Journal of Basic and Clinical Physiology and Pharmacology

DE GRUYTER Journal of Basic and Clinical Physiology and Pharmacology

EXPRESSION AND ROLE OF THE GENES INVOLVED IN THE TRANSPORT OF BILE ACIDS IN THE LIVER AND KIDNEYS IN MICE

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Abstract:

Background: Bile acids are synthesized in the liver from cholesterol.

Method: This study investigated the impact and the expression of different carriers of bile acid in the liver and kidneys. Eight (8) weeks male mice have been used, fed during fifteen (15) days and divided into two (2) groups: (15) mice fed with standard diet (control group); other (15) mice fed with a rich diet of 5%cholesterol (second group). Bile acids dosage is based on their oxidation by 7-α hydroxyl-steroid dehydrogenize. The mRNA expression was quantitatively analyzed by RT-PCR and the expression of the renal carrier bile acids protein was analyzed by Western blot.

Result: The expression of Bile Salt Export Pump **(**Bsep**)** involved in the uptake of bile acids in the basolateral membrane of hepatocytes revealed no differences in the two groups of mice. But the expression of Multidrug Resistance-associated Protein 2 **(**Mrp2) is reduced in mice of second group. However the expressions of Organic Anion transporting Polypeptide 4 (Oatp4), Organic Anion transporting Polypeptide 1 (Oatp1), and Sodium Taurocholate Co Assurant Polypeptide (Ntcp) involved in the uptake of bile acids in the apical pole of hepatocytes are suppressed in mice of second group. The expression of Multidrug Resistance-

associated Protein 3 (Mrp3) involved in the secretion of bile acids in the apical membrane of hepatocytes, revealed no significant differences between the two groups. In mice of the second group, blood concentration of bile acids on the last day has increased. In those mice, the expression of intestinal bile acid - transport is reduced in the kidneys comparing to control mice.

Keywords: bile acid, liver, kidneys, mouse

Abbreviations: **chol:** Cholesterol; **Ibat:** Intestinal bile acid - transport; **Bsep:** Bile salt export pump; **Oatp4**: Organic anion transporting polypeptide 4; **Oatp1:** Organic anion transporting polypeptide 1; **Ntcp:** Sodium taurocholate co assurant polypeptide; **Mrp2:** Multidrug resistance-associated protein 2; **Mrp3:** Multidrug resistance-associated protein 3; **Wt:** wild type.

Introduction

Cholesterol induced in the liver the transcribed rate of Cholesterol 7α-hydroxylaze (Cyp7a1), a limiting enzyme of bile acids synthesis (Attakpa S.E et al**).** The standard expression of Ileal bile Acid transporter (Ibat) has reduced in mice subjected to rich diet of cholesterol (Attakpa S.E et al**)**. In rodents, this rate limiting enzyme-which promotes the conversion of cholesterol into bile acids (Russell DW., 2003) is up regulated by sterols through an LXRα dependent pathway. (Gupta S et al. 2002; Peet DJ, Turley SD, Ma W, et al. 1998).

Conversely, the Cyp7a1 gene is down-regulated by bile acids through a feedback loop involving SHP dependent and independent pathways.(Kerr TA, Saeki S, Schneider M, et al.,2002; Abrahamson A, Gustafsson U, Ellis E, et al.,2005).

Bile acids are synthesized in the liver from cholesterol. At the end of that synthesis, more than 98% of synthesized bile acids are conjugated to glycine or taurine (Philippe Besnard et al., 2004), and then excreted in the bile. Bile is stored in the gallbladder until its release in the duodenum in food intake. After having played their role in the digestion and absorption of fats and fat-soluble vitamins, about 95% of the bile acids are reabsorbed along the intestine to be recycled to the liver via the portal vein (Philippe Besnard et al., 2004). The remaining 5% will be eliminated by faeces. It has been shown that bile acids represent a major route of elimination of cholesterol. The bile acid transport conjugates in the body key metabolism which requires several carriers. Intestinal reabsorption of non-conjugated bile acids occurs through passive and facilitated way at the duodenum and jejunum level, but the majority of bile acids is reabsorbed at the ileum level by active transport. In iléocyte brush border, bile acids are transported by the intestinal bile acid - transport (Ibat), a membrane protein called Apical Sodium - dependent Bile Acid transport (Asbt) (Schneider BL et al., 1995). In human, some mutations in gene encoding the Ibat lead to severe bile acids malabsorption which is the cause of profound disturbance of lipid metabolism (Oelkers P et al., 1997). That protein is very important in ileac bile acid uptake. At this level, bile acids are borne by the Ileal Bile acid Binding Protein (Ibabp). Bile acids are then secreted into the portal vein

by three transport systems including also a truncated form of Ibat, the t-Ibat (Lazaridis KN et al., 2000). Are also involved: the Protein Multidrug Resistance-associated Protein 3: Mrp3 (Kiuchi Y et al., 1998), and heterodimers formed by Organic Solute Transporters (OSTα and OSTβ) (Dawson PA et al., 2003). Bile acids are then recapte at the apical membrane of hepatocytes via the Na Taurocholate co - delinquency Polypeptide (Ntcp), also thanks to the Organic Anion transporting Polypeptide, Oatps 1, 2 and 4 at the basolateral membrane of hepatocytes.

Bile acids are spilled in bile by the bile salt export pump (Bsep) and in lesser extent by the Multidrug Resistance-associated protein 2 (Mrp2). Usually, few of none reabsorbed bile acids or secreted by the liver into the general circulation via Mpr3 is filtered by the kidney. At the proximal tubule's level epithelial cells and at the apical membrane, proteins involved in the transport of bile acids are Ibat, Oatp1, Mrp2 and Mrp3 and the Ost at the basolateral membrane. At the ileac and renal apical membranes, Mrp2 may be involved to the re-excretion of bile acids. The modulation of the excretion of these carriers may explain bile acids excretion by cholesterol elimination.

At hepatic level, a heterodimer formed by ATP - Binding Cassette g5 and g8 (Abcg5& g8) secretes cholesterol in the bile (Plösch T et al., 2002). The aim of this first work is to study the expression and the role of the genes involved in hepatic and renal bile acid transport.

Materials and Methods

Animals

Eight (8) weeks male mice C57BL/6J have been used. Those mice have been fed during fifteen (15) days and divided into two (2) groups:
- (15) control mice group fed with standard diet: wheat, fish and vitamins
- Other (15) mice second group fed with a rich diet of 5%cholesterol called: group of 5%cholesterol then, mice are killed. Livers and kidneys are collected and frozen at -80°C.

Total bile acids dosage:

A simple, rapid and sensitive bioluminescence method used to measure primary bile acids has been developed and validated. The method is based on enzymatic dehydrogenation of bile acids using a bacterial 7α-hydroxysteroid dehydrogenize which is co-immobilized on Sepharose 4B beads with NADH: FMN oxido-reductase and a bacterial luciferase. The assay is specific for 7α hydroxy bile acids and has a detection limit of 0.5 pmol/tube, with a linear range of 0.5-50 pmol/tube. The assay has shown good accuracy (6-8% intra-assay; 8-1 0% inter-assay). The values obtained with the bioluminescence assay are comparable with those obtained by gas-liquid chromatography, radioimmunoassay, or endpoint enzymatic assays. Used to measure the concentration of serum bile acids, there was no interferences with serum albumin, and the effect of other dehydrogenize activity in serum could be eliminated by heating the sample before the assay. Since the method is rapid (1 minute), extremely sensitive (requires only 10 µl of serum), and specific, it appears to be the best method currently available for the measurement of primary serum bile acids (Roda A et al., 1982).

RT-PCR quantification assay:

Total mouse liver RNA was isolated by extraction using Trizol reagent (Invitrogen Life Technologies, Groningen, the Nederland) according to the manufacturer's instructions. The integrity of RNA was electrophoretically checked by ethidium bromide staining and by the OD absorption ratio OD_{260nm}/OD_{280nm}..A microgram of RNA was reversibly transcribed with Superscript II RNAze H-reverse transcriptase using oligo (dT) according to the manufacturer's instructions (Invitrogen Life Technology, France)
Real PCR time was performed on an iCycler ,real detection time system (Bio-rad, Hercules, CA, USA),and amplification was done by using SYBR Green I detection(SYBR Green Jumpstart, Taq Ready Mix for quantitative PCR, Sigma-Aldrich, St Louis; MO USA). Oligonucleotide primers (***Table 1)***, used for mRNA analysis, were based on the sequences of mice gene in the Gene Bank database.

Table 1 *Primer and sequences used in mRNA quantification by real-time PCR*

GENES	PRIMER'S SEQUENCES	
	Forward	Reverse
β-actin	5'- GGCACCACACCTTCTACAATGAGC -3'	5'- CGACCAGAGGCATACAGGGACAG -3'
BSEP	5'- GAGGTTACTTAATAGCCTACG -3'	5'-CATCTATCATCACAGTTCCC-3'.
MRP2	5'- CCTAGACAGCGGCAAGATTGT -3'	5'- TTACAGGGTGGTTGAGACCAG-3'.
OATP4	5'-CACATTCGGTCCCAAGTTCT -3'	5'- CAAGCTGCCTTTAGGTCCAG -3'.
OATP1	5'- GCACAGAGAAAAAGCCAAGG -3'	5'- GGATAGGCAGAATGCCATGT -3';
NTCP	5'-CCTCAGTGCTTTCCTTCTGG -3'	5'- ACAGCCACAGAGAGGGAGAA-3'
MRP3	5'- AAGTCCTCCAGCACAGCCTA-3'	5'- AGCTCCAGCTTGGTACCTGA -3'
IBAT	5′-TGGGTTTCTTCCTGGCTAGACT-3′	5′-TGTTCTGCATTCCAGTTTCCAA-3′

The reverse transcriptase reaction was diluted and an aliquot was subjected to amplification by PCR with the gene-specific primers listed. To compensate the variations in mRNA quality and quantity as well as random tube-to-tube variation in reverse transcription and PCR, an `competimer' /primer mix specific for 18 S ribosomal RNA (Ambion, Austin, TX, U.S.A.) was employed (Fuchs M et al., 2001). The ratio of 18 S `competimers' to primers was adjusted in accordance with the manufacturer's instructions such that the amount of 18S ribosomal RNA products was within the same range as that of the mRNA species of interest.

The amplification was carried out in a total volume of 25µl containing 12.5µl SYBR Green Taq Ready Mix, 0.3µM of each primer and diluted cDNA. Cycling condition consisted to an initial denaturation step of 95°C for 3 minutes a hot start followed by 40 cycles of 95°C for 30 sec or at 60°C for 30sec with a simple fluorescence detection point at the end of the relevant annealing or extension segment.

At the end of the PCR, the temperature was increased from 60 to 90°C for 15sec and at 582°C for 60sec, and the fluorescence was measured every 15sec to draw the melting curve. The standard curves were generated for each protein or β-actin using serial dilution of positive control template in order to establish PCR efficiencies. All determinations were performed at least in duplicates using two dilutions of each assay to achieve reproducibility. Results were evaluated by iCycler iQ software including standard curves, amplification efficiency(E) and threshold cycle(Ct).Relative quantization of mRNA expression was determine using the ΔΔCt in which ΔΔCt= ΔCt (gene of interest) −ΔCt(β-actin). ΔCt= Ct (interest group) – Ct (control group). Relative quantity (RQ) was calculated as follow: $RQ= (1+E)^{(-\Delta\Delta Ct)}$.

This RT-PCR method has been described and validated by Eugène Attakpa et al., (2009)

Protein expression detection by Western Blotting:

Levy of kidney mucosa were lysed in one hour time on ice with 40µLof buffer of cold lyse
(Tris HCl, PH=8.2; 20mM final; NaCl 140mM final; NP-40 1%; Sodium orthovanadate PH=10,2mM final ; NaF 10mM; EDTA PH=8 5mM final; PMSF 1mM final, antiproteaze cocktail(sigma) 2µL for 1ml of final volume).
After centrifugation (15000rpm, 15 min at 4°C) the floated part were recovered for protein dosage (spectroscopic dosage BCA) before their separation by electrophoretic migration. Twenty five micrograms (25µg) of proteins have been denatured during 5 minutes at 100°C in presence of buffer, deposited on SDS-Polyacryl amide gel bis acryl amide (8%), and transferred on PVDF membrane (Millipore, Bedford, MA).
The membrane were blocked for 2hours in dilapidated milk solution diluted to 5% in TBS /(Tween- 20, 0.05%) and after a brief rinse in a solution of TBS-Tween-20, has been reincubate for 2hours with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).
The Ibat was detected using rabbit primary antibody anti-Ibat. These were diluted to 1/5000th in buffer (PBS, 0.1% Tween 20, 1% BSA). This operation aims to saturate the protein membranes, to reduce non-specific binding of antibodies

The membrane undergoes four (04) washings of five (5) minutes in Tween-20 0.05% buffer after the blots were stripped and re-probed with a 1:1000 dilution of a mouse anti-rabbit antibody conjugated to an enzyme HRP (Horse Radish Peroxides) diluted in the same previous solution (Santa Cruz Biotechnology, Santa Cruz, CA).
The revelation of banding protein is finally performed thanks to the substrate solution of HRP enzyme .To ensure that the quantity of the protein detected by immunoassay were deposited uniformly, we have made the revelation of β-actin protein on the same membrane after dehybridization . Visualization of blots was accomplished with a chemiluminescence kit after washing, according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA).
Every Western Blotting is a representative of six experiments realized from six independent preparations. This Western Blotting method has been described and validated by Eugène Attakpa et al., (2009)

Statistical analysis

Results are shown as means±SEM. The significance of the differences between mean values was determined by two-way ANOVA (STATISTICA, Version 4.1, Stat soft, Paris, France), followed by the least significant difference (LSD) test. Differences were considered significant at $P < 0.05$.

Results

- Bsep and Mrp2 expression involved in bile acids uptake in the basolateral membrane of hepatocytes

In order to examine Bsep expression, we employed a high cholesterol diet. We observed that Bsep mRNA expression in the basolateral membrane transporters does not vary. Furthermore, the expression of Mrp2 mRNA (in arbitrary units $p<0.001$) is reduced in mice that it received a high cholesterol diet. The parameters were determined as described in materials and methods section. Values are means ± SEM, n = 15 per group of animals (***Fig. 1***).

- Expression of Oatp4, Oatp1, and Ntcp involved in bile acids uptake of in the apical membrane of hepatocytes.

Since high cholesterol diet modulated expression of Oatp4, Oatp1, and Ntcp involved in bile acids uptake in the apical membrane of hepatocytes, we measured the expression of Oatp4, Oatp1, and Ntcp mRNA. We observed that expressions of the Oatp4, Oatp1, and Ntcp, are suppressed in mice subject to the 5% cholesterol, reducing the penetration of bile acids in hepatocytes. The expression of mRNA was quantitatively analyzed by using RT-PCR as described in materials and methods section. Values are means ± SEM, n = 15 per group of animals. Each value is the mean of six determinations ***(Fig.2).***

- Expression of Mrp3 involved in bile acids secretion in the apical membrane of hepatocytes

The level of Mrp3 mRNA was quantified. The hepatic expression of Mrp3 was up-regulated in mice subjected to high cholesterol diet compared to control mice. The Mrp3, mRNA expression was quantitatively analyzed by using RT-PCR as described in materials and methods section. Values are means ± SEM, n = 15 per group of animals. Each value is the mean of six determinations ***(Fig. 3)***

- Plasma expression of bile acids on the 15th day of regime

The plasma concentration in bile acids on the 15th day was higher in mice subjected to high cholesterol diet comparing to the controls mice. Plasma concentration in bile acids was performed as described in materials and methods section. Values are means ± SEM, n = 15 per group of animals. Each value is the mean of six determinations. Data were analyzed by two-ways ANOVA. *significant differences ($p<0.001$) between the two groups of mice.
(Fig. 4).

- Expression of the renal Ibat carrier and its protein

The densitometry analysis of western blots revealed that at the apical membrane of epithelial cells proximal tubules in mice subjected to 5% cholesterol diet, the Ibat and its protein expression is decreased comparing to control group.
The expression of mRNA was quantitatively analyzed by employing RT-PCR as described in materials and methods section. Ibat protein levels were analyzed by Western blot. Each value is the mean of

six replicates. * significant differences ($p<0.001$) ***(Fig.5).***

Discussion

While it is clear that cholesterol directly affects bile acid synthesis in rodents, (Russell DW., 2003; Gupta S et al., 2002; Peet DJ, Turley SD, Ma W, et al., 1998) its impact on intestinal uptake and its subsequent fecal route elimination is still unclear.

We have highlighted in our experimental conditions, the expression of the genes involved in bile acids uptake in the basolateral membrane of hepatocytes. Only Bsep expression in basolateral membrane does not vary. But the expression of Mrp2 is reduced in mice that received a high cholesterol diet. Oatp4, Oatp1, and Ntcp, expressions are strongly suppressed in mice subject to the 5% cholesterol regime. However, it has been shown that in mice subjected to high diet in fat (18%) and cholesterol (1%), only the level of expression of Oatp1 decreased, while that of Ntcp, Mrp2 or Bsep varies not (Erickson RP et al.,2005). At the same time, cholesterol increases. It can be concluded that in our study that: modulation of hepatic carriers, would be involved in the resistance to high cholesterol.

Bile acids are synthesized from cholesterol in the liver; their production is a major mechanism of cholesterol elimination and important for the maintenance of cholesterol homeostasis (Vlahcevic ZR et al.1999). Down regulation of Asbt expression in obstructive cholestasis might therefore be a clinical interest. The origin of that regulation remains elusive in vivo, as a mediated cholesterol rise in hepatic bile acid synthesis itself (Chen F, Ma L, Dawson PA, et al). Liver receptor homologue-1 mediates species and cell line specific bile acid-dependent, negative feedback regulation of the apical sodium-dependent bile acid transporter. (J Biol Chem 2003; 278:19909–16.). This concomitant repression might explain the high efficiency of fecal bile acid wasting and hence contribute to the well known resistance of the mouse to diet induced hypercholesterolemia. (Dietschy JM et al.,2002 ; Chen JY , Levy-Wilson B, Goodart S, et al.,2002; Schwarz M, Davis DL, Vick BR, et al.,2001; Phan J, Pesaran T, Davis RC, et al.,2002).

In the kidney, bile acid excretion in urine increased progressively both in animal cholestatic models but also in clinical cholestatic disorders (Lee J et al.2001; Stiehl A et al., 1995).

In Wistar rats, a marked reduction in Asbt protein expression in microsomal membrane fractions from whole kidney

after Cbdl was observed, resulting in reduced Asbt expression on the luminal membrane of the proximal tubule of the kidney, a change that is associated with a decreased reabsorb reabsorption capacity of bile acids from the glomerular filtrate (Lee J et al., 2001). In the same study, an up regulation of Mrp2 protein expression in the apical membrane of rat renal proximal tubule has been shown.

Mrp2 is associated to an increased ability to excrete divalent organic anions such as bile salt sulphates and glucuronides (bilirubin) that accumulate during cholestasis and would therefore facilitate extra hepatic pathways for bile acid and bilirubin excretion during cholestasis. This adaptive response of Asbt and Mrp2 in the kidney of the rat does not seem to be regulated by luminal bile acids. Here we showed that Asbt mRNA expression correlated inversely with bilirubin as well as with plasma bile acid concentrations, which were both used as markers for obstructive cholestasis.

In previous publication, it was shown that inhibition of Ileac Bile Acid Transport with SC-435, a competitive inhibitor of Asbt, lowered plasma cholesterol levels: (Koop I et al.1996) by inactivating the hepatic farnesoid x receptor (Meier PJ et al. 2002) and by stimulating cholesterol 7a-hydroxylase (Cyp7a1) (Li H et al. 2004). This cytochrome is the rate limiting enzyme of chenodeoxycholic acid synthesis.

These findings also raise the possibility of a coordinated regulation of intestinal and hepatic bile acid transfer following cholesterol feeding, as hepatic bile acid transporters such as Ntcp or Oatp1 are HNF-1a target genes. (Trauner M et al., 2003 ; Meier PJ et al.,2002 ; Shih DQ, Bussen M, Sehayek E, et al.,2001 ; Jung D, Hagenbuch B, Fried M, et al.,2004; Kullak-Ublick GA et al.,2004).

Molecular dissection of such regulatory pathway might provide new insights for the development of original hypocholesterolaemic treatments in the future.

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