP1B3 have a large overlap in their substrate specificity. It is therefore difficult to predict the individual contribution of either of the two transporters for the uptake of a given bile salt or a given drug. For example, in heterologous expression systems all hepatocytes OATPs mediate transport of rosuvastatin [28]. Most interestingly, in a recent genome wide SNP association study with patients on a high dose simvastatin treatment only OATP1B1 variants were identified as a risk factor for myopathy [29]. This can be taken as evidence that OATP1B1 is the functionally

relevant simvastatin (and most likely also other statin) uptake system in hepatocytes. The transport mechanism of OATPs is not known in detail, but they are believed to act as organic anion exchangers. Glutathione, glutathione-conjugates, oxidized glutathione as well as bicarbonate have been demonstrated to act as counteranions [30-33]. In a recent study, evidence was presented that many OATPs indeed exchange bicarbonate for anions during the transport step and that most OATPs show higher transport rates at low extracellular pH [34]. Elucidation of the exact transport mechanism of the OATPs is however important as this will allow to predict, whether OATPs have the potential to transport drugs against a concentration gradient into hepatocytes. Such a mechanism would certainly contribute to drug toxicity in hepatocytes. In this context, coadministration of the OATP inhibitor rifampicin with glibenclamide in healthy volunteers leads to an increase of the AUC and of Cmax of glibenclamide [35]. In rat studies, glibenclamide was found to be 50 times higher concentrated in the liver as compared to the serum, suggesting a concentrative uptake mechanism into hepatocytes [36].

BSEP has a narrow substrate specificity and transports mainly monanionic, conjugated bile salts [11,12]. There is a variation in its substrate pattern between species, as for example human BSEP, but not rat Bsep transports the bile salt metabolite taurolithocholate-3-sulfate [37,38]. Bsep transports barely any unconjugated bile acids [39]. This in vitro finding is supported in vivo by the observation that patients with a defect in bile acid conjugation have very little unconjugated bile acids in their bile [40]. BSEP is an electrogenic transporter and requires hydrolysis of ATP for transport activity [11,41]. BSEP is the sole transporter for monoanionic bile salts across the canalicular membrane. This becomes evident in patients with mutations in the BSEP gene. Such patients develop progressive familial intrahepatic cholestasis or BSEP deficiency syndrome type 2 and have less than 1 % of primary bile salts in their bile [4,42,43]. Furthermore, a comparison of rat Bsep and Mrp2 revealed no overlap in substrate specificities [37]. These findings suggest that

inhibition of BSEP, e.g. by drugs, should lead to reduced bile salt secretion and their retention within hepatocytes and consequently lead to cholestasis. Several drugs have been implicated in drug induced cholestasis and examples of such drugs like cyclosporine, rifampicin, rifamycin, glibenclamide or bosentan have been found to be competitive inhibitors of BSEP [37,44,45]. The list of BSEP inhibitors is continuously growing [46]. The estradiol metabolite estradiol-17β-glucuronide induces acute cholestasis in rats. It was therefore also tested for its inhibitory potential of rat Bsep. Interestingly, estradiol-17β-glucuronide does not inhibit Bsep expressed in Sf9 cells. If however Mrp2 is coexpressed with Bsep in Sf9 cells, estradiol-17β-glucuronide leads to a concentration and time dependent inhibition of Bsep [37]. This finding was confirmed and extended to sulfated progesterone metabolites [47,48]. In addition, Mrp2 could also interact directly with Bsep in the canalicular membrane in the presence of estradiol-17β-glucuronide and thereby inhibit Bsep [49].

INCIDENCE AND DIAGNOSTIC CRITERIA OF CHOLESTASIS OF PREGNANCY

Intrahepatic cholestasis of pregnancy (ICP) is an acquired form of cholestasis, which is observed in otherwise healthy pregnant women with a normal medical history. It usually occurs in the second and third trimester of pregnancy, when serum concentrations of estrogens and progesterone reach their peak [50,51] and is characterized by pruritus, elevated concentrations of bile salts, transaminases and rarely also of bilirubin in serum [52-55]. The suggested pathogenic key role of female sex hormones is further supported by the rapid cessation of cholestatic symptoms after delivery [56], the higher incidence of ICP in twin pregnancies [50] and the increased susceptibility of affected patients to develop intrahepatic cholestasis under oral contraception [57].

The incidence of ICP varies widely, originally ranging from 0.05 to >20% between different ethnic groups and geographical locations, with highest incidence rates reported for women with Araucanian Indian descent in Chile (for review see [54]). In Caucasian populations of the United States and Europe, incidence rates vary between 0.5 and 1.5%, with highest rates observed in Sweden and the Baltic countries. Since 1980 the incidence of ICP in Chile has declined, which has mainly been attributed to changes in environmental factors. The importance of environmental factors in the pathogenesis of ICP is also supported by the higher disease prevalence during the winter months in Chile, Finland and Sweden.

Data collected in different geographic locations including Europe, North and South America and Australia, have reached consensus about the catalogue of clinical and laboratory criteria essential to the diagnosis of ICP (reviewed in: [58-60]). The onset of ICP is typically indicated by the development of pruritus starting in late pregnancy in the absence of a past medical history, physical or ultrasonographic signs of liver disease or biochemical, virological or autoimmune abnormalities that could reveal acute or chronic liver disease. Pruritus may precede laboratory abnormalities and shows a characteristic distribution pattern, starting in the palms and soles before generalizing to other zones of the body surface.

Elevation of fasting serum total bile acid concentrations > 10mol/L may be the first and only laboratory abnormality in ICP [61,62]. Specifically, serum cholic acid becomes the primary bile acid in ICP women in contrast to normal pregnant women and nonpregnant women, in whom its proportion is almost similar to chenodeoxycholic acid. This results in a marked elevation of the cholic/chenodeoxycholic acid ratio compared to pregnant women without ICP [63-65]. Other laboratory findings reflecting cholestasis include variable elevations in the serum concentrations of alkaline phosphatase, 5' nucleotidase, total and direct bilirubin and transaminases. Elevation of alkaline phosphatase levels is not specific of cholestasis during pregnancy due to the placenta

isoenzyme, and the extent of transaminases elevation varies between 2-fold to 10-fold the upper limit of normal [58].

Surprisingly, the serum concentrations of gamma glutamyl transpeptidase (GGT) are normal or only modestly elevated in most patients with ICP and might allow conclusions about the underlying defect in bile acid transport. Specifically, there is indication that elevated GGT levels indicate an impairment of MDR3 function, while GGT is normal in BSEP-related forms of estrogen-associated cholestasis [66]. Therefore, GGT might be useful to clinically distinguish between MDR3 and BSEP-related forms of estrogen-related cholestasis, as it is already done for progressive forms of inherited familial intrahepatic cholestasis [42,67].

PATHOPHYSIOLOGY OF STEROID INDUCED CHOLESTASIS

Clinical evidence based on serum levels of estrogens during pregnancy linked steroid hormones with intrahepatic cholestasis of pregnancy [68,69]. Progesterone and its metabolites could in addition contribute to the pathogenesis of cholestasis of pregnancy [51,64,70]. Alternately, oral contraceptives can also lead to cholestasis [71 69]. In animal experiments, the steroid metabolites estradiol-17β-glucuronide and progesterone sulfate have been demonstrated to lead to acute cholestasis immediately after application [48,72].

From studies with rats treated for 5 days with high (usually 50 mg/kg body weight) ethinylestradiol, a model for estrogen-induced cholestasis, the following pathophysiologic picture emerged: Sodium-dependent uptake of taurocholate into basolateral liver plasma membrane vesicles is reduced by about 40 % and the v_{max} of ATP-dependent bile salt transport into canalicular vesicles was reduced by 60 %. Also, the transport of dinitrophenylglutathione was markedly reduced. These functional data were paralleled by a 40 % decrease of bile flow [73]. Heterologous expression of cloned rat Mrp2 identified this transporter as responsible for

dinitrophenylglutathione transport [74] and Bsep as the canalicular taurocholate tranporter [39]. At the mRNA level, Ntcp, Oatp1a1 and Oatp1a4 are markedly down-regulated after 5 days of ethinylestradiol treatment, while Oatp2b1 remains unchanged, which is mirrored at the protein levels of the respective transporters [75-77]. The canalicular transporters Bsep and Mrp2 remain unchanged at the mRNA level in this rat model, while protein levels are down regulated by about 40 % for Bsep and by about 80 % for Mrp2, respectively [76,78]. Also, the canalicular water channel aquaporin-8 is down-regulated in the canalicular membrane leading to a reduced water permeability of this membrane [79]. The basolateral salvage transporter Mrp3 is massively upregulated in ethinylestradiol treated rats at the mRNA and protein level [80,81]. Therefore, high levels of estrogens clearly alter the expression pattern of key hepatocellular bile salt and drug transporters. Estradiol seems to act predominantly via estrogen receptor a, as in mice with a disrupted gene for this receptor neither the expression of the uptake transporters Ntcp, Oatp1a1 and Oatp1a4, nor the expression of the efflux transporter Bsep is affected [82]. In human females, the depot estrogen ethinylestradiol propanolsulphonate leads to a significant increase of total serum bile salts. Among the different bile salt species, the most pronounced effect was observed with taurine conjugates [83]. As this was paralleled with an increase in secondary bile salts, this study suggests a mild cholestatic phenotype due to the estrogen.

In a 5 day treatment regimen with ethinylestradiol of Wistar and TR^- rats, which lack functional Mrp2, cholestasis was observed in both strains [84]. In contrast, the acute cholestatic action of estradiol-17β-glucuronide critically depends on expression of Mrp2, as this estrogen metabolite does not cause cholestasis in TR^- rats [49]. In less than 30 minutes, treatment of rats with a bolus of estradiol-17β-glucuronide leads to a rapid internalization of a fraction of Mrp2 and Bsep into a

subapical, vesicular compartment [85-87]. This internalization is dependent on Ca^{2+}-dependent protein kinase C [88].

In summary, the molecular events in estrogen induced cholestasis affect many transport systems in the basolateral and canalicular membrane, whereby the uptake side seems to be mainly affected at the transcriptional level, while in the canalicular export site posttranscriptional processes predominate.

Steroid induced cholestasis is not exclusively associated with estrogens and/or progesterones. The usage of androgenic or anabolic steroids, for example by competitive and noncompetitive athletes or body builders [89-91] can lead to liver injury including bland cholestasis [92]. Liver injury by this class of steroids is typically induced by compounds, which are alkylated at the 17α-position. Androgenic or anabolic steroid induced cholestasis can lead to severe jaundice [93,94], which may be prolonged [95] and accompanied with severe pruritus [96]. The literature on pathogenetic mechanisms of androgenic or anabolic steroid induced cholestasis is scarce. Evidence from rat studies has been presented that the pericanalicular microfilaments are lost [97]. Given the structural similarity of androgenic or anabolic steroids to estrogens, it is tempting to speculate that additionally similar mechanisms as with estrogens may apply to the pathogenesis of androgenic or anabolic steroid induced cholestasis.

GENETICS OF CHOLESTASIS OF PREGNANCY

Besides hormonal and most likely environmental factors, genetic susceptibility constitutes a risk factor to develop ICP. A genetic predisposition has been suspected based upon the strong regional clustering, the higher prevalence in female family members of patients with ICP and the susceptibility of ICP-patients to develop intrahepatic cholestasis under other hormonal challenges such as oral contraception [57]. In the last decade, mutations and polymorphisms in the canalicular

transporter proteins BSEP and MDR3 have both been associated with the development of ICP. A pathogenic role of genetically determined MDR3 dysfunction was first discussed upon the observation that female members of a large consanguineous family with one family member suffering from progressive intrahepatic cholestasis experienced typical recurrent episodes of ICP [98]. These observations were subsequently verified in pedigree and case-control studies, investigating the pathogenic role of heterozygous *MDR3* mutations in different populations. Strong evidence for a role of *MDR3* genetic variation came from a Swiss cohort, were the extent of *MDR3* genetic variation in 21 unrelated Caucasian women with ICP was compared to that observed in healthy pregnant control women [66]. In this collective, 47% of ICP patients had elevated GGT levels and 77% of these patients carried ICP-specific *MDR3* mutations, including three splicing consensus mutation. These findings were later confirmed by a Swedish study, reporting the association of specific MDR3 haplotypes and severe cholestasis in 12% of 52 observed ICP cases compared to 0% in the control group [99]. Furthermore, a large Italian study in 80 women found heterozygous *MDR3* mutations in 4% of cases [100]. A *MDR3* splicing site mutation was detected as causative locus for the development of ICP in a large consanguineous family of Mennonite kinship [101]. Very interestingly, the same genetic locus was associated with the development of gallstone disease, which makes it very tempting to think that the higher prevalence of gallstone disease observed in ICP women could also be related to MDR3 dysfunction. In contrast, a study in Finland failed to demonstrate a pathogenic role of *MDR3* mutations in ICP, pointing towards the heterogenous pathogenic nature of this disease [102].

In contrast to *MDR3*, the pathogenic role of *BSEP* genetic variation in ICP has only recently emerged. Biochemical workup of ICP-patients subsequently allowed the differentiation between high and low GGT forms of ICP, suggesting the involvement of different transporter pathways. While high GGT values were present in the majority of ICP-patients with an *MDR3* mutation,

genetic BSEP dysfunction was postulated in low GGT cases [98,103]. Two Swiss studies conducted in independent ICP collectives first suggested the BSEP p.V444A polymorphism as ICP susceptibility factor, with the homozygous and heterozygous state for the alanine in position 444 being significantly more frequent in ICP women than in healthy pregnant controls [66,104]. Very interestingly, the *BSEP* genotype in position 444 also correlated with serum bile acid levels, with carriers of the alanine showing higher serum bile acid levels than carriers of the valine allele. These findings were recently confirmed in two independent ICP cohorts comprising a total of more than 400 patients, were alanine homo- and heterozygotes were significantly more frequent in the ICP collectives. In the same study, heterozygosity for the BSEP mutations p.E297G, p.D482G and p.N591S formerly associated with benign and progressive forms of familial intrahepatic cholestasis type 2 were found in four, one and two ICP patients, respectively, allowing the extrapolation that 1% of European ICP cases are caused by these mutations [105]. While the molecular and mechanistic basis for p.V444A and p.N591S were not apparent, in-silico structural and functional analysis suggests that p.E297G and p.D482G destabilizes the protein fold of BSEP leading to decreased taurocholate transport in case of p.E297G [105,106]. In addition, decreased hepatic BSEP expression [107,108], and very recently, significantly reduced hepatic mRNA levels [109] was reported in healthy human liver tissue carrying the alanine allele in position 444 of BSEP, which could predispose to the development of ICP by way of decreased canalicular availability of BSEP. Furthermore, four novel heterozygous variants (c.-1G>T, p.M1V, p.W80R and p.M173T [110]) of the farnesoid X receptor (FXR), a key transcription factor driving the expression of BSEP and MDR3 [111,112], were recently identified in a British cohort of 92 women with ICP. Of these variants, p.M173T, which is located in the nucleotide binding domain of the second zinc finger significantly associated with ICP and was shown to have a markedly reduced capacity to activate the *BSEP* promoter in vitro [110]. Although the effect of this variant on MDR3

expression has not been studied, it is likely that activation of the MDR3 promoter is also reduced. In line with this, a recent report of an ICP patient suffering from alterations in three genes: p.S320F in *MDR3* (previously described in a patient with ICP [113]), p.A444V in *BSEP* and c.-1G>T in *FXR* [114], again highlights the role of FXR as a factor associated with ICP.

These observations in intrahepatic cholestasis of pregnancy can be translated to other estrogen-related forms of cholestasis, such as cholestasis seen with the use of oral contraceptives. Specifically, a heterozygous p.G855R mutation in BSEP leading to highly impaired taurocholate transport was associated with non-inflammatory cholestasis and highly elevated serum bile acid levels in a young patient under the first use of an oral ethinylestradiol/gestodene combination for contraception [115]. Interestingly, the mother and the maternal grandmother of the patient, who carried the same mutation had a history of ICP. In another study, homozygosity for the alanine phenotype in position 444 of BSEP was seen in four individuals with cholestasis under oral contraceptives [104]. It is therefore tempting to think that genetically determined impairment of canalicular transporter function not only predisposes to ICP, but constitutes a risk factor to the development of bland cholestasis observed with the use of female sex hormones. It can only be speculated, whether the same genetic events are also involved in cholestasis associated with the use of anabolic steroids.

DIAGNOSTIC CRITERIA AND INCIDENCE OF DRUG INDUCED CHOLESTASIS

Drug induced liver injury including cholestasis is another form of acquired liver disease, accounting for approximately two to five percent of hospitalizations for jaundice, ten percent of cases of hepatitis in all adults and more than 40 percent of hepatitis cases in adults older than 50 [116-118]. Drug induced liver injury causes a significant number of hospital admissions and may in severe cases necessitate liver transplantation [119]. Also, drug induced liver injury is leading to the

attrition of a significant number of substances during drug development and has repeatedly been responsible for the withdrawal of drugs from the market [120,121]. For these reasons, drug induced liver injury poses a significant burden to patient safety and the costs of modern health care systems.

The liver pathology of drug induced liver injury covers a wide spectrum of lesions from bland cholestasis to hepatitis and mixed forms [122,123]. It occurs with many drugs through a variety of mechanisms, which might differ in their clinical presentations ranging from asymptomatic mild biochemical abnormalities to an acute illness with jaundice that resembles viral hepatitis [124]. While good epidemiological data exist on the entire spectrum of drug-induced liver injury (for review see: [125,126]), data on the incidence of cholestatic forms of drug-induced liver injury are scarce. Cholestatic liver injury is typically characterized by a predominant elevation in alkaline phosphatase and bilirubin levels while the extent of aminotransferase elevations varies upon the causative drugs and the histological pattern of liver injury. Bland canalicular cholestasis is typically associated with minimal hepatocellular inflammation and normal or only slightly elevated aminotransferase levels and is often seen with anabolic steroids or oral contraceptives. In contrast, portal inflammation is seen in hepatocanalicular cholestasis, often associated with an elevation of aminotransferases. Hepatocanalicular cholestasis has been linked with different types of drugs, including the ACE-inhibitor captopril, the antibiotics dicloxacilline, nafcilline, amoxicilline-clavulanate and erythromycine as well as chlorpromazine, naproxen and terbenafine.

The diagnosis of drug-induced liver injury, including cholestasis can be difficult, as the relationship between drug exposure and hepatic injury is not always clear due to concomitant medication or preexisting liver disease. Different assessment systems such as the criteria established by the Council for International Organization of Medical Sciences (CIOMS) have

been developed in an attempt to codify the diagnosis of drug-induced liver disease into objective criteria [127]. According to a recent review, key elements for attributing liver injury to a drug include (a) previous drug exposure, (b) exclusion of underlying liver disease, (c) improvement of liver injury after cessation of the drug and (d) recurrence after exposure.

PATHOPHYSIOLOGY OF DRUG INDUCED CHOLESTASIS

Prior to their adverse action on hepatocytes, drugs need to be taken up into the cells. A large variety of drugs is entering hepatocytes via the OATPs expressed in the basolateral hepatocyte membrane [10,26,27]. Interestingly and importantly, it was recently found that the transport activity of OATPs may be directly modulated by physiologic substrates such as prostaglandins [128] or estrone-3-sulfate [129] as well as the drug clotrimazole [130] or the drug metabolite estradiol-17bglucuronide [131]. Such interactions may potentially lead to different intracellular drug concentrations at comparable serum levels.

Unfortunately, the underlying pathogenetic mechanisms of drug induced liver injury often remain enigmatic. After the cloning of rat Bsep, it could be directly demonstrated that drugs known to be leading to cholestatic liver injury, such as for instance cyclosporine [132,133] are competitive inhibitors of Bsep. Hence, this mechanism is the likely cause of cholestasis of such drugs as for example cyclosporin, rifamycin SV, rifampicin, glibenclamide [37,44]. The Ki values of Bsep inhibition in the Sf9 cell expression system compare favorably with the Km values obtained in isolated rat liver canalicular plasma membrane vesicles [37]. Such inhibition of Bsep leads to intracellular retention of bile salts in hepatocytes, which at elevated concentrations are cytotoxic to hepatocytes [134]. Bosentan is a dual endothelin receptor antagonist, which is pharmacologically active together with one of its main metabolites. Bosentan elimination is predominantly via the biliary route. Bosentan and its metabolite enter hepatocytes by OATP1B1 and OATP1B3

mediated transport [135]. In clinical trials, it was found that bosentan caused asymptomatic, reversible transaminase elevations in some patients [136]. The incidence of bosentan induced liver injury was dose dependent and increases in plasma bile salt levels of affected individuals correlated with the aministered dose of bosentan. Furthermore, individuals, who were taking glyburide together with bosentan showed a higher incidence of liver injury than patients with a bosentan monotherapy. Experiments with rat and human BSEP expressed in Sf9 cell vesicles identified bosentan as a competitive inhibitor of BSEP [45,136]. Rats treated with bosentan displayed an elevation of plasma bile salt levels, which further increases upon coadministration of glibenclamide [136]. Hence, as serum bile salt levels in patients positively correlated with the bosentan dose and as the serum liver parameters after stopping of bosentan spontaneous normalized, it can be concluded that bosentan acts as a competitive BSEP inhibitor. This inhibition of BSEP seems to be rather specific, as no elevation of serum bilirubin was observed [136]. Most interestingly, in a follow-up investigation of the cholestatic mechanism of bosentan in rats it was found that contrary to the expectations bosentan leads to a stimulation of bile flow [137]. The increased bile flow was not caused by an increased bile salt output but was associated with an increased glutathione and bicarbonate secretion. This stimulation of bile flow was not observed in TR^- rats, which lack functional Mrp2. Hence, bosentan not only directly affects the function of Bsep as a competitive inhibitor, but also exerts indirect effects, which depend on Mrp2. In vitro characterization of rat and human Mrp2/MRP2 as well as Bsep/BSEP expressed in Sf9 cells confirmed the inhibition by bosentan of both isoforms of Bsep/BSEP [138]. Furthermore, this study demonstrated a direct stimulation of Mrp2/MRP2 transport activity by bosentan. This finding most likely presents the molecular explanation for the observed increase of bile salt independent bile flo1w in rats. Stimulation of MRP2 activity is not unique for bosentan.

Other examples include sulfinpyraznoe, penicillin G or indomethacin [139]. The consequence of drug-induced activation of MRP2 may be a lowering of the bile salt concentration in the canaliculus below a (yet unknown) threshold value followed by an alteration in canaliculaur phospholipid and cholesterol secretion [140]. Taken together, Bsep can either be inhibited directly by drug form the cytoplasm or indirectly, most probably from the canalicular side. This latter process seems to need the presence of functional Mrp2.

In addition, indirect mechanisms of BSEP inhibition requiring MRP2 have been described, such as for example for estradiol-17β-glucuronide, bosentan and for the HER1/HER2 inhibitor PKI166 [37,138,141]. In cases, where the acquired liver disease is caused by bland cholestasis, this process is rapidly reversible upon discontinuation of the drug, as illustrated for bosentan [136].

Taken together, many drugs as well as endogenous steroid metabolites have the potential to interfere with transport activity of Bsep. As this includes direct and indirect inhibition of BSEP as well as regulation of its carrier density in the canalicular membrane, the actual mechanism of drug induced reduction of BSEP activity may be complex for a given substance.

Troglitazone is a drug, which was withdrawn from the market due to its hepatotoxicity. The exact molecular mechanisms of its toxicity remains somewhat enigmatic, but a consensus has emerged with time that troglitazone is mainly toxic to mitochondria [142,143]. In addition to its direct adverse action on mitochondria, troglitazone administration leads to an acute reduction to bile flow in rats. Hence troglitzone is also cholestatic drug [144]. Troglitazone is mainly metabolized into troglitazone sulfate in rats, which is subsequently excreted into bile [145]. Both, the parent compound and its sulfated metabolite are competitive inhibitors of Bsep in rat canalicular plasma membrane vesicles. Recently, troglitazone was also demonstrated to be an inhibitor of dog and human BSEP [146,147]. Taken together, troglitazone can negatively impact mitochondria via direct toxicity as well as by inhibiting BSEP, which in turn leads to an accumulation of bile salts in

hepatocytes. They by themselves are at elevated intracellular concentrations toxic to mitochondria [134]. Often, drug induced liver injury results not only in cholestatic but in mixed (hepatocellular and cholestatic) liver injury [117]. A study investigating a potential class effect of thiazolidinediones on Bsep found that both rosiglitazone and ciglitazone are inhibitors of ATP-dependent taurocholate transport into rat canalicular plasma membrane vesicles [147]. This is a strong indication that the toxicity of troglitazone requires multiple mechanisms for exerting its cholestatic potential.

In summary, drug induced cholestatic liver injury is a complex pathophysiologic entity including both direct and indirect effects of BSEP inhibition.

GENETICS OF DRUG INDUCED CHOLESTASIS

Investigations of the genetics of drug-induced liver injury have proved taxing, both because of their low incidence and their difficulty in replicating observed associations. Nevertheless progress has now been achieved by both candidate-gene and genome-wide association approaches. In particular, associations between antituberculosis drug-related liver injury and the "slow acetylator" genotype for N-acetyltransferase 2, amoxicillin/clavulanate-related liver injury, and the human leukocyte antigen (HLA) class II DRB1*1501 allele and flucloxacillin-related injury and the HLA class I B*5701 allele are now established [148,149]. Although, associations are so far drug-specific, more general susceptibility genes for DILI may exist. However, elucidation of these links will requires further investigation, ideally by using large cohorts involving international collaboration.

Therefore, the functional and clinical impact of genetic variations in *BSEP* and *MDR3* for the development of drug induced liver injury and, more specifically, cholestasis, is currently under

investigation (for review see: [150]. A Swiss study in 36 patients with drug-induced cholestasis supports a role of *BSEP* and *MDR3* mutations and polymorphisms in this condition. Specifically, full-length sequencing of *BSEP* and *MDR3* revealed a heterozygous p.D676Y mutation in *BSEP* observed in a patient taking fluvastatin and a heterozygous p.I764L mutation in *MDR3* observed in a patient taking risperidone [115], which both suffered from hepatocellular cholestasis. The pathogenic implications of these mutations remain, however, unclear. In case of BSEP, in-vitro taurocholate transport was unchanged for the mutated protein whereas the impact of the p.I764L mutation on MDR3 expression and function was not investigated. In the same study, the BSEP p.V444A polymorphism was observed significantly more frequent in patients with drug-induced cholestasis than in patients with drug-induced hepatocellular injury and healthy controls, with the AA phenotype being encountered in 61% of cholestatic patients compared with 31% and 32% in patients with hepatocellular injury and healthy controls, respectively. Overall, carriers of the alanine phenotype carried a 3-fold increased risk to develop cholestatic drug side effect under treatment with different drugs, such as β-lactam antibacterials, psychotropic drugs and proton-pump inhibitors. However, the underlying mechanism remains still unclear, as none of these drugs could be shown to inhibit BSEP function in-vitro (*unpublished results*). It can be speculated, whether BSEP inhibiting drugs with known cholestatic potential such as cyclosporine, rifampicin, rifamycin, glibenclamide, troglitazone or bosentan [37,44,45,145] might predispose to the development of cholestasis in carriers of the alanine allele.

Only limited information is so far available on the functional consequences of genetic variation in basolateral transporter systems. Tirona et al. identified a total of 14 non-synonymous *OATP1B1* polymorphisms SNPs in a population of African- and European Americans [151], six of which exhibited reduced in-vitro uptake of the OATP1B1 substrates estrone-3-sulfate and estradiol-17β-glucuronide. OATP1B1 genetic variants have also been associated with interindividual

differences in hepatic disposition of pravastatin and irinotecan, respectively [152-155]. Furthermore, the cellular uptake of the lipid-lowering drug rosuvastatin is highly dependent on NTCP and OATP function and varies upon the underlying NTCP and OATP haplotypes [156]. While the impact of these observations for the development of cholestasis remains to be studied, it could be speculated that differences in NTCP and OATP mediated basolateral drug uptake predisposes to the development of cholestasis by determining intracellular drug levels and hence, the concentration of potential competitive inhibitors of apical efflux transporters.

CONCLUSIONS

From the examples delineated in this article it is apparent that genetically determined dysfunction of hepatocellular uptake and excretion of bile salts is an important pathogenic factor for the development of cholestasis. While the genetic components of intrahepatic cholestasis of pregnancy and estrogen-induced cholestasis has clearly emerged over the last decade, the role of genetics in drug-induced cholestasis is less evident. The heterogeneous and multifactorial nature of drug-induced liver disease makes it not only challenging to clearly link liver disease to a specific drug, but so far impossible to prove the pathogenic role of a specific genetic transporter variant. Future challenges will consist in integrating different genetic determinants of drug toxicity with different environmental and comorbidity-related factors and in a comprehensive system, allowing the cautious use of problematic drugs in susceptible patients.

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