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Specific Bile Acids Inhibit Hepatic Fatty Acid Uptake in Mice

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Bile acids are known to play important roles as detergents in the absorption of hydrophobic nutrients and as signaling molecules in the regulation of metabolism. We tested the novel hypothesis that naturally occurring bile acids interfere with protein-mediated hepatic long chain free fatty acid (LCFA) uptake. To this end, stable cell lines expressing fatty acid transporters as well as primary hepatocytes from mouse and human livers were incubated with primary and secondary bile acids to determine their effects on LCFA uptake rates. We identified ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA) as the two most potent inhibitors of the liver-specific fatty acid transport protein 5 (FATP5). Both UDCA and DCA were able to inhibit LCFA uptake by primary hepatocytes in a FATP5-dependent manner. Subsequently, mice were treated with these secondary bile acids *in vivo* to assess their ability to inhibit diet-induced hepatic triglyceride accumulation. Administration of DCA *in vivo* via injection or as part of a high-fat diet significantly inhibited hepatic fatty acid uptake and reduced liver triglycerides by more than 50%. *Conclusion:* The data demonstrate a novel role for specific bile acids, and the secondary bile acid DCA in particular, in the regulation of hepatic LCFA uptake. The results illuminate a previously unappreciated means by which specific bile acids, such as UDCA and DCA, can impact hepatic triglyceride metabolism and may lead to novel approaches to combat obesity-associated fatty liver disease. (HEPATOLOGY 2012;56:1300-1310)

Bile acids contribute to several essential functions, including cholesterol catabolism and intestinal lipid emulsification. In addition to their role as detergents, bile acids can also act as endocrine signaling molecules via activation of nuclear receptors, including farnesoid X receptor and pregnane X receptor,[1] as well as the G-protein coupled receptor TGR5[1] to achieve profound effects on hepatic lipid and glucose metabolism.[2]

Following their release from the gall bladder, the majority of bile acids are reabsorbed unchanged. However, secondary bile acids can be generated by intestinal bacterial species in the intestine through chemical modification of bile acids, including deconjugation, oxidation of hydroxyl groups (at C-3, C-7, and C-12), and 7/β-dehydroxylation,[1] and can also transported to the liver via the portal vein.

Secondary bile acids may have both detrimental and cell protective effects.[3] Particularly, the human secondary bile acid ursodeoxycholic acid (UDCA) has been found to have applications for clinical use.[4] UDCA is a major component of bear bile, which has been used extensively in traditional Chinese medicine for liver ailments.[5] It is currently employed in the clinical treatment of diverse hepatobiliary disorders, including primary biliary cirrhosis,[6] primary sclerosing cholangitis,[7] cystic fibrosis–associated liver disease,[8] and cholelithiasis.[4]

Subsequent to intestinal absorption, hepatic transport systems mediate the uptake of both bile acids[9]

Abbreviations: BSA, bovine serum albumin; CA, cholic acid; CDCA, chenodeoxycholic acid; CoA, coenzyme A; DCA, deoxycholic acid; DMSO, dimethyl sulfoxide; FATP, fatty acid transport protein; FXR, farnesoid X receptor; HBSS, Hank's balanced salt solution; KO, knockout; LCA, lithocholic acid; LCFA, long chain free fatty acid; PBS, phosphate-buffered saline; TAG, triglyceride; TCA, taurocholic acid; THCA, 3a,7a,12a-trihydroxy-5β-cholestanoic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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and hydrophobic nutrients such as dietary LCFAs[10] from the circulation. We have previously demonstrated that two members of the long chain free fatty acid (LCFA) transport protein (FATP) family of membrane proteins, FATP2 and FATP5, are important for normal hepatic LCFA uptake as well as the development of diet-induced hepatosteatosis.[11-14] To identify potential FATP inhibitors we developed a high-throughput–compatible homogeneous screening method[15] that was subsequently used in proof-of-concept screens of a limited number of compounds against human FATP5.[16] Interestingly, this screen identified a bile acid as a weak inhibitor of FATP5.

Previous findings have hinted at an unexpected link between bile acids and hepatic FATPs. Overexpression studies with hepatic FATPs have shown that they not only enhance LCFA uptake[11] but also activate the primary bile precursors, 3a,7a-dihydroxy-5β-cholestanoic acid and 3a,7a,12a-trihydroxy-5β-cholestanoic acid (THCA) as well as deconjugated secondary bile to bile acid–coenzyme A (CoA) derivatives.[17] We subsequently generated FATP5-null animals[11] and confirmed that they display an increase in unconjugated bile acids.[14]

However, whether these dual FATP functions exist in parallel or influence each other is unclear. Particularly, whether and how bile acids can interact with the protein-mediated uptake of LCFAs by the liver has remained largely unknown. To address this potentially important link between sterol and LCFA metabolism, we systematically tested for the ability of primary and secondary bile acids to inhibit FATP2- and FATP5-mediated LCFA uptake and identified secondary bile acids as potent inhibitors of LCFA uptake *in vitro* and *in vivo*.

Materials and Methods

Microplate-Based LCFA Uptake Assays. LCFA uptake assays were modified based on the published quencher technique.[15] Briefly, stable cells were plated in 96-well black wall/clear-bottom plates (Costar) in 100 μL of Dulbecco's modified Eagle's medium/fetal bovine serum for a density of 30,000-50,000 cells/well. Mouse primary hepatocytes were isolated and plated in 24-well collagen-coated plates (150,000 cells/well) in 2 mL Dulbecco's modified Eagle's medium/fetal bovine serum complemented with 1% 100× insulin-transferrin-selenium solution (Gibco #41400-045). Stable cells and primary hepatocytes in the plates were incubated overnight resulting in 50%-70% confluency. All bile acids were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and then diluted with 1× Hank's balanced salt solution (HBSS) with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer, 4.5 g/L glucose, and 0.1% fatty acid free bovine serum albumin (HBSS bovine serum albumin [BSA] buffer). For 96-well and 24-well plates, 100 μL or 1 mL of HBSS BSA buffer with or without bile acids was added, respectively, to the cells followed by a 30-minute incubation in 5% CO_2. The buffer was then removed from the cells. For inhibitor screen assays, BODIPY-FA quencher mix (4.5 g/L glucose HBSS, 0.1% BSA, 2 μM fluorescently labeled LCFA C1-BODIPY-C12, 2 mM of the quencher AR 112: 760.58) with the bile acid was added. For inhibition reversibility assays, the cells were rinsed at this time point twice with phosphate-buffered saline (PBS), followed by addition of the prewarmed BODIPY-FA quencher mix to the cells without any bile acids. For all assays, immediately following the addition of the BODIPY-LCFA quencher mix, BODIPY-FA fluorescence was determined in real time using bottom excitation and detection. To this end, a Gemini plate reader was set to bottom read, highest sensitivity, excitation of 488 nm, and emission 515 nm (with a filter cutoff at 495 nm). Readings were taken at 2-minute intervals for 2 hours and uptake velocities were calculated based on linear regression through the data points.

Animal Experiments. The generation of FATP5 null mice has been described.[11,14] Mice were fed standard chow prior to the high-fat diet experiments (60% calories from fat; S3282, Bio-Serv). All experiments were performed with individually housed animals. For the bile acid injection experiments, mice were injected subcutaneously with 3.2 mg/kg deoxycholic acid (DCA) or lithocholic acid (LCA) in 20 μL DMSO on the back above the right hip once a day for 7 weeks while consuming a high-fat diet. For bile acid feeding experiments, 5 mg/g UDCA, 0.5 mg/g DCA, or 0.5 mg/g LCA were mixed into high-fat food. The mice were fed the supplemented high-fat diet for 7 weeks. Their food intake and body weight were recorded once a week. During this time, the mice had *ad libitum* access to water and the high-fat food. For tissue and plasma collection, mice were fasted 4 hours before being sacrificed by way of CO_2 asphyxiation. Subcutaneous injection sites were then examined to verify that no bile acid precipitates had accumulated. The liver and other organs were removed and lysed, and the protein and triglyceride (TAG) concentrations of organ lysates were assayed using the BCA protein assay kit and infinity TAG kit, respectively

(Thermo Scientific). All procedures were approved by the University of California Berkeley Animal Care and Use Committee.

Nonradioactive In Vivo Hepatic LCFA Uptake Assay. Animals were injected subcutaneously with a dose of 6.4 mg/kg DCA or taurocholic acid (TCA) prepared in 40 μL DMSO at 5 PM. The mice were then fasted overnight. At 10 AM, the following day, the mice were anesthetized with isoflurane and were given intraperitoneal injections of 100 μL of a 6.4 mg/kg DCA or TCA solution (dissolved in a 3:2 mixture of PBS/DMSO). This was followed immediately with another intraperitoneal injection with 20 μM C_1-BODIPY-C_{12} bound to 1% BSA in 100 μL PBS. Mice were sacrificed 20 minutes after the injection. Livers were harvested, weighed, and homogenized in 2 mL radio immunoprecipitation assay buffer. Protein concentrations of the homogenized samples were assayed using a BCA assay kit, and the remaining lysates were extracted with three volumes of Dole's reagent (heptane/2-propanol/2N sulfuric acid at 10:40:1 vol/vol/vol). After centrifugation at 18,000*g* for 10 minutes, the clear organic-phase supernatant (top layer) was collected. A total of 50 μL supernatant was added to a 96-well plate, and fluorescence at 488 nm excitation and 515 nm emission was determined. The fluorescence units were normalized to either protein concentrations or organ weights.

Statistical Analysis. The average of data and SEM are shown in the figures. Statistical significance was determined using a Student *t* test or one-way or two-way analysis of variance as appropriate.

See the Supporting Information for further details.

Results

We generated stable HEK-293–based cell lines following transfection of the cells with human FATP2, FATP5, or empty expression constructs. Using a quencher-based real-time uptake assay,[15] we were able to determine the correlation between FATP expression (Supporting Fig. lA, inset) and LCFA uptake rates (Supporting Fig. 1A). After optimization of uptake conditions (see Materials and Methods), linear regression of fluorescent values showed a significant increase in the BODIPY-LCFA (C1-BODIPY-C12) uptake rate in FATP5 (132-fold) and FATP2 (70-fold) expressing cells over vector controls, thus giving us a robust signal to background ratio for inhibitor screens. BODIPY-LCFA uptake was also visualized following uptake assays and showed intracellular accumulation of the fatty acid probe in intracellular structures with the morphological appearance of endoplasmic reticulum and small lipid droplets (Supporting Fig. 1B). This finding is in line with reports of BODIPY-LCFA uptake. C1-BODIPY-C12 fatty acids have been used extensively to study LCFA uptake (for examples, see Faergeman et al.,[18] Schaffer and Lodish,[19] and Hirsch et al.[20]), and their uptake can be competed with unlabeled fatty acids,[15] suggesting that it uses the same transporters. Also, BODIPY fatty acids can become incorporated into cellular lipids.[21]

We initially screened for the effects of the most common conjugated and unconjugated primary bile acids: cholic acid (CA), TCA, chenodeoxycholic acid (CDCA) and taurochenodeoxycholic acid. Whereas conjugated primary bile acids did not reduce LCFA uptake (Figs. 1 and 2), both CA and CDCA specifically inhibited FATP5-mediated LCFA uptake (Figs. 1 and 2), with IC_{50}s in the low micromolar range (Table 1). XTT assays demonstrated that none of the bile acids was toxic in the concentration range tested (Figs. 1 and 2). Interestingly, FATP5 inhibition by CA and CDCA showed complete reversibility (Supporting Fig. 2). A brief rinse of the cells treated with the bile acids was able to remove the inhibitory effect immediately. This pattern of inhibition was consistent with the hypothesis that bile acids that can serve as substrates for enzymatic activation by FATPs can inhibit LCFA uptake. To corroborate this notion, we tested the effect of the FATP2 and FATP5 substrate THCA, which was predicted to be a potent inhibitor of both FATPs. However, the addition of THCA resulted only in weak inhibition (FATP5) or no inhibition (FATP2) of LCFA uptake rates (Supporting Fig. 3B). Also, alpha-muricholic acid, one of the predominant unconjugated bile acids in mice, had no effect on LCFA uptake (Supporting Fig. 3A).

To compare these findings to those of published inhibitors of FATP2,[22] FATP2- and FATP5-overexpressing cells were treated with compounds CB-2 ((5E)-5-[(3-bromo-4-hydroxy-5-methoxyphenyl)methylene]-3-(3-chlorophenyl)-2-thioxothiazolidin-4-one), CB-5 (2-benzyl-3-(4-chlorophenyl)-5-(4-nitrophenyl)-1H-pyrazolo[5,1-b]pyrimidin-7-one), and CB-6 (2-[7-(trifluoromethyl)-2,3-dihydro-1H-1,4-diazepin-5-yl]naphthalen-1-ol) (Supporting Fig. 4). All three compounds inhibited FATP2-mediated LCFA uptake with IC_{50}s of 8 μM, 18 μM, and 12 μM, respectively. CB-5 (7 μM) and CB-6 (38 μM) also showed inhibition of FATP5-mediated uptake, although it should be noted that CB-5 elicited marked cytotoxicity (Supporting Fig. 4). Interestingly, inhibition by CB-6 but not CB-2 was

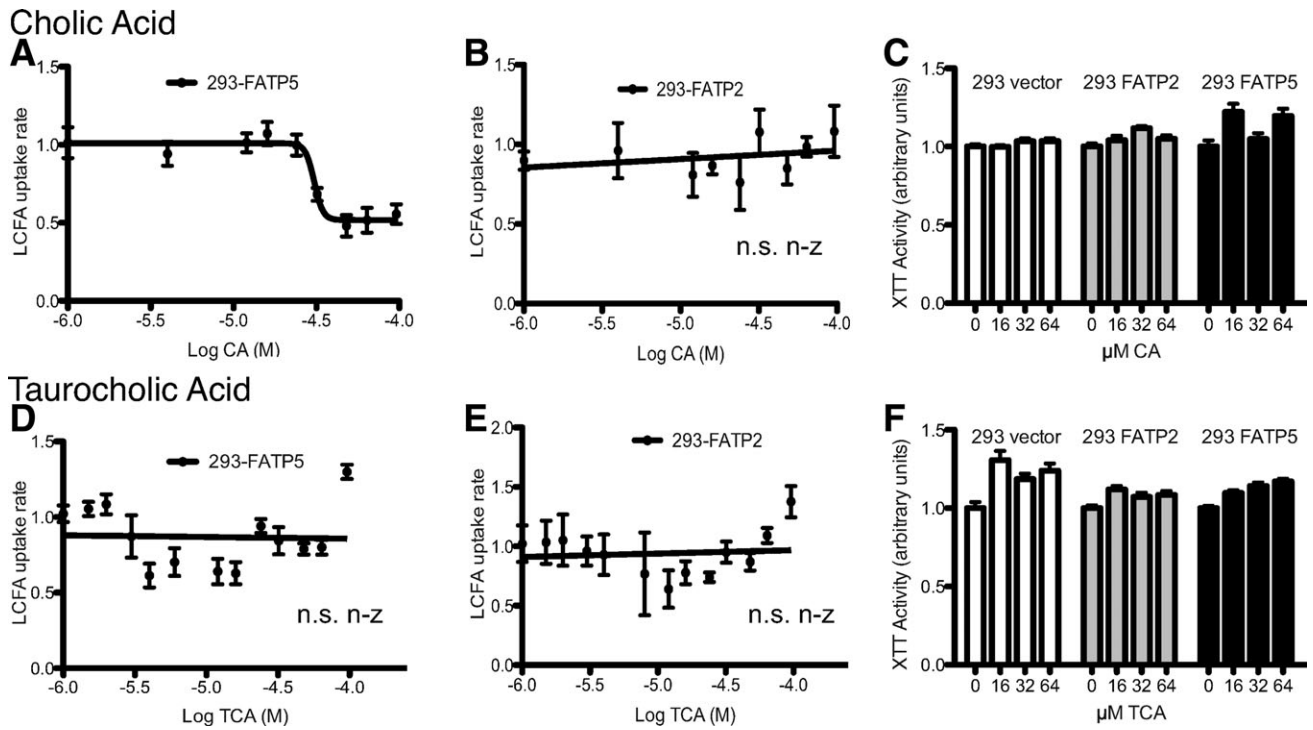

Fig. 1. Inhibition of hepatic FATPs by primary bile acids. Uptake assays with stable cell lines were performed as shown in Supporting Fig. 1A. Normalized LCFA uptake rates (in arbitrary units) by FATP5-expressing (first column) and FATP2-expressing (second column) cells are plotted against the indicated bile acids (in log M). The toxicity of each bile acid was tested against all three stable cell lines (column 3) using XTT assays. Lines show either nonlinear or linear regression through data points. IC_{50} for nonlinear regressions are shown in Table 1. N.s. n-z, not statistical non-zero. $*P < 0.05$, $**P < 0.01$ indicate whether the linear regression slopes differ significantly from zero.

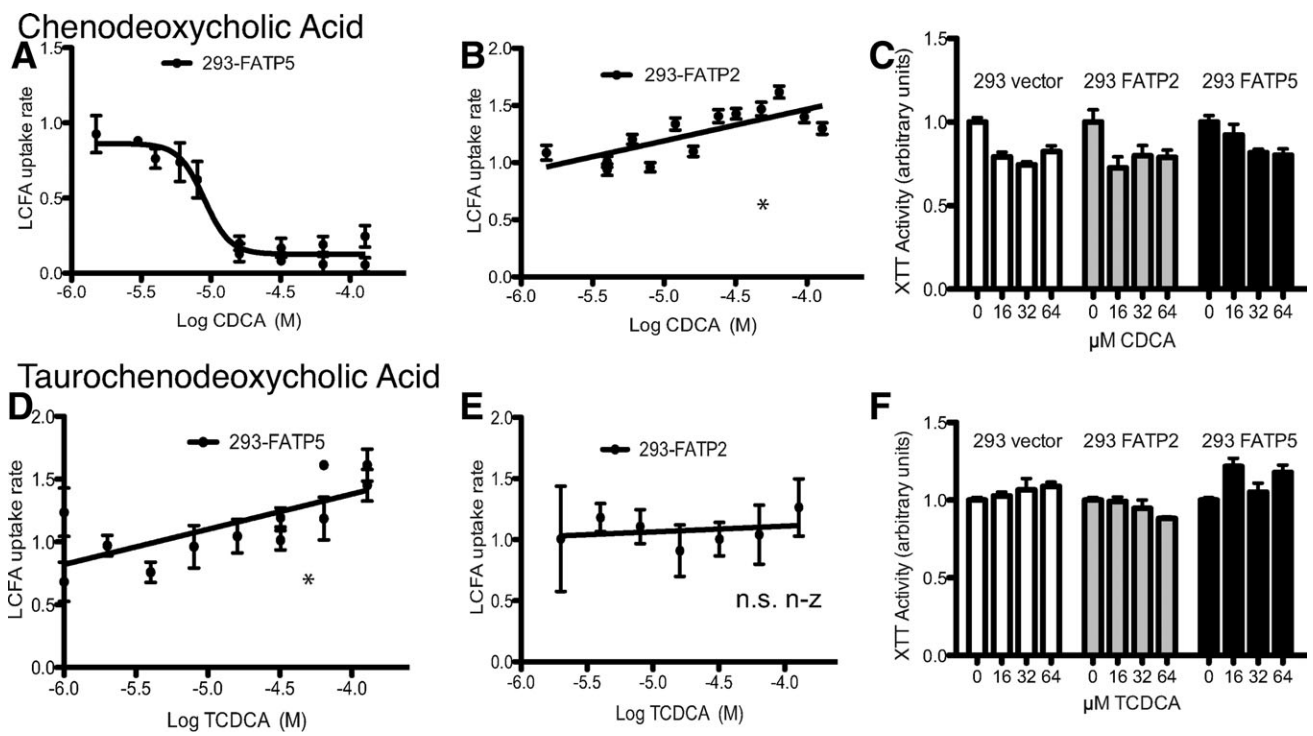

Fig. 2. Inhibition of hepatic FATPs by primary bile acids. Experiments were performed and analyzed as for Fig. 1.

Table 1. Data Summary for FATP5 and FATP2 Inhibitors

Bile Acid/ Compound	IC_{50}, μM		Physiological serum Concentration, μM	Reversible Inhibition
	FATP5	FATP2		
CA	31	–	0.135[22]-0.308[23]	Yes
TCA	–	–	0.105(0.021-0.433)[23]	–
CDCA	10	–	0.205[22]-0.502[23]	Yes
TCDCA	–	–	0.232 (0.022-0.621)[23]	–
THCA	–	–		–
UDCA	5	–	0.054[23]	Yes
TUDCA	–	–	0.004[23]	–
DCA	0.19	5	0.133[22]-0.415[23]	Yes
LCA	–	–	0.012[22]-0.057[23]	–
CB-2	–	8	–	Yes
CB-5	7 (toxic)	18 (toxic)	–	No (toxic)
CB-6	38	16	–	No

Abbreviation: TCDCA, taurochenodeoxycholic acid.

irreversible, hinting at a different inhibition mechanism from that of the bile acids (Supporting Fig. 2).

Next, we expanded our screening to the secondary bile acids ursodeoxycholic acid (UDCA), tauroursodeoxycholic acid (TUDCA), deoxycholic acid (DCA), and LCA. Whereas TUDCA did not inhibit FATP2- or FATP5-mediated uptake, UDCA was identified as a potent inhibitor of FATP5 (Fig. 3) with an IC_{50} of 5 μM (Table 1).

An even more potent inhibition of FATP5 and, to a lesser degree, FATP2 was observed following DCA addition (Fig. 4) with an IC_{50} of 0.19 and 5 μM, respectively. Although DCA is a naturally occurring secondary bile acid, it is also known to have cytotoxic effects, particularly at a higher concentration. Importantly, DCA did not display any cytotoxicity within the FATP5 inhibitory range (Fig. 4), and inhibition was found to be reversible (Supporting Fig. 2). In contrast, taurodeoxycholic acid (TDCA) inhibited neither FATP2- nor FATP5-mediated LCFA uptake (data not shown). Another naturally occurring secondary bile acid, LCA, showed no inhibition of LCFA uptake mediated by either FATP5 or FATP2 (Fig. 4).

To further explore the physiological and pharmacological implications of hepatic FATP inhibition by secondary bile acids, we tested the effects of UDCA on LCFA uptake by primary hepatocytes. Using a fluorescence-activated cell sorting–based LCFA uptake assay that allows for the gating of viable cells,[11] we found that UDCA but not TUDCA inhibited LCFA uptake by primary human hepatocytes (Supporting Fig. 5). UDCA also inhibited LCFA uptake by primary mouse hepatocytes from C57Bl/6 animals without any

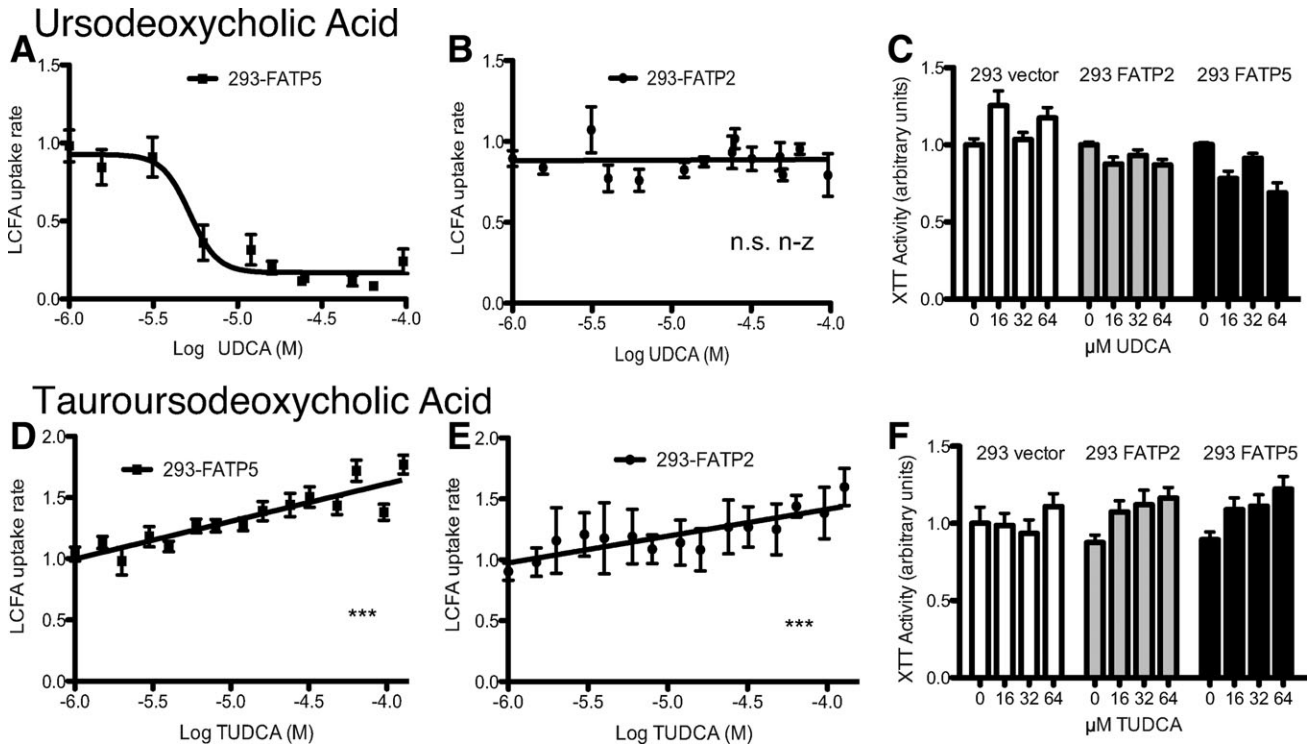

Fig. 3. Inhibition of hepatic FATPs by secondary bile acids. Normalized LCFA uptake rates (in arbitrary units) by FATP5-expressing (first column) and FATP2-expressing (second column) cells are plotted against the indicated bile acids (in log M). Toxicity of each bile acid was tested against three stable cell lines (column 3) using XTT assays. Lines show either nonlinear or linear regression through data points. IC_{50} for nonlinear regressions are shown in Table 1. N.s. n-z, not statistical non-zero. ***$P < 0.001$ indicates whether the linear regression slopes differ significantly from zero.

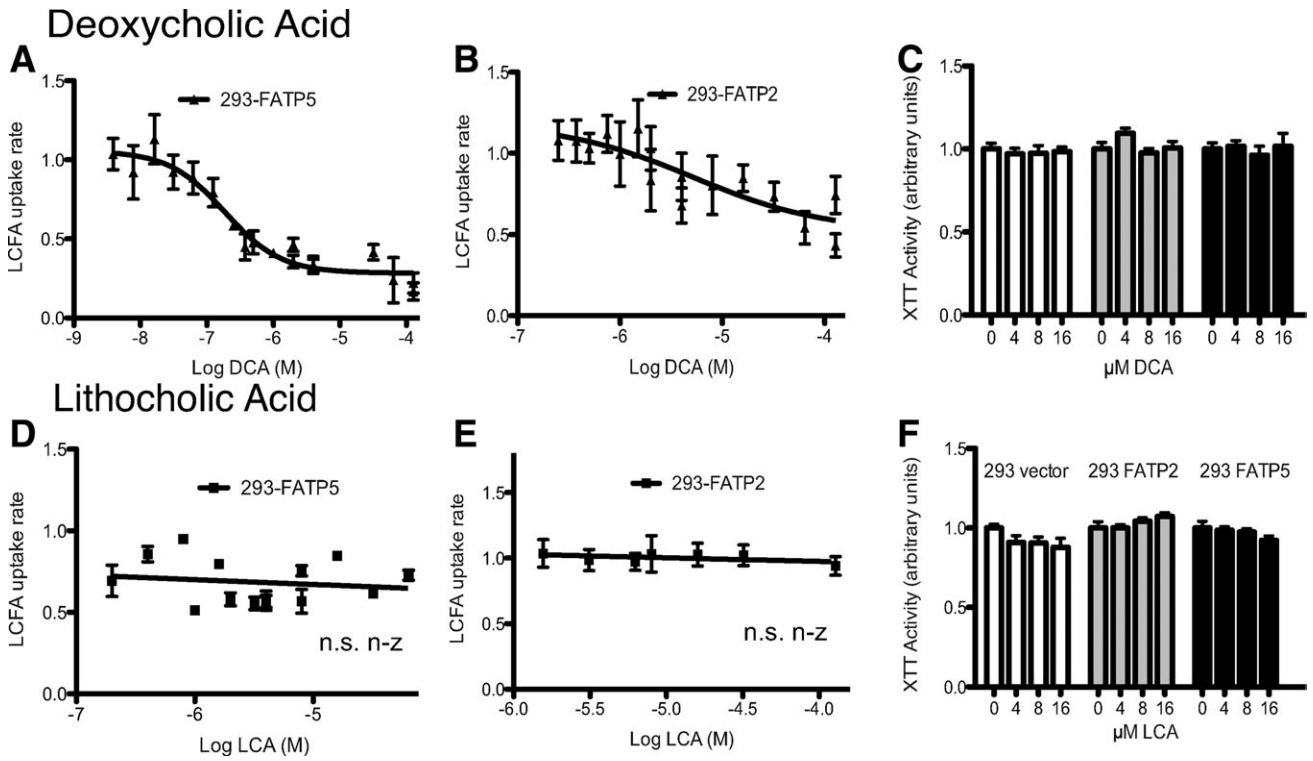

Fig. 4. Inhibition of hepatic FATPs by secondary bile acids. Experiments were performed and analyzed as for Fig. 3.

detectable cytotoxic effects (Fig. 5A, right). Importantly, this effect was entirely FATP5-dependent, as UDCA failed to inhibited LCFA uptake by primary hepatocytes from FATP5-null animals (Fig. 5A). As predicted form our stable cell line results, the secondary bile acid DCA inhibited hepatocyte LCFA uptake significantly (Fig. 5B), whereas LCA showed no inhibition of LCFA uptake by primary mouse hepatocytes (Fig. 5D). Furthermore, DCA-mediated inhibition was not associated with toxicity (Fig. 5B right) and, importantly, was primarily dependent on FATP5, as the effect was abolished in FATP5-null hepatocytes (Fig. 5B). We confirmed DCA inhibition of fatty acid uptake by primary hepatocytes using ^{14}C-labeled oleate (Supporting Fig. 6). Subsequent assays focused on the uptake of radiolabeled metabolites by primary hepatocytes and demonstrated that DCA can inhibit the uptake of a wide range of fatty acids without affecting uptake of a 2-deoxy-D-[^{3}H]glucose/glucose mix (Fig. 5C).

Next, we thought to determine the *in vivo* effects of the most potent bile acid, DCA. To this end, LCA and DCA were injected into animals both acutely and chronically, and the resulting effects on hepatic LCFA uptake and TAG accumulation, respectively, were measured. To this end, we established an *in vivo* assay of acute hepatic LCFA uptake based on the injection of a fluorescent fatty acid analog (C1-BODIPY-C12) followed by extraction of hepatic lipids 20 minutes postinjection. Using this approach, we were able to reproduce *in vivo* the ≈50% reduction in hepatic LCFA uptake in FATP5-null animals (Fig. 6A) previously shown only with isolated hepatocytes and ^{14}C-oleate.[11] We used this novel assay in conjunction with concomitant bile acid injections to establish *in vivo* a dose-response curve for DCA (Fig. 6B). As predicted from our cell-based studies, the inhibitory effect was observed with DCA but not TCA (Fig. 6C). Importantly, DCA inhibition was abolished in FATP5 knockout (KO) mouse hepatocytes (Fig. 6C). To determine the chronic effects of DCA on heptaic lipid accumulation, we used an intermediate dose (3.2 mg/kg) from our titration experiment and injected mice with DCA, LCA, or vehicle (DMSO) for 47 days while the animals were fed a high-fat diet. This injection routine neither triggered an increase in respiration (Fig. 6D) nor did it affect food consumption or body weight (Fig. 6E and Supporting Fig. 7A). DCA treatment did, however, have a profound effect on hepatic triglyceride accumulation. DCA caused a 68% reduction (6.6 versus 2.1 mg TAG/g protein) in the triglyceride content of the liver (Fig. 6F). As predicted from the lack of DCA inhibition of FATP5-null primary hepatocytes, DCA injections of FATP5-null animals did

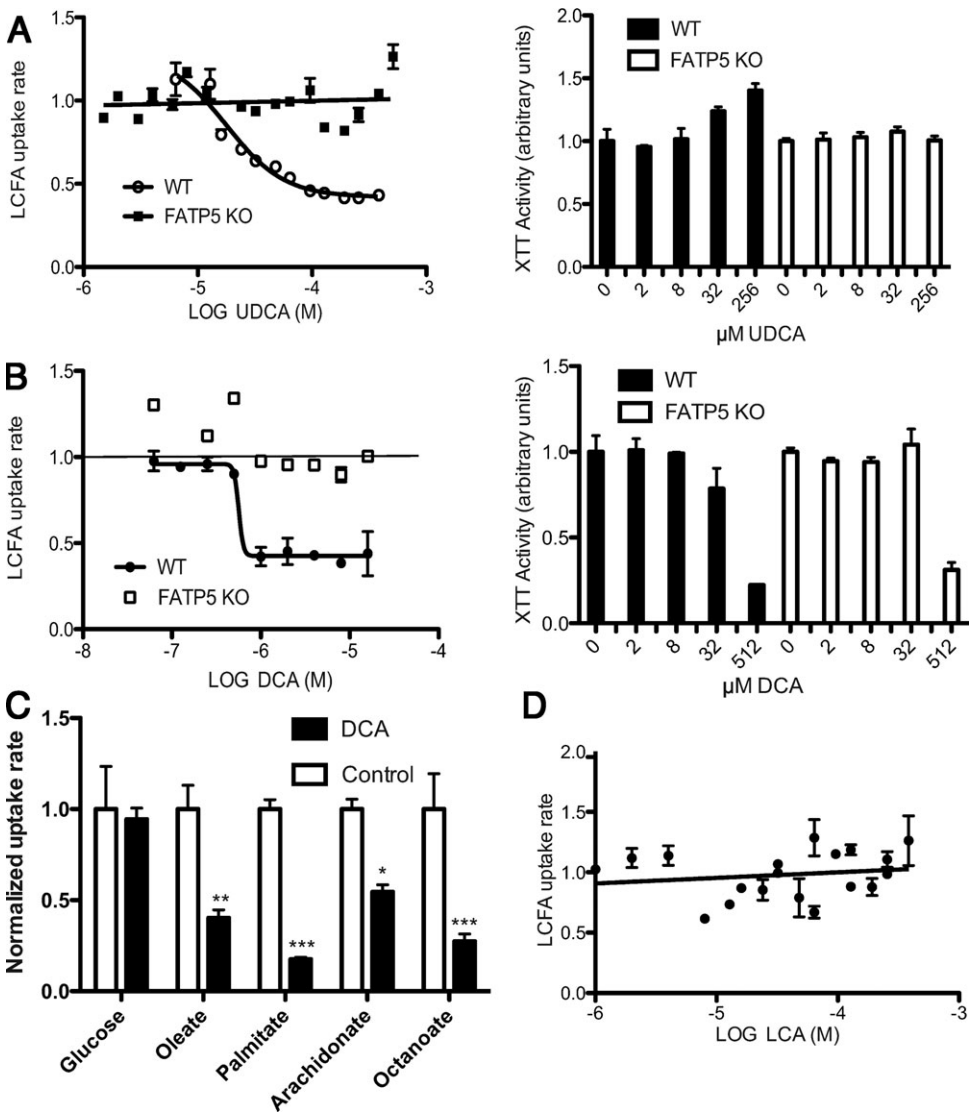

Fig. 5. *Ex vivo* inhibition of hepatic LCFA uptake by UDCA and DCA. Quencher-based LCFA uptake assay (in arbitrary units) with mouse primary hepatocytes from FATP5 wild-type or KO in 24-well plates exposed to the indicated bile acids (log M) for 2 hours during assay. (A) Left: The lines show a nonlinear regression through the wild-type (WT) data points and linear regression through the FATP5 KO data points. Right: XTT determined viability of primary mouse hepatocytes after 48-hour UDCA treatment. (B) Left: The lines show a nonlinear regression through the wild-type (WT) data; the horizontal line indicates basal uptake rate. Right: XTT determined viability of primary mouse hepatocytes after 48-hour DCA treatment. (C) Uptake assays with the indicated ^{14}C-radiolabeled fatty acids and a 2-deoxy-D-[^{3}H]glucose/glucose mix were performed with primary hepatocytes as shown in Supporting Fig. 6 for oleate. For comparison of the DCA effect across substrates, data were normalized to untreated controls. (D) The line shows a nonlinear regression through the wild-type data points. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

not cause a further reduction in liver TAG (Fig. 6F) beyond the effect of FATP5 deficiency by itself, which reduced diet-induced hepatic TAG accumulation by about 50%.[11]

In order to choose a more physiological delivery route for DCA and to assess in more detail the effect of this secondary bile acid on liver function, we supplemented high-fat diets with DCA. In pilot experiments, we determined that DCA addition in the range of 0%-0.2% caused a dose-dependent reduction in acute liver LCFA absorption without causing a detectable increase in circulating liver enzymes (Fig. 7A). Based on these results, we chose the lowest effective DCA dose (0.05%) for a 7-week high-fat diet–induced hepatosteatosis study. Neither DCA nor LCA feeding resulted in changes in respiratory rate (Supporting Fig. 7C), caloric output over a 36-hour period (Supporting Fig. 7B), food intake, or body weight (Supporting Fig. 7D,E). Furthermore, circulating lipids in the form of TAG and cholesterol were unchanged by dietary DCA supplementation in the range of 0.03%-0.4% (Supporting Fig. 7F-G). Notably, 0.05% DCA but not LCA supplementation resulted in an ≈50% reduction in hepatic TAG content (Fig. 7B) and markedly reduced lipid droplets in BODIPY-stained liver sections (Fig. 7D). Importantly, DCA treatment did not lead to hepatotoxicity or fibrosis based on alanine aminotransferase/aspartate aminotransferase assays (Fig. 7C) and liver morphology (Supporting Fig. 8). To address potential inhibition of intestinal LCFA transporters by bile acids, we determined that neither DCA, UDCA, nor LCA inhibited the principal intestinal fatty acid transporter FATP4[23] (Supporting Fig. 9A-C). Furthermore, direct measurements of intestinal fatty acid absorption with fat tolerance tests determined that the appearance of a labeled fatty acid in

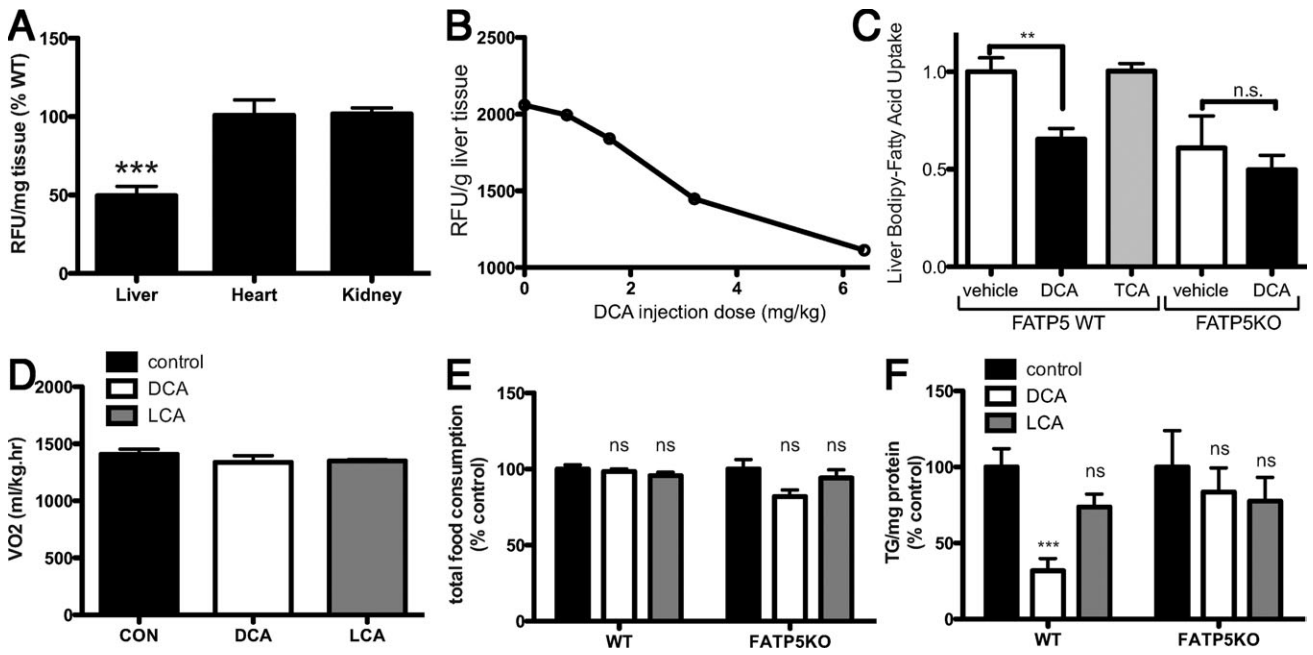

Fig. 6. FATP5-dependent reduction of hepatic LCFA uptake by DCA. (A) Validation of the nonradioactive hepatic LCFA uptake assay *in vivo* using wild-type (WT) and FATP5 KO mice. Comparison of hepatic LCFA uptake by wild-type and FATP5 KO animals after injection with BSA-bound fluorescent LCFA compound. At 20 minutes postinjection, fluorescence in organic extracts from the indicated tissues was determined, normalized to tissue weight, and expressed as % of wild-type (see Materials and Methods). (B) Acute reduction of hepatic LCFA uptake after two intraperitoneal injections of DCA or DMSO after 20 μM of BSA-bound fluorescent LCFA analog C1-BODIPY-C12. (C) Acute effects of injecting 6.4 mg/kg body weight DCA or TCA into WT or FATP5 KO mice on hepatic LCFA uptake rates (data from three independent experiments). (D) Chronic DCA/LCA injection. FATP5 wild-type and null animals were injected daily subcutaneously with 3.2 mg/kg body weight DCA (white bars), LCA (gray bars), or DMSO (control, black bars) for 47 days. O_2 consumption rates were determined using metabolic chambers. (E,F) At the end of the treatment, cumulative high-fat food consumption (E) and the TAG contents in livers (F) were determined. ns, not significant. $^{**}P < 0.01$. $^{***}P < 0.001$.

the circulation was not suppressed by DCA supplementation (Supporting Fig. 9D). Finally, we addressed the ability of DCA and LCA to activate hepatic farnesoid X receptor (FXR) target genes using quantitative polymerase chain reaction analysis and found that both secondary bile acids were FXR activators that suppressed Cyp7a1 messenger RNA levels to a comparable degree (Supporting Fig. 10).

Discussion

Our understanding of the physiological importance of bile acids for human health has been greatly expanded over the last decade and revealed new roles for bile acids as regulators of nuclear receptors and metabolic rates.[24] Bile acids regulate the enterohepatic recirculation and inhibit the expression of rate-limiting bile acid synthesis genes such as Cyp7a1[25] via activation of the nuclear hormone receptors farnesoid X receptor α (FXR-α)[26,27] and increased expression of the short heterodimer partner[28] which in turn can down-regulate hepatic fatty acid and triglyceride biosynthesis.[29] Bile acids can also activate the heterotrimeric G-protein receptor TGR5, which has been demonstrated to be involved in the regulation of glucose and energy homeostasis.[30] For example, CA has been shown to trigger the TGR5-cAMP-D2 signaling pathway and to enhance oxygen consumption independently of FXR-α *in vivo*.[24] We report a novel link between bile acids and metabolism—specifically, the ability of specific bile acids such as DCA and UDCA to inhibit hepatic LCFA uptake.

Other bile acids, including CA and CDCA, showed inhibition only at slightly supraphysiological levels based on circulating serum bile acid levels.[31,32] However, postprandial bile concentrations in the portal vein may be significantly higher and thus result in decreased hepatic LCFA uptake. Although chylomicron remnant uptake via endocytosis is thought of as the classical contributor to postprandial hepatic TAG uptake, inhibition of FATP5 has been shown to lead to a reduction in hepatic postprandial LCFA uptake, a redistribution of dietary lipids away from the liver, and reduced susceptibility to diet-induced hepatosteatosis.[10] Thus, one possible physiological function for this FATPs inhibition could be to channel dietary LCFA to

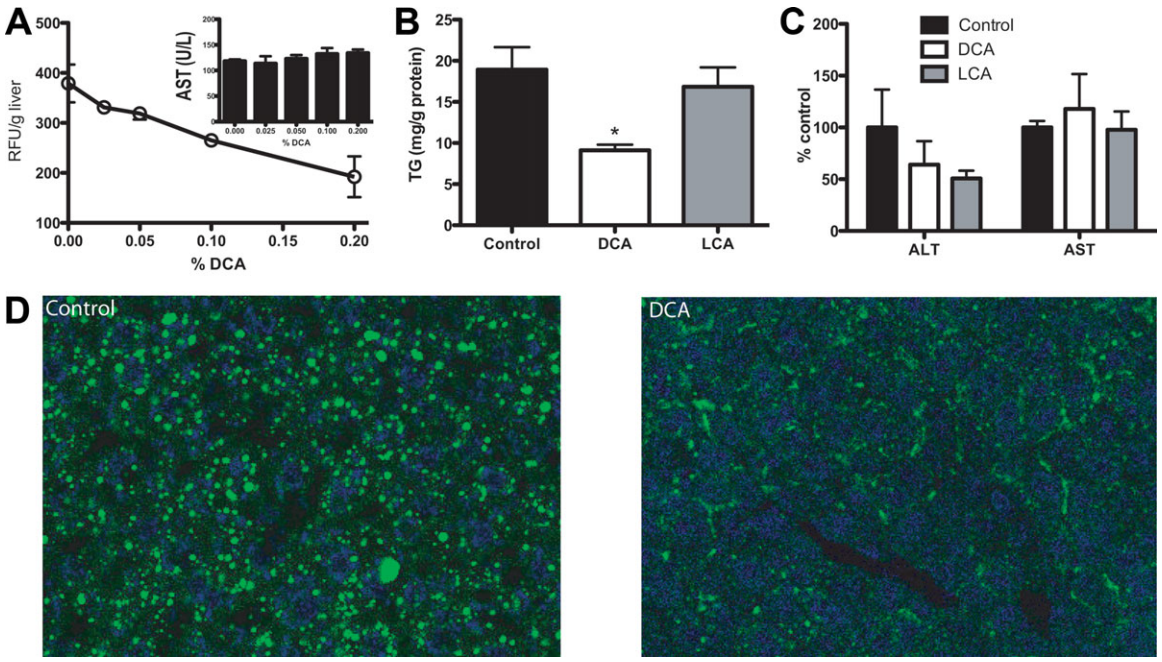

Fig. 7. Effects of DCA supplementation on liver physiology. (A) Mice were fed with the indicated concentrations of DCA added to high-fat food for 1 week followed by intraperitoneal injection with 100 μL of a 20-μM solution of the fluorescent LCFA analog C_1-BODIPY-C_{12} bound to BSA. At 40 minutes postinjection, livers were removed, solvent was extracted, and fluorescence at 515 nm was determined. The inset shows serum aspartate aminotransferase (AST) levels after 1 week of DCA/high-fat diet. (B-D) Wild-type animals were fed high-fat food supplemented with 0.5 mg/g DCA (white bars), 0.5 mg/g LCA (gray bars), or nonsupplemented high-fat chow (control, black bars) for 49 days. After the treatments, the TAG in livers (B) and serum aminotransferase activity (C) was determined. (D) Assessment of neutral lipid accumulation by confocal microscopy imaging (20× magnification) of BODIPY-stained neutral lipids in liver sections from DCA and control animals.

adipose stores in the postprandial phase when bile acid levels are high, but allow for maximal LCFA uptake to support ketogenesis during fasting conditions when LCFA are released by adipose tissue and circulating bile acid levels are low.

A particularly potent inhibitor of hepatic fatty acid transporters was the secondary bile acid DCA. DCA's inhibitory effect occurred at nanomolar concentrations well below the limit for cytotoxicity and was not only observed in cell lines and primary cells but also *in vivo* where DCA injections and feeding lowered hepatic TAG content in the context of high-fat–induced hepatosteatosis by 50%-70% and acutely reduced hepatic LCFA uptake by 31%. Notably, the secondary bile acid LCA, which did not inhibit FATP5/2-dependent cellular LCFA uptake, had no effect on liver TAG content or *in vivo* hepatic LCFA uptake. Clearly, bile acids can impact fatty liver disease through multiple mechanisms, including FXR and TGR5 activation. However, we would argue that the antisteatotic effects of DCA are, at least in part, due to its novel function as a FATP5 inhibitor. This assertion is based on several lines of evidence. First, the fact that LCA and DCA activate TGR5 equally[33,34] when only DCA lowered hepatic TAG, as well as the lack of enhanced respiratory rates following DCA injections or feeding, make a primary involvement of TGR5 unlikely. Second, both DCA and LCA activated FXR based on suppression of Cyp7a1 expression but differed in their effect on hepatosteatosis. Third, DCA treatment did not inhibit intestinal FATP4, nor did it reduce intestinal lipid absorption (Supporting Fig. 9) or cause hepatotoxicity (Fig. 7C-E). In contrast, DCA but not LCA inhibited LCFA uptake by primary hepatocytes in an FATP5-dependent fashion, and DCA injections were able to reduce the acute hepatic fatty acid uptake *in vivo* in a dose-dependent fashion (Fig. 6B). Finally, and potentially most importantly, DCA's antisteatotic effects were not observed in FATP5 KO animals, which recapitulates our findings in isolated hepatocytes and supports the model that inhibition of transporter-mediated hepatic LCFA uptake plays a key role in the prevention/reversion of diet-induced hepatosteatosis.

Physiological concentrations of DCA have been reported to be between 0.133 μM[31] and 0.415 μM,[32] placing it well within the observed IC_{50} 0.19 μM for FATP5. DCA is the only bile acid that has an IC_{50} within its physiological range. Importantly, as DCA is the product of specific bacterial strains in the colon,[35] predominantly belonging to the genera *Eubacterium*

and *Clostridium*,[36] changes in bacterial composition[37] and/or antibiotic use[38] would be predicted to impact hepatic LCFA absorption.

Although the physiological levels of UDCA in humans are likely to be too low (≈54 nM[32]) to impact hepatic LCFA uptake, oral UDCA administration has been widely used in the clinic for the treatment of several hepatobiliary disorders, including nonalcoholic steatohepatitis. Although passage through the enterohepatic circulation can lead to reconjugation and bioconversion, we expect that with chronic administration of DCA or UDCA, we can establish a mix of conjugated and unconjugated bile acids. Indeed, this has been shown for UDCA treatment in gallstone patients[39] and, importantly, feeding of DCA- and UDCA-supplemented diets has been shown to increase levels of both conjugated as well as unconjugated forms of these bile acids.[40] Oral administration of UDCA can also raise circulating UDCA levels to over 17 μM,[41] which is well above the observed IC_{50} for FATP5. Our mechanistic understanding of UDCA actions is incomplete but could include increased hydrophilicity of bile,[3] inhibition of apoptosis,[3] and conjugation to TUDCA, which has been shown to reduce endoplasmic reticulum stress in fatty liver disease animal models.[42] Here we show a potential novel mechanism by which UDCA might inhibit LCFA uptake through blockade of FATP5. We were able to show that UDCA inhibited hepatocyte LCFA uptake in an FATP5-dependent fashion. In comparison, results from human studies using UDCA to lower hepatosteatosis and nonalcoholic steatohepatitis showed that the administration of UDCA over 2 years at a dose of 13-15 mg/kg/day to 166 patients did not produce an effective result compared with placebo.[43] Results from another study indicated that administration of a high dose of UDCA (23-28 mg/kg/day) for 18 months to 185 nonalcoholic steatohepatitis patients failed to improve the overall histology but significantly improved lobular inflammation in males, younger patients (up to 50 years of age), slightly overweight patients, and in patients with hypertension.[44] We suggest that oral UDCA, similarly to DCA, acts at least in part by blocking hepatic uptake of free fatty acids and thus would predict that UDCA has a more pronounced effect in reducing the hepatic LCFA uptake in patients with high fat or high protein.

Although we present here clear data in support of the idea that select bile acids such as DCA can inhibit FATP5, the underlying structure/function relationships and biochemical mechanisms of inhibition will require further study. Besides the point that none of the tested conjugated bile acids inhibited FATP5 or FATP2, no clear lead compound structure emerged from the tested molecules. Furthermore, although FATP5 can act as a bile-CoA ligase,[14] we found no support for the hypothesis that all bile acids that are potentially activated to bile acid-CoA by FATP5 or FATP2 are inhibitors of LCFA uptake based on the lack of inhibition by THCA and LCA, which would be predicted to be substrates for FATP2/5 and FATP5, respectively.[14,17] Based on the rapid reversibility of inhibition, the lack of bile acid transporters in HEK-293 cells[45,46] and the absence of TGR5,[47] and FXR[48] in these cells, it is likely that the bile acids do not enter or signal in HEK-293 and rather act on the extracellular domain of FATP5/2 as competitive inhibitors. Alternatively, FATP5 could have a dual function as a high-affinity transporter for a specific subset of bile acids, overlapping with the pattern of inhibition. Further studies are needed to test this exciting possibility.

In conclusion, we have shown that the secondary bile acids UDCA and DCA are potent suppressors of hepatic LCFA uptake via reversible inhibition of FATP5. Based on this novel observation, we can make several interesting predictions, including (1) a new mode by which intestinal bacterial composition and activity may influence hepatic lipid metabolism and (2) the clinical use of UDCA as a more beneficial treatment of nonalcoholic fatty liver disease when the disorder is driven by hepatic uptake (rather than *de novo* synthesis) of LCFA.

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