A Combination of Transcriptomics and Metabolomics Uncovers Enhanced Bile Acid Biosynthesis in HepG2 Cells Expressing CCAAT/Enhancer-Binding Protein beta (C/EBP beta), Hepatocyte Nuclear Factor 4 alpha (HNF4 alpha), and Constitutive Androstane Receptor (CAR)

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A Combination of Transcriptomics and Metabolomics Uncovers Enhanced Bile Acid Biosynthesis in HepG2 Cells Expressing CCAAT/Enhancer-Binding Protein β (C/EBP β), Hepatocyte Nuclear Factor 4 α (HNF4 α), and Constitutive Androstane Receptor (CAR)

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S Supporting Information

ABSTRACT: The development of hepatoma-based in vitro models to study hepatocyte physiology is an invaluable tool for both industry and academia. Here, we develop an in vitro model based on the HepG2 cell line that produces chenodeoxycholic acid, the main bile acid in humans, in amounts comparable to human hepatocytes. A combination of adenoviral transfections for CCAAT/enhancer-binding protein β (C/EBP β), hepatocyte nuclear factor 4 α (HNF4 α), and constitutive androstane receptor (CAR) decreased intracellular glutamate, succinate, leucine, and valine levels in HepG2 cells, suggestive of a switch to catabolism to increase lipogenic acetyl CoA and increased anaplerosis to replenish the tricarboxylic acid cycle. Transcripts of key genes involved in bile acid synthesis were significantly induced by approximately 160-fold. Consistently, chenodeoxycholic acid production rate was increased by more than 20-fold. Comparison between mRNA and bile acid levels suggest that 12- α -hydroxylation of 7- α -hydroxy-4-cholesten-3-one is the limiting step in cholic acid synthesis in HepG2 cells. These data reveal that introduction of three hepatocyte-related transcription factors enhance anabolic reactions in HepG2 cells and provide a suitable model to study bile acid biosynthesis under pathophysiological conditions.

KEYWORDS: HepG2, bile acids, hepatic model, chenodeoxycholic acid, CDCA, C/EBP, HNF4, CAR, NMR spectroscopy, metabolomics, metabonomics

INTRODUCTION

HepG2 is probably the most widely used human hepatoma cell line. These cells maintain some liver-specific functions, but they are a poor alternative to primary cultures in the study of hepatocyte functions such as drug biotransformation and bile acid biosynthesis. The low biotransformation activity of hepatic cell lines is the consequence of very low gene transcription resulting in trace levels of mRNA.¹ Also, previous results suggest that the biosynthetic pathways to bile acids present in HepG2 are the same as those in humans, but the relative contribution to the final synthesis of bile acids differ substantially.^{2–4} The relative levels of transcription of the genes involved in bile acid synthesis could also be at the root of the reported differences.

Chenodeoxycholic acid (CDCA) and cholic acid (CA) are the primary bile acids in humans. They can be further conjugated with glycine and taurine rendering glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA) and taurocholic acid (TCA). CDCA is the main primary bile acid in humans. Bile acids are

produced de novo in hepatocytes from cholesterol through a series of reactions involving an initial hydroxylation at C₇ (75%) or C₂₇ (25%) followed by further modification of the steroid ring structure and by side-chain shortening.⁵ The hydroxylation step at C₇ is catalyzed by CYP7A1, the first step of the so-called “classical pathway” of BA biosynthesis, which is considered to be of great regulatory importance in both human and HepG2 cells.

The limited expression of drug-metabolizing enzymes in HepG2 cells can be attributed to the severe down-regulation of functional key transcription factors.^{6,7} In consequence, re-expression of such transcription factors could convert HepG2 cells in a more suitable model for hepatocyte pathophysiology studies in vitro.^{6,8,9} In this study we have extended the benefits of transcription factor re-expression to bile acid metabolism. HepG2 cells were transfected with three transcription factors using adenoviral vectors, and the associated changes in

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intracellular metabolism studied using metabolomics^{10,11} and transcriptomics. The global metabolic profile was measured using a ¹H NMR-based approach, previously utilized in other cell systems,^{12,13} and bile acids were measured using an LC–MS/MS-based method. The expression of CCAAT/enhancer-binding protein β (C/EBP β), hepatocyte nuclear factor 4 α (HNF4 α) and constitutive androstane receptor (CAR) balanced the relative mRNA level of the enzymes involved in bile acid biosynthetic pathway to those from human hepatocytes. In parallel, transfected HepG2 cells increased the production of CDCA, the main primary bile acid in humans, to levels within the range of cultured human hepatocytes.

MATERIALS AND METHODS

Chemicals

Methanol, water and acetonitrile were of LC–MS grade and were purchased from Fisher Scientific (Loughborough, U.K.). Formic acid, cholic acid (β -cholic acid-3 α ,7 α ,12 α -triol, CA), glycocholic acid [β -cholic acid-3 α ,7 α ,12 α -triol-*N*-(carboxymethyl)-amide, GCA], taurocholic acid [β -cholic acid-3 α ,7 α ,12 α -triol-*N*-(2-sulphoethyl)-amide, TCA], chenodeoxycholic acid (β -cholic acid-3 α ,7 α -diol, CDCA), glycochenodeoxycholic acid [β -cholic acid-3 α ,7 α -diol-*N*-(carboxymethyl)-amide, GCDCA], and taurochenodeoxycholic acid (β -cholic acid-3 α ,7 α -diol-*N*-(2-sulphoethyl)-amide, TCDCA) were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Deuterated internal standards (IS) deoxycholic acid-2,2,4,4-*d*₄ (DCA-*d*₄), cholic acid-2,2,4,4-*d*₄ (CA-*d*₄), glycochenodeoxycholic acid-2,2,4,4-*d*₄ (GCDCA-*d*₄) and glycocholic acid-2,2,4,4-*d*₄ (GCA-*d*₄) were purchased from Steraloid inc (Newport, USA).

Cell Culture and Adenoviral Infection

Human hepatoma HepG2 were maintained Ham's F-12/Leibovitz L-15 (1:1, v/v). Cultures were routinely supplemented with penicillin–streptomycin, L-glutamine, 2% bovine serum albumin and 10% heat-inactivated fetal bovine serum. For metabolomic studies, cells were plated in 6-well plates at 10⁶ cells per well. Next day, confluent plates were infected as described below. Cells and media were collected at 24, 48, and 72 h postinfection. For studies of de novo synthesis of bile acids, we used media without serum and without bovine serum albumin (BA-free). The HepG2 cell number and viability remained constant in complete and BA-free media. The recombinant adenoviruses encoding CAR, C/EBP β , and HNF4 α were developed in our laboratory as described elsewhere.^{7,14,15} HepG2 cells were infected with recombinant adenoviruses at a multiplicity of infection (MOI) ranging from 24 to 96 plaque-forming units/cell as described previously.⁸ Cells were washed, and fresh medium was added.

Reverse Transcription and Real-Time Quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (Qiagen) and reverse-transcribed using MMLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. PCR amplification was performed using Expand High Fidelity PCR system (Roche) following the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was run on a Light Cycler 480 II Real-Time PCR System (Roche) using the Light Cycler 480 SYBR Green I Master (Roche). The PCR reaction consisted of 7.5 μ L of SYBR Green Master I, 0.75 μ L of 6 μ M forward primer, 0.75 μ L of 6 μ M reverse primer, 3 μ L of water and 3 μ L of template cDNA (1/20) in a total volume of

15 μ L. Cycling was performed 10 min at 95 °C, followed by 40 rounds of 10 s at 95 °C, 10 s at 57–64 and 72 °C for 20 s, and extension at 72 °C for 5 min. The relative expression of each gene was normalized against human hydroxymethylbilane synthase (HMBS). The specificity of the amplified PCR products was confirmed by analysis of the melting curve and agarose gel electrophoresis. Primers used for the qRT-PCR are shown in Table S1 (Supporting Information).

Sample Preparation for ¹H NMR Spectroscopy

The aqueous soluble metabolites were extracted from the HepG2 and HepG2-Ad cell pellets using a dual phase extraction as described previously.¹² Sample preparation order was randomized, and 300 μ L of chloroform/methanol (2:1) was added to each tube containing the frozen cell pellet. The sample was vortex mixed for 30 s, and 300 μ L of ultrapure water added, before vortexing again for a further 30 s. The samples were then centrifuged (16000g, 10 min), and the aqueous and organic layers removed to separate sample tubes. The extraction was then repeated to maximize metabolite recovery, and the aqueous and organic layers pooled with the samples obtained from the first extraction. All samples were dried for 12 h in the fumehood to remove any organic solvent. The organic samples (not analyzed in this study) were stored (–80 °C) in this dried state, and the remaining solvent was removed from the aqueous samples by freeze-drying. The aqueous metabolites were reconstituted in phosphate buffer (600 μ L (0.2 M Na₂HPO₄, 0.043 M NaH₂PO₄, 100 μ M TSP, 3 mM NaN₃ in 100% D₂O)) and centrifuged (16000g, 5 min), and 550 μ L were transferred into a 5 mm NMR tube for spectroscopic analysis. All solvents and buffers used in the preparation of samples were checked for contamination by NMR spectroscopy prior to use, and extraction blanks were included to allow subtraction of background in the spectroscopic data.

¹H NMR Spectroscopy

All Carr–Purcell–Meiboom–Gill (CPMG) ¹H NMR spectra were acquired using a Bruker AVANCE DRX600C spectrometer (Bruker Biospin, Rheinstetten, Germany) at 14.1 T (600.13 MHz ¹H frequency), using a BACS 60 automated sample changer to introduce the samples into a 5 mm broadband-inverse tube probehead (Bruker Biospin, Rheinstetten, Germany) operating at a temperature of 300 K. The fixed echo time, τ , was set to 400 μ s, using the following pulse sequence: RD–90°–(τ –180°– τ)_{*n*}–AQ, and the free induction decay was recorded into 64 000 data points in the time domain, with a spectral width of 20 ppm. The spectra were recorded as the sum of 128 scans following 16 dummy scans. Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisitions using XWIN-NMR and ICON-NMR software (Bruker Biospin), which also controlled all aspects of automation and data acquisition. Assignment of spectral peaks to specific metabolites was based on published literature,¹⁶ statistical total correlation spectroscopy¹⁷ (STOCSY), in-house assignment databases and the Chenomx profiler in the NMR suite software (Chenomx Inc., Alberta, Canada).

Sample Preparation for UPLC–MS Analysis

Fifty microliters of culture medium were spiked with 25 μ L of a 1/100 dilution of deuterated IS stock solution, and 225 μ L of cold methanol were added for protein precipitation. After vortexing, samples were maintained at –20 °C for 15 min and

Figure 1. Comparison of intracellular metabolic profiles between HepG2 and HepG2-Ad cells. HepG2 cells were infected with 96 MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1). (A) Column scores plot of the OPLS-DA model summarizing the variation in the global metabolic profile of aqueous intracellular metabolites in the HepG2 and HepG2-Ad cells. The correlated component is shown on the y axis. (B) Column loadings plot of the OPLS-DA model summarizing the weighting of the selected variables for the correlated component. The OPLS-DA model was comprised of 3 components (1 correlation component and 2 orthogonal components) and data were Pareto scaled. The data were modeled using OPLS-DA and was assessed for validity by permutation analysis (Figure S1, Supporting Information). Error bars are jack-knife standard error of the scores or loadings weights computed from all rounds of cross validation. Metabolites marked with an asterisk (*) were overlapped with resonances that were unassigned to any metabolite/metabolites.

centrifuged at 10000g for 5 min at 4 °C. Supernatants were transferred to clean tubes and dried in a Savant speedvac concentrator (Thermo electron corporation, USA). The residue was then reconstituted by adding 50 μ L of methanol:water (50:50, v/v). After centrifugation at 10000g for 1 min at 4 °C, supernatant was transferred into 96-well plates for analysis.

To obtain cell extracts, each well was spiked with 25 μ L of a 1/100 dilution of the deuterated IS stock solution, 500 μ L of cold methanol were added, and samples were maintained at −20 °C for 5 min. Cells remaining were detached using a cell scraper and transferred to clean tubes; this procedure was repeated, and methanol extracts were mixed to ensure a total recovery. After centrifugation at 10000g for 5 min at 4 °C, supernatants were transferred to clean tubes and dried in a Savant speedvac concentrator. The residue was then reconstituted by adding 50 μ L of methanol:water (50:50, v/v) and was centrifuged at 10000g for 1 min at 4 °C. Finally, the supernatant was transferred into 350 μ L volume 96-well plates for its analysis. UPLC–MS analysis was performed as described previously.¹⁸

Data Handling and Statistical Modeling

The data were digitized and imported into Matlab (MathWorks) using an in-house script written and compiled by Dr. T. M. D Ebbels, Dr. H. C. Keun, Dr. J. T. M. Pearce, Dr. O. Cloarec and Dr. R. Cavill. The ¹H NMR spectra were automatically phased, baseline corrected and referenced to the internal standard (TSP). The spectral data were then visually assessed to ensure the automated processing and the water suppression had worked correctly. A targeted binning approach was used to prepare the data for multivariate analysis. The targeted binning saw the integration of every spectral feature in the NMR data, where manually defined regions of the full resolution spectral data were integrated. Where possible, regions of the spectra were defined so that an integral corresponded to a single specific metabolite, but because of overlapping spectral features, this was not always possible. These targeted bins were then normalized to the median fold change.¹⁹ For multivariate analysis, the data were exported to SIMCA-P+ V13 (Umetrics, San Jose, CA). Orthogonal partial least-squares-discriminant analysis (OPLS-DA) was applied to these data to model the association between the metabolic profile and the transfection status (i.e., transfected or not

Figure 2. Levels of selected gene transcripts involved in bile acid metabolism in HepG2 (diamonds) and HepG2-Ad cells. HepG2 cells were infected with 24 (HepG2-Ad1; squares) or 96 (HepG2-Ad2; triangles) MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1). Total RNA was extracted at 24, 48, and 72 h. mRNA levels of selected genes were normalized to hydroxymethylbilane synthase (HMBS) levels. Data correspond to the average $\pm$ SD from four independent experiments in duplicate relative to control HepG2 cells at 24 h. Horizontal dashed line corresponds to the average mRNA levels in human liver ($n = 5$).

transfected with the transcription factors) and to identify individual metabolites that were significantly contributing to any associations observed. In the loadings plot, only integrals that were confidently assigned to a metabolite or multiple metabolites were shown. The model was assessed for validity by permutation analysis (Figure S1, Supporting Information). Statistical p -values were estimated using a Mann–Whitney U test.

RESULTS

Intracellular Metabolic Profiling of HepG2-Ad Cells Suggests an Improvement of Biosynthetic Pathways

A metabolic profiling approach was used to determine if the intracellular metabolome of the HepG2 cells was altered by the transfection of the line with a combination of adenoviral vectors. The aqueous metabolites were isolated from the HepG2 cell line and the transfected line expressing C/EBP β , HNF4 α and CAR (HepG2-Ad). The metabolic profiles were generated using high-resolution ^1H NMR spectroscopy, and these data were analyzed using a pattern recognition approach based on OPLS-DA of normalized peak integrals for all observable and resolvable features. Expression of C/EBP β , HNF4 α and CAR clearly altered the global metabolic profile of HepG2 cells as shown by the OPLS-DA model “scores”, which

summarize variation across the NMR spectra (Figure 1A). The other orthogonal components are shown in the 2D scores plots in Figure S2 (Supporting Information). The metabolites causing this discrimination were identified from the OPLS-DA model “loadings” (Figure 1B), which indicate the weighting of each variable in the predictive model. For clarity, only loadings for integrated NMR spectral features that could be confidently assigned to a specific metabolite are shown. For the metabolites in the loadings plot the chemical shift regions integrated in the spectral data are shown in Table S2 (Supporting Information). A representative selection of the full resolution ^1H NMR spectra is also shown in Figure S3 (Supporting Information) to demonstrate the regions integrated for key metabolites. Metabolites observed at a lower intracellular concentration in the HepG2-Ad cells, compared to HepG2 cells, were glutamate, succinate, leucine and valine. Intracellular levels of myo-inositol were higher in the HepG2-Ad cells. The loadings plot also indicated that phosphocholine, alanine, lactate, acetate and threonine were influencing the separation of the two cells types, but these results were not consistent upon resampling.

Transduced HepG2 Cells Express Higher mRNA Levels of Key Genes from the Bile Acid Biosynthetic Pathway

Bile acid synthesis *de novo* is an anabolic pathway that requires high supply of ATP, NADPH+H⁺ and acetyl CoA. Recent work has demonstrated that reintroduction of C/EBP β , HNF4 α and CAR induces several CYP450 enzymes in HepG2 cells.⁸ Members of the CYP450 superfamily, such as CYP7A1 and CYP8B1, play essential roles in the biosynthesis of bile acids in the liver. Therefore, we examined the mRNA levels of a set of CYP450 genes involved in the production of bile acids from cholesterol. mRNA induction was evident 24 h after infection and increased in a time-dependent manner thereafter (Figure 2). Cholesterol 7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme in the synthesis of bile acids from cholesterol via the classic pathway, catalyzing the formation of 7- α -hydroxycholesterol. HepG2 cells transduced at 24 MOI exhibited an 18-fold induction of CYP7A1 mRNA, while increasing the adenoviral load to 96 MOI increased the value to 164-fold (Figure 2). CYP7A1 mRNA increase was not only significant with the dose of adenovirus but also with time within the same adenoviral dose ($P < 0.05$ between 48 and 72 h comparison). Sterol 12- α -hydroxylase (CYP8B1) catalyzes the 12- α hydroxylation of 7- α -hydroxy-4-cholesten-3-one, which is the critical step that determines the relative amounts of the two primary bile acids in humans, chenodeoxycholic acid and cholic acid. CYP8B1 mRNA levels showed an adenoviral dose-dependent increase with a maximum 19-fold compared to control HepG2. Finally, sterol 27-hydroxylase (CYP27A1) initiates the oxidation of C27 to an acidic group. CYP27A1 is induced by approximately 2-fold by the adenoviral combination. This preliminary result prompted us to extend our analysis to other genes of the pathway, such as AKR1D1, HSD3B7 and BAAT. AKR1D1 is responsible for the 5- β -reduction of bile acid intermediates carrying a Δ^4 -3-one structure. AKR1D1 mRNA levels increased by 7- and 29-fold when cells were infected with 24 and 96 MOI, respectively. HSD3B7 is an oxidoreductase that catalyzes initial stages of the synthesis of bile acids from cholesterol. HSD3B7 mRNA levels increased a maximum of 3-fold after adenoviral transduction. Finally, the mRNA of BAAT, the enzyme responsible for bile acid conjugation with glycine and taurine, did not significantly change in HepG2-Ad cells compared to HepG2.

Bile acid biosynthesis is essentially regulated through a complex transcriptional network, where FXR plays a pivotal role (reviewed in ref 5). In order to investigate if the observed mRNA modifications could be the consequence of FXR and/or LXR induction, we measured the mRNA levels of both nuclear receptors. FXR and LXR did not show any statistically significant change in time or dose (Figure 2). Also, no significant change of bile acid transporter MRP2 was detected (data not shown).

Exogenous Expression of C/EBP β , HNF4 α and CAR Increases Intracellular Levels and Secretion of Chenodeoxycholic Acid in HepG2 Cells

To confirm that increased mRNA levels result in enhanced bile acid synthesis, we measured the intracellular levels of primary human bile acids in HepG2 cells by target LC-MS/MS.¹⁸ We found a time and dose-dependent increase in the levels of intracellular CDCA (Figure 3A). Compared to control HepG2 cells at the specific time-point, we found a 2.5- and 7-fold for HepG2 cells infected at 24 and 96 MOI, respectively. CA,

Figure 3. Intracellular and extracellular levels of conjugated and unconjugated primary bile acids in HepG2 (black) and HepG2-Ad cells. HepG2 cells were infected with 24 (HepG2-Ad1; dark gray) or 96 (HepG2-Ad2; light gray) MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1). (A) Intracellular levels of bile acids were determined by LC-MS/MS at 24 and 48 h. (B) Accumulation rate (pmol/hour/plate) of CDCA in complete media of the cells in panel A. Data correspond to the average $\pm$ SD expressed in pmol per 3.5 cm cell culture plate (approximately 10^6 cells) from four independent experiments in duplicate.

GCDCA, GCA, TCDCA and TCA showed also a dose-dependent increase, reaching as much as 2-fold, but the values were not statistically significant except for GCDCA ($p < 0.05$). Bile acids are synthesized and excreted into the bile canalicula by hepatocytes. HepG2 cells grown in monolayer do not acquire the intricate network of bile canalicula; as a consequence, bile acids are simply secreted and accumulated into the culture media. Analysis of complete media revealed that media supplements already contained significant amounts of the primary bile acids (Figure S4, Supporting Information). Given the low levels of CDCA in complete media, we could estimate the accumulation of CDCA in complete media recovered from cultured HepG2 cells by subtracting the basal content. During the initial 24 h of infection, the accumulation rate in the control was 2 pmol/plate/h compared to 2.4 of transduced cells. At 48 h the values were 4 and 28, respectively, and at 72 h, 3.6 and 42.5 pmol/plate/h (Figure 3B). This result indicates that there is an increase in the levels of CDCA inside HepG2 cells as well as the accumulation rate of CDCA in the culture media parallel to the adenoviral expression of C/EBP β , HNF4 α and CAR.

De Novo Synthesis of Bile Acids in Transduced HepG2 Cells

In order to evaluate *de novo* synthesis and secretion of all primary bile acids in HepG2, we ran a series of experiments

Table 1. Comparison of mRNA Levels of Selected Gene Transcripts in HepG2 Cells Cultured in Complete or BA-free Media^a

CYP7A1			CYP8B1		
	BA-free	complete		BA-free	complete
HepG2-control	1.0 ± 0.4	1.0 ± 0.2	HepG2-control	1.0 ± 0.0	1.0 ± 0.2
HepG2-Ad1	6.7 ± 0.6	23.6 ± 7.7	HepG2-Ad1	0.8 ± 0.1	3.9 ± 0.3
HepG2-Ad2	30.2 ± 4.9	218.8 ± 53.8	HepG2-Ad2	2.6 ± 0.0	19.3 ± 8.6
CYP27A1			AKR1D1		
	BA-free	complete		BA-free	complete
HepG2-control	1.0 ± 0.1	1.0 ± 0.3	HepG2-control	1.0 ± 0.2	1.0 ± 0.6
HepG2-Ad1	1.3 ± 0.0	2.0 ± 0.6	HepG2-Ad1	8.2 ± 0.5	6.9 ± 1.3
HepG2-Ad2	1.7 ± 0.0	2.4 ± 1.1	HepG2-Ad2	27.8 ± 0.3	28.8 ± 12.2
HSD3B7			BAAT		
	BA-free	complete		BA-free	complete
HepG2-control	1.0 ± 0.2	1.0 ± 0.4	HepG2-control	1.0 ± 0.1	1.0 ± 0.3
HepG2-Ad1	1.3 ± 0.1	2.2 ± 0.3	HepG2-Ad1	1.3 ± 0.0	1.2 ± 0.4
HepG2-Ad2	1.8 ± 0.1	3.0 ± 0.4	HepG2-Ad2	1.9 ± 0.0	1.4 ± 0.9
FXR			LXR		
	BA-free	complete		BA-free	complete
HepG2-control	1.0 ± 0.3	1.0 ± 0.1	HepG2-control	1.0 ± 0.1	1.0 ± 0.3
HepG2-Ad1	1.6 ± 0.5	1.3 ± 0.1	HepG2-Ad1	0.7 ± 0.2	0.5 ± 0.0
HepG2-Ad2	1.6 ± 0.4	1.8 ± 0.2	HepG2-Ad2	0.6 ± 0.1	0.5 ± 0.2

^aHepG2 cells were infected with 24 (HepG2-Ad1) or 96 (HepG2-Ad2) MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1). mRNA levels of selected genes were normalized to hydroxymethylbilane synthase (HMBS) levels. Data correspond to the average $\pm$ SD from four independent experiments in duplicate relative to control HepG2.

Figure 4. Comparison of intracellular levels of conjugated and unconjugated primary bile acids in HepG2 and HepG2-Ad cells cultured in complete or BA-free media. HepG2 cells were infected with 24 (Ad1) or 96 (Ad2) MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1). Cells were cultured in complete media or media without serum and supplements (BA-free). Intracellular levels of bile acids were determined by LC-MS/MS at 24 (black), 48 (dark gray) and 72 (light gray) hours. Data correspond to the average $\pm$ SD expressed in pmol per 3.5 cm cell culture plate (approximately 10^6 cells) from four independent experiments.

where HepG2 cells were cultured in media without supplements (BA-free media). BA-free media does not contain any cholesterol or derivatives, and thus HepG2 cells must synthesize de novo all bile acids from acetyl-CoA. The expression profile of the genes selected in our analysis did not change when HepG2 cells were cultured in BA-free media (Figure S5, Supporting Information). However, HepG2 cells

cultured without serum contained significantly lower levels of CYP7A1, CYP8B1 and HSD3B7 (Table 1).

We next measured the intracellular levels in BA-free conditions (Figure 4). Strikingly, intracellular levels of CDCA were 5-fold higher. The intracellular content of CA and the conjugated forms GCA, TCA and TCDCA were hardly detectable, suggesting that the presence of these bile acids in

HepG2 cells incubated in the presence of serum is the result of the cellular uptake rather than biosynthesis. An exception is GCDCA, whose intracellular levels in HepG2 cells incubated with or without serum were comparable.

Finally, the synthesis and excretion of bile acids by HepG2 cells was studied by determining the release of bile acids into the BA-free medium during the first 72 h of incubation. The results are shown in Figure 5. The production of CDCA

Figure 5. Accumulation rate of conjugated and unconjugated primary bile acids in BA-free incubation media from HepG2 (black) and HepG2-Ad cells. HepG2 cells were infected with 24 (HepG2-Ad1; dark gray) or 96 (HepG2-Ad2; light gray) MOI of adenoviral vectors expressing C/EBP β , HNF4 α and CAR (4:3:1) and cultured in BA-free media. Data correspond to the average $\pm$ SD expressed in pmol per hour per 3.5 cm cell culture plate (approximately 10^6 cells) from four independent experiments in duplicate.

increased with time and adenoviral dose. In parallel, we also found an increase in the glycine conjugated form of CDCA (GCDCA) with time, but the effect was independent of adenoviral infection. The production rate of CA, GCA, TCDCA and TCA were barely above the limit of detection.

DISCUSSION

Human hepatoblastoma cells HepG2 growing in culture are a simple and helpful model to study hepatocyte pathophysiology in vitro. However, HepG2 cells lack many hepatocyte functions, and others have been altered substantially. HepG2 cells synthesize bile acids, but the biosynthetic pathways, the relative bile acid ratios and the absolute quantities differ significantly when compared to human hepatocytes. In the present study, we show that expression of only three transcription factors in HepG2 cells restores the production of CDCA, the main bile acid in humans, and balances the mRNA levels of the main genes involved in bile acid biosynthesis.

Transfection of the HepG2 cells with the three transcription factors altered the global metabolic profile when compared to the controls. If the HepG2-Ad cells are expressing a more liver like phenotype then it may be expected that key liver specific metabolic processes will be increased, such as bile acid, lipid or glutathione synthesis. Cholesterol biosynthesis is a liver-specific pathway that provides the precursor for bile acids and requires

two key components, cytoplasmic acetyl CoA and NADPH. The tricarboxylic acid cycle (TCA) is an important source of biosynthetic precursors in cancer cells,²⁰ as well as providing a source of ATP. Cytoplasmic citrate, exported from the mitochondria, and acetate are both precursors of acetyl CoA. In the present study, decreased succinate (derived from the TCA cycle) and acetate in the HepG2-Ad cells may represent increased lipid and cholesterol synthesis. ATP citrate lyase (ACYL) is the enzyme that cleaves citrate to generate acetyl CoA in the cytoplasm²¹ and is key to glucose derived lipid synthesis.²² Oxaloacetate is also produced in the reaction catalyzed by ACYL, which can be converted back to pyruvate (via malate by malic enzyme) to satisfy the requirement for NADPH and also replenish the TCA cycle. Glutaminolysis can also provide a source of NADPH and an anaplerotic mechanism to replenish the TCA cycle when citrate is in demand.²³ This could lead to a decrease in glutamate in HepG2-Ad cells, a change that we observed in the present study. Acetyl-CoA synthetase short-chain family member 2 (ACSS2) is the enzyme responsible for producing acetyl-CoA from acetate.²⁴ Even though synthesis of acetyl CoA via ACYL is normally favored, production via ACSS2 has been shown to be an important pathway in hepatocellular carcinoma both in vivo and in vitro.²⁵ The observed decrease in leucine and valine could be due to increased catabolism; leucine is known to contribute acetyl CoA for cholesterol synthesis in hepatocytes²⁶ and valine can contribute to anaplerosis of the TCA cycle.²⁷ The key metabolic pathways involved in cholesterol biosynthesis (specifically production of acetyl-CoA and NADPH) are summarized in Figure S6 (Supporting Information). The metabolites described in the present study are indicated in the figure to show the relevance to bile acid synthesis and the production of NADPH and acetyl-CoA.

Phosphocholine is a precursor of the membrane constituent phosphatidylcholine and may represent increased demand for membrane synthesis, or membrane turnover, due to proliferation. There is also evidence that intracellular phosphocholine can be increased because of viral transfection in tumor cells,²⁸ and the increase in intracellular phosphocholine in the HepG2-Ad cells in the current study may be a consequence of the transfection with the adenovirus and not related to proliferation.

HepG2 cells in culture synthesize CDCA and CA (this study and ref 3). In a previous report, human hepatocytes cultured in similar conditions than in our study,² synthesized CDCA and CA 17-fold and 10-fold faster than HepG2 cells. Moreover, media collected from cultured human hepatocytes is devoid of free bile acids (less than 1%), while media from HepG2 cells contain roughly 50% of free bile acids. Expression of C/EBP β , HNF4 α and CAR by adenoviral vectors increased the production of CDCA by 15-fold reaching around 86% of the production by human hepatocytes. The production of GCDCA increased by 2-fold. Given that the mRNA levels of BAAT, the bile acid conjugation enzyme, was not induced with the transduction, we suggest that GCDCA increase is the consequence of an increase in CDCA synthesis. In fact, the conjugation rate of other bile acids remained unchanged.

The observed changes in bile acid production after expression of C/EBP β , HNF4 α and CAR might be explained at the transcriptional level. The 200-fold increase in cholesterol 7 α -hydroxylase (CYP7A1) mRNA, the rate-limiting enzyme involved in conversion of cholesterol into bile acids (Figure 6), is probably the consequence of the positive transcriptional role

Figure 6. Biosynthetic pathways from cholesterol to cholic and chenodeoxycholic acids in HepG2. Values in brackets correspond to the relative levels of mRNA (HepG2/HepG2-Ad2) compared to cultured human hepatocytes (1.0). Relative low levels of CYP8B1 mRNA (highlighted) might explain the selective increase in CDCA production. The proposed pathway is based on the report by Axelson et al.³

exerted by C/EBP β , HNF4 α and CAR on CYP7A1 promoter. In fact, C/EBP β and HNF4 α are known to positively transactivate CYP7A1,^{29,30} and expression of Cyp7a1 is reduced 75% in CAR-null mice compared with wild type controls.³¹ The product of cholesterol 7 α -hydroxylase is then hydroxylated at either the 26 (CYP27A1) or 12 α (CYP8B1) position, apparently being the key bifurcation branch for the formation of CDCA and CA respectively. 26-Hydroxylation of this compound is followed by further oxidation, cleavage of the side chain and reduction of the A-ring yielding CDCA. Alternatively, 12 α -hydroxylation is followed by 26-hydroxylation and so on, yielding CA. CYP27A1 and CYP8B1 were induced several fold in transduced HepG2. However, while CYP7A1 and CYP27A1 mRNA levels were in the range of human hepatocytes, CYP8B1 was still 28% of that found in human hepatocytes. This is probably due to the fact that only HNF4 α and not CAR transactivates CYP8B1 promoter.³² This differential upregulation of CYP8B1 might explain why the increase of CYP7A1 results in an increase of CDCA and not CA production.

When the cells were cultured in BA-free media, we observed similar mRNA profiles, although levels were higher when HepG2 cells were grown in complete media. Consistently, bile acid levels in culture media were clearly lower. The reduced mRNA levels cannot be explained by a less efficient expression of the transgenes from the adenoviral vectors (Figure S7, Supporting Information). One reasonable explanation is that cells growing in BA-free media without cholesterol must synthesize bile acids from Acetyl-CoA, while cells grown in the presence of serum can use cholesterol as a cholestane source. Paradoxically, intracellular concentration of CDCA was higher

in cells grown with BA-free media (without serum), regardless of transduction. This can be explained by the role of serum albumin in bile acid transport. It is known that serum proteins such as albumin facilitate several fold the uptake and export of bile acids (preferably free bile acids) and steroid hormones in and out of the hepatocyte.^{33–36}

CONCLUSION

We have developed a new in vitro model based in the HepG2 cell line, which produces a total amount of CDCA similar to cultured human hepatocytes. This change is the consequence of selective induction of mRNA of genes present in bile acid biosynthetic pathway with an overall improvement of the ratio between the different mRNA compared to human hepatocytes.

ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2. Figures S1–S7. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACSS2, acyl-CoA synthetase short-chain family member 2; ACYL, ATP citrate lyase; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GCA, glycocholic acid; HMBS, hydroxymethylbilane synthase; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, LC-MS/mass spectrometry; MOI, multiplicity of infection; MMLV, Moloney murine leukemia virus; NMR, nuclear magnetic resonance; TCDCA, taurochenodeoxycholic acid; TCA, taurocholic acid

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