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ROLE OF MULTIDRUG-RESISTANCE PROTEIN 2 IN COPROPORPHYRIN TRANSPORT: RESULTS FROM EXPERIMENTAL STUDIES IN BILE FISTULA RAT MODELS

V. MORIONDO, S. MARCHINI, P. DI GANGI, M.C. FERRARI, F. NASCIMBENI, E. ROCCHI AND P. VENTURA ✍

Department of Medicines and Medical Specialities, University of Modena and Reggio Emilia
✍ Paolo Ventura, MD, Department of Medicines and Medical Specialities
University of Modena and Reggio Emilia, Italy
Policlinico di Modena Via del Pozzo 71, 41100 Modena, Italy
Tel. 039-059-4222807, Fax. 039-059-4225542, E-mail: paoloven@unimore.it

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Abstract – Coproporphyrin (CP) is one of the main by-products of heme biosynthesis and its abnormal accumulation is associated with different forms of porphyria. Indirect data obtained from animal and human models have suggested a possible role for Multidrug Resistance–associated Protein 2 (MRP2) and other MRPs in hepatocyte excretion of CP. Using normal, MRP2-deficient and a cholestatic rat model, we have assessed the role of MRPs in CP disposition. MRP levels were assayed using immunofluorescence. Biliary and urinary excretion patterns of CP and conjugate bilirubin were measured during equimolar infusions of CP isomers with and without phenoldibromopthalein sulfonate (BSP), a well-known MRP2 substrate. Our results suggest a role for the MRP system as a possible regulator of CP traffic and accumulation in normal and pathological conditions. Alteration in this systems (as observed in cholestatic disease) may play an important role in triggering clinical expression of porphyria in individuals with underlying mutations leading to porphyrin accumulation and may help explain the phenotypic heterogeneity in patients affected by different forms of porphyrias.

Key words: Coproporphyins, Porphyrias, Multidrug resistance proteins, Transporters, Isomers, Cholestasis, Dubin-Johnson syndrome

INTRODUCTION

Heme has a key role in many different critical biological processes; thus, it is not surprising that its biosynthesis is finely tuned according to the different requirements, which vary significantly among various cells and tissues (39). Compared with other cells, rapid rates of heme biosynthesis occur in liver and erythroid cells for mitochondrial cytochromes and hemoglobin production, respectively (13,14,39). Biosynthesis of heme is a multistep process that starts with simple molecules (succinyl-CoA and glycine) and involves 4 cytoplasmic and 4 mitochondrial enzymes (Fig.1). Each step of this biosynthesis pathway is directed by its own task-specific enzyme (1,45,48). Free porphyrins formed along the biosynthetic process are thought to have no biologic utility in humans, and are generally considered only as accidental by-products; nevertheless, in normal conditions, heme biosynthesis is normally remarkably efficient, with near-complete utilization of porphyrin intermediates (45). The relative low amount of free porphyrin produced as surplus during the synthetic process are mostly eliminated via emunctories systems (liver, kidney and also gut) (5,6,16,41,45). On the other hand, conditions characterised by abnormal tissue accumulation of free porphyrins are known to be associated to different forms of tissue damage. This situation is the main characteristic of the porphyrias, a group of human diseases caused by a defect in one of the eight enzymes of the heme biosynthesis pathway. As a consequence, heme precursors accumulate causing variable clinical manifestations in the affected subjects. Porphyrias are mostly inherited conditions, but

Abbreviations: CP, Coproporphyrin; **CPI**, Coproporphyrin isomer I; **CPIII**, Coproporphyrin isomer III; **URO-D**, uroporphyrinogen decarboxylase, **CPGO**, coproporhyrinogen oxidase, **BSP**, phenoldibromopthalein sulfonate; **MRP**, Multi drug resistance protein; **DJS**, Dubin-Johnson Syndrome; **WT**, Wild Type; **EI**, Eisai; **EE**, ethynil-estradiol

they may be triggered by exposure to many different environmental conditions (especially chemicals or drugs) or concomitant diseases able to interfere with the heme biosynthetic pathway (1,17,48). As above mentioned, the liver has a major role in heme synthesis, however, it also plays a key role in the regulation of porphyrins excretion. Excretion profiles, in the urine and faeces, of porphyrins and their metabolites may be altered in the presence of hepatocyte dysfunction and/or cholestatic diseases (10,34-36). Coproporphyrin (CP) is one of the by-product of heme synthesis and it is probably the most important among those produced in little surplus amount along the very efficient heme biosynthetic pathway (45). This is the reason why small amounts of CP are physiologically excreted by the liver and the kidney and may be detected in urine and bile (faeces) (5,16), mostly as two isomer forms, namely isomer I and isomer III (Fig.2). CP I is formed through decarboxylation (by uroporphyrinogen decarboxylase, URO-D) of uroporphyrinogen I, a spontaneous ("abortive") product that escapes the side-chain rearranging reaction of uroporphyrinogen III synthase, whose normal product is uroporhyrinogen III (which is, in turn, decarboxylated by URO-D to CPIII); differently from CPIII (that is normally produced in some excess with respect to CPI), CPI cannot be used as a substrate by the next step enzyme (coproporhyrinogen oxidase, CPGO) (Fig.1) (45).

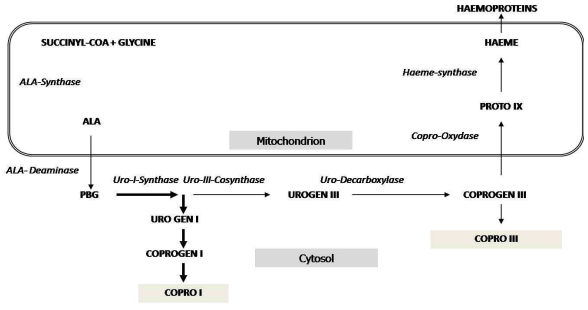

Figure 1. Coproporphyrin synthesis during Heme synthesis.
Coproporphyrin is normally formed during the synthesis of heme. **ALA**= δ-aminolevulinic acid; **PBG**= Porphobilinogen; **UROGEN**= Uroporphyrinogen; **COPROGEN**= Coproporphyrinogen; **COPRO**= Coproporphyrin; **PROTO IX** = Protoporphyrin IX.

In healthy adult subjects, total urinary excretion of CP is about 30-150 μg per gram of urinary creatinine per day (< 200-250 μg/day). The isomer ratio of CP (CPI/CPIII ratio) about 1:2 (5,16,40). In bile, about 65-70% of total CP is present as isomer I and the remaining as isomer III. Studies in humans have confirmed the role of liver in CP excretion: congenital adult and infant liver cholestatic diseases cause a derangement in porphyrins metabolism and excretion. Some forms of inherited jaundice are due to a defect in bilirubin excretion. Dubin-Johnson syndrome, DJS is associated with abnormal CP and biliary excretion patterns in both the urine and faeces, suggesting a link between the biliary excretion of both conjugated bilirubin and CP (3,9,19,25).

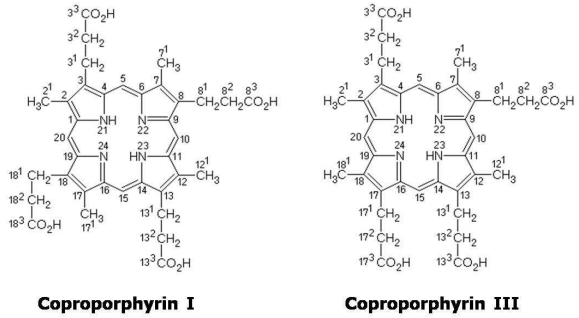

Figure 2. Chemical structure of coproporphyrin isomers. The different spatial disposition of four anionic (propionic acid) residues on pyrrole groups (symmetrical in isomer I, asymmetrical in isomer III) has been suggested to be important in influencing CP isomers excretion by hepatocyte canalicular (biliary) carrier (see details and references in the text).

Hepatocyte uptake and efflux processes involved in general metabolism and in bile formation are maintained by distinct transport systems at the two polar surface domains [i.e. basolateral (sinusoidal) and apical (canalicular)] of liver cells. Each of these transporter systems acts on different substrates, with different grade of specificity (Fig.3) (37). The Multidrug Resistance–associated Proteins (MRPs, also indicated as ATP-Binding Cassette ABCC) have been implicated in the cellular efflux of many different compounds (11,15,31,37). MRP2, the only canalicular member of the MRP family, mediates the transport of glucuronidated and sulfated bile salts (2,4,15,22,43). Furthermore, MRP2 is involved in biliary excretion of a wide spectrum of other organic anions (Fig. 4). While the substrate specificity has been well studied for many of the MRP family members there is functional overlap (with a possible vicarious function in case of intracellular accumulation of substrates), that has been observed between MRP2 and other basolateral trasporters (4,15,32,37).

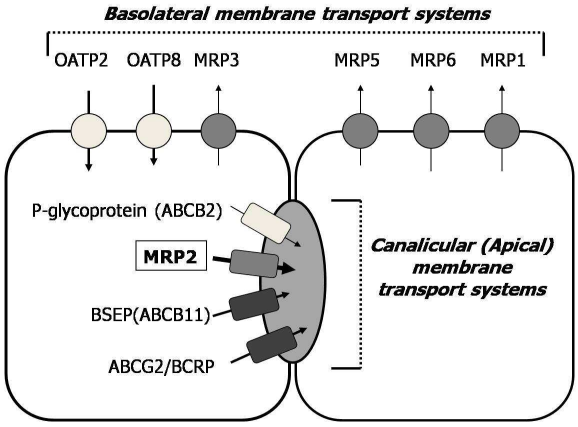

Figure 3. Membrane localization of main efflux pumps in hepatocytes : MRP2 is the only MRPs localized at the biliary pole of hepatocyte: it regulates the biliary efflux of many different substrates (see fig. 4); the other MRPs are localized at basolateral pole of hepatocyte and regulate the efflux of many different substrates in the sinusoidal space (and hence in plasma).

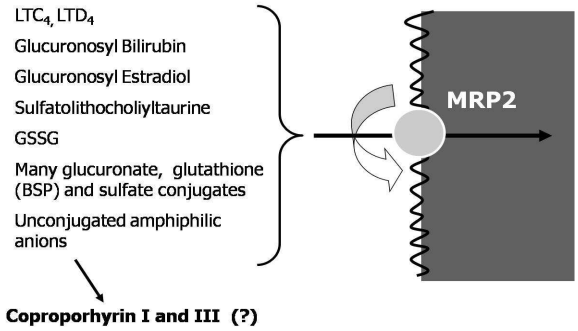

Figure 4. MRP2 substrates.

Indirect data from animal and human models of Dubin-Johnson syndrome in which there is decreased MRP2 expression, have suggested a possible role for MRP2 (biliary efflux) and other MRPs (sinusoidal efflux) in hepatocyte excretion of CP (19,46,49). MRP expression in other emunctories (gut and kidney) may also be important in regulation of CP excretion in urine and feces (7,28,43).

In order to assess the role of MRPs in CP excretion, we used a bile fistula rat model to compare conjugate bilirubin and CP excretion in bile and urine during equimolar infusions of CP isomers and phenoldibromopthalein sulfonate (BSP), a well-known MRP2 specific substrate. The results of CP excretion were evaluated taking in account the cellular expression of MRPs (MRP1,MRP2 and MRP3) in liver and kidney in different rat models (Wild type, MRP-2 knock-out and cholestatic rats).

MATERIALS AND METHODS

12 Male Wistar Rats, six with no pretreatment (Controls) and six ethinylestradiol-treated (1.25 mg/100 g of body weight in propylene glycol) (EE-rats) [treatment was made in order to induce a condition resembling an intrahepatic cholestasis, as previously described (23,24)] and 6 male Eisai rats (knock-out for MRP2) (EI-rats) were studied. All rats weighed between 220 and 310 g and were maintained awake in restraining cages after surgical preparation with intravenous and biliary cannulae.

For CP infusion, CPI and CPIII tetramethyl esters (Sigma-Aldrich Chemical Co., St.Louis. MO. USA, 95% pure) were hydrolyzed in 8 N HCl for 16 hr; after adjusting pH to 3.5, the free acid was extracted into ethyl acetate. It was then vacuum distilled to dryness and redissolved as the infusion mixture in 0.9% sodium chloride 0.01M phosphate buffer, pH 7.4 (final concentration 50 µg/mL for each isomer). No significant difference in water solubility between two isomers was shown.

CP isomer levels in serum, bile and urine were determined by HPLC, as previously described (10,34,35), conjugate bilirubin levels in bile and urine was measured by routine laboratory methods (Thermo Fisher Scientific Inc. Middletown, VA USA) .

After 3 hr saline intravenous infusion (2 mL/hr), all rats underwent a 4 hr equimolar CP infusion (50 µg/hr) followed by concomitant 3 hr phenoldibromopthalein sulfonate (BSP) (Sigma-Aldrich Chemical Co., St.Louis. MO. USA, 99% pure) intravenous infusion (0.4 µM/hr).

At intervals of 1 hr from the beginning of infusion, we measured CP and conjugate bilirubin levels in bile and urine (CP values were measured as µg/mg creatinine).

Before infusion, all rats underwent a liver and kidney biopsy in order to quantify MRP1, MRP3 and MRP2 tissue expression by tissue immunofluorescence, as previously described (18,20,21,22,44,47). In this analysis, due to the small amount of tissue obtained by the needle biopsy, we used a semiquantitative assessment of the immunostaining. We used the monoclonal antibody (MAb) QCRL1 against MRP1 and the MAb M2III-6 against MRP2 (Alexis Biochemicals, San Diego, CA). Polyclonal antibodies to MRP3, raised in rabbits against the 24-amino acid carboxyl terminus (FDS) and prepared as described by Konig et al. (22).

The immunofluorescence stainings of liver and kidney cryosections was performed as previously described (18,20,21,22,44,47). Antibodies were diluted in phosphate buffered saline containing 5% fetal calf serum as follows: (MAb) QCRL1 against MRP1, and the MAb M2III-6 and the fluorochrome-conjugated anti-antibodies at 1:100; for FDS at 1:50. Pictures were taken on an Axiovert S100TV microscope (Carl Zeiss, Jena, Germany) using the Improvision software (Coventry, UK) on a confocal laser scanning microscope (LSM510, Carl Zeiss).

Tissue sections were stained for each of the transporters. Confocal images of four different fields per sample were obtained with identical laser intensities and scanning parameters (e.g. contrast, brightness, confocal aperture diameter) for all samples, so that staining intensities were comparable. The mean level values of controls were considered as a measure of reference staining intensity (=1). Reported values are the ratio of the number of pixels of the respective transporter staining to the number of pixels of reference staining category. Figure 5 reports an example of results of immunofluorescency assay for MRP2 and MRP3 expression on liver tissue.

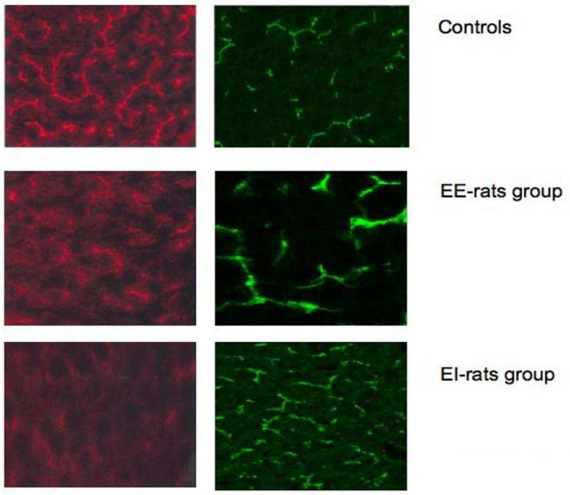

Figure 5. Example of MRP2 (red) and MRP 3 (green) expression, as resulted by immnunofluorescence staining, in liver tissue from normal, EE and EI rats.

Statistical Analysis

All data are presented as mean (± Standard Deviation, SD). After verifying data distribution, we used one-sample t–test (Bonferroni corrected for multiple comparisons) to compare normalized data concerning MRPs expression (a value of 1 was used as the reference expression in normal rats). In samples with repeated measurements ANOVA (with LSD post-Hoc) was used to compare bile, CP and bilirubin excretion rates during infusion experiments.
In all statistical analysis a value of $P < 0.05$ was considered significant. All analyses were conducted using SigmaStat® v.3.5 statistical software (Systat Software Inc., Richmond, California, USA).

RESULTS

Expression of MRP2, MRP1 and MRP3 in normal rat liver and kidney are presented in Table 1. Basal excretion of biliary CP resulted in CPI/CPIII ratio at 2-3:1. Apon intravenous infusion of equimolar amounts of CP isomers there was a significant increase in biliary excretion of CP, always in a 2-3:1 ratio, that is favoring CPI (Fig. 6 A). Urine obtained during the study contained relatively little of the administered CP, the CPI/CPIII ratio was 1:2. During the infusion periods bile flow did not show significant reduction and it remained within ±10% of the pre-infusion rate (Table 2). During BSP infusion, a significant reduction in biliary CP excretion rate was observed, even if maintaining a CPI/CPIII at 2-3:1 ratio. In this phase a significant increase in CP urinary excretion was observed, with a progressive shift in the CPI/CPIII ratio from 1:2 to 1.3-1.5:1. BSP infusion induced little modification in measured total bile flow.

In *EI-rats* (MRP2 -/-; with higher expression of MRP1 and MRP3 in liver and kidney, see data from Table 1) basal biliary CP excretion was lower than in controls and the CPI/CPIII ratio was near to 1:1. After intravenous infusion with equimolar amounts of CP isomers there was a significant increase in CP excretion in bile. However, this was still significantly lower, in terms of absolute values, when compared to controls. The CPI: CPIII ratio remained at approximately 1:1, (Fig. 6, B). Urine obtained before CP infusion contained relatively higher amounts of the CPI isomer, with a CPI/CPIII ratio of about 3-4:1. During CP infusion, the urinary excretion of CP significantly increased, maintaining a CPI/CPIII ratio favoring CPI isomer.

During BSP infusion phase, no significant reduction in biliary CP excretion rate, nor significant increase in CP urinary excretion were observed. During the infusion phase, bile flow did not show any significant reduction (it remained within ±10% of the preinfusion rate) (Table 2).

In the *EE-rat* model MRP2 was expressed at approximately 40% of control levels in the liver and expression was significantly increased in the kidney. Expression of MRP1 and MRP3 was significantly higher in the liver and kidney (Table 1). Basal excretion of CP into the bile was significantly lower than in controls and the ration of CPI/CPIII was altered to a 1.5-2:1 ratio. Intravenous infusion of equimolar amounts of CP isomers was followed by a significant increase in their biliary excretion, favoring CPI, with a CPI/CPIII ratio about 2:1, (Fig. 6, C). Urine obtained during the study contained increased levels of CP with the CPI/CPIII ratio shifted towards CPI, (CPI/CPIII ratio about 1.5-2:1). During the infusion phase bile flow did not show significant reduction (it remained within ±10% of the pre-infusion rate) (Table 2). During BSP infusion periods, a significant further reduction in biliary CP excretion rate was observed, even if maintaining the CPI/CPIII ratio at 1.5-2:1. In this phase, a further increase in urinary CP excretion was observed, with a progressive shift in the CPI/CPIII ratio from 1.6:1 to 2:1. BSP infusion induced little modification in measured total bile flow, which was significantly lower than that observed in control, as expected (Table 2).

DISCUSSION

Patterns of CP excretion in bile in many different pathological condition has been the object of several studies in both animals and man

Table 1. Basal expression of MRP1, 2 and 3 in liver and kidney.

	Liver§				Kidney§			
	MRP1	MRP2	MRP3	GAPDH	MRP1	MRP2	MRP3	GAPDH
Controls	1	1	1	1	1	1	1	1
EI-rats	2.3±0.2**	-- --	2.5±0.3**	1	2.1±0.2**	-- --	2.3±0.2**	1
EE-rats	1.5±0.3*	0.38±0.2**	1.6±0.2**	1	1.7±0.2**	1.6±0.3**	1.8±0.2**	1

§=**Values are normalized to expression in control animals.** **p<0.01; *p<0.020 vs. controls (Bonferroni correction significant for p<0.025)

Table 2. Effect of infusion on Bile flow (g/hr)

	Time (Hours)							
	Basal§	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	Saline	**CP Infusion**				**CP+BSP Infusion**		
Controls	0.89	0.92	0.91	0.91	0.89	0.95	0.94	0.89
EI-rats	0.90	0.90	0.92	0.89	0.85	0.89	0.92	0.88
EE-rats	0.42	0.45	0.46	0.38	0.44	0.42	0.45	0.46

§= Mean value of 3 hours detection. The values are the mean value registered in 5 rats per group. Standard deviation was always lower than 10% of reported mean. No significant values with respect to basal values were observed.

(12,19,25-27,36,38). Our data confirm that in normal conditions approximately 60% of the normal daily CP excretion is found in bile with a I to III ratio of about 2-3:1. The remaining CP is excreted in urine with the I to III isomer ratio being approximately 0.5:1 (5,16). In cases where impairment of hepatic excretory function is observed (cholestasis) there is an increase in the total CP excreted in urine, with a rise in the proportion of the CP isomer I.

The role MRPs play in CP excretion may be argued considering the patterns of CP excretion into bile and urine, considering the differential expression levels of the varios MRPs (Table 1). The absence of MRP2 (Fig. 6B) is characterized both by a significant decrease in bile CP excretion, and by a significant alteration in the CPI/CPIII biliary excretion pattern. The proportion of the CP I isomer in urine is increased to approximately 70% of the total. Similarly, in the experimental model of cholestasis (Fig. 6C), the decrease of MRP2 expression is associated with a significant reduction in CP biliary excretion, with a modification of the excretion pattern somehow intermediate among normal and MRP2-null conditions. These data strongly support a key-role for MRP2 in CP biliary excretion.

The reason for the differences in the proportions of the two isomers which are excreted in bile and urine is not readily apparent: a difference in serum protein-binding favoring the ultrafilterability of the III isomer could explain the relatively greater renal excretion in normal animals. However, no evidence for a difference in binding to the serum or liver cytosol fractions was observed in previous studies (8,16,33,42). Moreover, such a mechanism would not explain the increase in the proportion of the I isomer excreted in the urine during experimental cholestasis, nor would it explain the preponderance of CP I excreted in bile during the equimolar infusions of the isomers. These findings support, on the contrary, the hypothesis

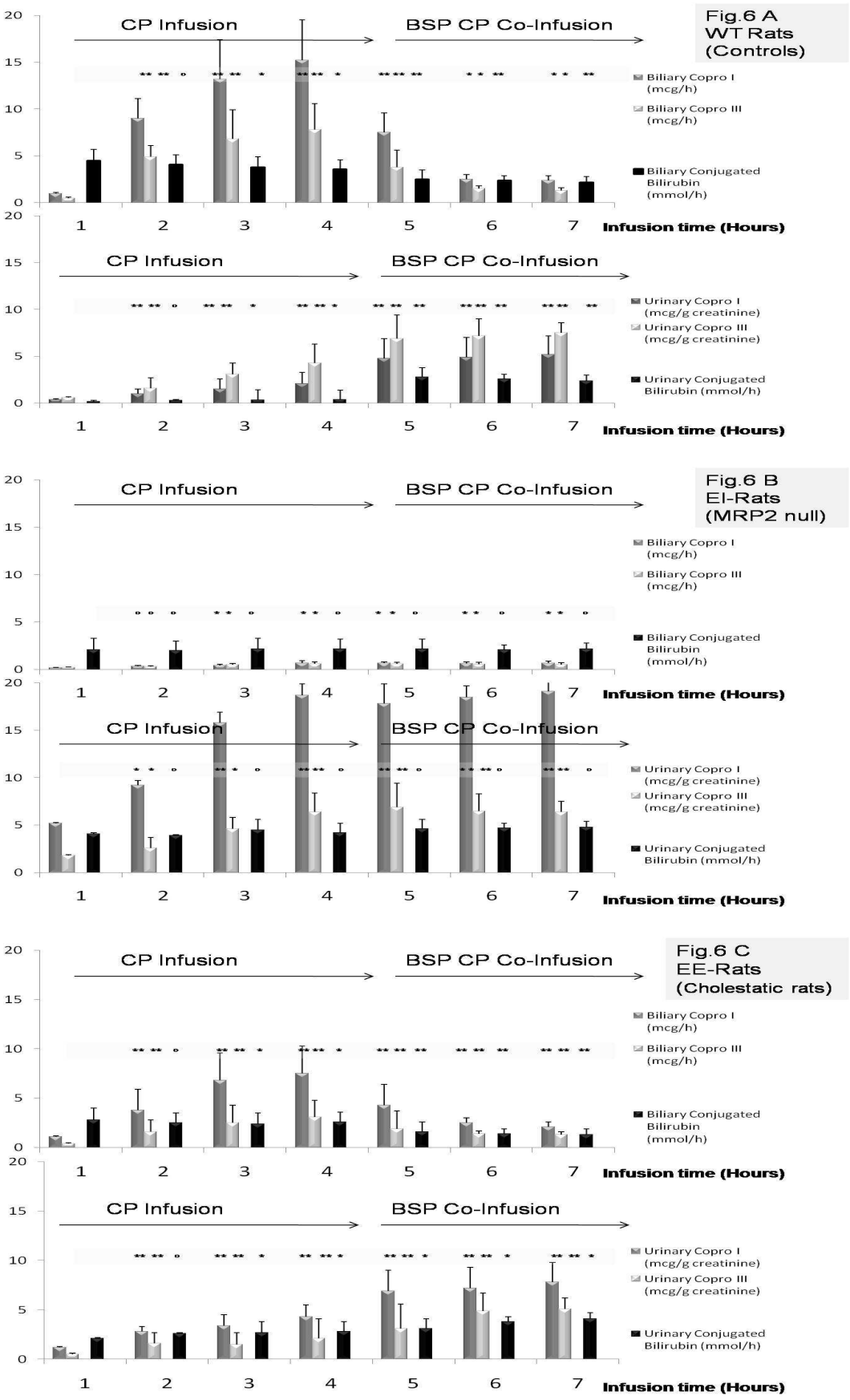

Figure 6. Conjugate bilirubin and CP isomers excretion in bile and urine during CP and CP/BSP infusion experiment in Controls (6A), EI (6B) and EE (6C) rats. **p<0.01; *p<0.05: °=not significant with respect to correspondent basal value.

that there is a discrimination between CP isomers and that excretion may depend on inherent characteristics of the transporter.

Since neither isomer is further metabolized by the liver (16), the discrimination in hepatic excretion may occur either at the uptake or excretory step. Discrimination at the uptake step might be reflected by a change in the plasma isomer ratio, since one isomer would have a much larger volume of distribution than the other. Furthermore, selectivity at the uptake step should give rise to similar ratios of isomers I to III in both liver and bile. However the ratio of CPI to III in liver is almost the reciprocal of that in bile. Thus the proportionally greater biliary excretion of the I isomer is likely not related to a more rapid liver uptake process, but rather, more rapid excretion as reflected by the relative increase in the isomer remaining in liver. These findings, thus, favor the excretory rather than the uptake step as the major determinant of the unequal proportions of isomers excreted in bile. Since the CP isomers are organic anions, it is reasonable to consider their transport into bile within the framework of current concepts about carrier transport systems, which are believed to exist for a variety of other organic anions (3,9). Even at the relatively low infusion rates used in our study, the concentration of each isomer in bile was always greater than in plasma and their relative rates of excretion were different.

The unequal excretion rates of the isomers may be in turn attributed to either separate transport systems with different affinities for each isomer or a single carrier which apparently favors the excretion of the I isomer.

The data obtained from our infusion studies (Fig.6, A, B and C) can not directly discriminate between a single carrier or two independent carrier mechanisms for the transfer of each isomer into bile; nevertheless, if two independent carriers were present, it is surprising that BSP, infused at rates considerably greater than that of either of the CP, caused a proportional reduction in the excretion of each isomer in bile. Under the latter circumstances, a greater reduction in the less efficient transport system for the III isomer might have been expected; instead, we observed a relatively greater reduction in the number of molecules transported in the more efficient CPI carrier system with a resultant preservation of the usual isomer ratio in bile.

Ethinylestradiol, which markedly impairs hepatic excretory mechanisms (Fig.6 C), had a similar effect (23,24). These observations, thus, all favor the view that a single carrier mechanism for both CP isomers operates in the liver cell and, considering the observations made in the absence of MRP2 (Fig. 6 B), this carrier is likely to be identified as MRP2.

The existence of a common transport system for both the CPI and CPIII isomers would readily explain the ratios found in bile and urine under normal circumstances and in the various conditions impairing hepatic excretory mechanisms. Thus, a statistical relationship between the transport of the two isomers may be envisaged in which there exists a single hepatic carrier having a two-point binding site, or fit, for the CP molecules. The two points of the binding site on the carrier may be postulated to be at a critical distance which corresponds to the distance between each of four propionic acid side groups of the symmetrical type I isomer. This isomer could, therefore, interact with the carrier in any one of four presentations corresponding to the four sides of the planar coproporphyrin I molecule. The III isomer, however, because of the reversal of the acidic substituents on one of its pyrrole rings, is asymmetrical and thus could fit the carrier in only two presentations, i.e., the two sides of the molecule having propionic acid groups at this same critical distance.

Assuming equal access of the isomers to a fixed carrier site, the probability of transport of the asymmetric III isomer would therefore be only one-half that of the symmetrical I isomer. An impairment in the postulated common hepatic carrier transport mechanism would cause a reduction in total coproporphyrin transport into bile with consequent diversion of more of the type I than the type III compound from the biliary to the urinary route of elimination. In either event, the same statistical considerations would still apply. Thus, to explain the findings in our studies and those reported in human liver disease, it is unnecessary to postulate separate transport systems for each CP isomer, but rather, a stereospecific common carrier which, on a statistical basis, results in a predominance of coproporphyrin I excretion in bile.

In our experiment no data were measured about coproporhyrinogen transport. Nevertheless no evidence exists that coproporphyrinogens rather than coproporphyrins are excreted in urine or bile: coproporhyrinogen are in fact very instable compounds, highly prone to spountaneously transform in their correspondent oxidized product (coproporphyrins) (this is also the reason why the measurement of reduced

porphyrins are difficult and , also for clinical purposes, the more stable oxidized compounds are commonly measured) (45,48) .

Other important remarks may be drawn by our infusion study. The first concerns the high affinity of the carrier (MRP2) for CPs. BSP infusion in fact induces a really important decrease of CP biliary excretion, significantly greater (even three-fold greater with respect to bilirubin decrease) than that observed for conjugate bilirubin (Fig. 6 A and C). The second remark concerns the role of other transporters in CP biliary excretion. MRP2 absence in fact (cfr results from EI -rats group) did not completely abolish the CP biliary excretion. In EI-rats group (but also in EE-rats groups) CP excretion is significantly reduced but not abolished, this suggesting that other transporters may "supply" the MRP2 function, even if they are less efficient (lower export of CP) and probably without the above-postulated stereo-specificity (CPI:CPIII ratio near 1:1).

Besides these considerations, to date, our data represent the first observation concerning the role of cell system transports as an important regulatory system able to influence the porphyrin metabolites traffic and accumulation into the cell (hepatocytes).

Alteration in this systems may have an important role in inducing or triggering the diseases due to porphyrin intermediates accumulating in patients prone to develop porphyria (as in porphyrias' carrier patients) and may have importance in explaining the different course of diseases (incomplete penetrance of genetic defect) in patients affected by porphyrias (1,17).

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