Engineered Three-Dimensional Liver Mimics Recapitulate Critical Rat-Specific Bile Acid Pathways

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A critical hepatic function is the maintenance of optimal bile acid (BA) compositions to achieve cholesterol homeostasis. BAs are rarely quantified to assess hepatic phenotype *in vitro* since existing analytical techniques have inadequate resolution. We report a detailed investigation into the biosynthesis and homeostasis of eight primary rat BAs in conventional *in vitro* hepatocyte cultures and in an engineered liver mimic. The three-dimensional (3D) liver mimic was assembled with layers of primary rat hepatocytes and liver sinusoidal endothelial cells. A high-pressure liquid chromatography and mass spectrometry technique was developed with a detection limit of 1 ng/mL for each BA, which is significantly lower than previous approaches. Over a 2-week culture, only 3D liver mimics exhibited the ratio of conjugated cholic acid to chenodeoxycholic acid that has been observed *in vivo*. This ratio, an important marker of BA homeostasis, was significantly higher in stable collagen sandwich cultures indicating significant deviation from physiological behavior. The biosynthesis of tauro- β -muricholic acid, a key primary rat BA, doubled only in the engineered liver mimics while decreasing in the other systems. These trends demonstrate that the 3D liver mimics provide a unique platform to study hepatic metabolism.

Introduction

THE LIVER PLAYS a critical role in the metabolism of carbohydrates, alcohol, fatty acids, and lipids.¹ Among these functions, the metabolism of cholesterol is critical for maintaining optimal health. Any impairment of this important hepatic function can lead to serious medical complications.^{2,3} The hydroxylation of cholesterol initiates a cascade of enzymatic reactions that synthesize bile acids (BAs). The hepatic biosynthesis and regulation of BAs is critical to the adsorption and clearance of dietary lipophilic molecules and environmental toxicants from the body.⁴ Intricate feedback inhibition pathways regulate BA homeostasis, which in turn controls the adsorption of lipophilic nutrients, cholesterol, and fat soluble vitamins by the body.^{2,5,6} Significant deviations in optimal concentrations and composition of the BA mixture *in vivo* can result in serious health complications, including cholestasis, gall stones,^{3,7–9} and organ damage.^{4,10}

BAs have been shown to participate in their own regulation through nuclear and membrane receptor feedback interactions controlling BA biosynthesis and enterohepatic circulation.^{4,11} Enterohepatic circulation begins with hepatic BA secretion through the bile canaliculi to the gall bladder for storage (Fig. 1). BAs are reabsorbed into the blood stream along with cholesterol and other lipophilic molecules and transported back to the liver. Up to 85% of BAs are recycled

through this process, thereby minimizing daily BA production¹² and necessitating its efficient regulation to maintain a tight control over the BA pool size and composition. The role of BAs in cholesterol clearance and catabolism and their emergent role in their own regulation underscore the necessity of monitoring BA biosynthesis and transformation.

There have been some studies on BA pathways in animal models.^{6,7,13–16} However, such models are expensive and the resulting data can be difficult to interpret. Moreover, as a result of insufficient sensitivity, the composition of BAs has been quantified largely in biological fluids such as blood, urine, bile, and tissue homogenates.^{5,14,17,18} These approaches have relied on limits of detection ranging from 5 to 50 ng/mL,^{17–19} a range in which small differences in BA biosynthesis are indiscernible.

In vitro hepatic models that are capable of mimicking *in vivo* liver functions exhibit tremendous potential to elucidate unknown or cryptic BA metabolic pathways.¹ Previous work has shown that two-dimensional hepatocyte cultures, specifically the collagen sandwich (CS), are capable of maintaining key liver-specific functions.^{20–22} Cellular co-cultures and three-dimensional (3D) hepatic constructs have also exhibited enhanced hepatic phenotypic function.^{23–32} However, the biosynthesis and the resulting homeostasis of BAs, a critical aspect of liver function, have been inadequately studied in these *in vitro* systems.^{9,33–35} Since

FIG. 1. A schematic of the enterohepatic circulation through which bile acids are transported into and away from the liver.

hepatocyte cultures do not contain high concentrations of BAs, a variety of approaches have been used to obtain precise information on conjugated and unconjugated BAs. Some previous studies have quantified BAs using gas chromatography, which requires extremely laborious sample preparation.^{9,14,33–36} In other reports, high-pressure liquid chromatography (HPLC) was utilized to simultaneously analyze both unconjugated and conjugated BAs.^{5,17,18,37,38} However, analytical techniques developed for *in vitro* analysis of BAs have inadequate sensitivity (limits of detection were >50 ng/mL) precluding meaningful analysis of the results.^{33,34,39} Further, the loss of phenotypic function exhibited by hepatocyte monolayers (HMs) because of cellular de-differentiation is likely to result in significant deviations in BA profiles from *in vivo* values.

We have recently reported that the assembly of 3D engineered liver tissue mimics comprised of primary rat hepatocytes (parenchymal cells) and liver sinusoidal endothelial cells (LSECs, nonparenchymal cells).⁴⁰ In these 3D cultures, hepatic parenchymal and nonparenchymal cells were arranged in stratified layers similar to *in vivo* structure resulting in stable liver-related functions over a 2-week period. More importantly, these 3D mimics exhibited a 16-fold increase in CYP1A1/2 (cytochrome P450) enzymatic activity, in comparison to just a twofold increase in CS cultures.⁴⁰ Although CYP1A1/2 does not mediate cholesterol metabolism, other CYP enzymes do so. Therefore, our goal was to test the engineered liver mimetic constructs to determine if they are a suitable platform to conduct detailed studies on BA and cholesterol homeostasis.

We report the development of a HPLC/mass spectrometry (MS) methodology that can simultaneously separate and quantify BAs with a 1 ng/mL limit of detection. To the best of our knowledge, this is the first study that reports the quantification of conjugated and unconjugated primary rat

BAs in *in vitro* hepatocyte cultures with such a level of sensitivity, which is an order of magnitude better than previous approaches. We achieved increased sensitivity by grouping the BAs based upon their hydrophobicity, before detection. Consequently, not all BAs have to be monitored simultaneously, thereby increasing the sensitivity of the method. A significant advantage of the 1 ng/mL detection limit was that we could identify products of key intermediate steps in the metabolic pathways of primary rat BAs. Mammalian species typically have two primary BA products of cholesterol metabolism, cholic acid (CA) and chenodeoxycholic acid (CDCA). In the rat, beta-muricholic acid (β MCA) is considered an additional primary BA since it is directly synthesized by rat hepatocytes from CDCA.^{41–44} The final step before functional BA secretion from the liver is conjugation to either glycine or taurine to increase their solubility *in vivo*.^{8,12,45} In this study, we exploited the 1 ng/mL detection limit of our HPLC/MS methodology to quantify the following primary rat BAs: CA, glycocholic acid (GCA), taurocholic acid (TCA), CDCA, glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), β MCA, and tauro-beta-muricholic acid (T β MCA).

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, phosphate-buffered saline (PBS), penicillin-streptomycin, and trypsin-ethylene diamine tetra acetic acid was purchased from Invitrogen Life Technologies. Type IV collagenase, HEPES (4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid), glucagon, and hydrocortisone were purchased from Sigma-Aldrich. Unless otherwise noted, all chemicals were purchased from Fisher Scientific and used as received.

Hepatocyte and LSEC isolation and culture

Primary rat hepatocytes and LSECs were harvested from female Lewis rats (Harlan) that weighed between 170 and 200 g. Animal care and surgical procedures were conducted as per procedures approved by Virginia Polytechnic Institute and State University's Institutional Animal Care and Use Committee. A two-step collagenase perfusion protocol was used to digest the liver before organ removal.^{21,22} Briefly, animals were anesthetized with a gas mixture of 3% (v/v) isoflurane/97% oxygen at a flow rate of 3 L/min (Veterinary Anesthesia Systems Co.). Liver perfusion was performed with Krebs Ringer Buffer containing 1 mM ethylene diamine tetra acetic acid, followed by a 0.1% w/v collagenase solution in Krebs Ringer Buffer containing 5 mM calcium chloride.

A suspension of cells containing both hepatocytes and nonparenchymal cells was obtained through the mechanical disruption of the excised liver, followed by subsequent filtration through nylon meshes (Small Parts, Inc.) ranging from 250 to 62 μ m in pore size. Hepatocytes were further purified using a Percoll (Sigma-Aldrich) density gradient. Cell viability was determined using trypan blue exclusion. Before hepatocyte use, cell viability was verified to be >90%.

Hepatocytes were seeded at a density of 1 million cells/well on Type 1 collagen coated six-well tissue culture plates (Becton Dickinson Labware) and maintained in the hepatocyte culture medium consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 200 U/mL penicillin-streptomycin, 20 ng/mL epidermal growth factor (BD Biosciences), 0.5 U/mL insulin (USP), 14 ng/mL glucagon, and 7.5 μ g/mL hydrocortisone. Collagen-coated six-well plates were prepared by first mixing a collagen solution consisting of nine parts of Type I collagen solution (BD Biosciences) and one part of 10 $\times$ DMEM; 0.5 mL of this solution was then added to each well and allowed to gel during an incubation period of 1 h at 37°C. CS cultures were formed through the deposition of a second layer 24 h later. Hepatocytes maintained in CS and confluent HM cultures (hepatocytes cultured solely on a single underlying layer of collagen) served as positive and negative controls, respectively. Cultures were maintained at 37°C in a humidified gas mixture of 90% air/10% CO₂. The culture medium was replaced every 24 h, and spent culture medium aliquots were stored -20°C for further analysis.

LSECs were purified from the nonparenchymal cell fraction using a Percoll step gradient.^{29,46,47} LSECs were separated from other nonparenchymal cells by differential adhesion involving a 30 min incubation period after which nonadherent LSECs were removed and subsequently cultured on fibronectin (Invitrogen)-coated substrates. The LSEC medium consisted of the endothelial cell basal medium supplemented with 5% (v/v) fetal bovine serum, 1% (v/v) endothelial cell growth supplement, and 100 U/mL penicillin-streptomycin in a humidified mixture of 95% air and 5% CO₂. All LSEC culture medium components were purchased from ScienCell Laboratories Inc. Studies to quantify BA production by LSECs only were conducted on LSECs cultured on fibronectin-coated six-well plates (~50,000 cells/well). For these studies, LSEC cultures were maintained in the hepatocyte culture medium,

which was replaced every 24 h, and the spent culture medium aliquots were stored at -20°C for further analysis.

Assembly of 3D liver mimics

The 3D engineered liver mimics were assembled using previously reported procedures.^{40,48} Primary rat hepatocytes cultures were allowed to spread for 72 h to form a confluent cell layer. Thereafter, polyelectrolyte multilayers (PEMs) consisting of alternately charged layers of chitosan and hyaluronic acid (HA) were deposited above the cells.⁴⁰ The PEM was obtained through the sequential deposition of chitosan (0.01% w/v) or HA (0.01% w/v) solutions.⁴⁰ LSECs were seeded at a density of either 25,000 (denoted as 25K) or 50,000 (denoted as 50K) cells per sample. Nonadherent LSECs were removed 1 h postseeding. Hepatocyte-PEM-LSEC constructs were maintained for up to 14 days in hepatocyte culture medium that was changed every 24 h. The spent culture medium aliquots were stored -20°C.

HPLC/MS

CA, TCA, GCA, and TCDCA were obtained from Calbiochem (EMD4Biosciences), CDCA and GCDCA from Sigma-Aldrich, and β MCA and T β MCA from Steraloids Inc. Culture medium samples analyzed using HPLC/MS were purified before analysis using Supelclean LC-18 1 mL SPE reverse phase sample preparation columns (Supelco). SPE columns were washed with HPLC-grade methanol and HPLC-grade water followed by 50 mM ammonium acetate (Sigma-Aldrich). Samples containing 20 ng of an internal standard (deuterated TCA; Toronto Research Chemicals Inc.) were loaded onto individual columns, washed, and subsequently dried. Eluted BAs were evaporated to dryness and resuspended in a mixture containing equal volumes of methanol and water.

All chromatographic separations were performed with a Luna C18 column (Phenomenex). Solutions were injected into the column with the use of a Thermo Survey autosampler. The sample was eluted from the column at a constant mobile phase flow rate of 0.2 mL/min using a gradient elution program in which mobile phase A consisted of 20 mM ammonium acetate (maintained at pH 4), and mobile phase B consisted of a liquid mixture of methanol/acetonitrile (67%/33% v/v). The gradient elution procedure was performed as follows; at $t=0$, the ratio was maintained at 45%/55% A/B; at $t=5$ min, 40%/60% A/B; at $t=25$, min 15%/85% A/B; at $t=25.6$ min, 45%/55% A/B; and at $t=30$ min, end MS detection followed by a 5 min postrun period to equilibrate the HPLC column. The HPLC column effluent was pumped directly into a Thermo Instrument TSQ triple quadrupole mass spectrometer (Thermo Finnigan) equipped with an electrospray ionization source operated in negative ion mode. Table 1 contains the mass of each BA ion when operated in negative mode with single ion monitoring.

System stability was quantified by the analysis of six successive identical injections of a sample containing 20 ng/mL of each BA and 80 ng/mL of internal standard (d4-TCA). The percent relative standard deviation of peak elution time, area, and area ratio (peak area normalized to internal standard area) were <10.7% and <5% for the majority of analytes demonstrating system stability (Table 1). A standard curve was obtained through the addition of known

TABLE 1. PROPERTIES OF BILE ACIDS MEASURED USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

Analyte	Ion mass (amu)	Peak elution time (min)	Mass segment	System stability peak % RSD			
				Elution time	Area	Area ratio	% Recovery
CDCA	391.2	20.0	3	0.21	10.7	8.3	73.0 ± 2.5
βMCA	407.2	10.4	1	0.16	5.2	3.7	96.3 ± 1.2
CA	407.2	14.8	2	0.19	4.5	3.3	97.7 ± 2.0
GCDCA	448.2	15.2	2	0.19	3.6	2.9	100.2 ± 2.7
GCA	464.2	10.4	1	0.21	2.3	3.3	102.2 ± 3.5
TCDCA	498.2	14.6	2	0.15	5.2	3.3	102.0 ± 0.5
TβMCA	514.2	5.6	1	0.38	5.2	5.2	97.3 ± 2.0
TCA	514.2	9.8	1	0.39	4.1	3.0	98.4 ± 2.0
TCA-d4	518.2	9.8	1	0.40	3.1	—	—

CA, cholic acid; CDCA, chenodeoxycholic acid; βMCA, beta-muricholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; RSD, relative standard deviation; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TβMCA, tauro-beta-muricholic acid.

concentrations of BAs to the fresh culture medium. Since bovine calf serum used in this study is known to contain BAs,^{17,18} the culture medium was first analyzed for individual BA concentrations.

Di-peptyl-peptidase IV immunostaining to image bile canaliculi

Hepatocytes were fixed in a solution of 2% v/v glutaraldehyde/PBS, followed by permeabilization in a 0.1% v/v Triton X-100 solution. The cells were incubated overnight at 4°C in a 3% goat serum/PBS (Millipore) solution. The samples were then incubated with the primary monoclonal antibody for Di-peptyl-peptidase IV (Cell Sciences) followed by incubation with an FITC-conjugated secondary antibody (Sigma-Aldrich). Images were obtained on an inverted Zeiss LSM510 confocal microscope (Carl Zeiss Inc.).

Statistical analysis

All data are reported as mean ± standard deviation. *t*-tests were conducted to detect differences in the mean values between day 4 and 12 for each culture and statistically significant samples at an α of 0.05 are denoted with an asterisk (*). At each time point, the statistical significance of BAs synthesized by the individual *in vitro* cultures was also determined by comparison to CS cultures. The Bonferroni correction for multiple hypothesis testing was applied to determine *p*-values, and statistically significant samples are denoted with an “#” symbol.

Results

Detection of BAs at 1 ng/mL resolution using HPLC/MS

The ability to detect and quantify BAs with high sensitivity was a critical requirement for our method since only 1 million hepatocytes were used in each *in vitro* hepatocyte culture in comparison to hepatic tissues used in previous studies.^{17,18,49,50} Our goal was to obtain a detection limit of ~1 ng/mL to accurately discern trends between different culture conditions. The eight BAs whose concentrations were measured from spent culture medium samples using HPLC/MS were CA, GCA, TCA, CDCA, GCDCA, TCDCA, βMCA, and TβMCA. Reverse-phase chromatography used to separate the BAs was based on hydrophobic interactions between

the individual analytes and the column resulting in a direct relationship between the retention time and hydrophobicity of each BA. The structure of the BAs, specifically, their side chain chemistries, the number and orientation of sterol ring hydroxyl substitutions, and conjugation contributed to their relative hydrophobicity (Fig. 2).^{5,12,17} Therefore, based upon retention times, BAs were ordered by decreasing hydrophobicity as follows: CDCA > GCDCA > CA > TCDCA > GCA > βMCA > TCA > TβMCA.

After effective separation, BAs were subsequently quantified using MS. In this study BAs were divided into three groups based on similar elution times listed in Table 1. The first group included TβMCA (514.2 amu), TCA (514.2 amu), TCA-d4 (518.2 amu), βMCA (407.2 amu), and GCA (464.2 amu) necessitating four masses to be monitored. The second group included TCDCA (498.2 amu), CA (407.2 amu), and GCDCA (448.2 amu), corresponding to only three masses being monitored. CDCA (391.2 amu) was monitored independently in the third segment because of a significantly higher elution time of 20 min. The limit of detection in the cell culture medium, defined as the lowest concentration at which the signal-to-noise ratio was >3:1, was observed to be below 1 ng/mL for each BA reported in this study. This level was significant, since this detection limit was at a minimum fivefold more sensitive than previous BA quantification techniques, which have reported detection limits ranging from 5 to 50 ng/mL.^{5,17–19}

Unconjugated BAs such as CA, CDCA, and βMCA were not found in fresh culture medium samples or in the spent culture medium obtained from the *in vitro* cultures. The identification of unconjugated BAs was not anticipated since they are only found *in vivo* because of enzymatic deconjugation of BAs during digestion.^{4,17,18,45} The intracellular concentrations of conjugated and unconjugated BAs (obtained from cell lysates) were found to be approximately zero. All other conjugated BAs of interest were present at appreciable concentrations in the fresh culture medium (Table 2). However, TβMCA was not identified in the fresh culture medium, since this is a rat-specific BA, synthesized only by rat hepatocytes.⁸ BAs were quantified from the spent culture medium for hepatocytes cultured as monolayers (HMs), in a CS, with chitosan-HA PEMs (denoted as Hep-PEMs), and hepatocyte-PEM-LSEC constructs. Hepatocytes were cultured with either 5 PE layers (denoted as Hep-5L) or

FIG. 2. The structure of bile acids (BA) and their glycine or taurine conjugates. CA, cholic acid; CDCA, chenodeoxycholic acid; beta-MCA, beta-muricholic acid.

15 PE layers (denoted as Hep-15L).⁴⁰ The 3D liver mimics were comprised of hepatocytes with an intermediate PEM and either 25,000 (denoted as 25K) or 50,000 LSECs (denoted as 50K). Specifically, the samples monitored were hepatocytes-5L-25K/50K LSECs and hepatocytes-15L-25K/50K LSECs.

BA production by LSECs

The spent culture medium from LSEC cultures was tested for the presence of BAs since LSECs are known to participate in cholesterol trafficking.²⁹ The metabolism or pro-

duction of the primary rat BAs by LSECs was quantified at both days 4 and 12 (Table 2). The differences in the composition and concentration of the BAs in spent LSEC and in the fresh culture medium were statistically insignificant. These data demonstrate that LSECs did not synthesize or metabolize BAs.

Biosynthesis of glycine and taurine conjugated CA

Spent culture medium samples for the *in vitro* hepatocyte samples obtained on days 4 and 12 in culture were analyzed

TABLE 2. BILE ACID COMPOSITION ON DAYS 4 AND 12 IN FRESH HEPATOCYTE AND SPENT LIVER SINUSOIDAL ENDOTHELIAL CELL CULTURE MEDIUM

Analyte	Culture medium (ng/mL)	LSEC day 4 (ng/mL)	LSEC day 12 (ng/mL)
GCDCA	93.7 ± 9.6	92.4 ± 3.8	111.1 ± 2.5
GCA	22.3 ± 2.2	26.1 ± 0.5	18.9 ± 0.6
TCDCA	119.4 ± 5.1	124.9 ± 0.9	126.9 ± 3.0
TβMCA	0	0	0
TCA	32.8 ± 3.6	37.8 ± 1.5	26.6 ± 0.7

LSEC, liver sinusoidal endothelial cell.

for the production of glycine-conjugated CA (GCA) and taurine-conjugated CA (TCA). GCA concentrations (Fig. 3A) were found to be similar across all samples with an increase in concentration observed from day 4 to 12, with the exception of HMs. The increase in GCA concentration was found to be ~9%, 19%, 16%, 15%, and 22% for CS, Hep-5L-25K LSECs, Hep-5L-50K LSECs, Hep-15L-25K LSECs, and Hep-15L-50K LSECs cultures, respectively. However, TCA concentrations (Fig. 3B) were found to decrease over time, with the exception of hepatocyte-PEM-LSEC constructs. The increase in TCA concentration was found to be ~17.3%, 15.4%, 16.3%, and 17.7% in Hep-5L-25K LSECs, Hep-5L-50K LSECs, Hep-15L-25K LSECs, and Hep-15L-50K LSECs cultures, respectively. Further, TCA concentrations were found to be statistically higher in the hepatocyte-PEM-LSEC samples on day 12, when compared with the TCA production in CS cultures. The production of

TCA by 3D liver mimic cultures suggests that the entire BA biosynthetic pathway is active on day 12. These results further demonstrate the advantage of developing an extremely sensitive quantification method, since the differences between culture groups was typically between 6 and 8 ng/mL for GCA and TCA. Previously reported BA quantification techniques with limits of detection >5 ng/mL^{5,17-19} would not have been able to discern the observed trends in GCA or TCA with sufficient precision to obtain meaningful results.

Metabolism of conjugated CDCA by phenotypically stable hepatocytes

The presence of bovine calf serum resulted in the fresh hepatocyte culture medium exhibiting high concentrations of GCDCA (93.7 ± 9.6 ng/mL) and TCDCA (119.4 ± 5.1 ng/mL) (Fig. 4A, B). Since hepatocytes in all culture conditions maintain their metabolic activity during the initial time points, these values decreased significantly by day 4 across

FIG. 3. GCA (A) and TCA (B) production measured on days 4 and 12. A * indicates a statistically significant difference in BA production from day 4 to 12 within a particular culture system. A # indicates a statistically significant difference between CS and another *in vitro* culture system at the same time point. HM, hepatocyte monolayer; CS, collagen sandwich; Hep-5L and Hep-15L, hepatocytes cultured with either 5 PE layers or 15 PE layers; GCA, glycocholic acid; TCA, taurocholic acid.

FIG. 4. GCDCA (A) and TCDCA (B) production measured on days 4 and 12. A * indicates a statistically significant difference in BA production from day 4 to 12 within a particular culture system. A # indicates a statistically significant difference between CS and another *in vitro* culture system at the same time point. GCDCA, glycochenodeoxycholic acid; TCDCA, taurochenodeoxycholic acid.

all cultures. This trend indicated that metabolically active hepatocytes converted CDCA, likely into the rat-specific BA T β MCA.⁴ However, this trend did not prevail over 12 days since cells in HMs and Hep-5L and Hep-15L cultures tend to de-differentiate and lose their ability to perform this conversion. *In vitro* cultures that exhibited a decrease in GCDCA and TCDCA concentrations from day 4 to 12 were identified as maintaining phenotype and the samples that exhibited the reverse trend were categorized as a decrease in hepatic phenotype. Specifically, cultures that maintained hepatocyte phenotype were CS, Hep-5L-25K LSECs, Hep-5L-50K LSECs, Hep-15L-25K LSECs, and Hep-15L-50K LSECs, and those that exhibited a decrease in phenotypic function were HM, Hep-5L, and Hep-15L.

In CS cultures the GCDCA concentration decreased from the value observed for the fresh medium by over 93% on day 4 and decreased by 95% on day 12. Similar trends were observed for the hepatocyte-PEM-LSEC samples as well. The GCDCA concentrations at day 12 (Fig. 4A) ranged

from ~5 to 23 ng/mL when hepatic phenotype was maintained, in contrast to 50–75 ng/mL when hepatic phenotype was impaired. The trends observed for GCDCA metabolism were mirrored in TCDCA metabolism (Fig. 4B). On day 12, CS and hepatocyte-PEM-LSEC cultures exhibited a TCDCA concentration ranging from ~4 to 13 ng/mL, whereas HM and hepatocyte-PEM samples contained 35–60 ng/mL, a threefold or higher increase of unmetabolized TCDCA over time. These trends indicated deterioration in hepatic phenotype over time in HM and Hep-PEM samples.

Correlating BA homeostasis with the [CA]/[CDCA] ratio

One traditional metric for determining the optimal composition of the BA mixture has been the [CA]/[CDCA] ratio. This ratio is usually determined by taking into consideration the total contribution from both conjugated and unconjugated forms of CA and CDCA. In healthy humans, the [CA]/[CDCA] ratio has been shown to vary between 2 and 3.^{51–54}

Values ranging from 4 to 5 have been observed in healthy rats.^{54–57} Bovine calf serum used in the current study contained appreciable amounts of GCA and TCA, and very high concentrations of GCDCA and TCDCA. Addition of this serum to the hepatocyte culture medium resulted in a significantly different ratio from values found *in vivo*. The [CA]/[CDCA] ratio in the fresh culture medium was 0.28, ~16-fold lower than values observed in normal rats. The [CA]/[CDCA] ratio in the *in vitro* cultures was calculated based on the additive concentrations of the conjugated BAs, [GCA+TCA]/[GCDCA+TCDCA], since the unconjugated form was not found to be present. Since a ratio of 0.28 is much lower than what has been detected *in vivo*, our goal was to investigate whether hepatocytes could correct this ratio to achieve physiological values. On day 4, the [GCA+TCA]/[GCDCA+TCDCA] ratio increased from 0.28 in the fresh culture medium up to 5.08 in the *in vitro* cultures, with the largest increase observed in CS cultures (Fig. 5). On day 12, this ratio ranged between 0.5 and 0.6 for HM, Hep-5L, and Hep-15L samples, indicating significant deterioration in metabolic activity. In comparison, three Hep-PEM-LSEC samples (Hep-5L-25K LSECs, Hep-5L-50K LSECs, and Hep-15L-50K LSECs) exhibited a [GCA+TCA]/[GCDCA+TCDCA] ratio ranging between 3.6 and 6.1, similar to values reported in healthy rats. On day 12, hepatocytes cultured in CS exhibited a significantly elevated ratio of 8.4. Previous reports have demonstrated that values of the [GCA+TCA]/[GCDCA+TCDCA] ratio larger than 4–5 were indicative of liver-related problems. For example, extremely high values between 8 and 15 were typically observed during the progression of hepatic carcinogenesis in rats^{55,56} or in aging rats with low metabolism.⁵⁷ The elevated values observed in CS cultures suggested a serious deviation from BA homeostasis.

Maintaining rat hepatic phenotype through the biosynthesis of MCA

MCA is a BA unique to rat cholesterol metabolism. It is synthesized from CDCA *in vivo* by phenotypically stable

hepatocytes.^{41–44} The production of T β MCA was monitored to determine the fate of metabolized CDCA reported in the previous section (Fig. 6). Unlike other conjugated BAs, no T β MCA was found in the fresh culture medium, since it is a rat-specific BA. On day 4, T β MCA was synthesized by all culture systems. HM, CS, Hep-5L, and Hep-15L cultures produced T β MCA in excess of 100 ng/mL, whereas Hep-5L-50K LSECs, Hep-5L-25K LSECs, Hep-15L-50K LSECs, and Hep-15L-25K LSECs exhibited T β MCA levels ranging from ~50 to 85 ng/mL. However, the trends were reversed at later time points for all culture systems other than the 3D liver mimics. Even CS cultures exhibited a decrease in T β MCA production from 175 to 136 ng/mL from day 4 to 12. In contrast, on day 12, hepatocytes cultured in all four 3D liver mimic constructs exhibited a statistically significant, approximately twofold increase in T β MCA production.

Presence of bile canaliculi in stable hepatocyte cultures

The presence of bile canaliculi is an important phenotypic feature of hepatocytes since these are the channels through which BAs are transported to and away from the liver.^{58,59} The presence of bile canaliculi was determined through the localization of the Di-peptyl-peptidase IV enzyme. Bile canaliculi were not observed in HM cultures (Fig. 7A). These channels were observed for Hep-15L and CS cultures; however, the fluorescence was diffuse (Figure 7B and C). However, in the engineered liver tissues (Hep-15L-50K LSECs) (Fig. 7D), well-defined bile canaliculi were observed. The results provide additional validation that hepatic phenotype was maintained in the liver mimetic cellular constructs.

Discussion

Current BA research has advanced beyond catabolism and clearance of cholesterol and other lipids from the body. Studies now show that BAs play key roles in their own regulation.⁴ This self-regulation mediated through specific

FIG. 5. The [GCA+TCA]/[GCDCA+TCDCA] ratio on days 4 and 12.

FIG. 6. Biosynthesis of TβMCA measured on days 4 and 12. A * indicates a statistical difference in BA production from day 4 to 12 within a particular culture system. A # indicates a statistical difference between CS and another *in vitro* culture system at the same time point. TβMCA, tauro-beta-muricholic acid.

receptor-mediated interactions results in important metabolic and tissue protective consequences.⁴ Since BA homeostasis is a critical hepatic function, it becomes increasingly important to quantify and understand their phenotypic contribution. *In vitro* hepatic cultures provide an attractive

and inexpensive medium to systematically measure and monitor changes in BA biosynthesis. However, HMs de-differentiate and CS cultures do not capture key heterotypic cellular interactions found *in vivo*. Engineered liver tissues comprised of stratified layers of primary hepatocytes and

FIG. 7. Immunostaining of hepatocytes with Di-peptyl-peptidase IV 6 days post-isolation to image bile canaliculi in hepatocyte monolayer (A), hep-15L (B), CS (C), and hep-15L-50K liver sinusoidal endothelial cells (D) cultures. Scale bar 50 μm. Color images available online at www.liebertonline.com/ten.

LSECs are a new avenue to obtaining comprehensive physiologically relevant metabolic data. In this study, limiting the number of masses that were monitored simultaneously increased the relative time period for the detection of individual BAs, which in turn enhanced the sensitivity of measurements.

In our earlier work, hepatic function has been shown to be low in the engineered liver mimetic constructs on day 4 (24 h after PEM and LSEC seeding)^{40,48,60} since the cells take time to recover from the PEM deposition. On day 4, the artificially high concentrations of GCDCA and TCDCA observed in the fresh culture medium (due to the addition of bovine serum) dramatically decreased after incubation with hepatocytes. However, this decrease was less significant in the 3D liver mimics than in the other culture systems since at this time point 3D liver mimics were still recovering from the PEM deposition procedure. However, by day 12, due to the unique heterotypic interactions in the liver mimics, significantly enhanced BA metabolism was observed. In particular, the conversion of conjugated CDCA to β MCA, a rat-specific BA, was observed to increase only in 3D liver mimics. We hypothesize that this affirmation of maintenance of hepatic phenotype occurs through CYP enzymes, a phenomenon that is well documented *in vivo* (Fig. 8).

A mass balance calculation conducted for the data obtained on day 4 in HM, Hep-5L, and Hep-15L cultures showed that >74% of the metabolized conjugated CDCA formed T β MCA. This value was 88% in CS cultures and 40%–50% in the engineered liver tissues. The same analysis conducted on BA composition obtained on day 12 revealed a twofold increase in CDCA metabolism only in the 3D liver mimics. In contrast, on day 12, the conversion of CDCA to T β MCA was found to be only 39%, 63%, 70%, and 66%, in HM, Hep-5L, Hep-15L, and CS cultures, respectively. These trends suggested significant deterioration in BA homeostasis over extended time periods even for CS cultures. However, the 3D liver mimics exhibited enhanced BA homeostasis and the percent conversion increased to 83%, 82%, 72%, and 64% for Hep-5L-25K LSECs, Hep-5L-50K LSECs, Hep-15L-25K LSECs, and Hep-15L-50K LSECs, respectively. Most important to note was the twofold increase in the conversion of CDCA to T β MCA in the engineered liver mimics from days 4 to 12. Based upon reports in the literature, T β MCA is the dominant form in rodents.^{4,61} Specifically, when MCA is synthesized, the concentration of the taurine-conjugated form is six times more concentrated than the corresponding glycine conjugate.⁶¹ For these reasons, we chose to focus our attention upon T β MCA. We hypothesize that the incomplete conversion of CDCA to MCA is the potential conversion into G β MCA (glyco-beta-muricholic acid). This will be the focus of future investigations.

A critical finding from these studies was that BA homeostasis was only exhibited by the 3D liver mimics on day 12, validated by the physiological ratio of [GCA+TCA]/[GCDCA+TCDCA]. In contrast, the ratio was highly elevated in CS cultures, and significantly lower in HMs. These data suggest that CS and HM systems are unsuitable to study BA homeostasis over long time periods. This deviation from homeostasis in CS cultures and the presentation of high ratios is potentially a sign of senescence,⁵⁷ whereas depressed ratio values have been linked to advanced liver cirrhosis and cholestatic liver disease.^{62,63} The mechanisms and

FIG. 8. Cytochrome P450 enzymes catalyze multiple steps in the biosynthesis of bile acids from cholesterol. The pathway depicts the rate-limiting steps in the classical and alternative pathways, and the step that results in the conversion of chenodeoxycholic acid to beta-muricholic acid.

pathways that result in this deviation will be the focus of future investigations.

The majority of BA synthesis from cholesterol (Fig. 8) proceeds through the classical pathway controlled by the rate limiting conversion of cholesterol to 7 α -hydroxycholesterol by CYP7A1.^{3,4,64} However, the cholesterol metabolism intermediate, 4-cholesten-7 α -3-one, which undergoes enzymatic conversion by CYP8B1, determines the rate of production of CA.^{3,4,7,54} These two CYP enzymes play an important role in the overall regulation of BA synthesis resulting in an optimal value of 4–5 for the ratio of [GCA+TCA]/[GCDCA+TCDCA] in healthy rats. Future investigations into the relative ratio of these CYP enzymes will reveal insights into their role in modulating this important ratio.

The successful implementation of a HPLC/MS method with 1 ng/mL quantification limits has led to the realization that hepatocytes cultured in 3D liver mimics recover crucial

BA metabolic pathways that recapitulate *in vivo* liver function, in contrast to other widely used culture systems included HMs and CS. The unique capability of our method to identify BAs in nanogram concentrations exhibits tremendous potential in future analyses of *in vitro* 3D liver mimics to provide new insights into the regulatory and biosynthetic pathways of BAs.

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Disclosure Statement

No competing financial interests exist.

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