High Doses of Ursodeoxycholic Acid Up-Regulate the Expression of Placental Breast Cancer Resistance Protein in Patients Affected by Intrahepatic Cholestasis of Pregnancy

Francesco Azzaroli1,2*, Maria Elena Raspanti1,2, Patrizia Simoni1, Marco Montagnani1,2, Andrea Lisotti1, Paolo Cecinato1, Rosario Arena1, Giuliana Simonazzi1, Antonio Farina1, Nicola Rizzo1, Giuseppe Mazzella1,2

1 Department of Medical and Surgical Sciences, S.Orsola-Malpighi Hospital, University of Bologna, Italy, 2 Centre for Applied Biomedical Research, S.Orsola-Malpighi Hospital, Bologna, Italy

Abstract

Background: Ursodeoxycholic acid (UDCA) administration in intrahepatic cholestasis of pregnancy (ICP) induces bile acids (BA) efflux from the foetal compartment, but the molecular basis of this transplacental transport is only partially defined.

Aim: To determine if placental breast cancer resistance protein (BCRP), able to transport BA, is regulated by UDCA in ICP.

Methods: 32 pregnant women with ICP (14 untreated, 34.9 ± 5.17 years; 18 treated with UDCA - 25 mg/Kg/day, 32.7 ± 4.62 years,) and 12 healthy controls (33.4 ± 3.32 years) agreed to participate in the study. Placentas were obtained at delivery and processed for membrane extraction. BCRP protein expression was evaluated by immunoblotting techniques and chemiluminescence quantified with a luminograph measuring emitted photons; mRNA expression with real time PCR. Statistical differences between groups were evaluated by ANOVA with Dunn’s Multiple Comparison test.

Results: BCRP was expressed only on the apical membrane of the syncytiotrophoblast. A significant difference was observed among the three groups both for mRNA (ANOVA, p = 0.0074) and protein (ANOVA, p<0.0001) expression. BCRP expression was similar in controls and in the untreated ICP group. UDCA induced a significant increase in placental BCRP mRNA and protein expression compared to controls (350.7 ± 106.3 vs 100 ± 18.68% of controls, p<0.05 and 397.8 ± 56.02 vs 100 ± 11.44% of controls, p<0.001, respectively) and untreated ICP (90.29 ± 17.59% of controls, p<0.05 and 155.0 ± 13.87%, p<0.01).

Conclusion: Our results confirm that BCRP is expressed only on the apical membrane of the syncytiotrophoblast and show that ICP treatment with high dose UDCA significantly upregulates placental BCRP expression favouring BA efflux from the foetal compartment.

Introduction

Intrahepatic cholestasis of pregnancy (ICP), a liver disorder unique to pregnancy, predominantly occurs during the third trimester of gestation. ICP is characterized by pruritus and biochemical alterations in liver tests which fully resolve after delivery [1,2], and is associated with an increased risk of foetal distress, preterm delivery and sudden intrauterine foetal death [3]. Given the substantial foetal risk, ICP is widely considered a serious clinical problem [3].

Various hypothesis involving environmental, genetic and hormonal factors have been investigated to elucidate the pathogenesis of ICP. While no certain cause has been identified, impaired bile acids (BA) metabolism leads to increased BA accumulation in the foetal compartment [4]. Elevated maternal BA levels affect placental transport, placental hormone production and chorionic vessel constriction [5–8] and increase myometrial sensitivity to oxytocin [7,9].

The placenta plays a major role in the pathophysiology of adverse foetal effects because of its capacity to transport BA. This capacity may somehow be regulated by ursodeoxycholic acid (UDCA), the treatment of choice for ICP [3,10]. Previous studies have shown that UDCA may improve bile acid transport across the placenta and upregulate the expression of transporters...
localized on the apical side of the trophoblast membrane. However, the classical transporters studied to date are not good candidates for primary bile acid. The breast cancer resistance protein (BCRP) has been reported to transport BA [11] but its placental expression in ICP has not been evaluated. Therefore, our study aimed to determine BCRP expression in ICP and its regulation by UDCA administration.

Materials and Methods

Ethics Statement

All women gave verbal informed consent to the study that was carried out according to the Declaration of Helsinki. All subjects were older than 18 years and able to give informed consent. The study was limited to the placenta which is routinely discarded as waste material and there was no change to the current standard medical and obstetric management of the patients. In addition, the study involved no sensitive genetic data or investigations with clinical consequences on the newborn or the mother. For these reasons, ethics approval was not required according to the Italian legislation (Law n.211, 24 June 2003, Directive 2001/20/CE). However, a letter from the gastroenterologist to the gynaecologist/obstetrician reporting patients’ verbal consent was attached to the clinical records.

Patients

Thirty-two consecutive pregnant women with ICP were enrolled in the study. A control group comprised 12 consecutive healthy women with physiological pregnancies. The following criteria were required for the diagnosis of ICP: elevated fasting serum BAs (>10 μmol/L), elevated aminotransferases (>40 IU/L for ALT and >37 IU/L for AST), and pruritus. Exclusion criteria were infectious, metabolic or drug-related liver disease, and a history of alcohol or drug abuse. Blood tests and abdominal ultrasonography were performed to exclude obstructive cholestasis and viral [anti-HCV antibodies (III generation enzyme-linked immunosorbent assay (ELISA)], HBsAg, anti-Epstein-Barr virus, anti-CMV, anti-HSV, anti-HIV], metabolic [blood cholesterol, glucose, triglycerides and iron] or autoimmune (ANA, SMA, AMA, p-ANCA and LKM) liver diseases.

Information on available treatments and their implications in ICP was provided by the same doctor (GM) to all patients on enrolment. Despite literature data suggesting that UDCA is the candidates for primary bile acid. The breast cancer resistance protein (BCRP) has been reported to transport BA [11] but its placental expression in ICP has not been evaluated. Therefore, our study aimed to determine BCRP expression in ICP and its regulation by UDCA administration.

Materials and Methods

Western Blot

Protein concentrations were determined according to the method of Lowry et al. [14] and equal amounts of membrane proteins (100 μg) were separated by standard SDS-PAGE electrophoresis. Actin was used as an internal control (AC-10, Sigma-Aldrich, St Louis, MO, USA), giving a constant signal in all samples. After transfer onto nitrocellulose membrane, immunoblotting was performed with an overnight incubation with anti-BCRP antibodies [anti ABCG2 (BXP21)x-cs-58222, anti ABCG2 (H705sc-25521dil 1:100 in TBS-Tween, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). After a 1 h incubation at room temperature with the peroxidase-conjugated secondary antibodies (Envision antisumouse IgG; K4001 and Envision antirabbit, K4003, Dako, Glostrup, Denmark), chemiluminescent signals (ECL, Amersham Pharmacia Biotech, Milan, Italy) were revealed by a Luminometer (Molecular Light Imagery, Berthold Technologies, Bad Wildbad, Germany). The chemiluminescent signal was quantified by Winlight 32 analysis software (Version 2.91, Berthold Technologies).

Real-time PCR

Total RNA was extracted from 50–100 mg frozen placental tissue using Trizol Reagent (Invitrogen, Milan, Italy) according to the manufacturer’s instructions. Purity of extracted RNA was measured by the absorbance ratio 260/280 nm (1.8–2.0) and its integrity was controlled on 1% agarose gel. Two μg of RNA were converted to first-strand cDNAs with the Superscript III reverse transcriptase kit (Invitrogen, Life Technologies Europe BV, Monza, Italy) according to the manufacturer’s instructions.

Real-time PCR was performed with the following primer and probe sets (TaqMan Gene Expression Assays) diluted 1:20, purchased from Applied Biosystems Italia (Monza, Italy):B-Actin (Human beta actin, cat. N. 4333762F), BCRP (Hs01053786_m1). Two μl cDNA were added to TaqMan Universal Master Mix (Applied Biosystems) and amplified in duplicate in an Applied Biosystems 7000 real-time PCR cycler. Cycle conditions were 50°C for 2 min and 95°C for 10 min, then cycled to 95°C for 15 s and 60°C for 1 min for 40 cycles. All samples were normalized to B-actin expression levels and the relative quantification was performed using the Ct method.

Statistics

Statistical differences between groups were evaluated by ANOVA with Dunn’s Multiple Comparison test and the results were expressed as mean ± S.E. Chi-squared test and Mann-Whitney test were used when appropriate.

Results

The groups were comparable with regard to age, parity and type of delivery. As expected a significant difference was present between controls and the cholestatic groups with regard to week of delivery, serum bile acids and transaminase levels. No significant

Placenta Collection and Membrane Vesicle Preparation

Placentas were obtained from term delivery or caesarean section (38–41 weeks) together with samples of cord and maternal blood. All placentas were immediately washed with cold PBS, placed on ice and processed for membrane separation or frozen in liquid nitrogen. Apical membranes were prepared according to Grube et al. [12] and the degree of enrichment in trophoblastic vesicles was assessed by determination of alkaline phosphatase and ATPase activity [13]. Briefly, pieces of placenta were homogenized (UltraTurrax) for 2 min in lysis buffer (250 mM sucrose, 10 mM Tris-HEPES pH 7, 4 mM EGTA, 5 mM EDTA) and centrifuged at 9000 g for 10 min; the supernatant was centrifuged at 100000 g for 35 min. The resulting pellet was resuspended in the lysis buffer containing MgCl2 (final concentration 10 mM), stirred on ice for 10 min and then centrifuged at 2500 g for 15 min. The supernatant, corresponding to the apical fraction and the pellet, containing basolateral membranes were rehomogenized (Potter), centrifuged at 100000 g per 35 min and the resulting pellets were resuspended in 20–80 μl of buffer. The membrane protein fractions were stored at –80°C until used.
differences were observed between the treated and untreated cholestatic groups (table 1).

Immunoblotting of apical and basolateral membranes confirmed that BCRP is expressed only on apical membranes of the syncytiotrophoblast (figure 1). Then the evaluation of apical membranes isolated from placentas collected from controls and groups 1 and 2 showed that the BCRP protein was significantly (p, 0.0001) increased in group 2 (397.8 ± 56.02%) compared to both group 1 and controls (155.0 ± 13.87% and 100.0 ± 11.44%, respectively) (figure 2). Similarly, BCRP mRNA expression was significantly (p = 0.0074) increased in group 2 (350.7 ± 106.3%) vs group 1 and controls (90.29 ± 17.59% and 100.0 ± 18.68%, respectively) (figure 3).

The type of delivery (vaginal or caesarean section) did not significantly influence placental BCRP mRNA or protein levels within the groups (see table 2).

**Discussion**

Our study shows that BCRP protein is upregulated in placentas from ICP women receiving UDCA compared to untreated patients and controls. We observed no significant difference in BCRP levels between samples from vaginal or caesarean section within each group suggesting that labour does not affect BCRP expression. This observation is more consistent for group 1 because of comparable numbers of caesarean and vaginal deliveries.

**UDCA Administration and BCRP Expression in ICP**

UDCA has been shown to improve bile acids (BA) transport across the placenta both via ATP-dependent and independent mechanisms [15]. The molecular basis of this transport remains unsettled. In humans, the only ATP-dependent molecule found modestly upregulated by UDCA in ICP is multidrug resistance protein 2 (MRP2) [16]. Furthermore, MRP2 predominantly transports bilirubin and to a minor extent sulphated BA [17,18],

**Table 1.** Baseline characteristics of the studied groups.

|                  | CONTROLS (N = 12) | ICP (N = 14) | ICP+UDCA (N = 18) | p≤ |
|------------------|-------------------|--------------|-------------------|----|
| **Age**          | 33.4 ± 3.32       | 34.9 ± 5.17  | 32.7 ± 4.62       | ns |
| **Week of delivery** | 39.17 ± 1.4*    | 36.64 ± 1.80 | 35.50 ± 2.59      | 0.0003 |
| **Transaminases** |                   |              |                   |    |
| GOT              | 23.0 ± 6.54*      | 139.1 ± 74.81| 135.4 ± 146.2     | 0.0001 |
| GPT              | 16.60 ± 5.98*     | 233.2 ± 45.85| 217.0 ± 238.6     | 0.0001 |
| **Bile acids**   |                   |              |                   |    |
| Cholic Acid      | 0.62 ± 0.76*      | 16.80 ± 17.52| 20.53 ± 14.46     | 0.0002 |
| Chenodeoxycholic Acid | 0.63 ± 0.66* | 8.15 ± 8.55  | 12.71 ± 8.50      | 0.0002 |
| **Type of delivery** |             |              |                   |    |
| Vaginal          | 9                 | 9            | 8                  | ns |
| Caesarean        | 3                 | 5            | 10                 | ns |
| **Parity**       |                   |              |                   |    |
| Primiparae       | 9                 | 12           | 14                 | ns |
| Multiparae       | 3                 | 2            | 4                  | ns |

*vs ICP and ICP+UDCA.

doi:10.1371/journal.pone.0064101.t001

**Figure 1.** Western blotting of syncytiotrophoblast membranes showing BCRP expression only on their apical side.

doi:10.1371/journal.pone.0064101.g001

**Figure 2.** Results of immunoblotting quantification showing BCRP protein expression. A: an example of western blotting of BCRP protein expression on the apical membranes of the syncytiotrophoblast in the different groups under study. B: results of BCRP protein quantification. *ANOVA – P < 0.0001 (Dunn’s Test: ICP+UDCA vs CTRL, P < 0.001; ICP+UDCA vs ICP, P < 0.01).

doi:10.1371/journal.pone.0064101.g002

**Figure 3.** Results of RT-PCR quantification showing BCRP mRNA expression on the apical membranes of the syncytiotrophoblast in the different groups under study. *ANOVA – P = 0.0074 (Dunn’s Test: ICP+UDCA vs CTRL and vs ICP, P < 0.05).

doi:10.1371/journal.pone.0064101.g003
suggesting that other proteins are involved in BA transplacental transport.

A few years ago, studies on BCRP-expressing bacteria showed that BCRP can transport primary BA [11], but subsequent studies in cells transfected with BCRP failed to show transport for taurocholic acid or taurodeoxycholate sulphate [19–21]. More recently, in vitro and in vivo evidence in knockout mice suggests that BCRP is able to transport BA across the epithelia that express it including the placenta [22]. Recent findings in knockout mice suggest that, despite a minimal/no role in the liver’s adaptive response to cholestasis, BCRP might be implicated in solute export in the kidney and intestine suggesting that its role in cholestasis might be more prominent in extrahepatic tissues [23]. In this regard, the placenta is a unique extrahepatic tissue that loses expression of the main BA transporters (BSEP and NTCP) during the course of gestation. Therefore, in the third trimester placenta, specific BA transport is likely to be covered in part by other transporters sharing a similar substrate specificity. ICP is typical of the third trimester when oestrogen levels peak and favour cholestasis while the putative BA transporters (BSEP and NTCP) are not expressed in the human placenta despite the need for BA transport.

References

1. Lammert F, Marschall HU, Glantz A, Matern S (2000) Intrahepatic cholestasis of pregnancy: molecular pathogenesis, diagnosis and management. J Hepatol 33: 1012–1021.
2. Ropponen A, Sund R, Ruokonen S, Yliorkola O, Aittomaki K (2006) Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based study. Hepatology 43: 723–728.
3. Greenes V, Williams M, C (2009) Intrahepatic cholestasis of pregnancy. World J Gastroenterol 15: 2049–2066.
4. Mazzella G, Rizzo N, Azzaroli F, Simoni P, Baglivo E, et al. (2007) Clinical and pathological characteristics of patients with intrahepatic cholestasis of pregnancy: effects on primary bile acids in babies and mothers. Hepatology 33: 504–508.
5. Meng LJ, Reyes H, Palma J, Hernandez I, Ribalta J, et al. (1997) Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy. J Hepatol 27: 346–357.
6. Altshuler G, Ariaswa M, Mokhtar-Nasab M (1992) Meconium-induced umbilical cord vascular necrosis and ulceration: a potential link between the placenta and poor pregnancy outcome. Obstet Gynecol 79: 760–766.
7. Sepulveda WH, Gonzalez C, Cruz MA, Rudolph MI (1991) Vasoconstrictive effect of bile acids on isolated human placental chorionic veins. Eur J Obstet Gynecol Reprod Biol 42: 211–215.
8. Monte MJ, Morales AI, Arevalo M, Alvaro I, Macias RI, et al. (1996) Reversible impairment of neonatal hepatobiliary function by maternal cholestasis. Hepatology 23: 1201–1217.
9. Germain AM, Kato S, Carvajal JA, Valenzuela GJ, Valdes GL, et al. (2003) Bile acids increase response and expression of human myoestetin oxytocin receptor. Am J Obstet Gynecol 189: 577–582.
10. Marin JJ, Marias RJ, Serrano MA (2003) The hepatobiliary-like excretory function of the placenta. A review. Placenta 24: 431–438.
11. Janvilisri T, Shahi S, Venot H, Balakrishnan L, van Veen HW (2005) Arginine-1402 is not essential for transport of antibiotics, primary bile acids and unconjugated steroids by the human breast cancer resistance protein (ABCG2). Biochem J 385: 419–426.
12. Grube M, Schwalbedien HM, Draber K, Prager D, Moritz KU, et al. (2005) Expression, localization, and function of the carminic acid transporter octm2 (Mr2243) in human placenta. Drug Metab Dispos 33: 31–37.
13. Bowers GN Jr, McComb RB (1966) A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. Clin Chem 12: 70–89.
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.
15. Serrano MA, Brits D, Laarena MG, Monte MJ, Bravo MP, et al. (1998) Beneficial effect of ursodeoxycholic acid on alterations induced by cholestasis of pregnancy in bile acid transport across the human placenta. J Hepatol 20: 829–839.
16. Azzaroli F, Mennone A, Flettri V, Simoni P, Baglio E, et al. (2007) Clinical trial: modulation of human placental multigrid resistance proteins in cholestasis of pregnancy by ursodeoxycholic acid. Aliment Pharmacol Ther 26: 1139–1146.
17. Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, et al. (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 1511: 7–16.
18. Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, et al. (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. Biochem J 327 (Pt 1): 305–310.
19. Kumkate S, Chuanchob S, Janvillari T (2008) Expression of ATP-binding cassette multidrug transporters in the giant liver fluke Fasciola gigantica and their possible involvement in the transport of bile salts and anthelmintics. Mol Cell Biochem 317: 77–84.
20. Imai Y, Asada S, Tsukahara S, Ishikawa E, Tsuruo T, et al. (2003) Breast cancer resistance protein exports sulfated estrogens but not free estrogens. Mol Pharmacol 64: 610–618.
21. Vaidya SS, Gerk PM (2006) Lack of interaction between tauroursodeoxycholate and ATP-binding cassette transporter isoform G2 (ABCG2). Mol Pharm 3: 303–306.
22. Blazquez AM, Briz O, Romero MR, Rosales R, Monte MJ, et al. (2011) Characterization of the Role of ABCG2 as a Bile Acid Transporter in Liver and Placenta. Mol Pharmacol.
23. Mennone A, Soroka CJ, Harry KM, Boyer JL (2010) Role of breast cancer resistance protein in the adaptive response to cholestasis. Drug Metab Dispos 38: 1673–1678.
24. Blazquez AG, Briz O, Romero MR, Rosales R, Monte MJ, et al. (2012) Characterization of the role of ABCG2 as a bile acid transporter in liver and placenta. Mol Pharmacol 81: 273–283.
25. Hahnova-Cygalova I, Ceckova M, Staed F (2011) Fetoprotective activity of breast cancer resistance protein (BCRP, ABCG2): expression and function throughout pregnancy. Drug Metab Rev 43: 53–68.
26. Mao Q (2008) BCRP/ABCG2 in the placenta: expression, function and regulation. Pharm Res 25: 1244–1255.
27. Grenes VL, Dixon PH, Chambers J, Raguz S, Marin JJ, et al. (2011) Characterisation of the nuclear receptors FXR, PXR and CAR in normal and cholestatic placenta. Placenta 32: 533–537.