New insights into the regulation of bile acids synthesis during the early stages of liver regeneration: A human and experimental study

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ABSTRACT

Background and aims: Liver regeneration is essential for the preservation of homeostasis and survival. Bile acids (BAs)-mediated signaling is necessary for liver regeneration, but BAs levels need to be carefully controlled to avoid hepatotoxicity. We studied the early response of the BAs-fibroblast growth factor 19 (FGF19) axis in healthy individuals undergoing hepatectomy for living donor liver transplant. We also evaluated BAs synthesis in mice upon partial hepatectomy (PH) and acute inflammation, focusing on the regulation of cytochrome-7A1 (CYP7A1), a key enzyme in BAs synthesis from cholesterol.

Methods: Serum was obtained from twelve human liver donors. Mice underwent 2/3-PH or sham-operation. Acute inflammation was induced with bacterial lipopolysaccharide (LPS) in mice fed control or antioxidant-supplemented diets. BAs and 7a-hydroxy-4-cholesten-3-one (C4) levels were measured by HPLC-MS/MS; serum FGF19 by ELISA. Gene expression and protein levels were analyzed by RT-qPCR and western-blot.

Results: Serum BAs levels increased after PH. In patients with more pronounced hypercholanemia, FGF19 concentrations transiently rose, while C4 levels (a readout of CYP7A1 activity) dropped 2 h post-resection in all cases. Serum BAs and C4 followed the same pattern in mice 1 h after PH, but C4 levels also dropped in sham-operated and LPS-treated animals, without marked changes in CYP7A1 protein levels. LPS-induced serum C4 decline was attenuated in mice fed an antioxidant-supplemented diet.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate amino transferase; BAs, bile acids; BHA, butylated hydroxyanisole; BMI, body mass index; C4, 7a-hydroxy-4-cholesten-3-one; CYP7A1, cytochrome 7A1; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GPBAR1, G protein-coupled BAs receptor 1; IL, interleukin; LDLT, living donor liver transplant; LPS, lipopolysaccharide; LR, liver regeneration; MnSod, manganese superoxide dismutase; PH, partial hepatectomy; TNF, tumor necrosis factor.

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1. Introduction

The liver plays fundamental roles in preserving systemic homeostasis, including central pathways in intermediary metabolism, the synthesis of serum proteins and bile acids (BAs), as well as the detoxification of waste metabolites and xenobiotic compounds [1]. This detoxification activity entails the handling of potentially cytotoxic molecules which may cause cellular damage and liver injury. To cope with this and other aggressions such as viral infections the liver has developed a unique and extraordinary regenerative capacity [2]. Liver regeneration (LR) can be triggered by parenchymal damage and also upon partial liver resection, being LR a highly regulated process in its onset and termination. A successful LR is essential to meet systemic metabolic demands, avoid liver failure and preserve survival. Remarkably, LR starts rapidly after injury or resection and precisely stops when the size of the functional parenchyma meets the metabolic needs of the individual, an intrinsic property known as the "hepatostat" [3,4]. Many signals have been involved in orchestrating LR, ranging from hemodynamic changes, hormones, components of the innate immune system, cytokines, growth factors and metabolites [2,5]. Indeed, given the liver is a central organ in general metabolism it is plausible that fluctuations in the systemic and intrahepatic levels of certain metabolites could influence the overall regenerative process [6]. BAs are increasingly recognized as crucial metabolites in LR, a notion supported by findings in humans and experimental models [7–10]. Besides their essential role in the intestinal absorption of fats and vitamins, BAs behave as signaling molecules through nuclear and cell surface receptors such as the farnesoid X receptor (FXR) and the G protein-coupled BAs receptor 1 (GPBAR1, also known as TGR5), respectively [9,11]. BAs-mediated activation of FXR in hepatocytes rewrites energy metabolism and promotes cell proliferation, while intestinal FXR activation stimulates the production of fibroblast growth factor 19 (FGF19, F15 in rodents) in ileal enterocytes, which also play a role in regulating hepatic BAs synthesis in the liver [8,16]. BAs production from cholesterol is carried out by several cytochrome P450 (CYP) hydroxylase enzymes, of which cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first and rate-limiting step in the major BAs synthetic route [20,21]. It is known that the CYP7A1/Cyp7a1 gene transcription is transcriptionally repressed shortly after PH by pleiotropic mechanisms, including the hepatic BAs-FXR-dependent pathway and the BAs-FXR-FGF19/FGF15 gut-liver axis, which would contribute to shutting down BA synthesis [12,22-24]. On the other hand, CYP7A1 activity also impacts on hepatic cholesterol levels and, importantly, cholesterol availability is needed for hepatocellular growth and proliferation during LR [20,25]. Therefore, preservation of hepatic BAs homeostasis during LR is essential to prevent injury while enabling compensatory liver growth. Unraveling the precise regulation of BAs synthesis during LR is important for the mechanistic understanding of LR, but also for the development of therapeutic strategies to prevent post-resection liver failure [26–29]. In this study we have combined observations in a unique cohort of healthy patients undergoing liver resection for living donor liver transplant (LDLT) [30,31], with studies performed in mouse models of PH and acute inflammation. Our results provide novel insights into the response of the BAs-FXR-FGF19 axis during LR in healthy humans. Moreover, they suggest the existence of previously unrecognized mechanisms controlling CYP7A1 enzymatic activity that may be relevant for liver BAs homeostasis under different stress conditions, including LR.

2. Materials and methods

2.1. Human samples

Human serum samples were collected from healthy patients (n = 12) undergoing right hepatectomy for LDLT at the University Clinic of Navarra at baseline (preoperative stage) and at different times after LR. We also included serum samples from patients with sepsis (n = 20). Their infections had diverse anatomical origins (kidney, lung, urinary system, abdomen, skin and gallbladder). Serum samples were collected at admission to the hospital, at day two and upon hospital discharge (5–12 days since admission). Serum samples were stored at the Bio-bank of the University of Navarra. The collection of these samples and the associated clinical information was approved by the Ethics Committee of the University of Navarra (protocols #064/2010, #146/2017 and #061/2015). The study was conducted in compliance with the ethical standards formulated in the Helsinki Declaration of 1996 (revised in 2000), upon obtention of the informed consent from all patients. Part of this cohort has been preliminary characterized in a recent report from our groups [32].

2.2. Animal models

PH (2/3 of liver mass) was performed in C57BL/6J male and female mice, 10 weeks of age (n = 6 per time point), as previously described [12,29]. A matched control group of sham-operated mice that underwent laparotomy and liver exposure without resection was included. Serum and liver tissue samples were collected for analyses at the indicated time points. For the acute inflammation model, C57BL/6J male mice, 8–10 weeks of age (n = 6 per time point) received a single i.p. injection of bacterial lipopolysaccharide (LPS) (#L2880, Sigma, St. Louis, MO, USA) or saline as described [33]. In a second protocol, prior to LPS injection mice were fed a control chow diet or the same diet supplemented with the antioxidant butylated hydroxyanisole (BHA, 0.7 %) (#W218405, Sigma) for 4 days, as described [34]. Serum and liver tissue samples were collected at the indicated time points for analyses. Experiments were conducted in accordance with the U.K. Animals Act and the EU Directive 2010/63/EU for animal experiments. Procedures were approved by the Ethics Committees for Animal Experimentation of University of Navarra (project #063-19E1) and Université Paris Saclay “CEEA 59” (project 2014-10#3417).

2.3. Cellular culture and analyses

The mouse immortalized hepatocyte cell line AML12 [35] was obtained from the ATCC and was cultured as previously described [36]. Cells were treated with cycloheximide (#C7698, Sigma) at 20 μg/mL for the indicated periods of time, and CYP7A1 protein levels were evaluated by immunoblot analysis as indicated below.

2.4. RNA isolation and quantitative real-time RT-qPCR

Total RNA was extracted from mouse liver tissues using the automated Maxwell system from Promega (Madison, WI, USA) as described

Conclusions: In human liver regeneration FGF19 upregulation may constitute a protective response from BAs excess during liver regeneration. Our findings suggest the existence of post-translational mechanisms regulating CYP7A1 activity, and therefore BAs synthesis, independent from CYP7A1/Cyp7a1 gene transcription.
[37]. For retro-transcription, RNA samples were exposed for 1 min at 90 °C for denaturalization followed by 1 h at 37 °C using a mix containing: 50 mM Tris-HCl pH 8.3, 75 mM KCl and 3 mM MgCl2, 10 ng/μL of random primers, 0.5 mM of each deoxyribonucleic triphosphate (dNTP), 5 mM of diethiothreitol (DTT), 1.2 μL/RNase inhibitors (RNase out) and 6 μL/U of M-MLV inverse transcribe enzyme. All reagents were from Invitrogen (Carlsbad, CA, USA), except dNTPs that were from Roche Diagnostics (Mannheim, Germany). Quantitative reverse transcription PCR (RT-qPCR) was performed as reported [37]. Primer sequences are available upon request.

2.5. Immunoblot analyses

Cells and tissues were lysed in RIPA buffer as described previously [38]. Homogenates from cells and tissues were subjected to immunoblot (Western blot) analysis as reported [39]. Blots were probed with anti-CYP7A1 (#MABD42, Merck, Rahway, NJ, USA) and anti-β-Actin (#ab6276, Abcam, Cambridge, UK) to demonstrate equal loading. Representative images are shown throughout the study.

2.6. Biochemical analyses

Levels of FGF19 in human serum samples were measured by sandwich ELISA using the anti-FGF19 antibody #AF969 as capture antibody, and the biotinylated anti-FGF19 #BAF969 antibody (both from R&D Systems, Minneapolis, MN, USA) for signal development essentially as described [29].

For BAs extraction and HPLC-MS/MS analyses the BAs used as standards were: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), α and β muricholic acids (α/βMCA) and hyodeoxycholic acid (HDCA) acid, as well as their tauroconjugate (TCA, TCDA, TDCA, TLCA, TUDC, TaMCA, TjMCA, THDCA) and glycoconjugate (GCA, GCDA, GDCA, GLCA, GUDC) forms, and tauroursodiolcholic acid (TSLCA), all from Sigma. 7α-hydroxy-4-cholesten-3-one (C4) was from Avanti Polar Lipids (Alabaster, Alabama, USA). Nor-deoxycholic acid (nor-DCA) used as internal standard was from Toronto Research Chemicals (North York, ON, Canada). According to the suppliers the purity of these compounds was ≥ 97 %. All other chemicals were of analytical grade. For BAs analysis, after addition of nor-DCA, BAs were extracted from serum by solid-liquid extraction using silica-based bonded phase cartridge (C18 Sep Pack cartridges, Waters-Millipore, Madrid, Spain). BA species were analyzed by a modification of the method described by Ye et al. [40], using an HPLC-MS/MS (6420 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA) as we previously reported [41].

Chromatographic separation was achieved with gradient elution using a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm) kept at 35 °C and a flow rate of 500 μL/min. Initial mobile phase was 73:27 methanol/water, both containing 5 mM ammonium acetate and 0.01 % formic acid, pH 4.6, and it was changed to 97:3 methanol/water over 10 min and then returned to 73:27 in 1 min. Electrospray ionization (ESI) in negative mode was used, with the following conditions: gas temperature 350 °C, gas flow 11 l/min, nebulizer 45 psi, capillary voltage 2500 V. MS/MS acquisition was performed in multiple reaction monitoring (MRM) mode using the specific m/z transitions: [M-H]− ion to 80.2 for taurine-conjugated bile acids and [M-H]- ion to 74 for glycine-conjugated bile acids. Unfortunately, free bile acids do not generate characteristic ion fragments, and transition from unfragmented precursor molecular ions 407.1 to 407.1, 391.3 to 391.3 and 375.3 to 375.3 were selected for trihydroxylated, dihydroxylated and monohydroxylated free BAs, respectively. Transition 377.0 to 331.3 was followed for the internal standard nor-DCA.

The bile acid precursor 7α-hydroxy-4-cholesten-3-one (C4) was determined in serum after acetonitrile precipitation/extraction by a modification of a previously described HPLC-MS/MS method [42], and in mouse liver tissues as reported [43,44].

Serum levels of the hepatic enzymes alanine aminotransferase (ALT) and aspartate amino transferase (AST), and bilirubin were measured as described [45].

2.7. Statistical methods

Data are presented as means (± SEM or ± SD where indicated). We conducted a repeated measures analysis using the mixed effects model fitted using the restricted maximum likelihood (REML) method with time and group as fixed factors. To determine significant difference between groups at specific time points, we performed post hoc multiple comparisons for each time point (Fisher LSD’s test) (Figs. 1, 3B, 4, 5A, C and supplementary Figs. 2 and 5). When comparing one group over time, we applied a repeated measures One-way analysis of variance (ANOVA) (Figs. 2, 3A, and 6) and aspartate amino transferase (AST), and bilirubin were measured as described [45]. For multiple group comparison, One-way analysis of variance (ANOVA) was used, followed by Tukey’s post hoc multiple comparisons test. (Fig. 7 and supplementary Fig. 4). When more than one experimental factor was analyzed, two-way ANOVA was applied (Fig. 8). Throughout the study a p value of < 0.05 was considered statistically significant. Data analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) version 10.1.

3. Results

3.1. Serum levels of BAs, FGF19 and C4 in healthy patients undergoing partial hepatectomy for LDLT

A total of 12 healthy patients were included in this study. Their demographic and clinical characteristics are described in Table 1. All preoperative liver tests were normal. Serum samples were collected prior to hepatectomy (t = 0) and at early time points after the intervention (t = 2, 12, 24, 48, 72, and 120 h) when the wave of highest rate of LR occurs [46]. No complications were reported during hospital admission, nor during extended follow-up of patients (at least 6 months). Consistently with previous reports [10,17] serum BAs levels rapidly increased shortly after resection (Fig. 1A). Interestingly, by looking at the individual kinetics of serum BAs concentrations increase after PH we could identify two groups of patients. These two groups could be arbitrarily separated by a threshold of 25 μM total BAs concentration at 48 h post-surgery, the time point at which peak concentrations were reached in patients with higher serum BAs levels (Fig. 1A and Supplementary Fig. 1). Noteworthy, these patients with serum BAs > 25 μM, which we have named here “hypercholanemics” (n = 6), were predominantly men, had higher body weight, and by 72 h after resection showed higher bilirubin levels than those classified below this threshold, or “normocholanemics” (n = 6), who were predominantly women (Table 1 and Fig. 1B). No significant differences in the pre-operative levels of serum BAs were found between these two groups (1.35 ± 0.55 and 1.94 ± 0.62 μM in normo- and hypercholanemic patients, respectively). We also analyzed the serum concentrations of free and conjugated primary (CA, CDCA) and secondary (DC, LC) BAs. The analysis of secondary versus primary BAs species both for glycoconjugated and tauroconjugated forms (Fig. 1C), indicated that the accumulation of BAs in the hypercholanemic group was mainly due to primary BAs, which were only moderately elevated in normocholanemic patients. These findings suggest a lower degree of BAs biotransformation by intestinal bacteria, which is consistent with a certain degree of cholestasis in these patients. The differential evolution of the ratio between serum primary and secondary BAs after LR in hypercholanemic and normocholanemic patients further supports this suggestion (Supplementary Fig. 2).

We next evaluated the serum levels of the ileal hormone FGF19. Prior to the intervention there were no differences in FGF19 serum levels between normocholanemic and hypercholanemic patients. After the intervention we found an early and progressive decline in the circulating
concentrations of this hormone in the normocholanemic patients (Fig. 2A). However, in those liver donors that were classified as hypercholanemic we observed a marked elevation in serum FGF19 levels shortly after liver resection (Fig. 2B).

As mentioned above, CYP7A1 transcriptional repression is considered a central hepatoprotective mechanism from BA toxicity after PH [12,22–24]. Importantly, hepatic CYP7A1 activity can be reliably monitored by analyzing the plasma levels of 7α-hydroxy-4-cholesten-3-one (C4), a stable product of the enzymatic reaction subsequent to that catalyzed by CYP7A1 [47,48]. Therefore, we measured serum C4 levels in our cohort of patients before and after liver resection (Fig. 2B).

3.2. BAs synthesis and regulation during mouse liver regeneration after PH

To better understand the mechanisms underlying the regulation of BAs synthesis during LR we performed equivalent studies in the well-characterized mouse model of PH [2].

Fig. 1. Serum levels of BAs, ALT, AST and bilirubin in patients undergoing PH for LDLT. (A) Serum levels of total BAs in the two groups of patients identified as normocholanemic or hypercholanemic (> 25 μM BAs, 48 h post-resection) at the indicated time points after surgery. (B) Serum levels of ALT, AST and bilirubin in these two groups of patients at the indicated time points after surgery. (C) Serum levels of secondary and primary BAs species both for glycoconjugated and tauroconjugated forms in normocholanemic and hypercholanemic patients at the indicated time points after surgery. Values are means ± SEM. * p < 0.05 and ** p < 0.01 vs normocholanemic patients.

Fig. 2. Serum levels of FGF19 in (A) normocholanemic (n = 6) and (B) hypercholanemic (n = 6) patients prior to liver resection (t = 0 h) and at the indicated time points after surgery. Values are means ± SEM. * p < 0.05 vs t = 0 h.

Interestingly, the pre-operative levels of serum C4 were significantly higher in hypercholanemic compared with normocholanemic patients, and both groups reached similar low concentrations two hours after PH (Fig. 3B). These findings suggest that among the healthy population some individuals may have a higher basal rate of BAs synthesis. While the reason for this difference is currently unknown, we found a direct correlation between basal serum C4 levels and patients’ weight (Supplementary Fig. 3).

Notably, in a parallel study on patients undergoing acute systemic inflammation (patients with sepsis) we also observed very low serum C4 levels at the time of hospital admission, with a fast and progressive recovery towards the day of their discharge (Supplementary Fig. 4). These findings indicate that in humans BAs synthesis is markedly shut down in situations of acute stress and inflammation. Nevertheless, a drop in serum C4 levels could be anticipated in the context of LDLT as these patients undergo a significant removal of liver mass, the main source of circulating C4. However, the magnitude and rapidness of this response apparently exceeded what could be expected by the extent of the resection, and by the level of CYP7A1 mRNA downregulation which in humans was recently reported to be of approximately 40 % two hours after PH [10].
mechanism during LR to reduce BAs synthesis in the context of BAs intervention (Fig. 4 B). As previously mentioned, downregulation of (Fig. 5 A and Supplementary Fig. 5E). Remarkably, a profound reduction in overload [8]. We confirmed this response in our model, observing a liver resection), with C4 levels returning to basal values by 12 h after the points in sham-operated mice (mice that underwent laparotomy without (Fig. 4 B). Surprisingly, we found a very similar response at early time PH has not been examined before, and the observed changes certainly merit further investigations. Next, we measured the circulating levels of C4 as an estimation of hepatic BAs synthesis and CYP7A1 activity [47,48]. In line with our findings in human liver donors a sharp decrease during mouse liver regeneration after PH, the latest time-point analyzed, in the latter one only in female mice (Supplementary Fig. 5D), which is in agreement with previous reports showing the upregulation of this ideal gene after PH [49]. The expression of other genes involved in BA transport such as Asbt and Ibabp was unchanged, and that of the BA receptors Fxr and Tgr5 was transiently elevated after PH, albeit this and intrahepatic BAs concentrations was observed quickly after liver resection compared with sham-operated mice (Fig. 4A and Supplementary Fig. 5A and B). Consistently with the elevation of intrahepatic BAs after PH, and aligned with previous reports [8], we observed a downregulation in Ntcp expression and the upregulation of Foxm1b mRNA levels both in male and female mice, albeit with different kinetics (Supplementary Fig. 5C). In ileal tissues, we detected the upregulation of Fgf15 gene expression by 12 h after PH, the latest time-point analyzed, in female mice, but still not in male animals (Supplementary Fig. 5D), which is in agreement with previous reports showing the upregulation of this gene after liver resection (Fig. 6), further supporting the idea of an impaired CYP7A1 activity during the very early stages of LR.

Taken together, these observations indicate that downregulation of CYP7A1 expression would not significantly contribute to the decrease in BAs synthesis during the early stages of LR after PH. While circulating C4 levels are a reliable readout of CYP7A1-mediated BAs synthesis, serum C4 concentrations might be influenced by other processes such as its hepatocellular secretion or systemic excretion. Therefore, we measured the intrahepatic concentrations of C4 after PH. Most interestingly, we also found a significant reduction in the levels of this metabolite shortly after liver resection (Fig. 6), further supporting the idea of an impaired CYP7A1 activity during the very early stages of LR.

### 3.3. Hepatic Cyp7a1 expression and CYP7A1 activity in acute inflammation

The fact that serum C4 levels were reduced not only after liver resection but also upon sham-operation, which like PH also triggers an acute phase inflammatory response [36,50], suggested the existence of mechanisms that could modulate CYP7A1 enzymatic activity under stress conditions. To further test this hypothesis, we evaluated serum C4 levels and hepatic Cyp7a1 expression in mice treated with bacterial lipopolysaccharide (LPS), which mimics the acute inflammatory response taking place during laparotomy and liver resection [36]. As shown in Fig. 7A, concomitant with the upregulation of the expression of inflammatory cytokines, and the oxidative-stress induced Mn superoxide dismutase (MnSod) gene, LPS injection led to a dose-dependent decrease in serum C4 levels as assayed 6 h after endotoxin injection. At this same time point, mice treated with LPS showed a profound downregulation in Cyp7a1 mRNA abundance (Fig. 7B), while CYP7A1 protein levels remained essentially unaltered (Fig. 7C).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patients’ characteristics.</th>
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<tr>
<td></td>
<td>Normocholanemics</td>
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<tr>
<td>Gender (female/male)</td>
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</tr>
<tr>
<td>Age (years)</td>
<td>37.90 ± 11.05</td>
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<tr>
<td>Weight (Kg)</td>
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<td>BMI</td>
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<td>Graff weight (% of body weight)</td>
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</tr>
<tr>
<td>ALT (U/L)</td>
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<tr>
<td>AST (U/L)</td>
<td>11.40 ± 7.76</td>
</tr>
<tr>
<td>GGTP (U/L)</td>
<td>10.05 ± 4.69</td>
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<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.88 ± 0.41</td>
</tr>
<tr>
<td>Days of hospital admission</td>
<td>6.16 ± 1.83</td>
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Data are expressed as mean ± SD.

and intrahepatic BAs concentrations was observed quickly after liver resection compared with sham-operated mice (Fig. 4A and Supplementary Fig. 5A and B). Consistently with the elevation of intrahepatic BAs after PH, and aligned with previous reports [8], we observed a downregulation in Ntcp expression and the upregulation of Foxm1b mRNA levels both in male and female mice, albeit with different kinetics (Supplementary Fig. 5C). In ileal tissues, we detected the upregulation of Fgf15 gene expression by 12 h after PH, the latest time-point analyzed, in female mice, but still not in male animals (Supplementary Fig. 5D), which is in agreement with previous reports showing the upregulation of this gene after liver resection (Fig. 6), further supporting the idea of an impaired CYP7A1 activity during the very early stages of LR.

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Few studies have evaluated the regulation of the \textit{CYP7A1}/\textit{Cyp7a1} gene beyond its transcriptional control and its mRNA stability [21]. Previous reports demonstrated the regulation of CYP7A1 enzymatic activity by phosphorylation reactions mediated by signaling kinases [51–53], while others showed the high sensitivity of the purified enzyme to be inhibited under oxidative conditions [54, 55]. Oxidative stress is a hallmark of acute liver inflammation [33]; therefore, we evaluated the response to LPS administration in mice that have been previously fed with chow diet or the same diet supplemented with the antioxidant BHA [34]. We found that LPS markedly reduced \textit{Cyp7a1} mRNA levels in mice fed chow and BHA-supplemented diets (Fig. 8A), while the abundance of CYP7A1 protein was comparable between the two groups of animals (Fig. 8B). However, the reduction in the serum levels of C4 elicited by LPS was significantly attenuated in mice fed the BHA-supplemented diet (Fig. 8C). These findings support the idea that CYP7A1 enzymatic activity can be inhibited in vivo under oxidative stress conditions.

4. Discussion

In this study, we first evaluated the response of the BAs-FXR-FGF19 axis in healthy human subjects after partial hepatectomy for LDLT. In agreement with previous experimental and human studies we observed a rapid elevation of BAs in peripheral blood, a response that can be expected to be due to the reduced mass of remnant liver tissue that has to cope with the portal BAs load returning to this organ [7, 10, 17, 18, 56]. Although we acknowledge the limited size of our study population, in our cohort we could clearly identify a subgroup of patients, referred here to as hypercholanemics, in which this post-resection surge in serum BAs levels was markedly more pronounced. Most interestingly, when we assessed the circulating levels of FGF19 we observed a differential response between individuals in which the post-resection elevation in serum BAs was more modest and the hypercholanemics patients. The previous work of Koelfat et al. found either no changes or a reduction in serum FGF19 concentrations in two cohorts of oncologic patients undergoing liver resection for colorectal metastases [10]. Concordantly, our normocholanemic group showed a transient reduction in serum FGF19; however, the levels of this hormone were markedly elevated in hypercholanemic patients at very early time points after surgery, which were not analyzed in previous studies [10]. Experimental observations support an important role for FGF19/FGF15 during LR, including protection from BAs toxicity as well as a BAs-independent regulation of the overall regenerative process [12, 13, 24]. However, the behavior and role of FGF19 during human LR is still not well understood. In humans, FGF19 is normally produced by ileal enterocytes to be released into the portal and systemic circulation, and transintestinal BAs flow is considered an essential driver for FGF19 upregulation [57–59]. Of note, a significant activation of FGF19 gene expression has been observed in the liver of patients with extrahepatic cholestasis in association with increased FGF19 circulating levels [60]. This suggests that upon BAs overload, liver cells may become a source of FGF19, likely as part of an autocrine/paracrine hepatoprotective response [60–64]. Although we cannot establish the origin of circulating FGF19 during human LR is still not well understood. In humans, FGF19 is normally produced by ileal enterocytes to be released into the portal and systemic circulation, and transintestinal BAs flow is considered an essential driver for FGF19 upregulation [57–59]. Of note, a significant activation of FGF19 gene expression has been observed in the liver of patients with extrahepatic cholestasis in association with increased FGF19 circulating levels [60]. This suggests that upon BAs overload, liver cells may become a source of FGF19, likely as part of an autocrine/paracrine hepatoprotective response [60–64]. Although we cannot establish the origin of circulating FGF19 in our patients due to the unavailability of hepatic and ileal tissue samples, it is interesting to note that the serum BAs levels reached by hypercholanemic donors were not far from those reported in cholestatic patients in which FGF19 was expressed by liver tissues [60]. On the other hand, it has been observed
that in primary human hepatocytes BAs can induce FGF19 gene expression at concentrations between 10 and 30 μM [65, 66]. Intrahepatic concentrations within this range could be found in hypercholanemic patients shortly after resection, even before maximal serum BA levels are reached, thus contributing to explain the rapid rise in serum FGF19 levels found in these patients. Furthermore, the observed apparent decrease in the bacterial biotransformation of primary and conjugated BAs strongly supports the impairment in the flow of BAs through the intestine and, hence, a lower FXR-dependent activation of FGF19 secretion by ileal epithelial cells. These findings would not be consistent with an intestinal origin for the rising serum levels of FGF19 observed after PH, suggesting the liver as the most likely source of this endocrine signal in these patients. On the other hand, the notion of potential changes in the enterohepatic circulation of BAs during the early stages of liver regeneration, already suggested in experimental studies [67], may have functional consequences for BA signaling, given the fundamental role played by gut microbiota in BA metabolism and in the regulation of liver regeneration [68, 69].

We also studied the dynamics of serum C4 levels in our cohort of liver donors, which as previously mentioned are a good reflection of hepatic BAs synthetic activity. Interestingly, preoperative serum C4 concentrations were significantly higher in those patients who after resection developed hypercholanemia and had higher bilirubin levels. Although the reasons for this variability in basal C4 concentrations are unknown, it was early observed that BAs synthesis rates, and thus C4 serum levels, positively correlated with body weight [47], which is in line with our current findings. Since our cohort of liver donors only included one obese patient (BMI 30.9), the preoperative evaluation of C4 serum levels may be informative on the risk of developing hypercholanemia after liver resection. Alternatively, it has been reported that serum C4 levels are generally higher in men than in women [70], and in spite of the small size of our study cohort we also observed that male individuals were more frequent among patients with higher basal C4 levels, those who after the intervention constitute the hypercholanemic group. Interestingly, in this context it is worth mentioning that liver regeneration after living donor hepatectomy is significantly faster in male than in female patients [46, 71].

Upon surgery all patients showed a remarkable reduction in circulating C4 concentrations. Clearly this can be attributed to the removal of a significant portion of liver tissue, where CYP7A1 activity resides. However, the fact that the lowest serum C4 levels were already reached

![Fig. 6. Intrahepatic C4 levels in mice prior to and at the indicated early time points after PH. Values are relative to levels found at t = 0 h, and are means ± SEM. * p < 0.05. vs t = 0 h.](image)

![Fig. 7. (A) Analysis of the mRNA levels of inflammatory cytokines, and the oxidative stress-responsive gene MnSod, in the livers of mice 6 h after i.p. injection of saline (control) or the indicated doses of LPS. Right panel shows the C4 serum concentrations in these mice. Analysis of Cyp7a1 mRNA (B) and protein (C) levels in mice livers 6 h after i.p. injection of saline or LPS (80 μg/Kg). Blots were probed with anti-actin antibodies to show equal loading. Representative images are shown. Values are means ± SEM. ** p < 0.01, *** p < 0.001 vs controls.](image)
at a very short time post-PH (2 h) suggested the potential involvement of signals inhibiting CYP7A1 enzymatic activity further to CYP7A1 expression downregulation. Indeed, earlier clinical observations already proposed the existence of CYP7A1 enzyme inhibitory mechanisms occurring in cholestatic patients [72]. Moreover, in vitro studies demonstrated that CYP7A1 enzymatic activity can be rapidly modified by post-translational phosphorylation/dephosphorylation reactions, and also quickly inhibited under oxidative stress conditions [52-54]. Thus, it is plausible that CYP7A1 activity could be regulated in vivo particularly during the early stages of liver regeneration. Our following experimental findings support this hypothesis: i) in mice undergoing PH circulating and intrahepatic C4 levels are markedly reduced after resection, when CYP7A1 protein levels are still unchanged; ii) in sham-operated mice, in which no liver mass is removed and CYP7A1 protein levels are preserved, serum C4 concentrations are rapidly reduced after the fall in serum C4 concentrations elicited by LPS injection can be significantly attenuated in mice fed and antioxidant-supplemented diet.

Pro-inflammatory cytokines and sustained inflammation have been reported to downregulate Cyp7a1 gene expression, and, eventually, CYP7A1 activity, in vivo and in vitro models [73-76]. Our present observations showing a significant reduction in serum C4 levels in patients with sepsis during the acute inflammatory phase indicate that this response may also occur in humans. Importantly, sustained inhibition of Cyp7a1 expression markedly influences hepatic lipids and glucose metabolism, and has been proposed as a pathogenic mechanism in chronic conditions such as infection, obesity, and type 2 diabetes [76]. Importantly, CYP7A1 expression and activity also have a crucial impact on cholesterol homeostasis from the early stages of the mevalonate pathway to its catabolism into BAs [20,73,77-79]. Preserving hepatic cholesterol availability is important for defensive responses during systemic infections [73,80,81], but it is also essential to enable cell growth and proliferation as occurs during liver regeneration [25,82,83]. Our present observations suggest that hepatic cholesterol and BAs homeostasis may be modulated in part by the regulation of CYP7A1 enzymatic activity, which could involve redox mechanisms. In fact, CYP7A1 contains eight cysteine groups, two of which have been found essential for its enzymatic activity in cellular assays [55]. While redox regulation of hepatic enzymes is increasingly recognized as a physiological mechanism [84,85], including the control of hepatocellular proliferation [86], persistent oxidative stress is a well-known driver of chronic liver diseases [87]. Given the potential physiopathological implications of controlling CYP7A1 enzymatic activity we believe that further studies addressing the post-translational regulation of CYP7A1, and directly evaluating CYP7A1 enzymatic activity during liver regeneration and inflammation, are worthwhile. In conclusion, FGF19 upregulation may constitute a protective response from BAs excess during liver regeneration. Our findings suggest the existence of post-translational mechanisms regulating CYP7A1 activity and, therefore, BAs synthesis, independent from CYP7A1/Cyp7a1 gene transcription.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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