

Diplomarbeit

The influence of bile acid conjugation on hepatic and intestinal metabolism

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Declaration of Academic Honesty

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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List of Abbreviations

Abbreviation	Meaning
36b4	ribosomal protein, large, P0 (gene)
Acox1	acyl-coenzyme A oxidase 1 (gene)
AKRD1	aldo-keto reductase family1, member 1
Akt	protein kinase B
ALP	alkaline phosphatase
ALT	alanine aminotransferase
α -MCA	alpha-muricholic acid
ANOVA	analysis of variance
ASBT	apical sodium-dependent bile salt transporter
Asbt	apical sodium-dependent bile salt transporter (gene)
AST	aspartate aminotransferase
BA	bile acid
BAAT	BA-CoA amino acid N-acetyltransferase
BACS	BA-CoA synthase
BAT	brown adipose tissue
BSEP	bile-salt export pump
Bsep	bile-salt export pump (gene)
BSH	bile salt hydrolase
β -MCA	beta-muricholic acid
CA	cholic acid
CAR	constitutive androstane receptor
CDCA	chenodeoxycholic acid
COUP-TFII	COUP transcription factor 2
Cp	crossing point value
CYP	cytochrome P450 isoenzyme
Cyp	cytochrome P450 (gene)
CYP2B	cytochrome P450, family 2, subfamily B
CYP2B10	cytochrome P450, family 2, subfamily B, polypeptide 10
Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10 (gene)

CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6
CYP2b6	cytochrome P450, family 2 subfamily b, member 6 (gene)
CYP3A	cytochrome P450, family 3, subfamily A
CYP3A11	cytochrome P450, family 3, subfamily A, polypeptide 11
Cyp3a11	cytochrome P450, family 3, subfamily a, polypeptide 11 (gene)
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4
Cyp3a4	cytochrome P450, family 3 subfamily a, member 4 (gene)
Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14 (gene)
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1
Cyp7a1	cytochrome P450, family 7, subfamily a, polypeptide 1 (gene)
CYP8B1	cytochrome P450, family 8, subfamily B, polypeptide 1
Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1 (gene)
CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1
DNA	deoxyribonucleic acid
e.g.	exempli gratia
FGF15	fibroblast growth factor 15
Fgf15	fibroblast growth factor 15 (gene)
FGF19	fibroblast growth factor 19
Fgf19	fibroblast growth factor 19 (gene)
FGFR4	fibroblast growth factor receptor 4
Fig.	Figure
Figs.	Figures
FoxO-1	forkhead box O-1
FXR	farnesoid X nuclear receptor
G0s2	G0/G1 switch gene 2 (gene)
GR	glucocorticoid receptor
HNF	hepatocyte nuclear factor
HNF4- α	hepatocyte nuclear factor 4 alpha

HSD3B7	3 beta-hydroxysteroid dehydrogenase type 7
i.e.	id est
I-BABP	ileal bile acid-binding protein (gene)
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
Igfbp	insulin-like growth factor binding protein (gene)
IGFBP1	insulin-like growth factor binding protein 1
Igfbp1	insulin-like growth factor binding protein 1 (gene)
JNK	c-Jun N-terminal kinase
LCA	lithocholic acid
LXR	liver X receptor
LXR- α	liver X receptor alpha
MDCA	murideoxycholic acid
mRNA	messenger ribonucleic acid
MRP3	multidrug resistance-associated protein 3
Mrp3	multidrug resistance-associated protein 3 (gene)
MRP4	multidrug resistance-associated protein 4
Mrp4	multidrug resistance-associated protein 4 (gene)
NAFLD	non-alcoholic fatty liver disease
n.d.	not detectable
NR	nuclear receptor
NTCP	Na ⁺ -taurocholate cotransporting polypeptide
Ntcp	Na ⁺ -taurocholate cotransporting polypeptide (gene)
OATP	organic anion transporting polypeptide
Oatp	organic anion transporting polypeptide (gene)
OST- α	organic solute transporter subunit alpha
Ost- α	organic solute transporter subunit alpha (gene)
OST- β	organic solute transporter subunit beta
Ost- β	organic solute transporter subunit beta (gene)
P	p-value
Ppar	peroxisome proliferator-activated receptor
Ppar- α	peroxisome proliferator-activated receptor alpha (gene)
Ppar- β	peroxisome proliferator-activated receptor beta (gene)

Ppar-γ	peroxisome proliferator-activated receptor gamma (gene)
PXR	pregnane X receptor
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
Shp	small heterodimer partner (gene)
SXR	steroid and xenobiotic receptor
TBST	Tris-buffered saline with Tween20
TCA	taurocholic acid
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UDCA-LPE	Ursodeoxycholyl lysophosphatidylethanolamide
UGT	uridine diphosphate-glucuronosyltransferase
VDR	vitamin D (1,25-dihydroxyvitamin D3) receptor
VDR	vitamin D receptor
vs.	versus
WAT	white adipose tissue
wt	weight

Zusammenfassung

Hintergrund

Gallensäuren (GS) betreten das Duodenum beinahe ausschließlich in konjugierter Form. Im Tierexperiment und im klinischen Alltag werden jedoch vorrangig unkonjugierte GS oral verabreicht.

Ziele und Methoden

Diese Studie untersuchte den Einfluss von Taurin-Konjugation zweier GS auf biometrische Zielgrößen und auf die Genexpression sowie Proteinsynthese von Schlüsselenzymen im Gallensäure-, Lipid- und Kohlenhydratstoffwechsel. Dazu wurden Mäusen die unkonjugierten GS Cholsäure (CA) und Ursodeoxycholsäure (UDCA) sowie deren Taurin-Konjugate Taurocholsäure (TCA) und Tauroursodeoxycholsäure (TUDCA) über 7 Stunden, 4 Tage und 3 Wochen gefüttert. Körper- und Gewesgewicht wurden gemessen und Leberfunktionsparameter im Serum bestimmt. Die Gallensäuresynthese, -hydroxylierung, und -exkretion wurde durch RT-PCR-Messungen von Cytochrom P-450 (Cyp)7a1, Cyp8b1, Cyp3a11, Cyp2b10 und Ost- β in Lebergewebe abgeschätzt. Die Expressionen eines Schlüsselregulators im hepatischen Lipidstoffwechsels (Ppar- α) und dessen Zielgene (Acox1, G0s2, Cyp4a14) wurden ebenfalls in Lebergewebe gemessen. Zudem wurde Igfbp1-mRNA in der Leber quantifiziert und der Igfbp1-Proteinspiegel im Serum durch Western Blot Analyse ermittelt. Ferner haben wir im Ileum die Expression von Fgf15, Shp, I-BABP und Ost- β untersucht.

Ergebnisse

Taurin-Konjugation beider GS verringerte das Lebergewicht nach 3 Wochen. TUDCA erhöhte die Körpergewichtszunahme im Vergleich zu UDCA nach 3 Wochen. Taurin-Konjugate zeigten einen Trend zu verminderter Repression der Gallensäuresyntheseenzyme Cyp7a1 und Cyp8b1. TUDCA reprimierte Ost- β in der Leber stärker als UDCA. Die Expressionslevel der Phase I Entgiftungsenzyme Cyp3a11 und Cyp2b10 wurden durch Taurin-Konjugation tendenziell vermindert. Ppar- α und Acox1 wurden durch TUDCA induziert, nicht hingegen durch UDCA. Dies deutet auf eine konjugationsabhängige Modulierung der Ppar- α .

Signalübertragung durch GS hin. Igfbp1-mRNA-Spiegel und -Proteinspiegel wurden durch TCA drastisch erhöht durch CA dagegen kaum beeinflusst. Taurin-Konjugation wirkte sich nur geringfügig auf Expressionslevel von Fgf15, Shp, I-BABP und Ost- β im Ileum aus.

Konklusion

Die berichteten konjugationabhängigen Effekte auf Schlüsselenzyme des Gallensäure-, Lipid- und Kohlenhydratstoffwechsels könnten die physiologische Bedeutung bisheriger Experimente mit unkonjugierten GS einschränken.

Abstract

Background

Bile acids (BAs) are formed in the liver, stored in the gallbladder and secreted into the intestine. Under physiologic conditions, almost exclusively conjugated BAs are secreted into the intestine. However, unconjugated BAs are used widely in BA-feeding experiments and solely in clinical practice.

Aims and Methods

This study aimed to investigate the impact of taurine-conjugation on hepatic and intestinal metabolism. In an animal experiment mice were fed the unconjugated BAs cholic acid (CA) and ursodeoxycholic acid (UDCA) or the corresponding taurine-conjugated BAs taurocholic acid (TCA) and tauroursodeoxycholic acid (TUDCA) for 7 hours, 4 days and 3 weeks. Biometric measurements and liver functions tests were performed. Hepatic BA synthesis, hydroxylation and excretion were assessed by Q-PCR for cytochrome P-450 (Cyp)7a1, Cyp8b1, Cyp3a11, Cyp2b10 and organic solute transporter (Ost)- β . Expression levels of peroxisome proliferator-activated receptor alpha (Ppar- α) and its target genes (Acox1, G0s2 and Cyp4a14) were quantified in the liver. Further, we determined insulin-like growth factor binding protein 1 (Igfbp1) mRNA levels in the liver and protein levels in serum by Western blot analysis. Ileal expression of fibroblast growth factor 15 (Fgf15), small heterodimer partner (Shp), ileal bile acid-binding protein (I-BABP) and Ost- β was also measured.

Results

Taurine-conjugation of both CA and UDCA reduced liver weight after 3 weeks. TUDCA increased body weight gain compared to UDCA after 3 weeks. Conjugated BAs tended to repress Cyp7a1 and Cyp8b1 less without reaching statistical significance in most groups. TUDCA repressed Ost- β in liver tissue stronger than UDCA. Cyp3a11 and Cyp2b10 expression tended to be lower in conjugated groups. Ppar- α and Acox1 were induced by TUDCA but not by UDCA after 4 days and 3 weeks, suggesting a conjugation-dependent modulation of Ppar- α signaling by BAs. Igfbp1 mRNA levels in the liver and protein levels in

serum were massively elevated by TCA but hardly affected by CA. Taurine-conjugation had only minor effects on ileal expression of Fgf15, Shp, I-BABP and Ost- β .

Conclusion

The observed conjugation-dependent effects on the expression of major regulatory enzymes of BA, lipid and glucose metabolism may question the transferability of experiments using unconjugated BAs to a physiologic condition.

1. Introduction

Conjugation comprises a group of reactions in the liver that result in enhanced solubility of excretion products to facilitate biliary or urinary excretion (1). In contrast to all other conjugated compounds which are secreted into bile, conjugated bile acids (BAs) are actively reabsorbed in the terminal ileum and taken up by hepatocytes from portal venous blood (2). The resulting circular flow of BAs, termed enterohepatic circulation, is elaborately regulated, serves various physiological functions and leads to a number of diseases, if interrupted in the intestine, the liver or the biliary tree (3,4,5).

Since 1999, endocrine functions of BAs as endogenous ligands for nuclear receptors (NRs) have been increasingly elucidated, consolidating their position as regulators of lipid, glucose and energy homeostasis (3,6). Similar to glucocorticoids BAs bind intracellular receptors, influence gene expression and thereby mediate metabolic functions (3). Above all, the revelation of molecular interactions enhanced our understanding of how BAs regulate their own synthesis and how tissues adapt to high concentrations of potentially toxic BAs.

For almost two decades the effect of BAs on the transcription of major regulatory genes in BA, lipid and glucose homeostasis has been extensively studied *in vivo* and *in vitro*. However, in BA feeding studies, important models for investigating BAs in health and disease, BAs are mostly applied in their – physiologically inappropriate – unconjugated form. Hence, there is limited knowledge about possibly different effects of applying conjugated BAs. Furthermore, the validity of BA feeding models upon physiologic conditions might be limited due to the application of unconjugated BAs.

The animal studies performed in the context of this diploma thesis therefore aimed to investigate the effect of taurine-conjugation on hepatic and intestinal mRNA and protein levels by feeding two physiologically and clinically relevant BAs in conjugated and unconjugated form. Basic concepts of BA physiology and the metabolic role of NRs are summarized in *Chapter 2*. The aims of the study are

further specified in *Chapter 3* and applied methods are delineated in *Chapter 4*. Finally, obtained results are presented in *Chapter 5* and discussed in *Chapter 6*.

2. Background

2.1. Structure of bile acids

BAs are amphipathic end products of cholesterol metabolism with a vast variety of chemical structures occurring in nature (7). In modern mammals predominantly C_{24} BAs are found, under which cholic acid (CA), chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) are the most abundant in humans and many other species (3,7). Figure 1 shows the structure of mammalian BAs that consist of a C_{20} sterol nucleus with a variable number and orientation of hydroxyl groups and a C_4 side chain containing a carboxyl group (7). Among the four rings of the sterol nucleus, A and B rings are in cis-configuration, leading to a convex hydrophobic and a concave hydrophilic side of the molecule (3,7). Alpha-hydroxyl groups are orientated towards the hydrophilic side, β -hydroxyl groups toward the hydrophobic side (3).

The biological activity (e.g. micelle formation, cell signaling, lithogenicity and toxicity) of BAs is tightly linked to the number and orientation of hydroxyl groups and their conjugation (3). CA, a $3\alpha,7\alpha,12\alpha$ -trihydroxy BA with intermediate cytotoxicity, is one of two primary BAs synthesized from cholesterol in the human liver, the other being CDCA ($3\alpha,7\alpha$) (2,4,7). Ursodeoxycholic acid (UDCA), a $3\alpha,7\beta$ -dihydroxy BA, is a major BA in bears, nutrias and beavers but less common in humans, where it is formed out of CDCA by bacterial enzymes in the colon or out of 7-oxo precursors in the liver (2,7). By epimerization of the 7α - into a 7β -hydroxyl-group polarity is shifted to the hydrophobic side of the molecule and hydrophilicity is increased (3). In effect, UDCA lacks cytotoxicity in most model systems (4). UDCA is used as safe and effective medication in cholestatic liver disease (4).

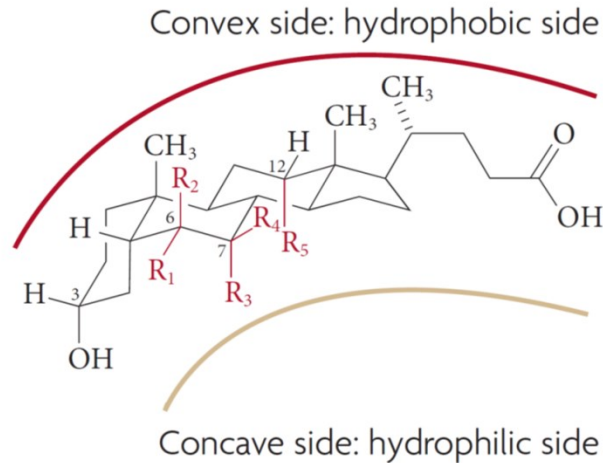


Fig. 1. Structure of bile acids. From Thomas C. et al. (3).

2.2. Bile acid formation

BA synthesis occurs exclusively in hepatocytes via two different multienzyme pathways: the classical (or neutral) pathway and the alternative (or acidic) pathway (3,8). As shown in Figure 2, both pathways result in the formation of CA and CDCA as their main end products (3). At least 75% of the total BA pool can be attributed to the classical pathway, which produces CA and CDCA in roughly equal amounts (3,8). Less than 18% of BAs in humans are synthesized via the alternative pathway that delivers predominantly CDCA (8). Humans synthesize only the two primary BAs CA and CDCA, whereas mice additionally synthesize α -muricholic acid (α -MCA) and β -muricholic acid (β -MCA) (9).

Cholesterol conversion via the classical pathway includes four steps: initiation (i.e. 7α -hydroxylation), sterol ring modification, oxidation and shortening of the side chain, and conjugation (3). 7α -hydroxylation, the first and only rate-limiting step of the pathway, is conducted by the microsomal cytochrome P450 (CYP) isoenzyme CYP7A1 (8). Subsequently, 3 beta-hydroxysteroid dehydrogenase type 7 (HSD3B7) converts 7α -hydroxycholesterol to 4-cholesten- 7α -3-one, the common precursor of CA and CDCA (3,8). Afterwards, a 12α -hydroxylation, catalyzed by CYP8B1, may or may not occur determining whether CA or CDCA is formed in further consequence (3). In regard to BA-composition, this is a crucial step

affecting the ratio of CA to CDCA and subsequent secondary BAs (2,10). Further sterol ring modifications, catalyzed by aldo-keto reductase family1, member 1 (AKRD1) precede side chain oxidation, catalyzed by mitochondrial CYP27A1, and side chain shortening (3,8,10). In a two-step process, the resulting primary BAs CA and CDCA are then conjugated (i.e. amidated) with amino acids (3,7). BA-CoA synthase (BACS) forms BA-CoA before amidation with glycine or taurine by BA-CoA amino acid N-acetyltransferase (BAAT, also BAT) (3).

The alternative pathway is initiated by oxidation and shortening of the side chain, catalyzed by CYP27A1 (8). Thus, sterol ring modification occurs after side chain shortening in this pathway (8). In summary, CYP7A1 determines the size and CYP8B1 the composition of the BA pool (3).

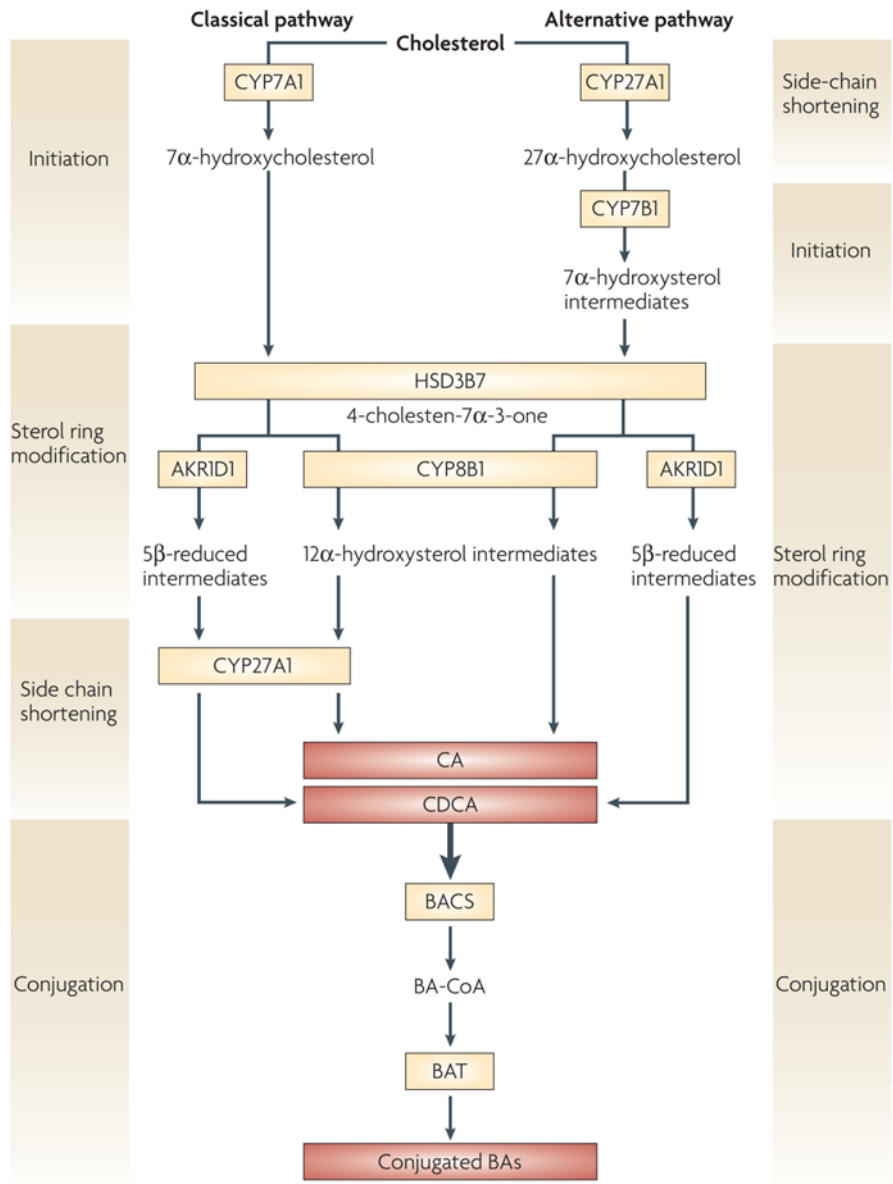


Fig. 2. Bile acid synthesis pathways. From Thomas C et al. (3).

2.3. Conjugation and enterohepatic circulation

BAs are formed in the liver, stored in the gallbladder, secreted into the duodenum upon meal ingestion, actively reabsorbed in the terminal ileum and transported back to the liver through the portal venous system (2,8). The resulting circular flow of BAs is termed enterohepatic circulation (5).

Primary BAs are synthesized from cholesterol in the liver, secondary BAs are formed out of primary BAs by bacteria in the intestine and tertiary BAs result from further modification of secondary BAs after their return to the liver (3,4). The most abundant BAs in the respective species are the human primary BAs CA and CDCA and the murine primary BAs CA and β -MCA (2,9,11).

Before excretion from the hepatocyte, CA, CDCA, α -MCA and β -MCA are N-acyl amidated (i.e. conjugated) with glycine or taurine (16,33). Humans conjugate BAs predominantly to glycine, rodents to taurine (12). Other modifications at various locations of the BA molecule that can occur under certain circumstances like cholestasis are sulfation, glucuronidation and conjugation with glutathione, N-acetylglucosamine or glucose (16). Glycine- and taurine-conjugation increase the acidic strength of the side chain, thereby enhances ionization at the pH present in the small intestine and therefore prevent passive diffusion across cell membranes (16,33). In effect, high intraluminal concentrations of conjugated BAs are achieved warranting fat digestion and absorption (16,33).

Because glycine- and taurine-conjugated BAs are mainly ionized under physiological pH and therefore impermeable to cell membranes and because canalicular BA concentrations exceed hepatocellular concentrations over 1000-fold, excretion into the bile canaliculi requires the canalicular bile-salt export pump (BSEP) (3,7). Conjugated BAs are subsequently stored in the gallbladder (2). Unconjugated BAs are more toxic and less capable of facilitating lipid absorption and are found only in trace quantities in gallbladder bile (2,6). When the gallbladder contracts after a meal conjugated BAs are released into the small intestine where they enhance dietary lipid absorption before being extensively recycled (2,5,8). Only a small fraction of conjugated BAs is taken up via passive diffusion throughout the intestine, the majority is actively absorbed in the terminal ileum (2,13,14). Unconjugated BAs are much more permeable to the intestinal wall than glycine conjugates which, in turn, are more permeable than taurine conjugates (13). Not surprisingly, unconjugated BAs have been shown to be absorbed more efficiently than their taurine conjugates in jejunum, ileum and colon (14).

While BAs travel through the distal part of the ileum the bacterial flora begins to increase and biotransformation by bacterial enzymes may occur (2). The first major biotransformation and a precondition for further modification is deconjugation and occurs in both ileum and large intestine (2,15). Deconjugation is catalyzed by intracellular enzymes termed bile salt hydrolases (BSHs) and yields unconjugated BAs and glycine or taurine (2,15). BSHs are found in a large number of mainly gram-positive bacteria including *Enterococcus*, *Clostridium* and *Bacteroides* spp. (15). Deconjugation of taurine conjugates is slower than of glycine conjugates (16). The second major biotransformation is dehydrogenation of a 3 α - or 7 α -hydroxyl to a 3- or 7-oxo group resulting in the formation of “oxo bile acids”(2,7,17). Dehydrogenation is also performed by a wide range of intestinal bacteria (17). A 3-oxo group can consequently be reduced by further bacterial enzymes (isomerases) to a 3 β -hydroxyl group which leads to the formation of “iso bile acids” (2). Both oxo and iso BAs may undergo “repair” during hepatic passage, i.e. reduction and reepimerization, respectively (2). The third and final major biotransformation carried out by a restricted number of anaerobes in the large intestine is 7-dehydroxylation (2,17). 7-dehydroxylation transforms CA (3 α ,7 α ,12 α) into DCA (3 α ,12 α) and CDCA (3 α ,7 α) into lithocholic acid (LCA) (3 α); in rodents 7-dehydroxylation additionally transforms α -MCA (3 α ,6 β ,7 α) and β -MCA (3 α ,6 β ,7 β) into murideoxycholic acid (MDCA) (3 α ,6 β) (2,7,18).

Within one cycle of enterohepatic circulation, about 95% of BAs are reabsorbed and 5% egested in feces (8). Reabsorbed BAs regulate the replacement of the lost BAs (i.e. de novo synthesis) via several pathways that affect Cyp7a1 expression (3,7).

When BAs are taken up by the hepatocyte from portal venous blood they mediate a feed-back regulation of their synthesis involving several transcription factors and NRs (i.e. ligand-regulated transcription factors) that regulate Cyp7a1 at a transcriptional level (3,8,10,19). The BA-activated NR farnesoid X nuclear receptor (FXR) is highly expressed in liver and intestine and plays a major role in the regulation of the enterohepatic recycling and synthesis of BAs (3,10). BAs bind FXR and thus induce the transcription of small heterodimer partner (Shp) and other FXR target genes (19). Shp, in turn, represses Cyp7a1 transcription in

hepatocytes by interfering with the activity of several NRs that under basal conditions activate Cyp7a1 transcription (3,19).

Feed-back inhibition of BA synthesis also happens via another FXR-mediated but partially Shp-independent pathway initiated the intestine (3). BAs that enter the villus epithelium of the ileum activate FXR and thereby induce the expression of fibroblast growth factor (Fgf)15 (in mice) and Fgf19 (in humans) (19). Secreted FGF15/19 interacts with hepatic fibroblast growth factor receptor 4 (FGFR4) leading to a c-Jun N-terminal kinase (JNK)-dependent repression of Cyp7a1 (19,20,21).

2.4. The significance of bile acid conjugation

Under physiologic conditions, unconjugated BAs are present only in trace amounts in gallbladder bile when secreted into the duodenum (2). The biological relevance of conjugation lies in augmentation of the acidic strength which enhances ionization and thus inhibits passive absorption of BAs in the biliary tract and the small intestine (16,33). Consequently, pharmacokinetics of orally administered unconjugated BAs profoundly differs from their taurine- or glycine-conjugates (2). Although feeding of unconjugated BAs is widely used in animal studies for the investigation BA physiology, the extent to which collected data are conferrable to conjugated BAs is ill defined. This study aimed to elucidate differential effects of administering taurine-conjugates and their corresponding unconjugated BAs.

Therapeutic use of UDCA in the treatment of primary biliary cholangitis is nearly completely limited to its unconjugated form (3,4). There is only one recent study (22) which compares UDCA to its conjugated counterpart in these patients demonstrating similar efficacy of both BAs. The potential role of TUDCA in the treatment of cholestatic liver diseases necessitates elucidation of its potentially deviating mechanism of action.

2.5. Bile acids and nuclear receptors

Ligand-activated transcription factors are termed nuclear receptors (NRs) and regulate gene expression (10). There are three NRs that are activated by BAs: the farnesoid X receptor (FXR), the pregnane X receptor (PXR) or its human ortholog the steroid and xenobiotic receptor (SXR) and the vitamin D receptor (VDR) (10). FXR plays a major role in BA homeostasis, PXR and SXR regulate lithocholic acid and drug metabolism and VDR regulates calcium and phosphate metabolism (3,10). Further, BA metabolites activate the oxysterol receptor liver X receptor (LXR)- α playing a prominent role in lipid metabolism (10). Together with peroxisome proliferator-activated receptors (Ppar- α,γ,δ) and hepatocyte nuclear factor 4 α (HNF4- α) these NRs coordinately regulate energy metabolism, BA metabolism, lipoprotein metabolism and triglyceride metabolism and therefore have been referred to as the “metabolic receptors” (10).

2.6. Ppar- α governs hepatic lipid metabolism

Ppars are a group of NRs involved in the regulation of lipid metabolism, adipocyte differentiation, cell proliferation and inflammation (23). They were identified to mediate the transcriptional effects of synthetic compounds called peroxisome proliferators which gave them their name (24). Soon it became obvious that eicosanoids and certain other fatty acids are endogenous ligands for Ppars with different degrees of affinity (23,24). There are three Ppar isotypes: α , β (also called δ) and γ (24). Ppar- α is mainly expressed in liver and brown adipose tissue, Ppar- β in gut, kidney and heart and Ppar- γ in adipose tissue and colon (24). Ppars control various genes in several pathways of lipid metabolism, such as fatty acid transport, cellular uptake and intracellular activation, catabolism and storage (23).

In the liver, Ppar- α serves as master regulator of lipid metabolism during fasting and mediates anti-inflammatory processes (25). In the fasting state, fatty acids are transported from adipose tissue to the liver where they induce Ppar- α (26). Activation of hepatic Ppar- α facilitates hepatocellular fatty acid uptake, fatty acid activation and peroxisomal and mitochondrial fatty acid-oxidation at the

transcriptional level (23). Fatty acid oxidation in the liver generates ketone bodies serving as alternative energy source for peripheral tissues (26). In one study (27) Ppar- α has also been shown to repress Cyp7a1 transcription *in vitro*. Expression of Ppar- α is upregulated by HNF-4 and Ppar- α itself and downregulated by the transcription factor COUP transcription factor 2 (COUP-TFII) (28).

2.7. FXR and bile acid homeostasis

There are two members of the FXR subfamily of NRs: FXR- α and FXR- β (3). FXR- α occurs in mice and humans whereas the murine FXR- β is a pseudogene in humans (3). As a result of alternative promotor usage and alternative splicing of a single gene, there are four isoforms of FXR- α in mice and humans: FXR- α 1, FXR- α 2, FXR- α 3 and FXR- α 4 (3). These isoforms vary in their distribution to liver, intestine, kidney and adrenal cortex and their properties of activating gene expression *in vitro* (3). All members of the FXR subfamily are further collectively referred to as FXR.

More than 80 endogenous and synthetic compounds have been identified as potential FXR ligands, acting as agonists or antagonists with varying degrees of affinity (29). Important endogenous ligands include BAs, steroids and fatty acids (29). CDCA, DCA, LCA and CA have been shown to activate FXR *in vivo* and *in vitro* in decreasing order of potency: CDCA > DCA > LCA > CA (29). The role of UDCA as direct FXR agonist is doubtful. Lew et al. (30) showed that UDCA regulates the expression of some FXR target genes in an FXR transactivation assay and Zollner et al. (31) demonstrated attenuation of effects on FXR targets in FXR knock out mice, however *in vitro* effects are weak and UDCA has not been proven to bind the receptor *in vitro* or *in vivo*.

The physiological function of FXR is to protect against the accumulation of potentially toxic BAs which appears to be most crucial in the liver (3,6). In response to high transhepatic BA flux, activation of FXR- α downregulates BA uptake systems and synthesis while it increases BA conjugation and efflux (3,6). BAs enter the hepatocyte via the Na⁺-taurocholate cotransporting polypeptide

(NTCP) or via organic anion transporting polypeptides (OATPs) in the basolateral membrane (29). Conjugated BAs are taken up by NTCP, whereas unconjugated BAs are taken up by OATPs (29). The expression of both uptake systems is negatively regulated in a FXR-mediated fashion (6,29). The rate limiting enzyme in the classical BA synthesis pathway, CYP7A1, and the key regulatory enzyme for BA composition, CYP8B1, are regulated by two FXR-mediated pathways mentioned above (32). The intestinal FXR/Fgf15 pathway is crucial for the suppression of both Cyp7a1 and Cyp8b1, whereas the hepatic FXR/Shp pathway is important for suppressing Cyp8b1 and shows only a minor role in the suppression of Cyp7a1 (32). The alternative pathway of BA synthesis, initiated by CYP27A1, is mainly regulated by Cholesterol (33). Yet Cyp27a1 can also be suppressed by BAs, presumably via the FXR/Shp pathway (29). The two enzymes involved in glycine and taurine conjugation of BAs, BACS and BAAT, are also upregulated by FXR activation (29). Canalicular efflux of glycine and taurine conjugated BAs happens against a tremendous concentration gradient, necessitating active transport via BSEP (34). FXR also induces the expression of Bsep (3).

Like in hepatocytes FXR also counteracts exceeding BA levels in the ileal enterocyte by reduction of BA uptake, reduction of BA synthesis - via crosstalk with the liver - and enhancement of BA secretion (3). The apical sodium-dependent bile salt transporter (ASBT), a main BA transporter for glycine- and taurine-conjugated BAs, is negatively regulated by FXR in a Shp-dependent fashion (3,29). The ileal bile acid-binding protein (I-BABP) which shuttles BAs in the ileocyte from apical to basolateral and the basolateral organic solute transporter (OST)- α /OST- β that mediates the excretion of glycine- and taurine-conjugated BAs are both induced by FXR (29). As mentioned above, intestinal FXR is an inducer of Fgf15/19 that in turn represses BA synthesis (29).

2.8. Detoxification and alternative bile acid transport

CYPs not only mediate BA synthesis and cholesterol homeostasis but are primarily involved in the conversion of various exogenous and endogenous

compounds into more polar derivatives to enhance their elimination (6). Substrates to CYPs include environmental pollutants, carcinogens, prescription drugs and potentially toxic BAs (6,31). CYP3A family members like the human CYP3A4 or the murine CYP3A11 are expressed in liver and intestine and catalyze the hydroxylation of LCA, DCA and CDCA, which can be necessary to avoid liver damage (31,29,35). Enzymes of the CYP3A family metabolize more than half of all prescription drugs and thus are of high pharmaceutical significance (6). The CYP2B family, comprising the human CYP2B6 and the murine CYP2B10, also catalyzes the hydroxylation of foreign chemicals, steroids and potentially of BAs (31,36). CYP3A enzymes are regulated by a variety of NRs including PXR, CAR, FXR, VDR, LXR and HNF4- α (35,37,38). Expression of Cyp2b is regulated by PXR, CAR and by the glucocorticoid receptor (GR) (36). PXR and CAR are very promiscuous in their ligand specificity and are activated by a wide range of foreign chemicals (6). PXR, as one of the three BA-activated NRs, also binds LCA, DCA and CDCA (6,35,39).

Under pathologic conditions such as cholestasis, a shift in canalicular and basolateral transporter expression inverts the polarity of transhepatic BA transport and leads to basolateral BA excretion (34). Canalicular transport systems are then downregulated whereas basolateral multidrug resistance-associated protein (Mrp)3 and Mrp4 are induced (34,40,41,42). Further transporters in the basolateral bile salt export system are OATPs and OST- α /OST- β (3,31,34). Alternatively to canalicular efflux, glycine- and taurine-conjugated BAs are excreted by MRP4 and OST- α /OST- β . Sulphated- and glucuronidated BAs are secreted by MRP3 and OATP2 (3). BAs induce Ost- α /Ost- β in an FXR-dependent fashion (31).

2.9. The IGF/IGFBP system

Insulin-like growth factors (IGFs) are ubiquitously expressed anabolic peptides that influence cell growth and metabolism (43,44). Locally synthesized IGFs exert autocrine and paracrine effects, circulating IGFs exert systemic endocrine effects (44). During the circulation in blood, IGFs are bound to one of at least 6 insulin-like

growth factor binding proteins (IGFBP1-6) (45). In addition to carrying IGFs and thereby prolonging their half-life, IGFBPs also modulate IGF availability and activity and exert other IGF-independent functions (44).

IGFBP1 is mainly produced in liver, endometrium and kidney from where it is secreted into blood (46,47). Secretion from the liver is the main source of systemic IGFBP1 (47). IGFBP1 consists of 3 different domains: a conserved N- and C-terminal domain that bind IGF-1 and IGF-2 and a variable midsection (44,46). There is a strong inverse correlation between the circulating IGFBP1 level and the biologically active free IGF-1 level (47,48). Apart from determining IGF activity, IGFBP1 also increases glucose uptake in peripheral tissues, decreases hepatic glucose output and influences lipid metabolism (46). The main physiological function of IGFBP1 is to prevent from exceeding availability of IGFs that causes hypoglycemia (49). Further, IGFBP1 is a biomarker for metabolic and neoplastic diseases. (49,50,51). IGFBP1 constitutes the only IGFBP with tight regulation on a transcriptional level (47). It is a primary target gene of Ppar- α , - γ and - δ (49). In addition, *Igfbp1* expression is positively regulated via the glucocorticoid receptor (GR), HNFs, VDR and possibly via PXR (43,46,52,53,54,55). Downregulation of *Igfbp1* expression is mediated by insulin through protein kinase B (Akt) that abolishes forkhead box O-1 (FoxO-1) transcription factor from the *Igfbp1* promoter (47,56). Therefore *Igfbp1* mRNA levels in the liver dramatically rise during fasting and rapidly decline after feeding (43,46).

3. Aims

The experiment aimed to investigate the impact of taurine-conjugation on body- and tissue-weights as well as on the transcriptional regulation of BA, lipid and glucose metabolism. In particular BA synthesis, signaling, detoxification and transport were studied. Expression levels of key regulatory genes in BA, lipid and glucose homeostasis were quantified. Additional aims comprised serum levels of regulatory proteins in glucose metabolism and parameters of liver function.

To do this, bodyweight, liver weight, white adipose tissue (WAT) weight, brown adipose tissue (BAT) weight and 24-hour stool weight were measured. Serum was analyzed for biochemical parameters of liver function: ALT, AST, ALP, total serum BAs and total bilirubin. Hepatic mRNA expression levels of genes involved in BA synthesis (Cyp7a1 and Cyp8b1), signaling (Shp), hydroxylation (Cyp2b10 and Cyp3a11) and excretion (Ost- β) were quantified. Moreover, we studied ileal mRNA levels of genes affecting BA synthesis (Fgf15), signaling (Shp), transport (I-BABP) and excretion (Ost- β). Further measurements comprised mRNA levels of the key regulatory NR of hepatic lipid metabolism (Ppar- α) and classical Ppar- α target genes (Acox1, G0s2, Cyp4a14). We also determined hepatic transcription and secretion of a regulatory protein in hepatic and peripheral glucose metabolism (Igfbp1).

4. Materials and Methods

4.1. Animal experiments

Male 8 to 10-week-old C57BL/6J mice, bred at our own animal facility, were used for all experiments, with the exception of the 3-week-CA/TCA group which consisted of male C57BL/6N mice (purchased from Janvier Labs, Le Genest St. Isle, France). Mice were fed a diet supplemented with CA, TCA, UDCA and TUDCA for 4 days and 3 weeks as well as with CA and TCA for 7 hours before harvesting. Intervention groups were fed a BA-supplemented diet at 0.5% (wt/wt) of the respective compound, obtained by thoroughly mixing CA, TCA, UDCA and TUDCA with standard mouse chow. Control groups were fed standard mouse chow (Ssniff M-Z). To force postprandial regulation, mice fed for 7 hours were previously fasted overnight. BA-fed animals and their controls were housed separately in groups of 3-6, to which they were randomly distributed. All mice were held with a 12-hour light-dark-cycle under an ambient temperature of 24°C and were allowed unrestricted access to food and water. Fecal excretion was determined by stool collection, weighing, and stool pellet counting over 24 hours before sacrificing. In some groups body weight was monitored. At the mentioned time points mice were sacrificed towards the beginning of the 12-hour light phase and blood was collected from cervical arteries. Liver, ileum, BAT and WAT were harvested, weighed, snap-frozen in liquid nitrogen and stored at -80°C. All experiments were approved by the local animal care and use committees in accordance with the National Academy of Sciences.

4.2. Selection of feeding durations

During BA feeding input into the BA pool has two sources: uptake of ingested BAs and endogenous synthesis from cholesterol (2). BA loss happens predominately via fecal excretion (2). When the intestinal BA absorption increases at the beginning of BA feeding adaptation processes occur in liver and ileum in order to regain BA homeostasis (3). In a steady state, BA input via ingestion and synthesis equals BA loss (2).

In order to investigate the effects of different durations of BA feeding, mice were fed for 7 hours, 4 days and 3 weeks. 7-hour feeding was performed to determine the initial effect of the applied BAs with a minimum of BA modification and metabolite formation by bacterial and hepatic enzymes. Mice were harvested after 4 days in order to gain insights into hepatic and intestinal adaptation to BA stress. Long-term feeding of 3 weeks aimed to achieve a steady state of circulating BAs and their metabolites.

4.3. Serum biochemical analysis

Serum samples were analyzed for ALT, AST, ALP, total serum BAs and total bilirubin as described previously (57).

4.4. Determination of hepatic and ileal messenger RNA levels

RNA isolation, reverse transcription into complementary DNA and quantitative polymerase chain reaction were performed as previously described (58) with the exceptions that 2 µg of total RNA were reverse transcribed at 42°C for 90 minutes and that 36b4 was used as housekeeping gene.

4.5. Analysis of Igfbp1 by Western blotting

Whole blood was centrifuged at 4600 rpm for 10 minutes to obtain serum. Equal amounts of serum (4 µl) were loaded onto a 10% dodecyl sulfate-polyacrylamide gel for electrophoresis. Serum samples from control diet-, CA- and TCA-fed animals ran together on the same gel as did samples from control-diet-, UDCA- and TUDCA-fed animals. After transfer onto a nitrocellulose membrane, Ponceau S staining (not shown) confirmed similar amounts of protein. Membranes were washed with 0,1% TBST and blocked with 5% skimmed milk for 1 hour at room temperature before overnight incubation at 4°C with a polyclonal goat antibody against IGFBP1 (Santa Cruz, sc-6000) diluted 1:2000. Subsequently, the blots were incubated with the secondary antibody for 1 hour at room temperature. As described previously, binding was detected by using the enhanced chemiluminescence method western blotting detection system (57).

4.6. Statistical Analysis

Data are presented as arithmetic means \pm standard deviation of 3-6 animals per group, using Microsoft Excel 2010. Differences between groups were tested applying one-way ANOVA followed by Tukey's post-hoc test, using SPSS statistics (SPSS Inc., Chicago, IL). Level of significance was set at $P < 0.05$.

5. Results

5.1. Effect on body and tissue weights

Body weight progressively increased over 3 weeks under all diets except UDCA (Fig. 3). Absolute and relative body weight increased most in control mice (2.2 g, 8%), less in TUDCA fed mice (0.7 g, 3%) and decreased in UDCA fed mice (-0.8 g, -3%) ($P < 0.05$; Table 1). CA and TCA supplementation showed only minor effects on body weight change.

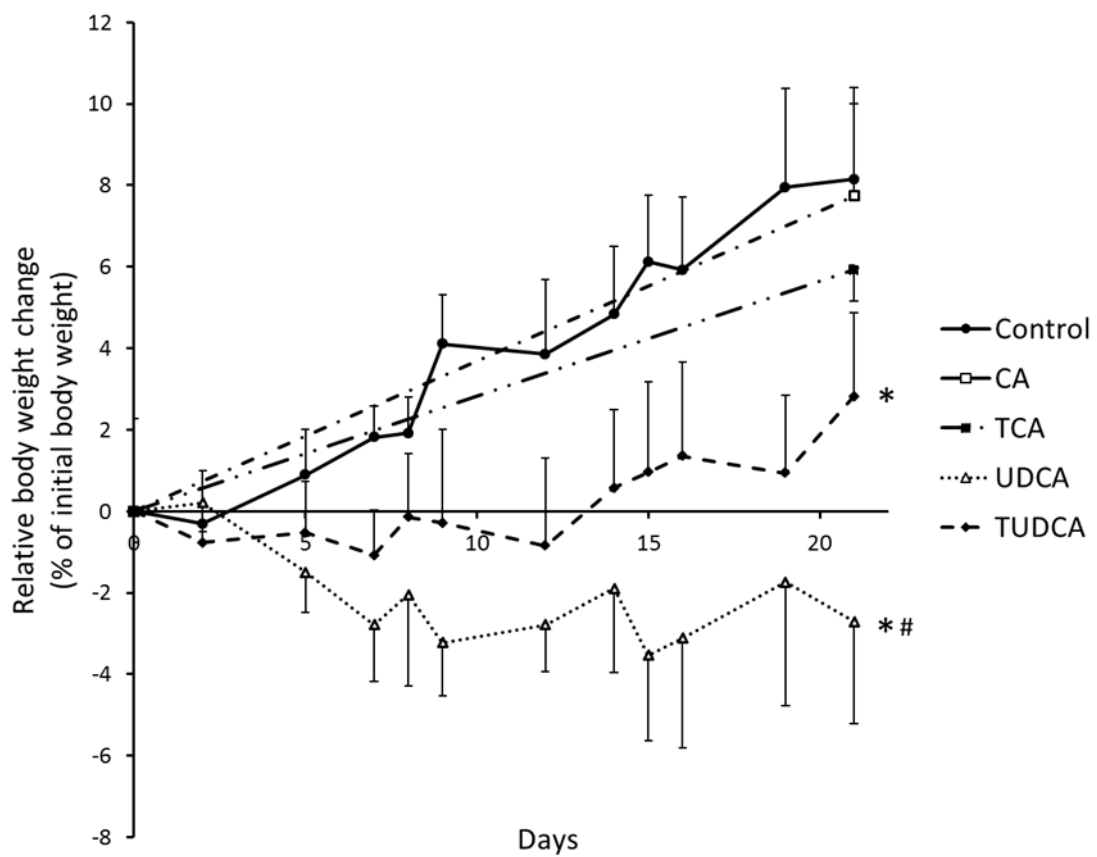


Fig. 3. Relative body weight change (percent of initial body weight) of mice fed control diet, a diet supplemented with unconjugated bile acids (0.5% wt/wt) or a diet supplemented with taurine-conjugated bile acids (0.5% wt/wt) for 3 weeks. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 4-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid.

Taurine-conjugation of both BAs reduced the liver weight (Table 1). After three weeks liver weight and liver to body weight ratio were markedly increased by CA, less increased by TCA, unaffected by UDCA and reduced by TUDCA. CA and TCA coordinately reduced WAT weight, whereas UDCA significantly elevated WAT weight compared to TUDCA. A parallel trend was observed in brown adipose tissue (BAT) weights. Overall, BA feeding had no effect on stool weight and stool pellet count.

	Control	CA	TCA	Control	UDCA	TUDCA
Body weight change (g)	2.2 ± 0.6	1.8 ± 0.5	1.4 ± 0.2	2.0 ± 0.5	-0.8 ± 0.8 *	0.7 ± 0.5 *#
Liver weight (g)	1.16 ± 0.06	1.67 ± 0.08 *	1.30 ± 0.08 #	1.22 ± 0.06	1.22 ± 0.09	1.01 ± 0.12 *#
Liver to body weight ratio (%)	4.0 ± 0.4	6.6 ± 0.2 *	5.2 ± 0.3 *#	4.5 ± 0.3	4.5 ± 0.1	3.8 ± 0.3 *#
WAT weight (mg)	375 ± 35	230 ± 22 *	233 ± 16 *	293 ± 68	438 ± 168	242 ± 46 #
WAT to body weight ratio (%)	1.4 ± 0.1	0.9 ± 0.1 *	0.9 ± 0.1 *	1.1 ± 0.2	1.6 ± 0.5	0.9 ± 0.2 #
BAT weight (mg)	73 ± 3	61 ± 12	47 ± 8 *	53 ± 7	59 ± 10	46 ± 5 #
BAT to body weight ratio (‰)	2.7 ± 0.1	2.4 ± 0.5	1.9 ± 0.3	1.9 ± 0.3	2.2 ± 4.5	1.7 ± 0.3
24-hour stool weight (g)	0.91	1.04	1.24	1.02 ± 0.06	1.15 ± 0.30	1.21 ± 0.13
24-hour stool to body weight ratio (%)	3.3	4.1	5.0	3.8 ± 0.3	4.1 ± 0.9	4.6 ± 0.5
24-hour stool pellet count	94	106	118	120 ± 7	89 ± 22	114 ± 18

Table 1. Effect of a diet supplemented with unconjugated or taurine-conjugated bile acids on body weight change, tissue weights and stool analysis after 3 weeks. White adipose tissue, WAT; brown adipose tissue, BAT. Data are mean ± SD of 4-6 mice per group. * P < 0.05 vs. control. # P < 0.05 vs. corresponding unconjugated bile acid.

5.2. Serum analysis

Serum ALT and AST activity tended to be elevated by CA and TCA after 4 days yet normalized after 3 weeks (Table 2). ALT and AST were not altered by UDCA or TUDCA at any time. CA slightly elevated ALP after 3 weeks, UDCA and TUDCA tended to reduce it. The total BA concentration in serum of animals fed unconjugated BAs always tended to exceed concentrations of animals fed conjugated BAs.

	ALT (U/l)	AST (U/l)	ALP (U/l)	Total bilirubin (mg/dl)	Total bile acids (μ mol/l)
Control	36 \pm 0	106 \pm 8	68 \pm 4	0.17 \pm 0.02	6 \pm 10
7 hours CA	332 \pm 243	944 \pm 905	82 \pm 3	0.22 \pm 0.08	134 \pm 168
7 hours TCA	27 \pm 6	107 \pm 32	71 \pm 13	0.10 \pm 0.04	8 \pm 9
Control	35 \pm 9	101 \pm 36	83 \pm 18	0.19 \pm 0.09	4 \pm 4
4 days CA	643 \pm 352	309 \pm 150	141 \pm 12	0.64 \pm 0.26 *	363 \pm 32 *
4 days TCA	1851 \pm 1498	1280 \pm 915 *	152 \pm 118	0.28 \pm 0.11	261 \pm 23 *#
Control	35 \pm 17	89 \pm 4	119 \pm 9	0.03 \pm 0.04	7 \pm 2
3 weeks CA	49 \pm 17	88 \pm 19	198 \pm 25 *	0.06 \pm 0.05	178 \pm 17 *
3 weeks TCA	61 \pm 23	87 \pm 10	152 \pm 17 #	0.08 \pm 0.00	136 \pm 36 *
Control	56 \pm 26	145 \pm 53	245 \pm 27	0.16 \pm 0.06	7 \pm 7
4 days UDCA	33 \pm 4	155 \pm 50	133 \pm 40 *	0.30 \pm 0.08	363 \pm 136 *
4 days TUDCA	36 \pm 5	158 \pm 43	93 \pm 11 *	0.21 \pm 0.11	175 \pm 201
Control	36 \pm 3	128 \pm 38	114 \pm 14	0.04 \pm 0.03	2 \pm 3
3 weeks UDCA	33 \pm 4	89 \pm 29	104 \pm 12	0.34 \pm 0.20 *	472 \pm 92 *
3 weeks TUDCA	30 \pm 0 *	100 \pm 15	93 \pm 7 *	0.20 \pm 0.17	280 \pm 247 *

Table 2. Serum analysis of mice fed control diet, a diet supplemented with unconjugated bile acids or a diet supplemented with taurine-conjugated bile acids for 7 hours, 4 days and 3 weeks. Data are mean \pm SD of 3-6 mice per group. * P < 0.05 vs. control. # P < 0.05 vs. corresponding unconjugated bile acid.

5.3. Effect on hepatic bile acid synthesis

In order to assess conjugation-dependent regulation of BA-synthesis Cyp7a1, Cyp8b1 and Shp mRNA levels were quantified in the liver.

Feeding CA and TCA for 7 hours resulted in insignificantly decreased Cyp7a1 mRNA levels (Fig. 4A). All BAs markedly repressed Cyp7a1 mRNA expression after 4 days and 3 weeks (n.s. for TCA after 3 weeks; Fig. 4). Feeding CA, TCA, UDCA and TUDCA for 4 days led to a repression of Cyp7a1 to $7 \pm 1\%$, $8 \pm 2\%$, $2 \pm 1\%$ and $17 \pm 12\%$ of control diet fed animals, respectively (all $P < 0.05$; Fig. 4, B and D). Long term feeding of CA, TCA, UDCA and TUDCA for 3 weeks tended to augment the repression of Cyp7a1 to $3 \pm 5\%$, $0 \pm 0\%$, $6 \pm 2\%$ and $13 \pm 8\%$ of controls, respectively (Fig. 4, C and E).

There was a slight tendency of attenuated repression of Cyp7a1 mRNA by conjugated BAs.

Cyp7a1

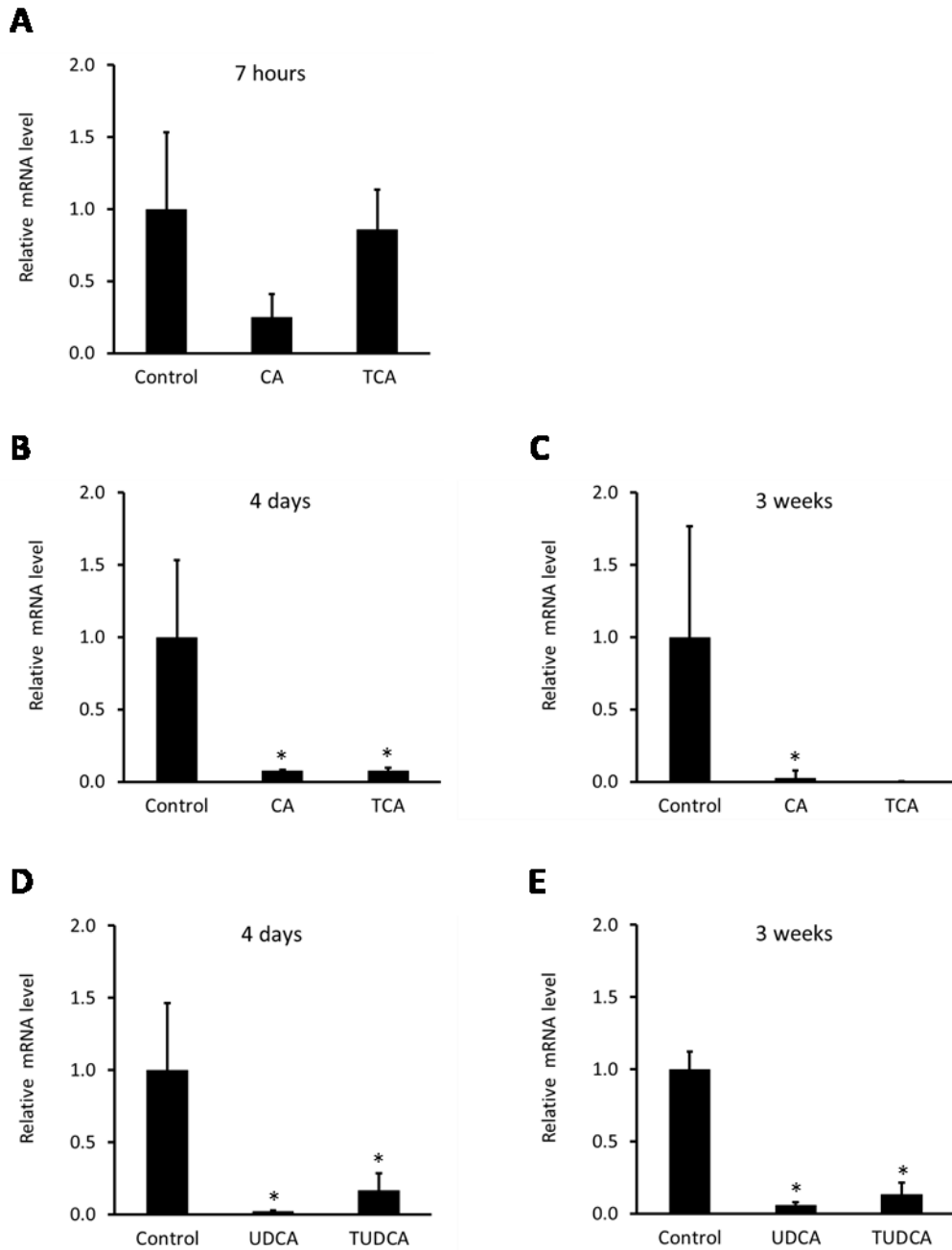


Fig. 4. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on hepatic cytochrome P-450 (Cyp)7a1 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. Cyp7a1 mRNA levels were insignificantly lowered by CA and TCA after 7 hours (A). Cyp7a1 was repressed by all bile acids after 4 days (B, D), by CA after 3 weeks (C) and by UDCA and TUDCA after 3 weeks (E). There is a slight tendency of weaker repression of Cyp7a1 by conjugated bile acids.

TUDCA repressed Cyp8b1 expression to a lesser extent than UDCA after 4 days ($65 \pm 29\%$ vs. $7 \pm 3\%$ of controls, $P < 0.05$; Fig. 5A). Both BAs repressed Cyp8b1 after 3 weeks ($P < 0.05$; Fig. 5B) with a tendency of weaker repression by TUDCA.

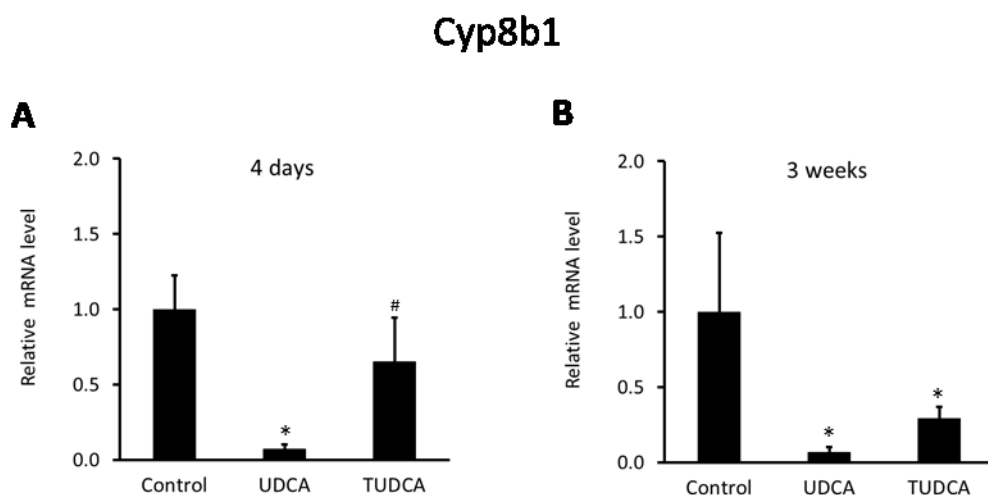


Fig. 5. Effect of feeding ursodeoxycholic acid (UDCA) or tauroursodeoxycholic (TUDCA) on hepatic cytochrome P-450 (Cyp)8b1 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Data are mean \pm SD of 4-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. UDCA. TUDCA represses Cyp8b1 to a lesser extent than UDCA after 4 days (A). Both bile acids repress Cyp8b1 after 3 weeks with a tendency of weaker repression by TUDCA (B).

Hepatic Shp mRNA expression was only moderately affected by BA feeding (Fig. 6). TCA tended to induce Shp slightly more than CA after 7 hours, 4 days and 3 weeks (Fig. 6, A, B and C). UDCA and TUDCA both tended to downregulate Shp mRNA after 3 weeks ($53 \pm 28\%$ and $38 \pm 18\%$ of controls, respectively, n.s.; Fig. 6E).

Shp

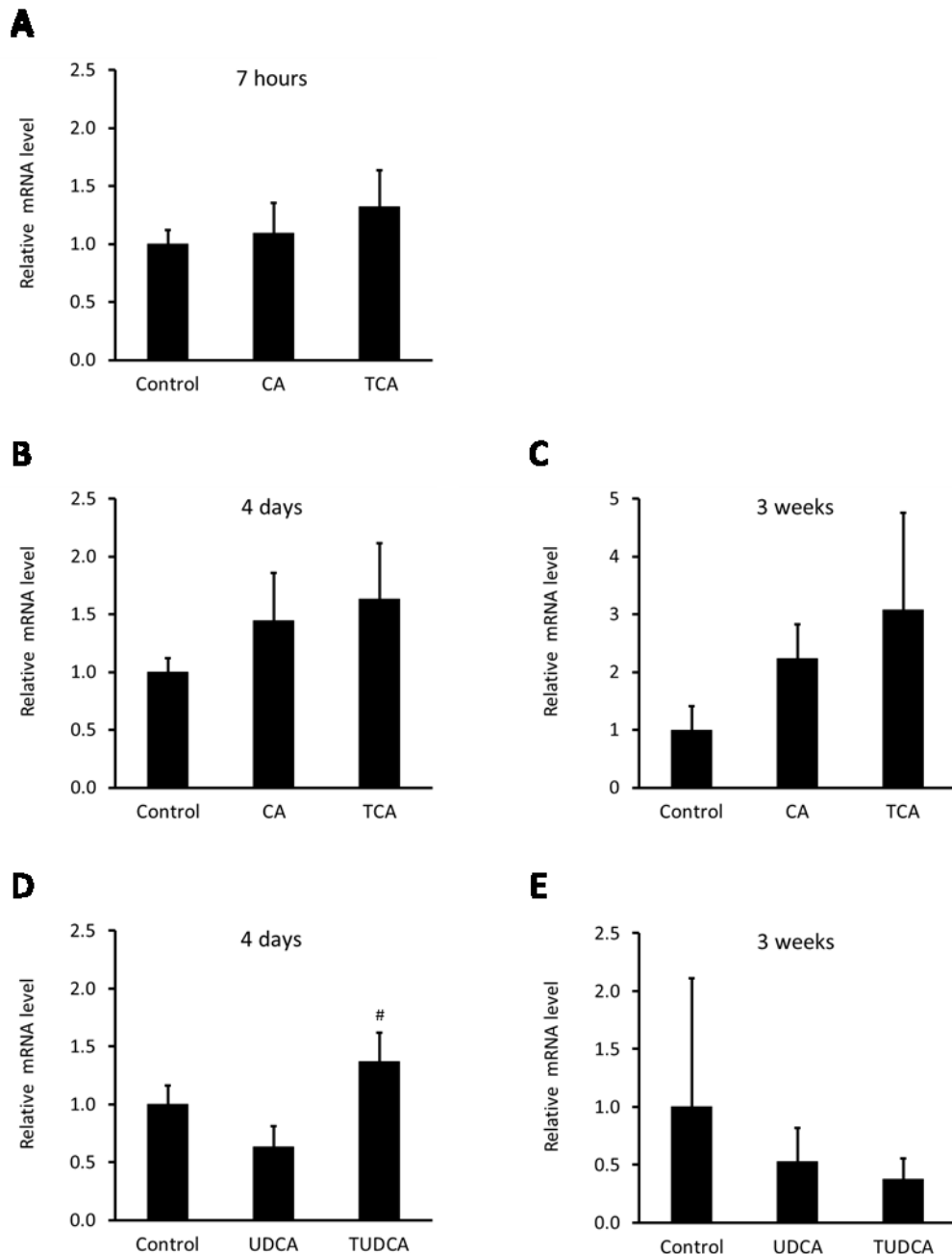


Fig. 6. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the hepatic mRNA level of small heterodimer partner (Shp) in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. # $P < 0.05$ vs. corresponding unconjugated bile acid. Bile acid supplemented diets had only moderate effects on Shp expression in the liver (A, B, C, D, E). TCA tended to upregulate Shp slightly more than CA after 7 hours (A), 4 days (B) and 3 weeks (C).

5.4. Hepatic bile acid transport and detoxification

Hepatic Ost- β mRNA levels were not affected by 7-hour feeding of CA and TCA (Fig. 7A). In line with previous findings (22), Ost- β expression after 4 days was strongly induced by CA and TCA to $1472 \pm 445\%$ and $1757 \pm 408\%$ of control mice, respectively ($P < 0.05$, Fig. 7B).

Surprisingly, Ost- β expression was repressed by both UDCA and TUDCA. TUDCA augmented the downregulation of Ost- β after 4 days compared to UDCA ($16 \pm 11\%$ vs. $51 \pm 14\%$, $P < 0.05$; Fig. 7C). The same trend could be observed after 3 weeks without reaching statistical significance (Fig. 7D).

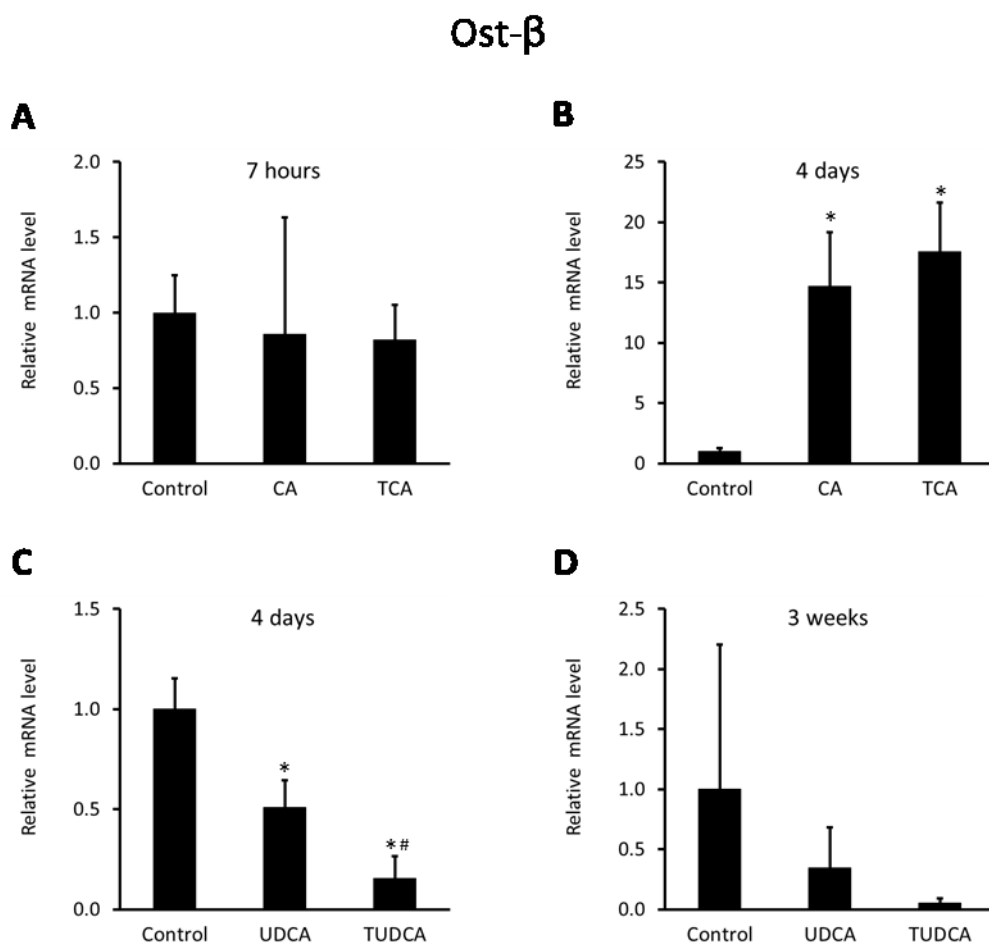


Fig. 7. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on hepatic organic solute transporter (Ost)- β expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time

PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. CA and TCA did not alter Ost- β expression after 7 hours (A) but led to a strong induction after 4 days (B). TUDCA augmented downregulation of Ost- β after 4 days (C) and insignificantly after 3 weeks (D) compared to UDCA.

CA but not TCA tended to induce Cyp3a11 after 4 days (Fig. 8A). Both CA and TCA induced Cyp3a11 after 3 weeks ($216 \pm 40\%$ and $268 \pm 66\%$ respectively, $P < 0.05$; Fig. 8B).

UDCA induced and TUDCA tended to slightly repress Cyp3a11 mRNA after 4 days to $234 \pm 56\%$ and $67 \pm 11\%$ of controls, respectively ($P < 0.05$, Fig. 8C). Similar relations of Cyp3a11 expression were measured after 3 weeks but failed to reach statistical significance (Fig. 8D).

Cyp3a11

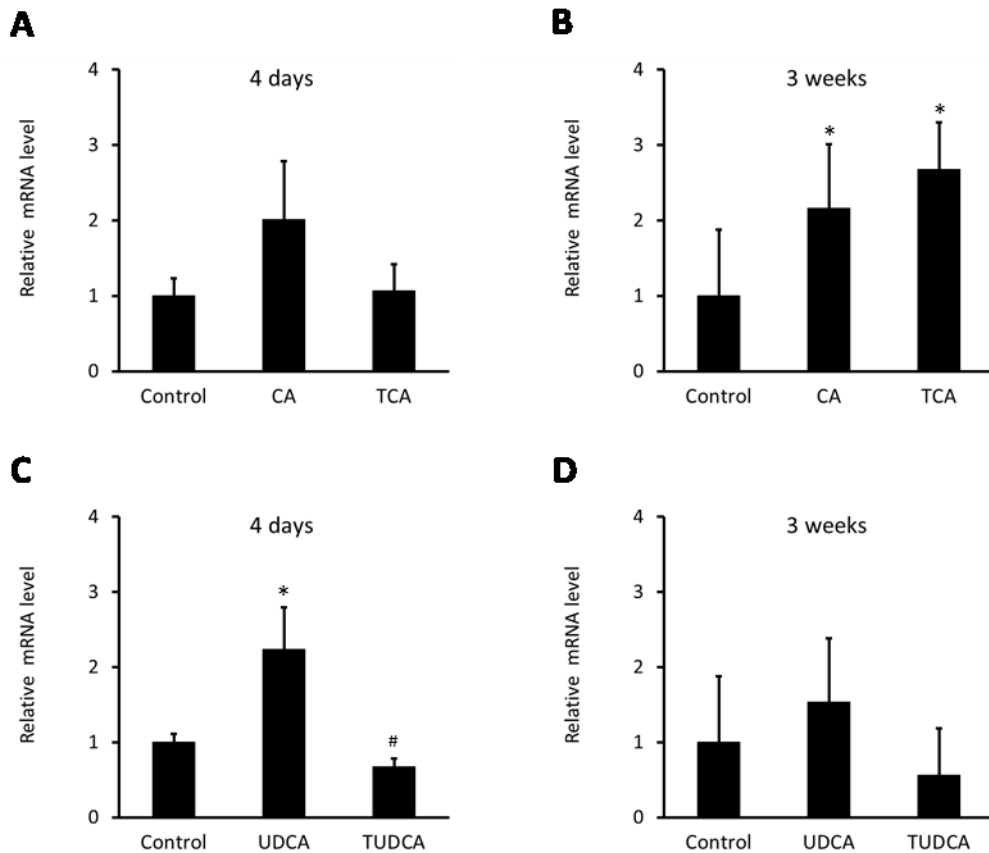


Fig. 8. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on hepatic cytochrome P-450 (Cyp)3a11 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. Unconjugated bile acids tended to upregulate Cyp3a11 (A, B, C, D). TUDCA led to lower Cyp3a11 mRNA levels than UDCA (C and D), significant after 4 days.

Taurine-conjugated BAs tended to reduce Cyp2b10 mRNA levels after 4 days and 3 weeks compared to unconjugated BAs (Fig. 9, B, C, D and E). CA induced Cyp2b10 strongly, but insignificantly after 4 days though TCA showed only minor effects ($2767 \pm 3198\%$ vs. $189 \pm 294\%$ of controls; Fig. 9B). CA induced Cyp2b10 in an attenuated fashion after 3 weeks but still exceeded TCA ($915 \pm 273\%$ vs. $457 \pm 192\%$ of controls; Fig. 9C). After 4 days UDCA had no effect on Cyp2b10

expression whereas TUDCA tended to repress it. After 3 weeks UDCA tended to induce and TUDCA did not affect Cyp2b10 expression.

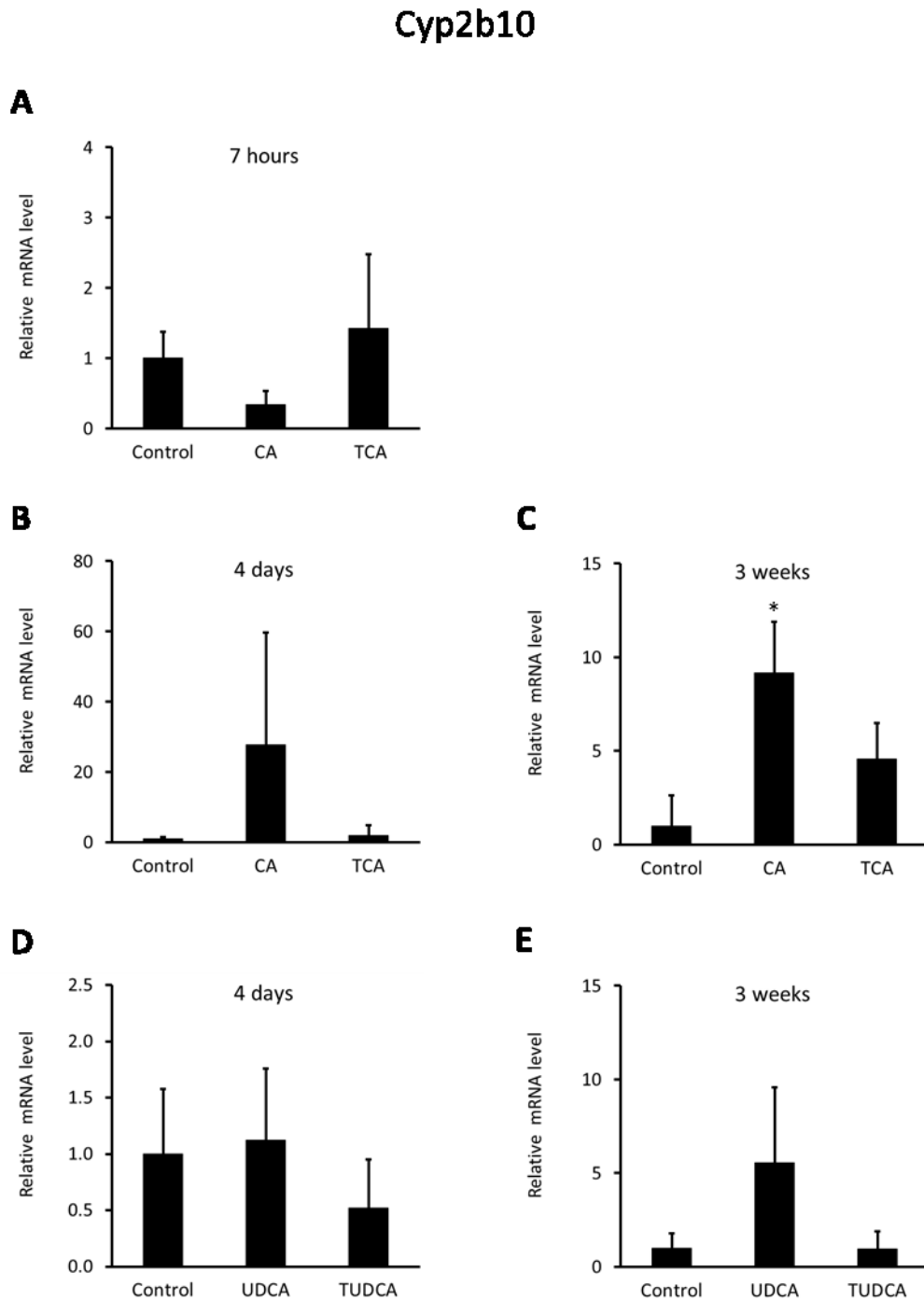


Fig. 9. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on hepatic cytochrome P-450 (Cyp)2b10 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic

acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. Only moderate effects on Cyp2b10 were observed after 7 hours (A). TCA tended to induce Cyp2b10 less than CA after 4 days (B) and 3 weeks (C). TUDCA led to lower Cyp2b10 mRNA levels than UDCA after 4 days (D) and 3 weeks (E) without reaching statistical significance.

5.5. Ileal bile acid response

In order to determine the intestinal contribution to the feed-back regulation of BA synthesis, Fgf15 expression levels in the terminal ileum were measured. 7-hours feeding already tended to increase Fgf15 expression without reaching statistical significance (Fig. 10A). After 4 days TCA led to a weaker induction of Fgf15 than CA ($1155 \pm 340\%$ vs. $2073 \pm 525\%$ of controls respectively, $P < 0.05$; Fig. 10B). After 3 weeks Fgf15 induction by both BAs is augmented but the same trend can still be observed (Fig. 10C). In contrast, TUDCA tended to induce Fgf15 more than UDCA after 4 days and 3 weeks. (Fig. 10, D and E).

Fgf15

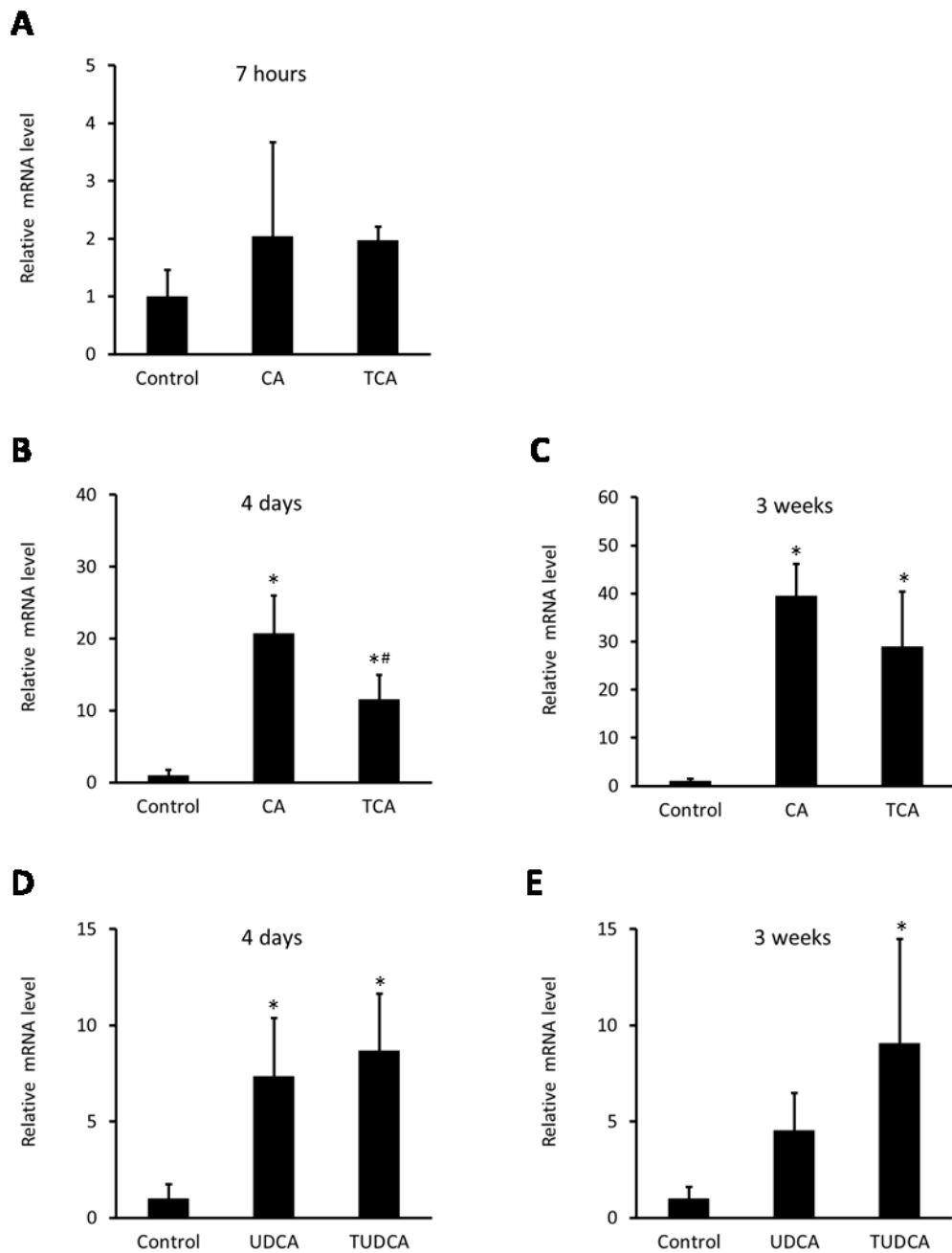


Fig. 10. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on ileal fibroblast growth factor (Fgf)15 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. No significant differences in the expression of Fgf15 were observed after 7 hours (A). TCA led to a weaker induction of Fgf15 after 4 days (B) and 3 weeks (C), statistically significant after 4 days. TUDCA showed a slight tendency of stronger Fgf15 induction than UDCA (D and E).

I-BABP expression levels were upregulated by long- and short-term treatment with UDCA and TUDCA (n.s. after 4 days; Fig. 11, A and B). After 4 days as well as after 3 weeks the taurine-conjugate tended to evoke an ameliorated upregulation.

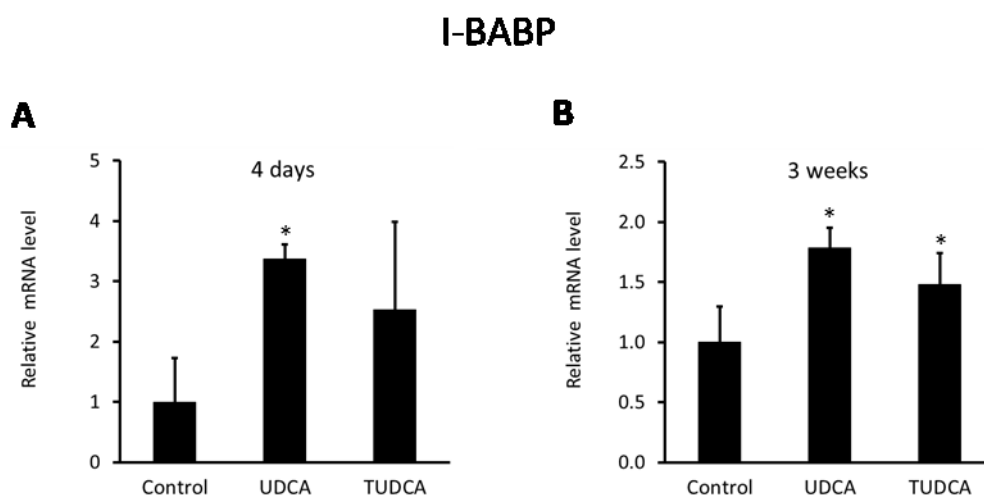


Fig. 11. Effect of ursodeoxycholic acid (UDCA) or tauroursodeoxycholic acid (TUDCA) feeding on ileal bile acid-binding protein (I-BABP) expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Data are mean \pm SD of 4-6 mice per group. * $P < 0.05$ vs. control. UDCA induced I-BABP after 4 days and 3 weeks (A and B). TUDCA led to a slight induction after 3 weeks (B).

The atypical NR Shp is expressed in the intestine and has been shown to be upregulated by ingested FXR agonists (70). Thus we measured Shp expression levels in the terminal ileum to determine FXR activation by the administered BAs. Ileal Shp mRNA expression levels were barely or not detectable in control diet fed mice (Fig. 12). CA tended to induce Shp slightly more than TCA after 7 hours (Fig. 12A). Both, CA and TCA, led to a robust coordinate induction of ileal Shp expression after 4 days and 3 weeks (Fig. 12, B and C). Although Shp was only just detectable after 4-day feeding of UDCA, neither UDCA nor TUDCA led to a marked induction of Shp mRNA levels in the ileum (Fig. 12, D and E).

Shp

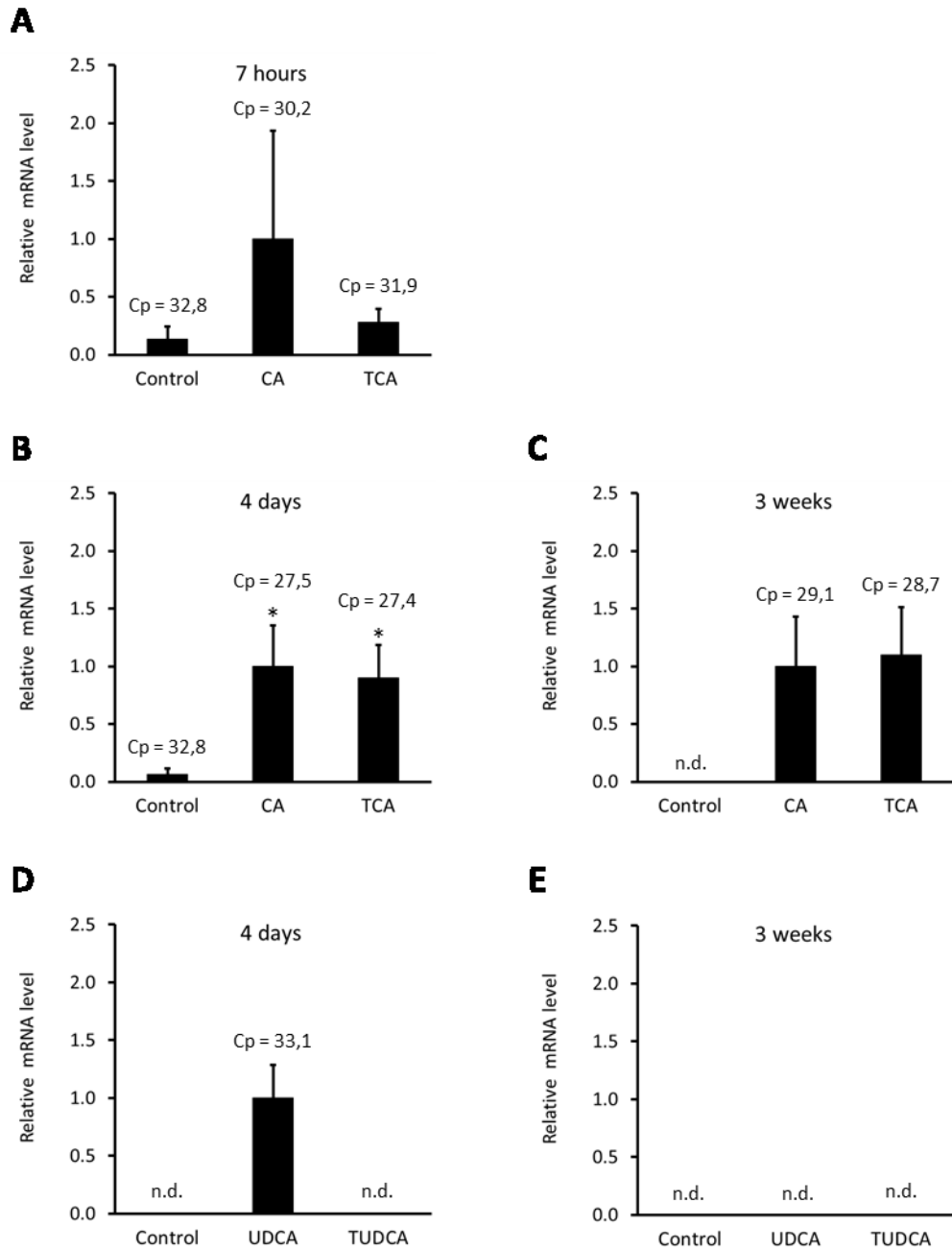


Fig. 12. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the ileal mRNA level of small heterodimer partner (Shp) in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 7 hours, 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to the respective unconjugated bile acid. Mean crossing point (Cp) values are depicted above corresponding bars. A Cp value of 35 or above has been denoted as not detectable (n.d.). Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. Shp mRNA expression levels were low (A and B) or not detectable in control mice (C, D and E). Ileal Shp expression was coordinate induced by CA and TCA after 4

days (B) and 3 weeks (C). Neither UDCA nor TUDCA led to a marked induction of Shp after 4 days (D) or 3 weeks (E).

Neither UDCA nor TUDCA did affect ileal Ost- β expression levels after both 4 days and 3 weeks (Fig. 13).

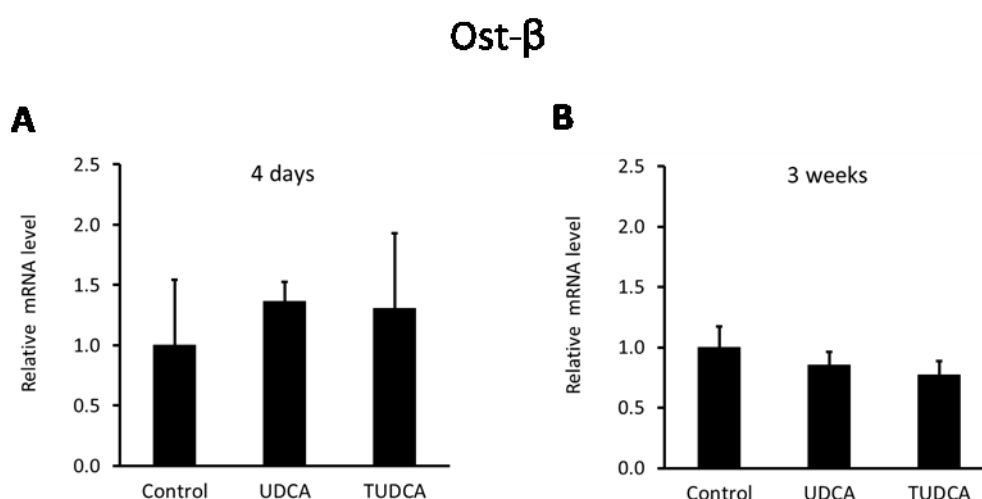


Fig. 13. Effect of ursodeoxycholic acid (UDCA) or tauroursodeoxycholic acid (TUDCA) feeding on ileal organic solute transporter (Ost)- β expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Data are mean \pm SD of 4-6 mice per group. Both bile acids had negligible effects on ileal Ost- β expression (A and B).

5.6. Taurine-conjugation alters Ppar- α signaling

Ppar- α is part of a complex regulatory network of transcription factors influencing BA synthesis on a transcriptional level (59). Here we show that BA administration has differential transcriptional effects on Ppar- α , partly dependent on taurine-conjugation.

CA and TCA feeding for 4 days repressed hepatic Ppar- α expression levels to $43 \pm 7\%$ and $46 \pm 9\%$ of controls, respectively (both $P < 0.05$; Fig. 14A). TUDCA

induced Ppar- α expression after 4 days and 3 weeks ($235 \pm 38\%$ and $165 \pm 36\%$ of controls, respectively, both $P < 0.05$; Fig. 14, A and B), whereas UDCA had no effect.

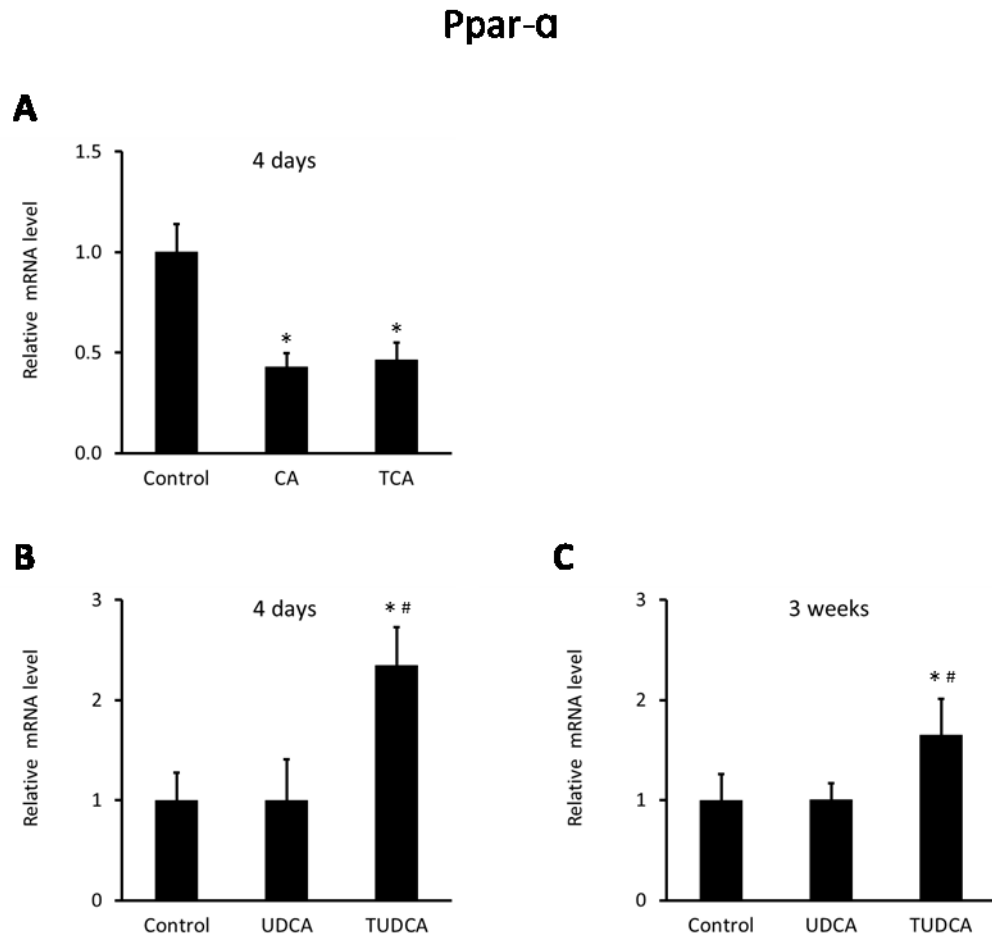


Fig. 14. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the hepatic expression of ligand activated transcription factor peroxisome proliferator activated receptor (Ppar)- α . Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. CA and TCA repressed Ppar- α expression after 4 days (A). TUDCA induced Ppar- α expression after 4 days (B) and 3 weeks (C), whereas UDCA had no effect.

In order to test the functional relevance of variations in Ppar- α mRNA levels, we examined the expression of Ppar- α target genes.

Acyl-coenzyme A oxidase 1 (Acox1) is the first and rate limiting enzyme in peroxisomal β -oxidation, physiologically upregulated by Ppar- α during fasting (26,60,61). Hepatic expression of Acox1 paralleled expression levels of Ppar- α (Fig. 15). CA and TCA repressed Acox1 expression levels after 4 days to $31 \pm 4\%$ and $29 \pm 16\%$ of controls, respectively (n.s. for CA; Fig. 15A). This effect was absent after 3 weeks (Fig. 15B). Consistent with the induction of Ppar- α , Acox1 was upregulated only by TUDCA after 4 days and 3 weeks to $176 \pm 25\%$ and $165 \pm 22\%$ of controls, respectively (both $P < 0.05$; Fig. 15, C and D). In contrast, UDCA feeding had no effect on Ppar- α expression.

Acox1

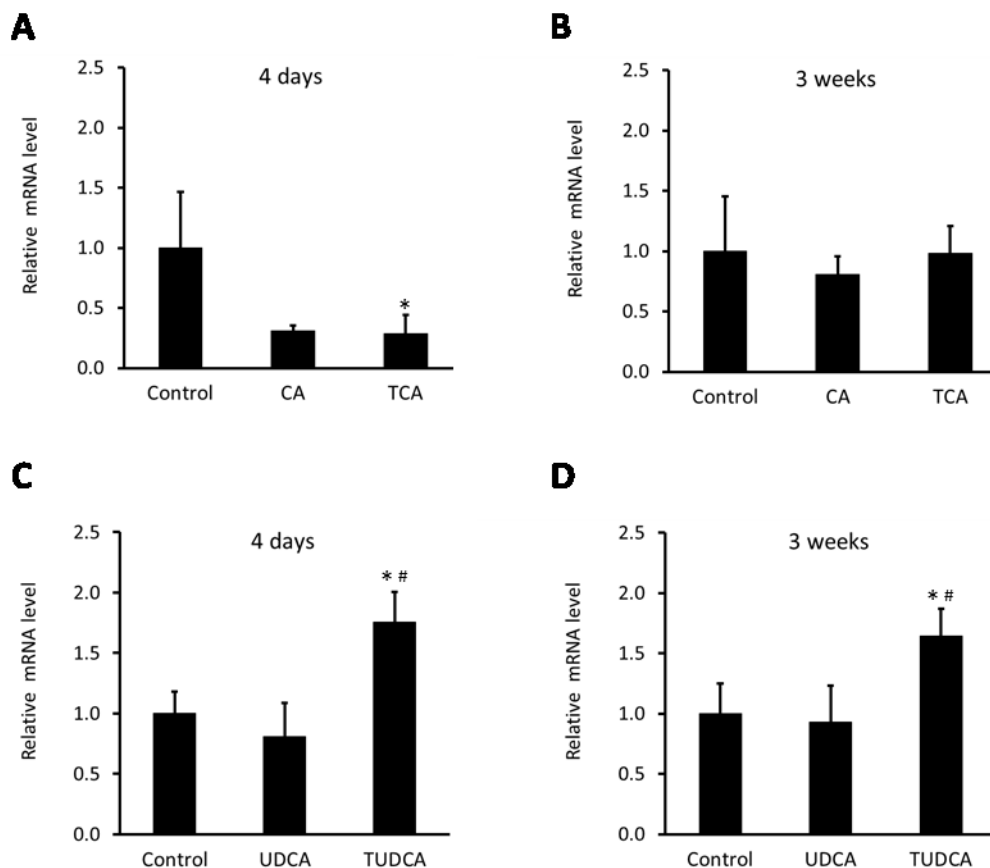


Fig. 15. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the hepatic expression of Ppar- α target gene acyl-coenzyme A oxidase (Acox1). Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. TCA repressed Acox1 expression after 4 days (A). Neither CA nor TCA affected Acox1 expression after 3 weeks (B). TUDCA induced Acox1 expression levels after 4 days (C) and 3 weeks (D), while UDCA showed no effect.

G0/G1 switch gene 2 (G0s2) functions as master regulator of triglyceride storage and is upregulated in the liver by Ppar- α during fasting (62,63). Hepatic G0s2 mRNA levels paralleled the expression of Ppar- α and Acox1 after 4 days but diverged after 3 weeks. CA and TCA repressed hepatic G0s2 mRNA levels after 4 days to $14 \pm 5\%$ and $8 \pm 1\%$ of controls, respectively (both $P < 0.05$; Fig. 16A). An insignificant trend of G0s2 repression by CA and TCA was still evident after 3 weeks ($17 \pm 7\%$ and $25 \pm 15\%$ of controls; Fig. 16B). In line with the effects on Ppar- α and Acox1, TUDCA induced G0s2 expression after 4 days (Fig. 16C). However, this effect disappeared after 3 weeks (Fig. 16D).

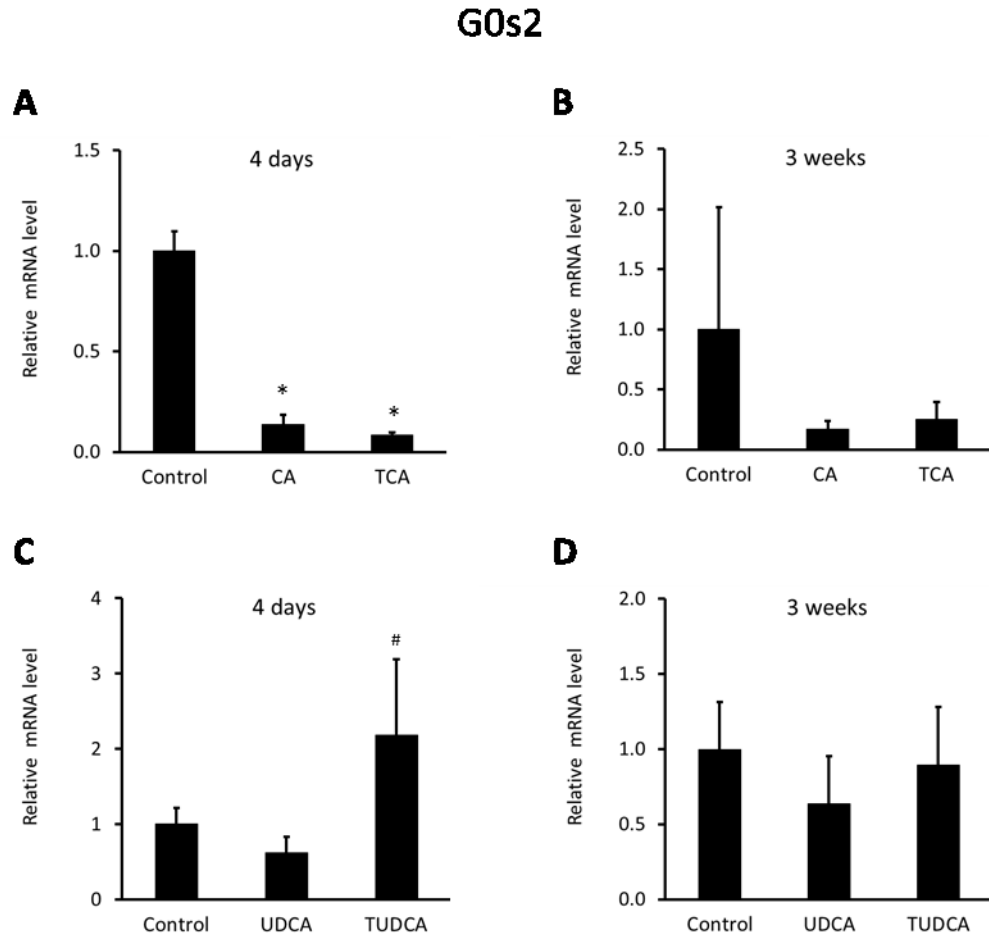


Fig. 16. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the hepatic expression of Ppar- α target G0/G1 switch gene (G0s)2. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. CA and TCA led to a repression of G0s2 after 4 days (A) and a tendency of downregulation after 3 weeks (B). TUDCA elevated G0s2 mRNA levels compared to UDCA after 4 days (C) but not after 3 weeks (D).

Cyp4a14 is another classical Ppar- α target, catalyzing microsomal hydroxylation of fatty acids before degradation by peroxisomal β -oxidation (60,64). Fitting the expression profile of Ppar- α , Acox1 and G0s2, CA and TCA dramatically suppressed Cyp4a14 mRNA levels after 4 days to $7 \pm 7\%$ and $2 \pm 1\%$ of controls, respectively (both $P < 0.05$; Fig. 17A). Interestingly, long-term feeding of CA and TCA markedly induced Cyp4a14 expression to $908 \pm 364\%$ and $398 \pm 401\%$ of

controls, respectively (n.s. for TCA; Fig. 17B). In contrast to other Ppar- α target genes, Cyp4a14 tended to be induced by both UDCA and TUDCA after 4 days and 3 weeks (Fig. 17, C and D).

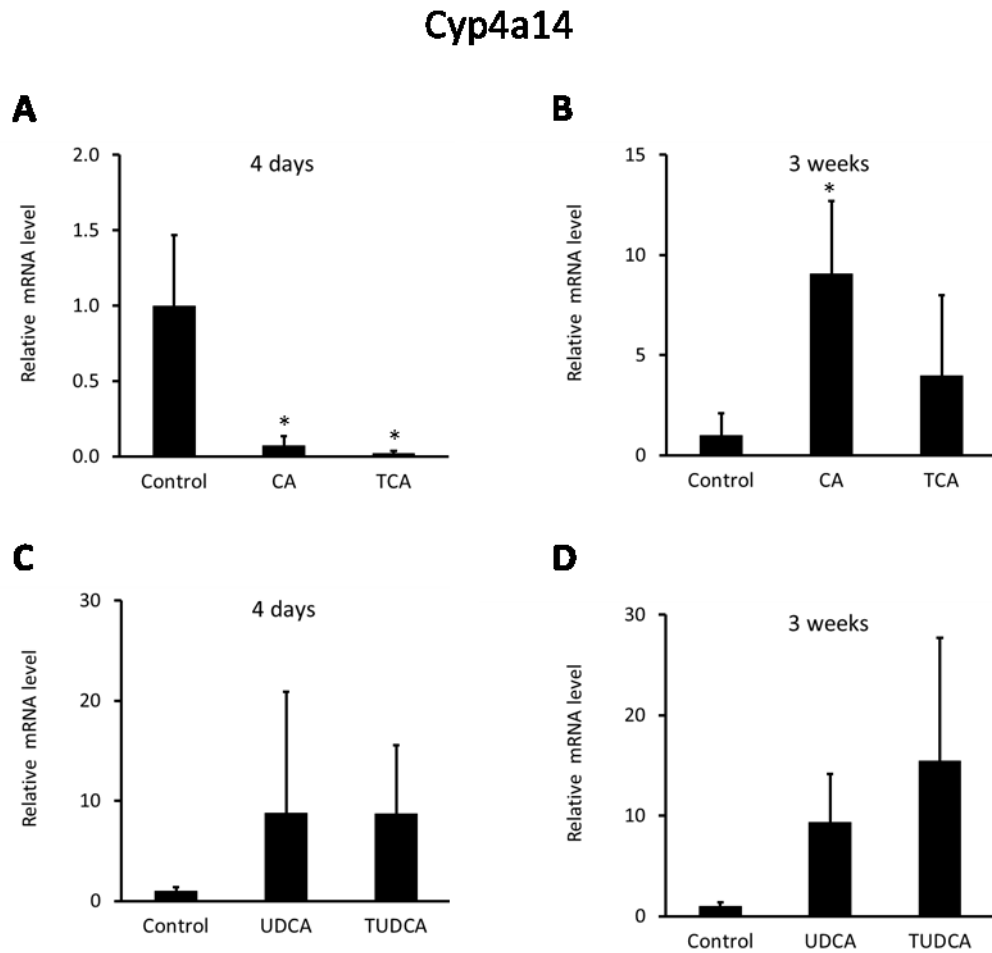


Fig. 17. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the hepatic expression of Ppar- α target gene cytochrome P-450 (Cyp)4a14. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days and 3 weeks. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-6 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. Short-term feeding of CA and TCA dramatically repressed hepatic Cyp4a14 expression (A), whereas long-term feeding led to an induction, significant for CA (B). UDCA and TUDCA tended to elevate hepatic Cyp4a14 mRNA levels after 4 days (C) and 3 weeks (D).

In summary, CA and TCA coordinately repressed Ppar- α and Ppar- α target genes except Cyp4a14 after long-term treatment. TUDCA induced Ppar- α and Ppar- α target genes, however, UDCA had no effect.

5.7. Taurine-conjugation boosts Igfbp1 expression and secretion

Hepatic expression levels of Igfbp1 differed dramatically from all other studied Ppar- α target genes. TCA induced Igfbp1 mRNA expression in the liver 31-fold after 4 days, whereas CA did not cause significant alterations (Fig. 18A). In contrast, both UDCA and TUDCA reduced Igfbp1 expression levels to $18 \pm 16\%$ and $27 \pm 9\%$ of controls, respectively ($P < 0.05$; Fig. 18B).

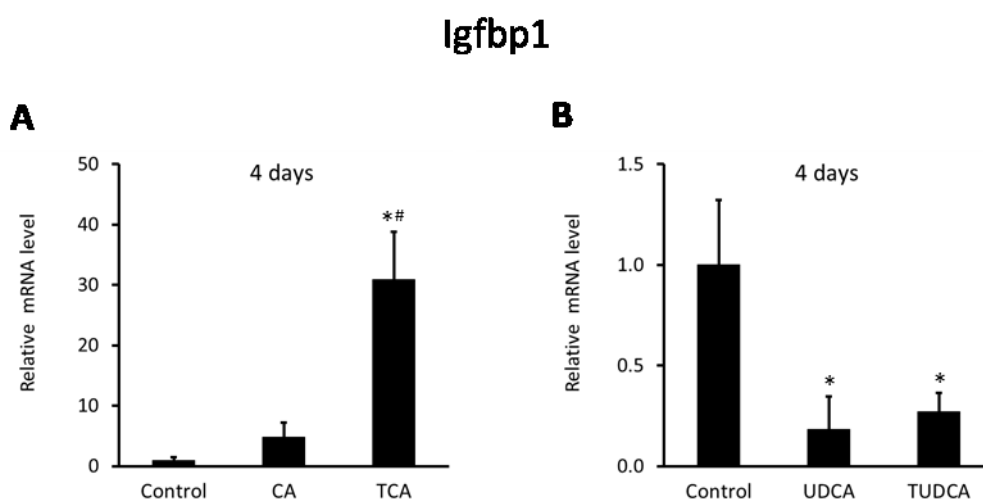


Fig. 18. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on hepatic insulin-like growth factor binding protein (Igfbp)1 expression in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days. mRNA levels were determined by real-time PCR, normalized to 36b4 and presented as values relative to controls. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. Data are mean \pm SD of 3-5 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. corresponding unconjugated bile acid. TCA led to a 31-fold induction of hepatic Igfbp1 mRNA expression, whereas CA had no significant effect (A). Both UDCA and TUDCA markedly repressed Igfbp1 (B).

To test the hypothesis that differences in hepatic Igfbp1 mRNA levels affect circulating protein, Western Blot analysis of IGFBP1 in serum was performed. In

line with hepatic mRNA expression, protein levels were strongly elevated by TCA, hardly affected by CA and markedly decreased by both, UDCA and TUDCA (Fig. 19).

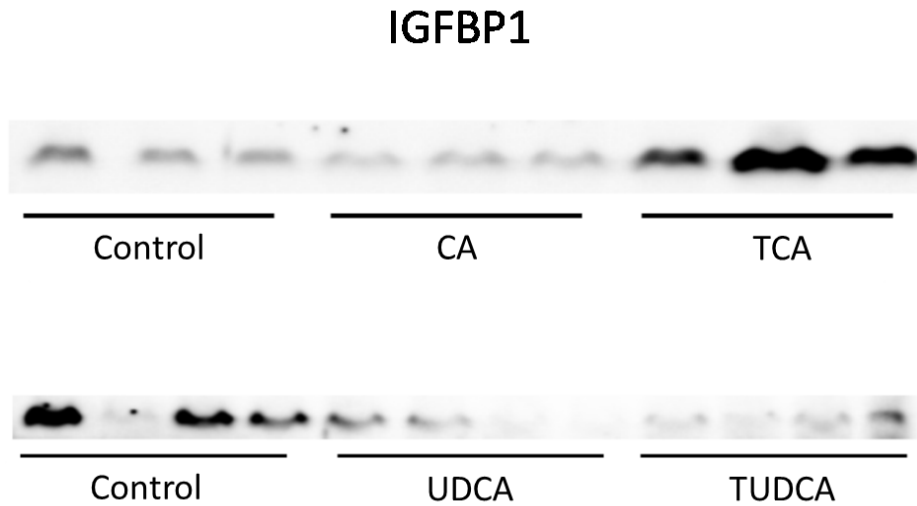


Fig. 19. Effect of feeding unconjugated or corresponding taurine-conjugated bile acids on the serum protein level of insulin-like growth factor binding protein (IGFBP)1 in mice. Control diet or bile acid supplemented diets (0.5% wt/wt) were fed for 4 days. Protein levels were determined by Western blot analysis as described in Materials and Methods. Two representative immunoblots are shown. Cholic acid, CA; taurocholic acid, TCA; ursodeoxycholic acid, UDCA; tauroursodeoxycholic acid, TUDCA. TCA, but not CA, induced Igfbp1 in serum, whereas UDCA and TUDCA led to a similar repression.

6. Discussion

6.1. Summary

The current study is the first to systematically examine transcriptional effects of BA conjugation *in vivo*. Two BAs of physiologic and clinical importance and of different degree of hydrophobicity, toxicity and affinity to NRs have been applied in unconjugated and taurine-conjugated form (3). Hepatic, intestinal as well as metabolic parameters were studied at various time points. Robust differences between free BAs and taurine-conjugates were observed in body-weight change, liver weight, WAT weight, BAT weight and in hepatic expression levels of genes involved in BA synthesis (Cyp8b1), BA excretion (Ost- β), lipid metabolism (Ppar- α , Acox1 and G0s2) and glucose metabolism (Igf1). In addition to a conjugation-dependent induction of Ppar- α by TUDCA, we found concordant effects on Ppar- α target genes (Acox1 and G0s2) suggesting a functional relevance. Further, we were able to demonstrate a dramatic conjugation-dependent induction of Igfbp1 expression and secretion by TCA. Moreover, we found statistically insignificant but consistent conjugation-dependent trends in mRNA expression levels of major regulatory genes in BA homeostasis in liver (Cyp7a1, Cyp2b10, Cyp3a4) and ileum (Fgf15, I-BABP) after diverse durations of BA feeding.

It is important to mention that the observed effects are not necessarily directly attributable to the fed BAs themselves but may be ascribed to BA derivatives formed in liver and intestine or to other metabolites originating from altered hepatic and intestinal metabolism.

6.2. Effect on biometric measurements and laboratory tests

Alterations in body and tissue weights induced by BA feeding have been consistently reported in previous studies, yet these data are mainly based on unconjugated BAs. We found a different effect of UDCA and TUDCA on body weight change (Fig. 3). After 3 weeks, animals fed UDCA tended to lose weight, while animals fed TUDCA tended to gain weight, resulting in a significant

difference in body weight change. Although the specific mechanism involved are unknown, our findings support the hypothesis that differences in energy expenditure as indicated by decreased BAT weight in the TUDCA group ($P < 0.05$ vs. UDCA; Table 1) may have importance. Variations in food intake, e.g. conferred by gustatory effects of taurine-conjugation could also have had a role. Since neither stool weight, nor stool pellet count differed between groups, bile acid diarrhea is an unlikely cause for the weight change. The effects of unconjugated BAs (UDCA and CA) and of TCA widely paralleled previous findings (65,66,67,68).

We found that taurine-conjugation of both BAs reduced liver weight and liver to body weight ratio (Table 1). In line with Song et al. (65), liver weight was markedly increased by CA compared to standard mouse chow. TCA led to an attenuated increase in liver weight compared to CA, which is consistent with a study of Wolters et al (68) showing that TCA feeding tended to elevate liver weight. The lacking effect of UDCA on liver weight is consistent with a study of Song et al. (65). A lowering effect of TUDCA on liver weight, as observed in the present study, was also reported by Oh et al. (66). Although we have no ready explanations for the observed variations in liver weight, we speculate that alterations in hepatic lipid, glycogen or peptide storage had importance. No differences in hepatic cell proliferation were observed in microscopy (data not shown).

CA and TCA feeding coordinately decreased WAT weight after 3 weeks (Table 1). These findings are consistent with several studies reporting protective effects of BAs and other FXR agonists against obesity and insulin resistance (67,69,70). Surprisingly, TUDCA lacked the marked increasing effect on WAT weight as observed in the UDCA group. It is interesting to note that UDCA decreased gonadal fat weight after long term feeding in mice (66) and that *in vitro* results suggest that UDCA but not TUDCA inhibits proliferation of human adipocytes (71). Energy expenditure in BAT, the thermogenically most important tissue in rodents, can be elevated by BA administration and therefore constitutes a potential target to treat obesity (3). BAT mass is increased and weight gain diminished in transgenic mice overexpressing Fgf15/19 (3). Therefore we also determined the influence of BA feeding on BAT weight. We found that changes in BAT weight paralleled those in WAT weight.

Our results of serum parameters are in good agreement with previous findings. Two classical serum parameters of liver injury, ALT and AST, tended to be elevated by 4- day feeding of both CA and TCA, similar to observations by others (65,72) (Table 2). In our experiment ALT and AST normalized after 3-week-feeding of CA and TCA, which may be attributed to hepatic adaptation processes, progressive conjugation and renal elimination of potentially toxic CA. We found that TUDCA, similar to UDCA, does not affect parameters of liver injury confirming its lack of hepatotoxicity (65). Dose-dependent increases in total serum BA concentration occur when mice are fed CA or UDCA at 0.3% wt/wt or above (65). Accordingly, total BA concentration was elevated by all BAs fed for 4 days and 3 weeks (n.s. for TUDCA after 4 days). Since taurine-conjugated BAs pass the intestinal wall less efficiently than unconjugated BAs and have a higher fractional hepatic extraction from portal venous blood (2,13,14), it is not surprising that unconjugated BAs led to higher total serum BA concentrations than their corresponding conjugates.

6.3. Bile acid homeostasis in the liver

Transcription of *Cyp7a1*, a gene coding for an enzyme catalyzing the first and rate-limiting step of the classical BA synthesis pathway, was strongly downregulated by all administered BAs after 4 days and 3 weeks (n.s. for TCA after 3 weeks; Fig. 4). This is consistent with the generally established concept of negative feed-back regulation of BA synthesis. Similar results for CA, TCA and UDCA have been presented previously (31,68,73,74). We observed that conjugated BAs in comparison to unconjugated BAs tended to exhibit a weaker suppressive effect on *Cyp7a1* transcription. Several potential mechanisms may have contributed to this effect. These include reduced absorption of conjugated BAs in the proximal intestine (13,14) and decreased *Fgf15* induction (Fig. 10). Further, decreased affinity of taurine-conjugates to NRs mediating *Cyp7a1* expression, effects of different BA metabolites formed in liver and intestine and reduced BA pool size in animals fed taurine-conjugates might play a role. The influence of gut bacteria on BA metabolism has recently been demonstrated by

Islam et al. (75) who found that CA feeding in rats causes tremendous alterations in cecal microbiota and fecal BA composition. To what extent these findings account for our observations and whether they are influenced by taurine-conjugation remains elusive. Neither intestinal FGF15 synthesis (Fig. 10) nor the effects of taurine itself, which has been shown to induce Cyp7a1 expression *in vitro* (76), satisfactorily explain the differences found.

Modulation of Cyp8b1 expression due to feeding of different BAs followed a similar pattern as Cyp7a1 expression described above (Fig. 5). TUDCA repressed Cyp8b1 to a lesser extent than UDCA, suggesting a shift of BA pool composition from CA towards CDCA and its derivatives. Since TUDCA conversely tended to increase intestinal Fgf15 expression, one may assume a hepatic mechanism.

Shp is an atypical nuclear receptor induced by FXR activation which in turn represses Cyp7a1 and Cyp8b1 (32,77,78). Previous studies (68,73) documented upregulation of Shp by CA, TCA and UDCA. We could observe a trend towards stronger Shp induction by TCA and TUDCA than by corresponding unconjugated BAs (Fig. 6). This opposes the trend of attenuated repression of Cyp7a1 and Cyp8b1 by taurine-conjugates discussed above.

Previous studies (31,73) have shown that the hepatic expression of the alternative BA transporter Ost- β is robustly induced by CA in an FXR-dependent fashion and hardly affected by UDCA feeding. The current study found that both CA and TCA strongly induce Ost- β expression levels, whereas both UDCA and TUDCA exert negative regulatory effects (Fig. 7). TUDCA repressed Ost- β stronger than UDCA. This leads to the hypothesis of weaker hepatic FXR activation by TUDCA feeding, e.g. by dilution of endogenous FXR ligands, which is conclusive with attenuated downregulation of Cyp7a1 and Cyp8b1 by the taurine-conjugate.

Cyp3a11 and Cyp2b10 encode hepatic phase I hydroxylation enzymes and stereotypical target genes of PXR and CAR, respectively (79). Phase I hydroxylation of BAs increases hydrophilicity, reduces toxicity and thus protects from BA-induced liver damage (31). CA and UDCA administration has been shown to upregulate both Cyp3a11 and Cyp2b10 mRNA expression (31,80). Contrarily,

Song et al. (73) using the same mouse strain, equal BA concentrations and identical feeding durations found no effect on Cyp3a11 expression by CA and a significant repression by UDCA.

We found that after 3 weeks TCA led to a similar upregulation of Cyp3a11 expression as CA (Fig. 8). TUDCA tended to repress Cyp3a11 expression, whereas UDCA resulted in an induction. Hence, our results confirm the findings of Zollner et al. (31) and additionally provide evidence of lacking Cyp3a11 induction by TUDCA.

Cyp2b10 tended to be induced by unconjugated BAs (significant for CA after 3 weeks; Fig. 9, B, C, D and E). By contrast, feeding of conjugated BAs, especially of TUDCA, tended to lower Cyp2b10 expression levels. These results are also in line with Zollner et al. (31) and suggest attenuated induction and even repression of Cyp2b10 mediated by taurine-conjugation.

In order to study the direct effects of BAs on phase I hydroxylation, two human liver cell lines (HepG2 cells and HUH7 cells) were incubated with CA, TCA, UDCA, TUDCA at our laboratory and analyzed for Cyp3a11 and Cyp2b10 expression levels (data not shown). *In vivo* effects could not be reproduced in this experiment, suggesting that the effects may be attributed to BA derivatives or other metabolites formed during enterohepatic circulation.

6.4. Transcriptional effects in the terminal ileum

FGF15 and its human ortholog FGF19 are synthesized in the intestine and exert crucial functions in the feedback inhibition of BA synthesis independent of hepatic Shp induction (3,32,81). According to a study of Song et al. (73), oral administration of CA leads to a robust dose-dependent increase in ileal Fgf15 expression. In accordance with Song (73), we found a marked induction of Fgf15 by unconjugated BAs (Fig. 10). We observed a differential effect of conjugated BAs: TCA led to an attenuated upregulation, whereas TUDCA tended to enhance upregulation of ileal Fg15.

I-BABP is a cytoplasmic protein in ileal enterocytes (82). It exclusively binds to BAs, mediates their transport to the basolateral membrane and is believed to be involved in the regulation of several intestinal effects of BAs (29,82,83). In line with Song et al. (73), we observed an induction of I-BABP expression by UDCA (Fig. 11). In contrast, the induction of I-BABP was absent (Fig. 11A) or tended to be attenuated (Fig 11B) under TUDCA feeding.

Ileal expression of both Fgf15 and I-BABP can be induced by intestinal FXR activation (70). Considering the absent induction of the ileal FXR target gene Shp by UDCA and TUDCA (Fig. 12, D and E) and the contrary effect of taurine-conjugation on the expression of Fgf15 and I-BABP, we suggest a FXR-independent effect of TUDCA on Fgf15 and I-BABP expression.

Ileal mRNA expression levels of the atypical NR and FXR target Shp were reported to be dramatically elevated by feeding CA, DCA or a intestine-restricted FXR agonist, but are unaffected by UDCA or LCA feeding (70,73). Consistent with that, we found a coordinate robust induction of ileal Shp expression by both CA and TCA (Fig. 12, B and C), but no marked induction by UDCA or TUDCA. The absence of a positive regulatory influence of UDCA and TUDCA on intestinal Shp expression questions their capacity of intestinal FXR activation and suggests that alternative signaling pathways mediate their effects in the intestine. This hypothesis is supported by the lacking upregulation of ileal Ost- β , another FXR target, upon UDCA and TUDCA feeding. Conforming to previous studies (31,73), we did not find regulatory effects of UDCA or TUDCA on ileal Ost- β expression (Fig. 13).

6.5. Conjugation-dependent regulation of Ppar- α signaling

Ppar- α governs the transcriptional regulation of hepatic lipid metabolism and additionally affects the adjacent network of BA homeostasis (25,84). In reverse, the effect of BA treatment on the expression of Ppar- α and its target genes is poorly investigated. Here we provide evidence for differential transcriptional

regulation of Ppar- α and several classical Ppar- α targets by BA administration *in vivo*. Moreover, we were able to demonstrate that taurine-conjugation alters the regulatory effects of BAs on Ppar- α expression and signaling.

CA and TCA coordinately repressed Ppar- α mRNA expression in the liver supporting their role as endocrine feeding signals (Fig. 14A). Downregulation of Ppar- α appears conclusive with increased liver weight in these animals since Ppar- α knockout mice develop hepatomegaly due to lipid accumulation (85).

TUDCA induced Ppar- α mRNA expression, while UDCA had no effect. This taurine-dependent induction of Ppar- α is in line with a previous study (86) that reports upregulation of Ppar- α and downstream targets by a UDCA phospholipid conjugate (UDCA-LPE) suggesting a conjugation-dependent mechanism. Considering that Ppar- α activation shifts BA pool composition towards the toxic FXR agonists CDCA and LCA, conjugation-dependent upregulation of Ppar- α may also partly account for conjugation-dependent transcriptional effects and trends in liver (Cyp7a1, Cyp8b1, Cyp3a11, Cyp2b10) and intestine (I-BABP) (87,88,89).

Data suggest that Ppar- α reduces BA toxicity, enhances BA glucuronidation via UDP-glucuronosyltransferase (UGT) enzymes, inhibits the expression of hepatic BA uptake systems (Oatps and Ntcp) and is essential for the expression of certain canalicular BA transporters, namely the ATP-binding cassette family (84,87,88,89,90). Furthermore, synthetic Ppar- α agonists exert beneficial effects on liver function tests in cholestatic patients (91,92,93,94,95). Thus it is tempting to hypothesize that taurine conjugation may augment the protective potential of UDCA in cholestatic livers via induction of Ppar- α .

In order to test the functional significance of variations in hepatic Ppar- α mRNA expression we determined mRNA levels of classical Ppar- α target genes and found concordant effects (Figs. 15 and 16). These results suggest that administered BAs not only exert conjugation-dependent transcriptional effects on Ppar- α but also regulate the expression of downstream target genes involved in hepatic lipid metabolism.

G0s2 encodes for a small protein with inhibitory effects on lipolysis in liver and adipose tissue and functions as key regulator of tissue-specific triglyceride storage (62,63). Transcription at the G0s2 promoter is induced by Ppar- α and LXR- α and can be suppressed by FoxO-1 (62). We observed a marked reduction G0s2 mRNA levels in the liver by CA and TCA feeding (Fig. 16A). Considering that G0s2 knockout mice are resistant to high fat diet-induced liver steatosis and exhibit improved whole body glucose tolerance and insulin sensitivity, G0s2 repression could alleviate metabolic diseases (62). In fact, G0s2 has already been proposed as therapeutic target for treating non-alcoholic fatty liver disease (NAFLD) and insulin resistance (62). In support of a conjugation-dependent effect of BAs on Ppar- α signaling TUDCA induced G0s2 expression, whereas UDCA had no effect.

6.6. Bile acids regulate Igfbp1 in a conjugation-dependent way

IGFs are single-chain protein homologues of insulin which stimulate cell proliferation, migration and differentiation and exert other insulin-like effects (96). The vast majority of IGFs are bound to IGFBPs (IGFBP1-6) that prolongate their half-life and modulate their biologic activity (96). Hence, IGFBPs are believed to play a role in the pathogenesis of metabolic diseases, neuronal diseases and cancer (97). Published data on potential regulatory effects of BAs on the expression of Igfbps are still missing.

In this study, we provide evidence that BA administration leads to major alterations in Igfbp1 mRNA expression and protein synthesis. Moreover, we were able to demonstrate robust conjugation-dependent effects on mRNA and protein level. TCA led to a 31-fold induction of hepatic Igfbp1 expression, while CA had no significant effect (Fig. 18A). In contrast, both UDCA and TUDCA strongly repressed hepatic Igfbp1 expression in line with their function as feeding signals (Fig. 18B). Western blot analysis revealed that serum protein levels paralleled hepatic mRNA expression levels (Fig. 19).

Degenhardt et al. (49) found that Igfbp1 is a target gene of Ppars, including Ppar- α . In the current study, however, Igfbp1 expression levels behaved contrarily to

those of Ppar- α or other Ppar- α target genes (Acox1, G0s2, Cyp4a14). This suggests alternative regulation of the observed effects, possibly via HNFs or FoxO-1. The hypothesis of FoxO-1 mediating the opposed regulation of Igfbp1 and other Ppar- α targets is supported by a recent study that showed a significant reduction in the hepatic level of FoxO-1 by feeding a synthetic Ppar- α agonist (98). Particularly the contrary effects of BA feeding on Igfbp1 and G0s2 may be attributed to FoxO-1 since FoxO-1 stimulates the expression of Igfbp1 and reduces the expression of G0s2 in the liver (47,99). Finally, elevated Igfbp1 expression could be interpreted as a sign of liver injury and together with a tendency of increased serum ALT and AST activity (Table 2) may suggest higher acute hepatotoxicity of TCA than of CA after 4 days (100).

6.7. Limitations

Restricted animal numbers constitute a severe limitation to this study. Group sizes of 3-6 mice are rather small, even for basic research. Larger mouse numbers presumably would have permitted weak but consistent conjugation-dependent trends in mRNA expression to reach statistical significance.

Another limitation concerns the mouse strain. Not all mouse groups were composed of exactly the same mouse substrain. 3-week feeding of CA and TCA was performed on C57BL/6N mice, whereas all other feedings were performed on C57BL/6J mice. Genetic variations between the substrains may have affected obtained data and confounded data interpretation. Differences in mouse substrains are generally considered to potentially account for deviations of a wide range of metabolic parameters. Thus, comparability of this particular group to other groups may be limited.

The informative value of big parts of the study is restricted to events on a transcriptional level since we predominately determined mRNA levels. Results do not allow drawing conclusions about protein levels and enzyme activities or to deduce implications for potential substrates. Moreover, transferability of the

observed effects to the human condition is restricted due to physiological differences between man and mouse.

Finally, it has to be mentioned that we cannot demonstrate mechanistic explanations for the obtained results. This study rather aimed to detect a wide range of conjugation-dependent effects without focusing on distinct mechanisms.

6.8. Conclusion

Reported data constitute the first comprehensive analysis of BA conjugation *in vivo*. We found that taurine-conjugation alters the transcriptional regulation of major regulatory enzymes of BA, lipid and glucose metabolism. Partly consistent, biometric measurements confirmed and revealed marked conjugation-dependent effects on body weight development and tissue weights. Taken together, decreased liver weight and attenuated expression of hydroxylation and excretion enzymes in the liver suggest lower hepatotoxicity of ingested taurine-conjugated BAs. The observed effects question the general transferability of experiments using unconjugated BAs to a physiologic condition and encourage reconsidering advantages and disadvantages of conjugated BAs in medical use.

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