Physiological characteristics of *allo*-cholic acid[1]

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Abstract The physiological characterstics of *allo*-cholic acid (ACA), a typically fetal bile acid that reappears during liver regeneration and carcinogenesis were investigated. [^{14}C] Tauro-ACA (TACA) uptake by Chinese hamster ovary cells expressing rat organic anion transporter polypeptide (Oatp)1 or sodium-taurocholate cotransporter polypeptide (Ntcp) was lower than that of [^{14}C]taurocholic acid (TCA). Although TACA inhibited ATP-dependent TCA transport across plasma membrane vesicles from Sf9 cells expressing rat or mouse bile salt export pump (Bsep), no ATP-dependent TACA transport was found. In rats, TACA was secreted into bile with no major biotransformation and it had lower clearance and longer half-life than TCA. In mice, TACA bile output was lower (−50%) than that of TCA, whereas TACA induced 9-fold higher bile flow than TCA. Even though the intracellular levels were lower for TACA, translocation into the hepatocyte nucleus was higher for TACA than for TCA; however, rate of DNA synthesis, expression levels of α-fetoprotein, albumin, Ntcp, and Bsep, cell viability, and apoptosis in rat hepatocytes were similarly affected by both isomers. In conclusion, TACA partly shares hepatocellular uptake system(s) for TCA. Furthermore, in contrast to other "flat" bile acids, TACA is efficiently secreted into bile via transport system(s) other than Bsep and is highly choleretic, hence its appearance during certain situations may prevent accumulation of cholestatic precursors.—Mendoza, M. E., M. J. Monte, M. A. Serrano, M. Pastor-Anglada, B. Stieger, P. J. Meier, M. Medarde, and J. J. G. Marin. **Physiological characteristics of *allo*-cholic acid. *J. Lipid Res.* 2003.** 44: **84–92.**

Supplementary key words bile • liver • steroid

Major bile acids (BAs) are mono or polyhydroxylated acidic steroids with a 5β-cholanoyl structure in which the A and B rings are approximately perpendicular; however, minor BAs with unsaturations affecting C5 or with a 5α-cholanoyl configuration (*allo*-BA) have both rings on the same plane, explaining their designation as "flat" BAs. They are uncommon in healthy adult mammals, but are present in fetuses as well as in lower species. For example, in some migratory species of fishes, such as *Petromyzon marinus, allo*-BAs are strong specific stimulants of the olfactory epithelium (1). These BAs may play an important role as conspecific migratory pheromones produced by larvae to attract adult individuals towards reproductive areas upstream in the river (2).

Allo-BAs were first described at the beginning of the previous century and little attention has since been paid to the physiological characteristics of these BAs in mammals (3). Because the cholestatic properties of other BAs that share with *allo*-BAs their flat structure have been described and the molecular bases of this effect have been studied (4), the first objective of the current study was to investigate the liver handling of *allo*-cholic acid (ACA) (see structure in the inset of **Fig. 1**).

Flat BAs reappear in both adult humans and in the rat during physiological and pathophysiological liver cell proliferation. They are easily detected in the serum and even more easily in the urine, presumably due to the low ability of the liver to handle them, in patients with hepatocellular carcinoma (5). In rats, flat BAs also reappear during hepatocarcinogenesis (6, 7) and are transiently elevated during the liver regeneration that follows two-thirds partial hepatectomy (8, 9). Whether this is a mere epiphenomenon or *allo*-BAs play a biological role is unknown. The discovery of BAs in the nuclei of rat hepatocytes (10) together with the observation that the composition of this sub-pool of BAs markedly differs from that of the cytosolic sub-pool (7, 9) and the fact that important changes occur in the nuclear sub-pool during liver carcinogenesis (7) and regeneration (9) suggest that *allo*-BAs may be involved in signaling mech-

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Abbreviations: ACA, *allo*-cholic acid; αFP, alpha-fetoprotein; Bsep, bile salt export pump; CA, cholic acid; GCDCA, glycochenodeoxycholic acid; GC-MS, gas-chromatography mass-spectrometry tandem; Ntcp, sodium-taurocholate cotransporter polypeptide; Oatp, organic anion transporter polypeptide; TACA, tauro-*allo*-cholic acid; TCA, taurocholic acid; UDCA, ursodeoxycholic acid.

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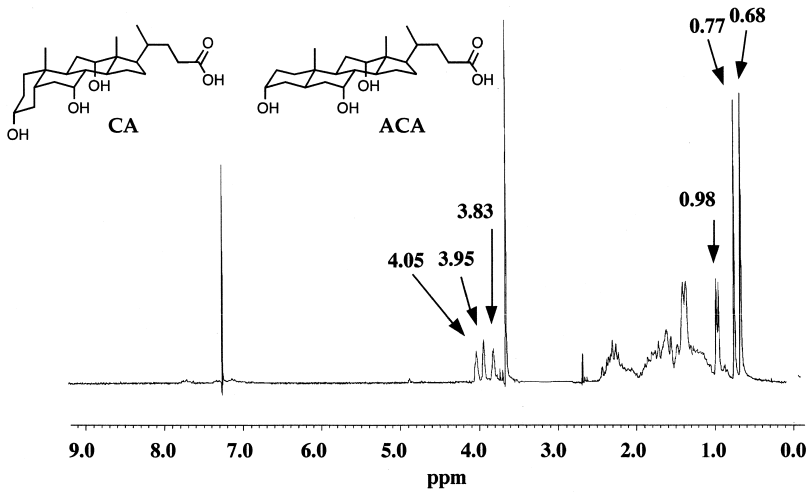

Fig. 1. ^{1}H-NMR spectrum of *allo*-cholic acid methyl ester (methyl-ACA) showing the most characteristic signals. The inset depicts the structure of the 5α (ACA) and 5β (CA) epimers of cholic acid.

anisms related to proliferation, differentiation, viability, or apoptosis of parenchymal liver cells. We therefore extended these studies in an attempt to decipher this question by investigating the ability of ACA to reach the hepatocyte nucleus and to affect the processes mentioned above.

MATERIALS AND METHODS

Chemicals

Bile acids or sodium salts and methyl cholate (more than 95% pure by TLC), taurine, 3α-hydroxysteroid dehydrogenase, sulfatase, β-glucuronidase, cholylglycine hydrolase, Hoechst-33258, neutral red, culture media, supplements, and antibiotics for hepatocyte and cell line cultures were from Sigma-Aldrich (Madrid, Spain). G418-sulfate (Geneticin) and fetal calf serum were from Boehringer Manheim (Barcelona, Spain). [^{14}C]Taurocholic acid (TCA, specific activity 49 mCi/mmol), [^{3}H]TCA (specific activity 3 Ci/mmol), [^{14}C]taurine (specific activity 108.5 mCi/mmol) and [methyl-^{14}C]thymidine (specific activity 57 mCi/mmol) were from PerkinElmer Life Science (Pacisa & Giralt, Madrid, Spain). 3α,7α,12α-trihydroxy-5α-cholanoic acid (ACA) was synthesized from methyl ester (9% yield) by the method described by Iida et al. (11). The trimethylsilyl ether derivative of methyl-ACA was prepared and analyzed by gas-chromatography mass-spectrometry tandem (GC-MS) (5) using commercial ACA (Toronto Research Chemicals, Ontario, Canada) as standard. Both the retention-times in GC (data not shown) and mass spectra (**Fig. 2**) were consistent with the assigned identity of the product. The purity was approximately 99%. The radiolabeled derivative [^{14}C]tauro-*allo*-cholic acid (TACA) (259 μCi/mmol specific activity) was obtained by conjugating ACA with [^{14}C]taurine (12). [^{14}C]TACA was purified to more than 98% by liquid-solid extraction in octadecylsilane cartridges followed by preparative TLC. Intermediate compounds in the reactions and the final products were dissolved in CDCl3 and characterized by proton nuclear magnetic resonance (^{1}H-NMR) at 200 MHz using tetramethylsilane as internal standard. Figure 1 depicts the ^{1}H-NMR spectrum for the methyl-ACA, indicating the most characteristics signals. The characteristic shifts (δ) for methyl-ACA and TACA: the hydrogens in β disposition geminal to the α-hydroxyl groups on three, seven, and 12 and the methyl groups (18, 19, and 21), as well as the taurine methylenes 25 and 26, are shown in **Table 1**. Moreover, as expected, the signal corresponding to the hydrogen geminal to the 3α-hydroxyl changed from a broad multiplet (axial disposition) in cholic acid (CA) (data not shown) to a narrow multiplet (equatorial disposition) in ACA (Fig. 1).

In vitro studies on TACA transport

Chinese hamster ovary (CHO) cells that had previously been stably transfected with the cDNA for rat sodium-taurocholate cotransporter polypeptide (Ntcp) (13) or organic anion transporter polypeptide (Oatp1) (14) and wild-type CHO cells were used in uptake studies. Cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml L-proline, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml amphotericin B at 37°C in an atmosphere of 5% CO_2-95% air. Selective media contained additional 400 μg/ml geneticin. The expression of above-mentioned carriers was induced by incubating the transfected cells for 24 h with culture medium supplemented with 5 mM butyrate (15).

Once grown to confluence on 60 mm diameter dishes, the cells were rinsed three times with pre-warmed (37°C) sodium- or choline-containing Earle's balanced saline solution supplemented with 5.5 mM D-glucose. Cells were then incubated at 37°C in the presence of 20 μM [^{14}C]TACA or [^{14}C]TCA for 15 min. Transport was

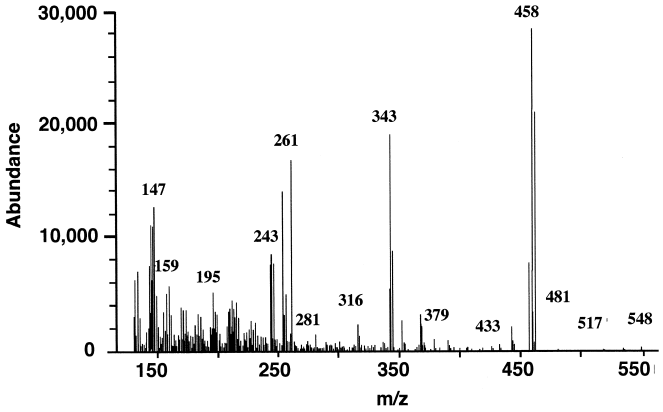

Fig. 2. Mass spectrum of the trimethyl silyl derivative of ACA (methyl ester) subjected to gas-chromatography mass-spectroscopy tandem (GC-MS).

TABLE 1. ^{1}H-NMR assignments

Group[a]	Number of H	Shift (δ) Multiplicity	
3α,7α,12α-Trihydroxy-5α- Cholanoic Acid Methyl Ester			
3β-H	1	4.05 m	
7β-H	1	3.83 m	
12β-H	1	3.95 m	
18-Me	3	0.68 s	
19-Me	3	0.77 s	
21-Me	3	0.98 d	J= 5.4 Hz
COOMe	3	3.66 s	
3α,7α,12α-Trihydroxy-5α-Cholanoyl-Taurine			
3β-H	1	3.85 m	
7β-H	1	3.67 m	
12β-H	1	3.81 m	
18-Me	3	0.59 s	
19-Me	3	0.69 s	
21-Me	3	0.90 d	J= 5.4 Hz
25-H	2	2.87 t	
26-H	2	3.47 t	

δ is given in ppm. Tetramethylsilane was used as internal reference.
[a]Numbering of groups refers to those of positions for C in the usual bile acid structure of a cholanoic acid. Solvent was $CDCl_3$.

stopped with 2 ml of ice-cold sodium- or choline-containing Earle's solution. After two additional washing steps with stop solution, the cells were digested in 1 ml Lowry solution (100 mM NaOH, 189 mM Na_2CO_3) for 2 h. Cells were harvested by scraping to measure radioactivity and total protein concentrations.

Plasma membrane vesicles from insect Sf9 cells expressing rat or mouse bile salt export pump (Bsep) were obtained as previously described (16, 17). Vesicles were incubated with [^{3}H]TCA, [^{14}C]TACA, or both in the absence or in the presence of 5 mM ATP plus an ATP regenerating system (3 mM phosphocreatine plus 100 μg/ml creatine phosphokinase) for 20 min, and uptake was stopped using a rapid filtration technique, as previously described (17, 18). The study was carried out at a high substrate concentration (100 μM) in order to increase the radioactivity retained by the vesicles on the filters up to an accurate level. Additionally, to accomplish this it was necessary to count three filters together.

In vivo studies

Non-fasting male and 21-day pregnant Wistar CF rats and male Swiss Albino mice were from the Animal House at the University of Salamanca, Spain. They were fed on commercial pelleted rat or mouse food (Panlab, Madrid, Spain) and water ad libitum. Temperature (20°C) and the light/dark cycle (12 h:12 h) in the room were controlled. All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 8023, revised 1985). Surgery and bile collections were carried out under sodium pentobarbital (Nembutal N.R., Abbot, Madrid, Spain) anesthesia (50 μg/g bwt, ip). Flexible catheters were inserted into the left carotid artery to collect blood samples and into the left jugular vein to administer BAs. The common bile duct was also cannulated to collect bile samples. In mice, the gallbladder was ligated. After administration of a single iv bolus of [^{14}C]TACA or [^{14}C]TCA, blood (in rats) and bile (in rats and mice) samples were collected at the indicated time-points.

In some experiments, a single dose of 10 nmol/g bwt [^{14}C]TACA was injected through the penis vein to rats under ether anesthesia. The animals were allowed to recover from anesthesia in a warmed cabinet and were then housed in individual metabolic cages where they had free access to food and water. Six hours after TACA administration, a blood sample was obtained from the tail vein. Serum was ultrafiltered using 10 kD cut-off filters (Amicon Micricon-10, Lexington, MA). Urine samples were collected for 14 days. After this time, animals were anesthetized with sodium pentobarbital and surgically prepared as described above to collect bile samples over 8 h (at 1 h intervals) in order to obtain most of the bile acid pool.

Nuclear translocation, differentiation, proliferation, viability, and apoptosis

Translocation of [^{14}C]TACA or [^{3}H]TCA into the nucleus of adult rat hepatocytes that were isolated by two-steps collagenase method (19) was measured as previously described (9). The degree of differentiation was studied in 21 days fetal liver cells freshly after isolation (20) or after 2 or 4 days in primary culture in the presence of none, 10 μM, or 50 μM ACA, CA, or ursodeoxycholic acid (UDCA). Purification of total RNA was performed using the RNAeasy mini kit from Quiagen (Izasa, Barcelona, Spain), following the instructions supplied by the vendor. RNA quality was determined by denaturing formaldehyde agarose gel electrophoresis and the amount was measured spectrophotometrically at 260 nm. Northern blot was performed using 20 μg of denatured RNA, which was size-fractionated by agarose gel electrophoresis. The fractionated RNA was transferred onto a nylon membrane (Biodyne B; Pall Gelman Sciences, Barcelona, Spain) and successively probed with rat Bsep, Ntcp, albumin, and α-fetoprotein (αFP) cDNAs under high stringency conditions using ExpressHyb hybridization solution from Clontech (BD, Madrid, Spain). β-actin cDNA was used as an internal calibrator for comparative purposes among the different experimental conditions.

The effect on proliferation, cell viability and apoptosis was determined in adult rat hepatocytes in primary culture. Cells in Williams' medium E, pH 7.4, were seeded at ~10^4 cells/cm^2 and cultured for 24 h as previously reported (21). The medium was then replaced by fresh one that contained 10 μM or 50 μM ACA, CA, or UDCA. Cells plated in the presence 0.1% DMSO were used as controls. At 69 h after seeding, [^{14}C]thymidine (50,000 dpm/plate) was added. Three hours later, plates were washed twice with PBS and cells were detached with Lowry solution in order to measure total DNA, protein, and radioactivity. Toxicity was determined at the end of the exposure period by the neutral red retention test, as previously described (21).

The pro-apoptotic effect of ACA was evaluated using four different approaches. In a first attempt, apoptosis measurements were carried out by the detection of histone-associated DNA fragments using a Cell Death Detection ELISA kit (Roche, Barcelona). Because even the positive control used in these experiments, i.e., glycochenodeoxycholic acid (GCDCA), did not induce a strong pro-apoptotic effect, additional experimental design and evaluation methods were used. Freshly isolated hepatocytes were plated, and after a 1.5 h attachment period they were exposed to 100 μM ACA, CA, or GCDCA for 4 h before carrying out morphological evaluation of apoptosis by: *i*) staining of nuclei with Hoechst-33258 (5 μg/ml, 20 min in the dark) in 4% paraformaldehyde-fixed cells, followed by fluorescence microscopy to visualize condensed chromatin as well as nuclear fragmentation (22); *ii*) DNA extraction and electrophoresis in 1.5% agarose gels to assess DNA ladder formation (23, 24); and *iii*) single cell electrophoresis (comet) assays (25).

Analytical methods

Bile flow was determined gravimetrically. Radioactivity in bile, total serum, ultrafiltered serum, urine, cell extracts, and isolated nuclei was measured on a Beckman LS-6500 liquid scintillation counter (Beckman Instruments, Madrid, Spain) using Universol Scintillation Cocktail from ICN (Biolink, Barcelona, Spain). The concentrations of 3α-hydroxyl-BAs in bile were measured enzymatically using 3α-hydroxysteroid dehydrogenase (26). Biotransformation of [^{14}C]TACA was investigated by TLC of BAs extracted

from bile and urine samples (5) using n-butanol-acetone-acetic acid-water (35:35:10:20) as eluent. The presence of total ACA in bile and urine samples was investigated by GC-MS (5) after liquid-solid extraction, enzymatic desulfation, deglucuronation, and deamidation (27, 28), and derivatization (29) of BAs. Proteins were measured using a modification of the Lowry method (30) with serum bovine albumin as standard. DNA was measured fluorometrically using Hoechst-33258 (31). The rate of DNA synthesis was determined by [^{14}C]thymidine incorporation into DNA (32).

Pharmacokinetic and statistical analysis

In order to compare the pharmacokinetics of [^{14}C]TACA and [^{14}C]TCA, model-independent methods based on the theory of statistical moments were used to analyze data on serum concentrations after iv administration of both of these BAs to anesthetized rats. AUCs (areas under the curve) and MRTs (mean residence times) were calculated by numerical integration using the trapezoidal rule, as described by Yamaoka et al. (33). Because this was observed over a limited period of time (120 min), extrapolation to infinity ($\propto$) was carried out using a monoexponential equation. Clearance (Cl) was calculated as the absolute dose divided by the AUC and the half-life ($T_{1/2}$) as Ln2/Ke, where Ke (elimination constant) is 1/MRT. Results are expressed as mean ± SE. To calculate the statistical significance of the differences, paired or unpaired Student's *t*-tests and the Bonferroni method of multiple-range testing were used, as appropriate.

RESULTS

TACA uptake, biotransformation, and secretion

Although wild-type CHO cells exhibited low uptake of TCA, this was not negligible for TACA (**Fig. 3**). Owing to the fact that TACA has a structural similarity with steroid hormones and because CHO cells originiate from a steroidogenic tissue, they may contain intrinsic transport systems for steroids able to carry out the TACA uptake that was observed in wild-type CHO cells. Nevertheless, the expression of Oatp1 or Ntcp by transfected CHO cells was accompanied by a significant enhancement in TACA uptake. The expression of these carriers induced Na^+-independent and Na^+-dependent uptake of TCA, respectively (Fig. 3). TACA uptake was similar to that found for TCA in Oatp1-expressing CHO cells; however, in Ntcp-expressing CHO cells, although TACA uptake was significantly higher than that seen for wild-type CHO cells, this was not reduced when Na^+ was replaced by choline in the incubation medium. Experiments carried out on rat or mouse Bsep-containing plasma membrane vesicles of Sf 9 insect cells revealed that ATP-dependent transport of TCA was inhibited by TACA; however, TACA was not transported by ATP-dependent systems present in these vesicles (**Fig. 4**).

The results obtained in in vitro experiments were consistent with those found in experiments carried out on in vivo models. After administration (iv, 4 nmol/g bwt) of TCA or TACA to anaesthetized rats, the disappearance of TACA from plasma was slower than that of TCA (**Fig. 5**). Pharmacokinetic analysis indicated that TACA clearance was lower and that it had a longer half-life than TCA (**Table 2**). Ultrafiltration of serum samples collected 6 h after iv administration of TACA in separate experiments revealed that 80% of this BA was present in the non-ultrafilterable fraction, i.e., presumably bound to serum proteins; however, TACA was rapidly secreted into bile. The total bile output of TACA (78% of dose administered in 2 h) did not differ significantly from that of TCA (Table 2). At the moment of maximum biliary output of ^{14}C, most (94.9 ± 1.2% by T.L.C, n = 4 animals) of the radioactivity secreted into bile was found to be non-biotransformed TACA. Free [^{14}C]taurine accounted for less than 0.5% of this radioactivity. Neither TCA nor TACA induced modifications in bile flow at the dose given to rats (data not shown). To investigate the effect of TACA on bile flow, a higher dose of 25 nmol/g bwt was used. Moreover, to reduce the amount of TACA required to reach this goal, these experiments were carried out on mice (**Fig. 6**). The amount of TACA secreted into bile in the ensuing 3 h (270 ± 53 nmol/g liver, i.e., 58 ± 5% of the dose) was approximately half ($P < 0.01$) that found for CA (492 ± 67 nmol/g liver, i.e., 94 ± 3% of the dose; $P < 0.01$). Conversely, the BA-induced increase in bile flow was much higher for TACA (99.3 ± 2.7 μl/μmol) than for TCA (11.3 ± 1.7 μl/μmol; $P < 0.001$).

Urinary excretion was investigated in conscious rats maintained in metabolic cages for two weeks after iv administration of TACA (10 nmol/g bwt). Recovery of the radioactivity in urine during this period accounted for 13% of the dose administered. The urinary output peak (5% of the dose administered) was reached at 12 h after administration. TACA accounted for only 2.5% (by TLC) of the ^{14}C found in urine at that time. The presence in urine of unconjugated ACA released from TACA, and hence unlabeled, was investigated in three animals by GC-MS analysis of urine samples collected 24 h after TACA administration. No unconjugated ACA was detected in any of these samples. Two weeks after administration of TACA, rats were anesthetized in order to collect as

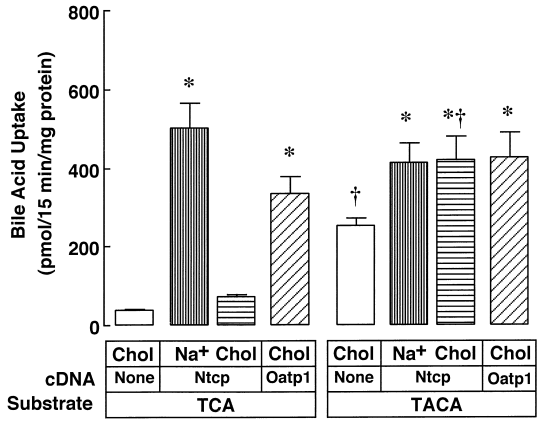

Fig. 3. Uptake of [^{14}C]taurocholic acid (TCA) and [^{14}C]tauro-*allo*-cholic acid (TACA) by CHO cells either of wild-type or stably expressing the rat carriers sodium-taurocholate cotransporter polypeptide (Ntcp) or organic anion transporter polypeptide (Oatp)1. Cells were incubated with 20 μM of one of the radiolabeled bile acids in medium containing either 116 mM NaCl (Na^+-bars) or choline-Cl (Chol-bars) for 15 min at 37°C. Values are means ± SE from four experiments (carried out in triplicate each). Comparisons were carried out by the Bonferroni method of multiple range testing. * $P < 0.05$, as compared to wild-type cells; † $P < 0.05$, as compared to uptake of TCA under similar conditions.

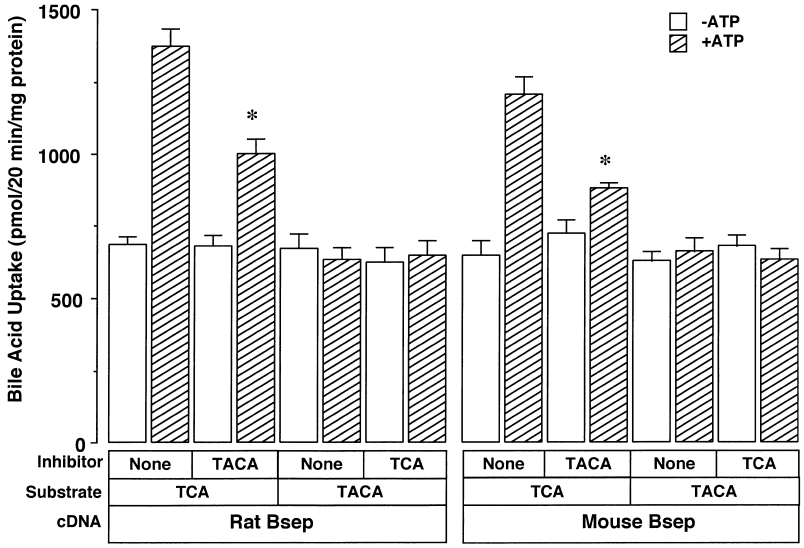

Fig. 4. Uptake of [^{3}H]TCA and [^{14}C]TACA separately or together by plasma membrane vesicles obtained from insect Sf9 cells expressing rat or mouse bile salt export pump (Bsep). Membrane vesicles were incubated with 100 μM of one or both of the radiolabeled bile acids in the absence or in the presence of 5 mM ATP plus an ATP regenerating system (3 mM phosphocreatine plus 100 μg/ml creatine phosphokinase) for 20 min at 37°C. Values are means ± SE from three experiments (carried out in triplicate each). * $P < 0.05$, as compared to uptake in absence of inhibitor in similar conditions by paired Student's *t*-test.

much as possible of the whole BA pool by bile drainage and to measure the proportions of TACA and ACA. The BA pool size, determined as the cumulated biliary output of 3α-hydroxyl-BAs for 8 h, was 12.8 ± 0.1 μmol/g liver (n = 3 rats). [^{14}C]TACA identified by TLC was 1.16 ± 0.07 nmol/g liver; i.e. less than 0.01% of the total BA pool. The presence of total (conjugated with [^{14}C]taurine plus non-amidated) ACA in these bile samples accounted for 0.27% of total BAs as determined by GC-MS.

Determination of biological effects

Translocation into the nuclei of freshly isolated rat hepatocytes in suspension was much higher for TACA

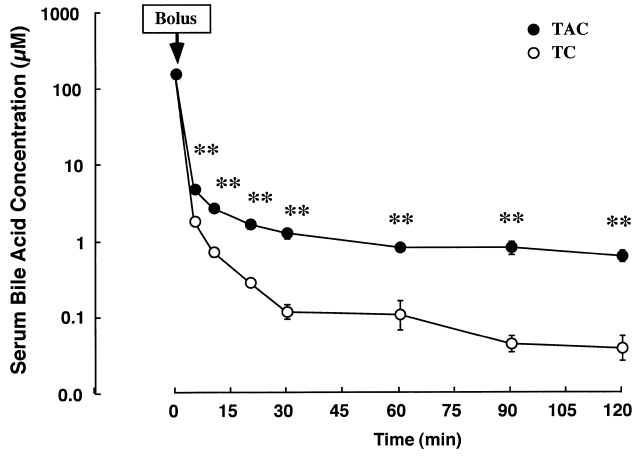

Fig. 5. Time-course of serum bile acid concentration in rats following intravenous administration of 4 nmol/g bwt of [^{14}C]TACA or [^{14}C]TCA. Blood was collected at the indicated times from the carotid artery and radioactivity in serum was measured. Values are means ± SE from four animals in each group. ** $P < 0.01$, significantly different by Student's *t*-test.

than for TCA, even though the intracellular level of the former BA was much lower than that of the latter (**Fig. 7**). Because of this characteristic and its transient reappearance during liver growth, several potential biological effects of ACA were investigated; namely, the ability to affect hepatocyte differentiation, proliferation, cell viability, and apoptosis. The expression level of proteins typical of adult hepatocytes, such as albumin and the BA carriers Ntcp and Bsep and that of a protein typical of fetal parenchymal liver cells, such as αFP, were used as an indication of the level of hepatic differentiation. **Figure 8** shows that even though the RNA loaded was lower in the case of adult hepatocytes (lane 1), as indicated by the amount of β-actin, the amounts of mRNA for albumin, Ntcp, and Bsep were markedly high. Freshly isolated fetal hepatocytes also contained high amounts of mRNA for these proteins, including αFP, which was not detected in adult hepatocytes. A decrease in the expression level of these four proteins was observed after 2 days in culture. This was slight for albumin and αFP, but very marked for Ntcp and Bsep. A partial recovery in the expression level for all three proteins was observed at 4 days. The presence of CA, ACA, or UDCA at 10 μM (data not shown) or 50 μM (Fig. 8) had no perceptible effect at 2 or 4 days on the amount of these proteins, except that Bsep that was higher both at 2 or 4 days in hepatocytes incubated with CA or ACA.

No ability of ACA to affect rat hepatocyte proliferation was found. The amount of DNA in the culture of adult rat hepatocytes increased up to ~200% after 72 h either in the absence or the presence of ACA, CA, or UDCA (**Table 3**). This was consistent with the absence of significant BA-induced effects on the rate of DNA synthesis (Table 3). To determine toxic and pro-apoptotic effects, an additional BA with well-known abilities in this respect, glycochenodeoxycholic acid

TABLE 2. Elimination into bile and pharmacokinetic parameters from serum concentrations curves obtained in anesthetized rats

	Serum				Bile	
	AUC	Cl	MRT	$T_{1/2}$	Output	Output
	μmol/l/min	*ml/min*	*min*	*min*	*nmol/g liver*	*% of dose*
TACA	622.8 ± 36.5	1.62 ± 0.09	49.3 ± 6.8	11.9 ± 2.3	86.2 ± 2.3	78.0 ± 4.4
TCA	412.0 ± 3.1[b]	2.43 ± 0.02[c]	4.4 ± 1.9[b]	3.03 ± 1.3[a]	80.4 ± 3.5	72.5 ± 4.7

AUC, area under the curve; Cl, clearance (dose/AUC); MRT, mean residence time; $T_{1/2}$, half-life (MRT.Ln2). Values are means ± SD from four experiments in each group, in which following intravenous administration of 4 nmol/g bwt of [^{14}C]TACA or [^{14}C]TCA to anaesthetized rats blood and bile samples were collected at the times indicated in Fig. 5. Statistical moments of the serum concentration curves were calculated by extrapolation to infinity. Statistical significance compared to TACA by Student's *t*-test.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

(GCDCA), was included in the study as a positive control. At high concentrations GCDCA, but not ACA or CA, was found to reduce cell viability (Table 3). GCDCA, but not ACA or CA, also induced the release of mono- or oligo-nucleosomes (Table 3) and significantly increased the proportion of apoptotic cells (**Fig. 9**, Table 3). As revealed by DNA-ladder formation in agarose gel electrophoresis and comet assays, DNA fragmentation was evident in GCDCA-treated cells but was very weak in hepatocytes incubated with CA or ACA (Fig. 9).

DISCUSSION

Biliary secretion

The ability of flat BAs of the unsaturated type to inhibit secretion into bile of major BAs might account for their deleterious effect in several liver diseases in which their increased level has been partly associated with the cause of cholestatic symptoms in these patients (4). However, our results indicate that, although TACA is also able to inhibit TCA transport by rat or mouse Bsep, while itself is not a substrate for this carrier, TACA induced a stronger choleretic effect than TCA. This was probably due to the fact that TACA was efficiently eliminated from the hepatocytes into bile. It must be kept in mind that TACA is synthesized by hepatocytes, hence its first and major route will be canalicular secretion before it enters enterohepatic circulation. Nevertheless, the contribution of this BA to bile formation, in the situations where it appears, is probably minor. Thus, considering the amount of TACA secreted into bile at the time of rat liver regeneration when such secretion is maximal, i.e., 3 days after partial hepatectomy (9), and assuming that the ability to induce bile flow is similar to that seen in intact animals, an assumption that probably involves a moderate under-estimation (34), it can be calculated that the contribution of TACA to bile formation would be approximately 10 nl/min/g liver, i.e., lower than 0.5% of total bile flow. Although this may seem

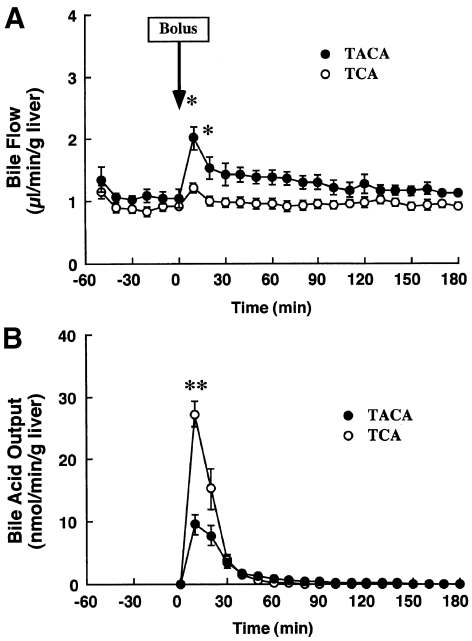

Fig. 6. Time-course of bile flow (A) and bile acid output (B) in mice after intravenous administration of 25 nmol/g bwt of [^{14}C]TACA or [^{14}C]TCA. Bile was collected 60 min before and 180 min after bile acid administration (arrow) and radioactivity in bile samples was measured. Values are means ± SE from four animals in each group. *$P < 0.05$, **$P < 0.01$, significantly different by Student's *t*-test.

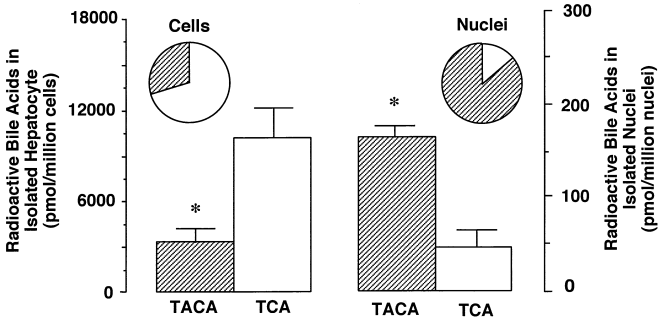

Fig. 7. Translocation of TCA and TACA into the rat hepatocyte nucleus. Isolated rat hepatocytes were incubated for 60 min with 45 μM [^{3}H]TCA and 45 μM [^{14}C]TACA before homogenization, nuclei isolation, and radioactivity measurement. Bile acid uptake by hepatocytes (left panel) and bile acid contents in the isolated nuclei (right panel) are shown as mean ± SE of three experiments. Comparisons (* $P < 0.05$) were carried out by the paired Student's *t*-test. The inset depicts the proportion of each bile acid in whole cells and in isolated nuclei.

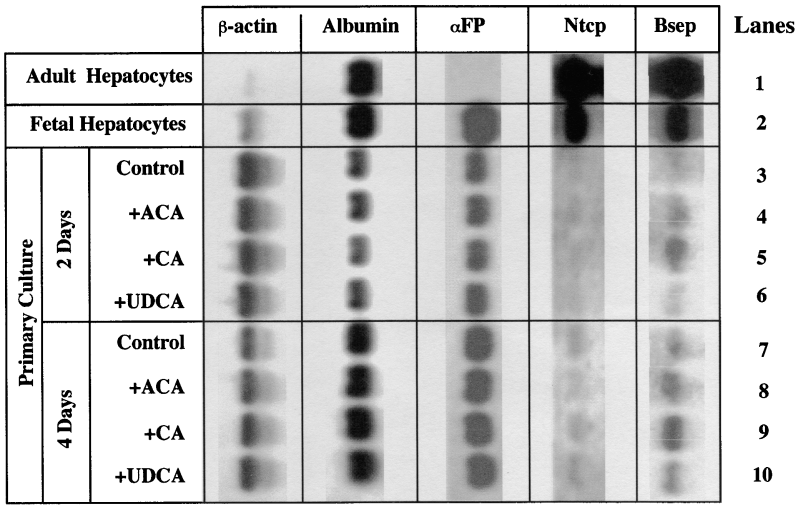

Fig. 8. Effect of the presence of bile acids in the medium used to culture fetal rat hepatocytes on the amount of mRNA for albumin, α-fetoprotein (αFP), Ntcp, and Bsep as determined by Northern blotting of 20 μg of total RNA loaded in each lane. The level of β-actin in the sample was used as internal calibrator. Fetal rat hepatocytes were cultured for 2 or 4 days in the presence of 50 μM ACA, CA, or ursodeoxycholic acid (UDCA). The level of expression of the indicated genes in freshly isolated adult and fetal hepatocytes was also determined for comparative purposes.

a poor physiological role, in principle it should not be neglected for the following reason. *Allo*-BAs are presumably synthesized from 3-oxo-Δ^4-BAs, which are normally transformed into 5β-BAs by 3-oxo-Δ^4-steroid 5β-reductase. In situations where inappropriate activity of this enzyme or overproduction of its substrate occurs the transformation of the cholestatic compounds into a highly choleretic counterpart, that can be readily eliminated into bile, might play a role in the protection of the hepatocyte against the accumulation of toxic products. The activity of Δ^4-steroid 5α-reductase, which is normally present in the liver and is presumably responsible for carrying out this metabolic transformation also on Δ^4-BAs (35), is probably only evident under the abnormal circumstances of high intracellular levels of these substrates, as mentioned above.

Liver handling

Observation of the high endogenous ability of wild-type CHO cells to take up TACA was not a complete surprise because of the structural similarity of TACA and steroid hormones and because of the tissue of origin of these cells, in which steroid hormone exchange across plasma

TABLE 3. Effect of BAs on replication, toxicity, and apoptosis in rat hepatocytes in primary culture

	DNA	Thymidine Incorporation	Neutral Red Retention	Apoptosis Index	Apoptotic Cells
	% of initial	*dpm/μg DNA*	*% of control*		*%*
Control	201 ± 6	275 ± 27	100	1	3.8 ± 1.9
CA					
10 μM	209 ± 14	259 ± 25	92 ± 3	0.90 ± 0.15	
50 μM	208 ± 18	281 ± 29	93 ± 2	0.83 ± 0.22	
100 μM					5.3 ± 3.2
ACA					
10 μM	207 ± 16	319 ± 38	93 ± 3	0.95 ± 0.22	
50 μM	187 ± 13	253 ± 20	92 ± 4	1.05 ± 0.91	
100 μM					3.3 ± 1.9
UDCA					
10 μM	182 ± 15	327 ± 20	89 ± 2	0.68 ± 0.18	
50 μM	180 ± 16	301 ± 21	88 ± 4	0.55 ± 0.16[a]	
GCDCA					
10 μM			88 ± 6	1.29 ± 0.16	
50 μM			79 ± 3[a]	1.47 ± 0.12[a]	
100 μM					18.8 ± 2.4[a]

Values are means ± SE from at least four measurements carried out in triplicate. Total DNA, radiolabeled thymidine incorporation into DNA (replication), and neutral red retention (toxicity) were determined in cultures exposed to the indicated BA for 72 h. Nucleosome release (Apoptosis Index) was evaluated after 6 h exposure. The proportion of apoptotic cells was determined by Hoechst-33258 after exposing the cells to higher BA concentration for shorter times (4 h).

[a] $P < 0.05$ as compared to control by paired Student's *t*-test.

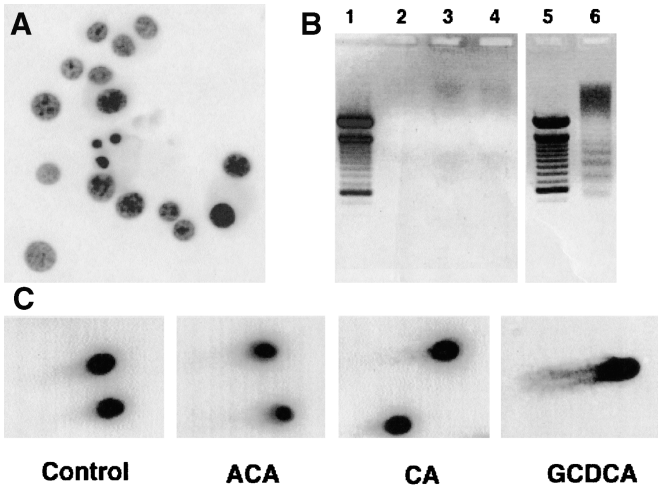

Fig. 9. Pro-apoptotic effect on adult hepatocytes. Following isolation and plating for 1 h 30 min, cells were exposed to none (control) or 100 μM ACA, CA, or glycochenodeoxycholic acid (GCDCA) for 4 h. A: Representative picture of intact and fragmented nuclei as detected by Hoechst-33258-staining of DNA. The proportions of apoptotic cells under each experimental condition are given in Table 3. B: DNA ladder in 1.5% agarose gel electrophoresis of DNA extracted from hepatocytes of the experimental groups: Control (lane 2), ACA (lane 3), CA (lane 4), and GCDCA (lane 6). A DNA molecular weight standard was loaded in lanes 1 and 5. C: Characteristic images from comet assays for the hepatocytes of each experimental group.

membrane is part of their normal function. Moreover, the ability to take up TACA was enhanced in CHO cells expressing rat Oatp1, a carrier that also transport steroid hormones (36). These results suggest that TACA can also act as a substrate for this liver transporter. With respect to Nctp, direct measurements of TACA uptake have revealed efficient uptake by CHO cells transfected with the cDNA of rabbit Ntcp (37). These findings are partly consistent with our own results. Indeed, although TACA uptake was enhanced in CHO cells expressing rat Ntcp, this transport was not affected by removal of Na^+ from the incubation medium.

Taken the results from in vitro experiments on TACA transport together, it may be concluded that TACA in part shares the pathways enabling its entry into the hepatocyte with major BAs, although in the case of TACA this probably occurs with lower efficiency. This concept is further supported by the pharmacokinetic analyses carried out. Moreover, previously reported finding of a relatively high abundance of this BA in the urine of patients with hepatocellular carcinoma (5) together with the results obtained in the present work regarding enhanced excretion of this bile acid and its derivatives in urine after iv administration to rats are in agreement with a poor liver clearance as compared with that of its 5β-epimer, partially counterbalanced by enhanced elimination by the kidney.

Because most of the TACA was found in bile with no further biotransformation, this BA, rather than any potential derivative, must be the substrate for a canalicular carrier different from Bsep. The exact identity of the transporter responsible for canalicular TACA secretion has not been elucidated in the present work, but it can be suggested that it could well be the same one as that used by steroid hormones with a similar flat structure, such as estradiol-17β-D-glucuronide, whose transport across the bile canalicular membrane is predominantly mediated by cMOAT/MRP2 (38). Despite this, however, other isoforms of this family of proteins can also cooperate in certain cases to export these compounds from the liver cells (39).

Physiological role

In previous studies we have shown that unconjugated and conjugated forms of CA have a similar ability to be translocated into the hepatocyte nucleus, while other hydrophilic BAs, such as muricholic acids, are not (7, 9). Therefore, the existence of specific mechanisms controlling the size and composition of the nuclear sub-pool of BAs was suggested. Other steroids, such as glucocorticoids, are targeted to the nucleus via a soluble receptor, which binds them in the cytosol and shuttles them toward the nucleus. Because of the structural similarity between steroid hormones and *allo*-BAs, it could be suggested that ACA might use this kind of pathway for its translocation into the hepatocyte nucleus, an event that occurs with much greater efficacy than that of its 5β-epimer. The question then arises as to whether this interesting property of ACA might have any repercussions in liver physiology. Because this BA appears during liver growth, a role in the complex concert of signals controlling liver tissue morphogenesis could be postulated; however, although such a hypothesis is undoubtedly appealing, in our work we were unable to confirm such a notion with the data available. The ability of ACA to affect hepatocyte proliferation, differentiation, viability, and apoptosis was no higher than that of other major BAs that have protective (40) or deleterious (21, 41) effects and that are commonly found in the BA pool at much higher concentrations than those of ACA. Nevertheless, because for efficient liver growth and shaping to occur there needs to be an interplay among mitogens, co-mitogens, and differentiation signals, which were not investigated in the present work, and because the list of genes involved in liver tissue differentiation studied by us was not exhaustive, this hypothesis cannot yet be ruled out. Moreover, in addition to a potential effect of ACA on hepatocytes acting in an autocrine manner, the interesting possibility of a paracrine effect of ACA on liver cells other than hepatocytes was not addressed either in the present study.

In conclusion, TACA partly shares hepatocellular uptake system(s) for TCA. Furthermore, in contrast to other flat BAs, such as Δ^4-BAs and Δ^5-BAs, which are believed to be in part responsible for the cholestasis observed in some metabolic liver diseases (4), TACA is efficiently secreted into bile via transport system(s) other than Bsep and is highly choleretic, hence its appearance during certain situations may prevent accumulation of cholestatic precursors.

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