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Short-Term Feedback Regulation of Bile Salt Uptake by Bile Salts in Rodent Liver

Stefanie Mühlfeld,[1] Olga Domanova,[2,3,4] Thomas Berlage,[2,3,4] Claudia Stross,[1] Angelika Helmer,[1] Verena Keitel,[1] Dieter Häussinger,[1] and Ralf Kubitz[1]

The sodium taurocholate cotransporting polypeptide (Ntcp) is the major bile salt uptake transporter at the sinusoidal membrane of hepatocytes. Short-term feedback regulation of Ntcp by primary bile salts has not yet been investigated *in vivo*. Subcellular localization of Ntcp was analyzed in Ntcp-transfected HepG2-cells by flow cytometry and in immunofluorescence images from tissue sections by a new automated image analysis method. Net bile salt uptake was investigated in perfused rat liver by a pulse chase technique. In Flag-Ntcp-EGFP (enhanced green fluorescent protein) expressing HepG2-cells, taurochenodeoxycholate (TCDC), but not taurocholate (TC), induced endocytosis of Ntcp. TCDC, but not TC, caused significant internalization of Ntcp in perfused rat livers, as shown by an increase in intracellular Ntcp immunoreactivity, whereas Bsep distribution remained unchanged. These results correlate with functional studies. Rat livers were continuously perfused with 100 μmol/L of TC. 25 μmol/L of TCDC, taurodeoxycholate (TDC), tauroursodeoxycholate (TUDC), or TC were added for 30 minutes, washed out, followed by a pulse of ^{3}[H]-TC. TCDC, but not TDC, TUDC, or TC significantly increased the amount of ^{3}[H]-TC in the effluent, indicating a reduced sinusoidal net TC uptake. This effect was sensitive to chelerythrine (protein kinase C inhibitor) and cypermethrin (protein phosphatase 2B inhibitor). Phosphoinositide 3-kinase (PI3K) inhibitors had an additive effect, whereas Erk1/2 (extracellular signal activated kinase 1/2), p38MAPK, protein phosphatase 1/2A (PP1/2A), and reactive oxygen species (ROS) were not involved. *Conclusion*: TCDC regulates bile salt transport at the sinusoidal membrane by protein kinase C- and protein phosphatase 2B-mediated retrieval of Ntcp from the plasma membrane. During increased portal bile salt load this mechanism may adjust bile salt uptake along the acinus and protect periportal hepatocytes from harmful bile salt concentrations. (HEPATOLOGY 2012;56:2387-2397)

Bile salts are essential for biliary cholesterol excretion and lipid absorption from the intestine. In addition, increased bile salt concentrations may alter cholesterol biosynthesis,[1] energy expenditure,[2] immune functions,[3] hepatic microcirculation,[4] and may induce apoptosis.[5] Therefore, liver cells express several bile salt transport proteins in order to maintain bile salt homeostasis. The sodium taurocholate cotransporting polypeptide (Ntcp/Slc10a1) is the major uptake system for conjugated bile salts from blood into liver parenchymal cells.[6] Together with several organic anion transporting polypeptides (Oatp1a1/Slc21a1, Oatp1a4/Slc21a5, and Oatp1b2/Slc21a10),[7] it controls bile salt uptake at the sinusoidal membrane. Secretion of bile salts at the canalicular membrane is mediated by the bile salt export pump (Bsep,

Abbreviations: cAMP, cyclic adenosine monophosphate; CDC, chenodeoxycholate; EEA-1, early endosome antigen 1; EGFP, enhanced green fluorescent protein; ERK1/2, extracellular signal activated kinase 1/2; Mrp2/3/4, multidrug resistance associated protein 2/3/4; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; p38MAPK, p38 mitogen activated protein kinase; PI3K, phosphoinositide-3-kinase; PP2A/B, protein phosphatase 2A/B; ROS, reactive oxygen species; TC, taurocholate; TCDC/GCDC, tauro-/glycochenodeoxycholate; TC/TCDC*, TC-/TCDC-concentration estimated by radioactivity measurements of ^{3}[H]-TC or ^{3}[H]-TCDC in effluent and bile; TDC, taurodeoxycholate; TLC, taurolithocholate; TLCS, taurolithocholic acid 3-sulfate; TUDC, tauroursodeoxycholate.*

From the [1]Department of Gastroenterology, Hepatology and Infectious Diseases, Medical Faculty, Heinrich-Heine University, Düsseldorf, Germany; [2]Fraunhofer Institute for Applied Information Technology FIT, Sankt Augustin, Germany; [3]RWTH Aachen, Department of Computer Science, Aachen, Germany; [4]Bonn-Aachen International Center for Information Technology B-IT, Bonn, Germany.

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ABCB11)[8] and to a lesser extent by the multidrug resistance associated protein Mrp2 (ABCC2).[9] Furthermore, bile salt export back into the blood may also occur at the sinusoidal membrane and may be mediated by Mrp3 (ABCC3) and Mrp4 (ABCC4), two members of the ABC subfamily C, as well as the organic solute transporter OST α/β.[10-12] Rat Ntcp is an integral membrane glycoprotein with putative seven transmembrane domains. On a long-term scale bile salts down-regulate Ntcp at the level of gene transcription, which is mediated by the farnesoid-X-receptor (FXR) and the short heterodimer partner 1 (SHP1).[13] On a short-term scale, transport capacity of Ntcp is controlled by regulated insertion of Ntcp into and retrieval from the sinusoidal membrane, a microfilament-dependent process.[14] Insertion into the plasma membrane is stimulated by cyclic adenosine monophosphate (cAMP)[15] and involves dephosphorylation of Ntcp at serine and threonine sites.[16] Recently, we could show in Ntcp-transfected HepG2-cells that activation of protein kinase C reduces surface expression of Ntcp by 30%-40% within 60 minutes and induces the formation of Ntcp-containing vesicles.[17] To date, little is known about the possible role of primary bile salts in short-term regulation of Ntcp, a process that appears to be favorable in terms of rapid adaptations to varying concentrations of bile salts in portal blood. In order to overcome the methodological problems of this issue, we established a ^{3}[H]-TC (taurocholate) pulse technique in perfused rat liver and introduced a new automated image-processing approach to study localization of basolateral and canalicular transport proteins in images from perfused rat livers. We demonstrated that physiological portal concentrations of taurochenodeoxycholate (TCDC), an endogenous substrate of Ntcp, down-regulate Ntcp by retrieval from the sinusoidal membrane.

Materials and Methods

Materials. The anti-Ntcp antibody (K4)[6] was a gift from Dr. Stieger (Kantonsspital Zürich, Switzerland). The anti-Bsep antibody (K24) was raised in rabbit.[18] The anti-FLAG antibody (M2) was from Sigma Aldrich (Deisenhofen, Germany), the Na^+-K^+-ATPase antibody (C464.6) from Millipore (Billerica, MA), the anti-Zo-1 antibody (ZO-1-1A12) from Invitrogen (Karlsruhe, Germany), the anti-EEA1 antibody (14/EEA1) from BD Transduction Laboratories (Franklin Lakes, NJ). Bile salts were from Sigma Aldrich, ^{3}[H]-TC from DuPont (Bad Homburg, Germany), ^{3}[H]-TCDC from Hartmann Analytic (Braunschweig, Germany). Apocynin, PD098059, cypermethrin, wortmannin, chelerythrin, and okadaic acid were from Calbiochem (Darmstadt, Germany), SB202190 from Alexis (Grünberg, Germany).

Flow Cytometry. The FLAG-Ntcp-EGFP plasmid was cloned as described previously.[17] HepG2 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) Nutrimix F12 (Invitrogen), 10% fetal calf serum (PAA, Coelbe, Germany). FLAG-Ntcp-EGFP was transfected into HepG2 cells using Lipofectamine2000 (Invitrogen), stable clones were selected by 0.5% Geneticin (Gibco, Karlsruhe, Germany). Three independent clones were established. FLAG-Ntcp-EGFP expressing HepG2 cells were stimulated with 100 μM of different bile salts (1 hour, 37°C), washed with ice-cold phosphate-buffered saline (PBS), and incubated with Accutase (Millipore, Schwalbach, Germany) at 37°C. Cells were centrifuged (30 seconds, 4,500*g*), resuspended in PBS, and filtered through 70 μm gauze. Cells were incubated with the anti-Flag M2 antibody (labeled with Alexa Fluor R-647-PE) at a dilution of 1:2,500 (30 minutes, 4°C). Fluorescence intensities of EGFP and Alexa Fluor-647-PE were measured in 20,000 single cells in a FACSCalibur Flow Cytometer (Becton Dickinson, Heidelberg, Germany) using an excitation wavelength of 488 nm. Cells were gated for their characteristic forward and sideward scatter. Fluorescent measurements were acquired at 530 $\pm$ 30 nm (EGFP) and >670 nm (Alexa Fluor 647-PE). Identical settings were used to compare cells from different conditions.

Immunofluorescence. Livers were perfused with 100 μmol/L of TC (control) or TCDC for 60 minutes. Sample preparation and immunostaining were performed according to a standard operating procedure to assure reproducibility. Before and after addition of TCDC or TC, liver lobes were excised and shock-frozen in liquid nitrogen. Cryosections (5 μm) were fixed in methanol (10 minutes, −20°C) and incubated with the

Address reprint requests to: Ralf Kubitz, M.D., Department of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine University of Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany. E-mail: kubitz@med.uni-duesseldorf.de; fax: (49) 211 8117517.

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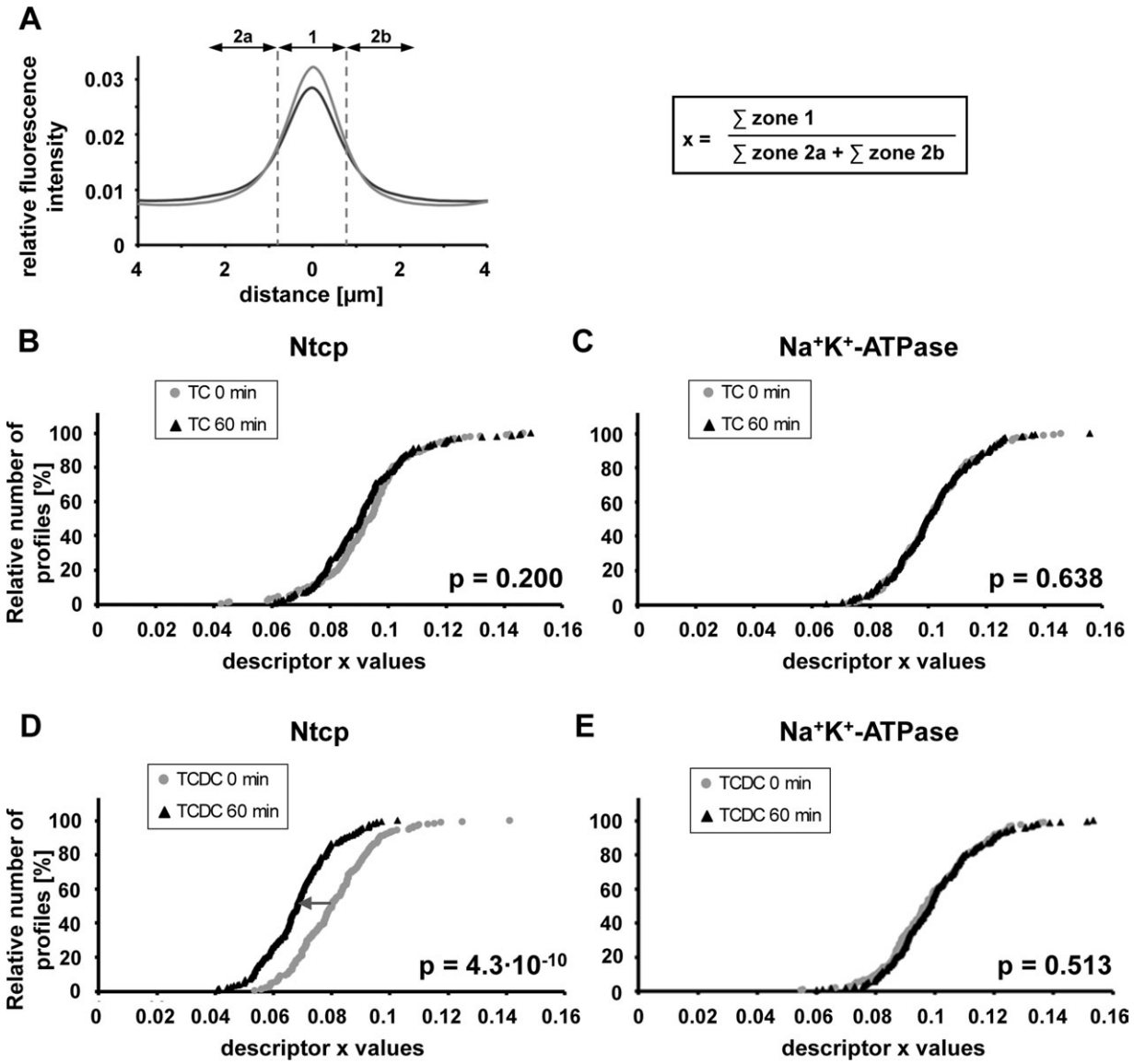

Fig. 1. Automated image analysis reveals retrieval of Ntcp from the plasma membrane in TCDC, but not TC, perfused rat liver samples. Cryosections from perfused rat livers were prepared for confocal microscopy by immunohistochemistry and analyzed by automated image processing. The numerical descriptor x was introduced to assess transport protein translocation from the basolateral membrane into the cells. It describes the ratio between fluorescence intensities at the sinusoidal membrane (zone 1) and fluorescence intensities on either side of the membrane (zone 2a and 2b) (A). Descriptor x was calculated for each individual Ntcp and Na^+K^+-ATPase intensity profile and the Wilcoxon rank sum test was applied to compare datasets. Automated intensity profile measurements revealed a significant change in Ntcp distribution in TCDC (D, $P < 0.005$), but not in TC (B) stimulated livers within 60 minutes. TCDC perfusion resulted in a shift of descriptor x to lower values (D), indicating the translocation of Ntcp into the cells. Na^+K^+-ATPase distribution remained unchanged under both conditions (C,E).

primary antibodies: anti-Ntcp (1:200) and anti-Na^+-K^+-ATPase (1:500) (or anti-Bsep [1:30], and anti-Zo-1 [1:750]), and secondary antibodies Alexa-Fluor546 goat anti rabbit (1:500), and Alexa-Fluor488 goat anti mouse (1:500) in a microarray hybridization station (Advalytix Slide Booster, Implen, Munich, Germany). Tissue samples were analyzed using a LSM-510 confocal laser scanning system (Zeiss, Jena, Germany).

Automated Densitometric Fluorescence Intensity Analysis. Cryosections of perfused rat liver were stained for Ntcp ("functional" marker) and Na^+-K^+-ATPase ("structural" marker). In confocal images, Ntcp and Na^+-K^+-ATPase distribution was analyzed by automated image processing. Basolateral membranes were identified by foreground-background detection and skeletonized. Fluorescence intensity profiles of the "structural" and "functional" marker proteins were extracted perpendicular to the basolateral membrane (Supporting Fig. 1). Na^+K^+-ATPase profiles were selected according to three conditions. Acceptable intensity profiles have a sufficiently high peak fluorescence in the central part (corresponding to the basolateral membrane) and low

intracellular fluorescence at the tails. Therefore, the first condition rejects profiles if:

$$\frac{\sum_{l=0}^{30} I_l + \sum_{m=50}^{80} I_m}{62} \bullet 2 > \frac{\sum_{n=31}^{49} I_n}{19}$$

where integral fluorescence intensities from different parts of the profile are compared.

The second condition eliminates asymmetric profiles, by comparing the integral fluorescence intensities of the tails:

$$\text{ratio } 1 = \frac{\sum_{i=0}^{30} I_i}{\sum_{j=50}^{80} I_j}$$

Profiles are rejected if:

$$((\text{ratio } 1 > 2.5)||(\text{ratio } 1 < 0.4))$$

is true. For the third condition a median filtering algorithm is applied to the intensity profiles. Local minima and maxima are determined. If:

$$\text{number of local maxima} = 0$$

profile is rejected as a straight line. If:

$$\text{number of local maxima} = 1$$

profile is accepted. Only one high peak is found. Profiles with more than one local maximum:

$$\text{Number of local maxima} > 1$$

are analyzed for relative position and height of these maxima. They are accepted if:

$$\text{second maximum} < (0.5 \bullet \text{first maximum})$$

or rejected if:

$$\text{vect F[minimum]} < (0.6 \bullet \text{second maximum})$$

where a significantly deep local minimum separates two maxima in close proximity. Profile selection reduces the initial number of intensity profiles per image from ≈20,000 to ≈4,000. Subsequently, accepted profiles are ranked according to an inverted relative peak height:

$$\frac{\sum_{p=0}^{19} I_p + \sum_{q=61}^{80} I_q}{\sum_{r=30}^{50} I_r}$$

The higher the peak relative to the tails, the smaller the ranking criteria and the higher the rank assigned. According to their rank, 20 Na^+K^+-ATPase profiles are taken per image. These profiles and the corresponding Ntcp intensity profiles are considered for statistical evaluation. The numerical descriptor x, which describes the ratio of Ntcp fluorescence intensity at the plasma membrane (zone 1; 1.6 μm in the center of the profile) and the cytoplasm near the plasma membrane (zones 2a and 2b; 3.2 μm each) (Fig. 1A), was calculated from the extracted intensity profiles for relative quantification.

Measurements of TC Uptake in the Perfused Rat Liver. Animals received care according to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, revised 1985; and APS guidelines). The study protocol was approved by the local authorities. Livers of male Wistar rats (150 g) were perfused in a nonrecirculating system as described[19] with a flow rate of 4 mL/g liver/min, where liver weight was estimated to be 4% of body weight. TC was present at a concentration of 100 μmol/L in the influent throughout the experiment in order to saturate bile salt transport. Samples from the effluent were collected every minute. The common bile duct was cannulated and bile samples were collected every minute. After 50 minutes, when biliary bile salt secretion reached a steady state, further 25, 40, or 100 μmol/L of TC (control), TCDC, TDC, TUDC, or taurolithocholate (TLC) were added for 30 minutes (Fig. 2). After a wash-out period of 10 minutes, ^{3}[H]-TC or ^{3}[H]-TCDC was added to the perfusate for 5 minutes in a nanomolar range (dissolved in 100 μmol/L of TC or TCDC, respectively). Samples from effluent and bile were collected for 60 minutes. Radioactivity was measured by a scintillation counter (Packard Instruments, Frankfurt, Germany). Measurements of radioactivity were used for the calculation of TC- (or TCDC-)concentrations in bile and effluent, in the following referred to as TC* (TCDC*). In inhibitor assays the inhibitors cypermethrin (1 μM), wortmannin (100 nM), SB202190 (150 nM), PD98059 (1 μM), chelerythrine (1 μM), apocynin (20 μM), okadaic acid (5 nM) were added 20 minutes before bile salt stimulation for a total time of 110 minutes (Fig. 2).

Statistics. Data were reproduced in three to five independent experiments. For the automated image analysis, data from 10 different areas per tissue sample were processed. Values are given as means ± standard deviations. Perfusion and colocalization experiments were analyzed using Student's t test. $P < 0.05$ was considered statistically significant. Densitometric analysis of protein distribution in immunofluorescence images were performed using

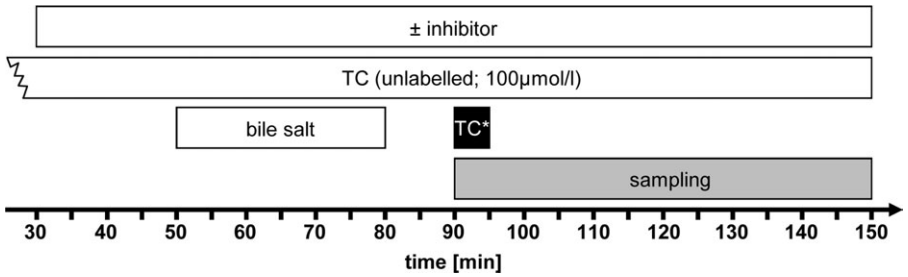

Fig. 2. Perfusion protocol. Livers were perfused with 100 μmol/L of TC throughout the experiment. Twenty-five μmol/L of different bile salts (TC, TCDC, TDC, or TUDC) was added to the perfusate on top for 30 minutes. After a washout period of 10 minutes ^{3}[H]-TC (=TC*; dark box) was added for 5 minutes. Samples from bile and effluent were collected for the subsequent 60 minutes. Inhibitor assays were performed by the addition of inhibitors 20 minutes before stimulation with bile salts. In these experiments TC or TCDC were added in a concentration of 100 μmol/L in order to achieve maximal stimulation.

Wilcoxon rank sum test. $P < 0.005$ was considered statistically significant.

Results

TCDC Stimulates Ntcp Retrieval from the Basolateral Membrane. HepG2 cell clones, expressing Ntcp with an intracellular EGFP- and an extracellular FLAG-tag, were treated with 100 μM of TC, TCDC, taurolithocholic acid 3-sulfate (TLCS), or TLC. Bile salts had no effect on forward scatter (cell size), side scatter (cell granulation) (data not shown), or general cell morphology (Supporting Fig. 2C,D). Although the total amount of NTCP, as evidenced by the green fluorescence from the intracellular EGFP-tag, was unaffected (Fig. 3B), extracellular FLAG-associated fluorescence was reduced by TCDC as compared to control (Fig. 3A). This suggests that TCDC reduces Ntcp at the plasma membrane by retrieval into intracellular compartments. Even stronger effects were observed for cells stimulated with more hydrophobic bile salts, such as TLCS or TLC (Supporting Fig. 2A).

TCDC Induces Redistribution of Ntcp in Perfused Rat Liver. In confocal images obtained from TCDC treated livers, intracellular Ntcp fluorescence intensity was markedly increased within the cells as compared to control livers (TC). However, there was no obvious change in Na^+/K^+-ATPase distribution at the basolateral membrane (Fig. 1C,E; Supporting Fig. 3). Subcellular Ntcp distribution was quantified by automated image processing. Within 60 minutes of TCDC, but not TC perfusion, descriptor x values (Fig. 1A) shifted significantly towards lower values in all datasets ($P < 0.005$, Fig. 1B,D). These results suggest that TCDC induces endocytosis of Ntcp into intracellular compartments in the intact organ.

TCDC Stimulates Ntcp Endocytosis in EEA1-Positive Vesicles. In confocal images of tissue sections from TC- or TCDC-perfused livers, Ntcp and EEA1 colocalizing pixels were quantified by calculation of the weighted colocalization coefficient according to a preset threshold. After TCDC perfusion colocalizing pixels in the cytoplasm were markedly increased

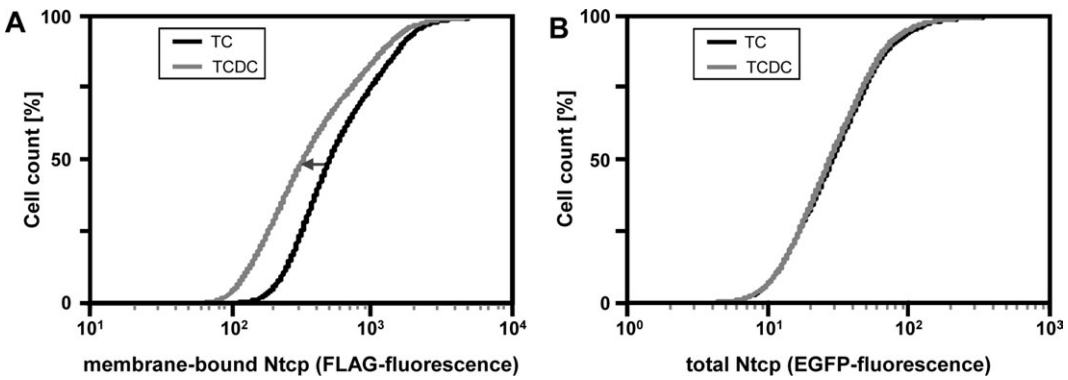

Fig. 3. TCDC induces retrieval of Ntcp from the membrane of HepG2 cells. HepG2 cells stably expressing FLAG-Ntcp-EGFP were treated with 100 μM of different bile salts for 1 hour at 37°C and analyzed by flow cytometry. Ntcp in the plasma membrane was quantified by a fluorochrome labeled FLAG-antibody, which detected the extracellularly localized FLAG-tag in unpermeabilized cells. Total Ntcp was measured by EGFP fluorescence. Fluorescence intensity distributions are shown in cumulative histograms. Cells showed significant reduction of FLAG-associated fluorescence after preincubation with 100 μM TCDC, compared to TC (=control; A), whereas green fluorescence (EGFP) was not affected (B).

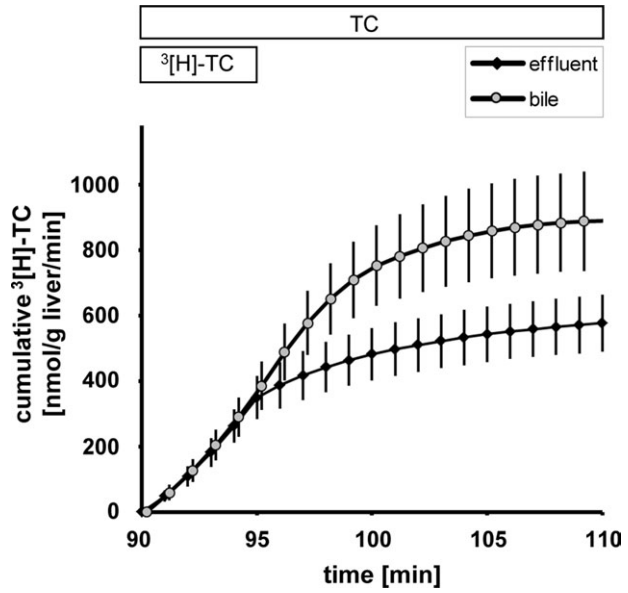

Fig. 4. TC* recovery from effluent and bile. TC was perfused at a concentration of 100 μmol/L throughout the experiment. After 90 minutes ^{3}[H]-TC was added for 5 minutes. Samples from effluent and bile were collected every minute and radioactivity was monitored over time. During a single acinar passage of the perfusate almost two-thirds of TC* is secreted into bile.

(Supporting Fig. 4F,H), indicating endocytosis of Ntcp-containing vesicles. The number of colocalizing Ntcp- and EEA1-positive pixels was significantly increased from 45 ± 17% to 74 ± 19% (TCDC), compared to 39 ± 7% to 37 ± 16% (TC) (Supporting Fig. 4I).

Subcellular Bsep Distribution Is Unaffected by TC or TCDC Stimulation. Livers were perfused with TC or TCDC for 60 minutes, cryosectioned, and stained. In confocal images of Bsep and Zo-1, subcellular Bsep distribution was quantified by automated image processing (for details, see Supporting material). Subcellular Bsep distribution was unchanged irrespective of bile salt treatment, as demonstrated by statistical evaluation of descriptor y values (Supporting Fig. 5F,H). This indicates that endocytosis of the canalicular transporter Bsep is not stimulated by TCDC and that short-term regulation of Ntcp is independent of Bsep.

Measurements of TC- or TCDC-Uptake in the Perfused Rat Liver. With 100 μmol/L of TC in the perfusate, bile flow reached a steady state within 50 minutes. Between 50 and 80 minutes of perfusion, up to 100 μmol/L of TCDC, TUDC, TDC, or TC (=control) were added on top. After a washout period of 10 minutes, ^{3}[H]-TC was added for 5 minutes (Fig. 2). Under control conditions almost two-thirds of TC are extracted from the perfusate and excreted into bile during a single azinar passage of the perfusate (Fig. 4). When 25 μmol/L of TCDC were perfused prior to ^{3}[H]-TC, the amount of TC* recovered from the effluent was 1.1 ± 0.3 μmol/g liver, which equals 55.9 ± 16.8% TC* from total input (Fig. 5). This was two times more as compared to control conditions (24.8 ± 6.2% TC* from total input). Vice versa, TC* in bile was reduced to 3.1 ± 1.1 μmol/g liver by TCDC stimulation (32.0 ± 12.8% of TC* from total input). When TC, TDC, or TUDC (each 25 μmol/L on top of 100 μmol/L of TC) were perfused for 30 minutes prior to the addition of ^{3}[H]-TC, there was no significant difference in the recovery of TC in effluent or bile as compared to control (Fig. 5; Supporting Fig. 6). On the other hand, the known cholestatic bile salt TLC led to complete cholestasis with a stop of bile flow at a concentration of 100 μmol/L, whereas 40 μmol/L of TLC induced a reduction of net sinusoidal uptake of the same magnitude as TCDC (62.6 ± 11.2% TC* from total input, compared to 55.6 ± 11.2% TC* from total input for TLC or TCDC, respectively) (Supporting Figs. 6, 7).

To exclude simple substrate competition, TC and ^{3}[H]-TC were replaced by TCDC and ^{3}[H]-TCDC. In case of substrate competition the TCDC-induced effect should be abrogated in an experimental setup without TC. However, the amount of TCDC* recovered from the effluent was even four times higher than in control experiments (93.1 ± 10.6% from total

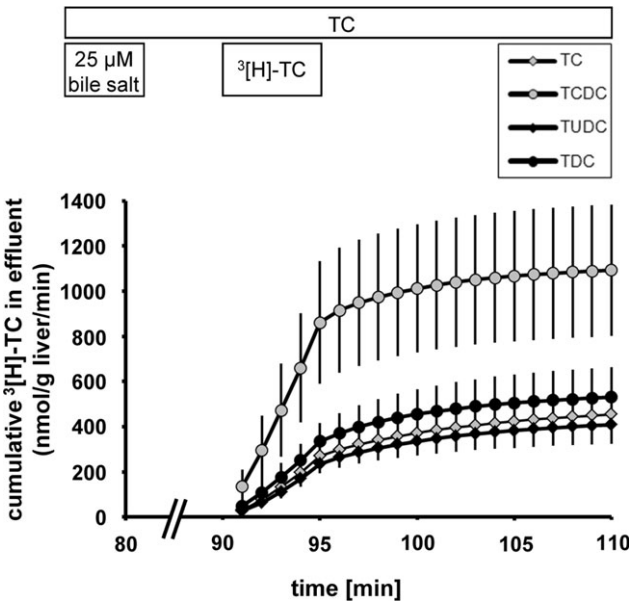

Fig. 5. Preperfusion with TCDC, but not TC, TDC, and TUDC, significantly increases TC* recovery from effluent. When 25 μmol/L of TCDC was perfused prior to the ^{3}[H]-TC pulse, the amount of TC* recovered from the perfusate was two times more as compared to control conditions. Preperfusion with TC, TDC, or TUDC (each 25 μmol/L) caused no significant difference in TC* recovery in effluent or bile.

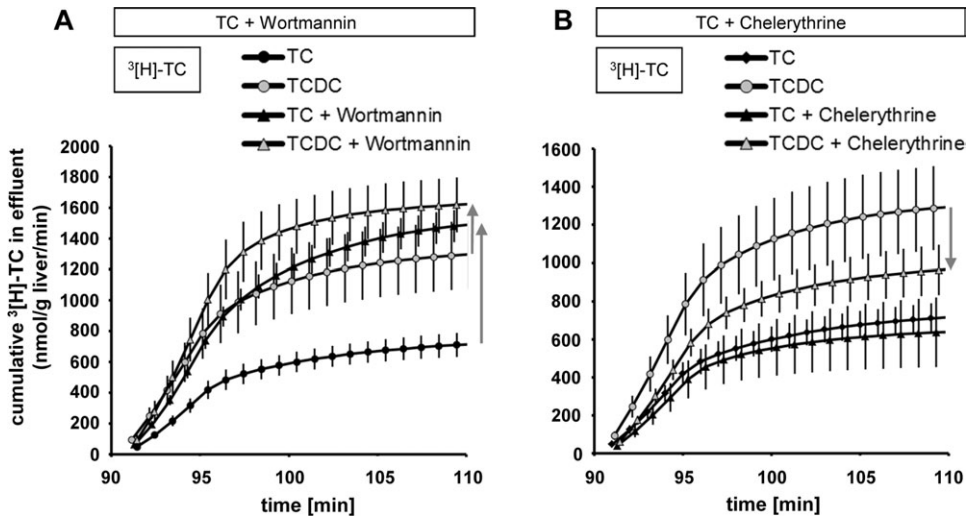

Fig. 6. The PI3K and PKC have divergent effects on TCDC-dependent Ntcp regulation. Inhibitors were added 20 minutes before stimulation with 100 μmol/L of TC or TCDC (see Fig. 2 for details), followed by a 5-minute pulse of ^{3}[H]-TC. Inhibition of PI3K by wortmannin significantly increased TC* recovery in the effluent in TC and TCDC stimulated livers (A). In contrast, the PKC inhibitor chelerythrine decreased the effect of TCDC on TC* uptake significantly, whereas TC stimulated TC* recovery was unchanged (B).

input) and bile flow was strongly reduced (Supporting Fig. 8), indicating that substrate competition was not responsible for reduced net TC or TCDC uptake. The importance of taurine conjugation in TCDC-induced reduction of net TC uptake was analyzed by chenodeoxycholate (CDC) and glycochenodeoxycholate (GCDC) perfusion. Although CDC had a less pronounced and statistically not significant effect on ^{3}[H]-TC-uptake (51.9 ± 11.3% TC* from total input in CDC versus 65.8 ± 8.7% in TCDC stimulated livers), the glycine conjugate GCDC elicited a very similar effect on TC* recovery in effluent and bile as compared to TCDC (68.3 ± 6.4% TC* from total input in GCDC versus 65.8 ± 8.7% in TCDC stimulated livers) (Supporting Fig. 6). Taken together, CDC, its conjugates, and TLC, the secondary bile salt derived from CDC by dehydroxylation, but not other tested bile salts, reduced net uptake, suggesting that the effect was specific for certain bile salts.

Phosphoinositide-3 Kinase (PI3K) Is Not Deactivated by TCDC. Activation of PI3K is known to stimulate Ntcp exocytosis and to increase TC uptake.[20]

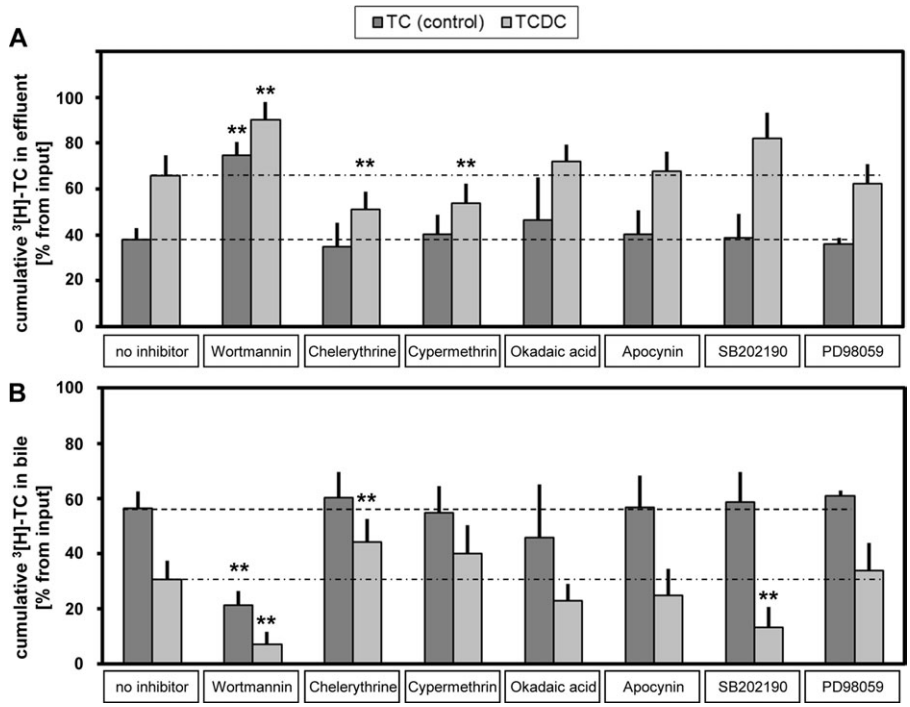

Fig. 7. Comparative analysis of the effects of different inhibitors on TC* recovery in effluent and biliary secretion. TCDC (light gray) increased TC* recovery in effluent (A) and decreased TC* secretion into bile (B) as compared to TC (dark gray). Inhibition of PI3K by wortmannin induced cholestasis independent of TC/TCDC. PKC-inhibition by chelerythrine and PP2B-inhibition by cypermethrin partly inhibited the effect of TCDC on sinusoidal uptake. Inhibition of PP2A (okadaic acid), ROS-scavenging (apocynin), inhibition of p38MAPK (SB202190), or Erk1/2 (PD98059) had no significant effects. For experimental details see Fig. 2 and Materials and Methods. Data are given as means ± standard deviations of the percentage of TC* from total input. **Statistically significant differences ($P < 0.05$).

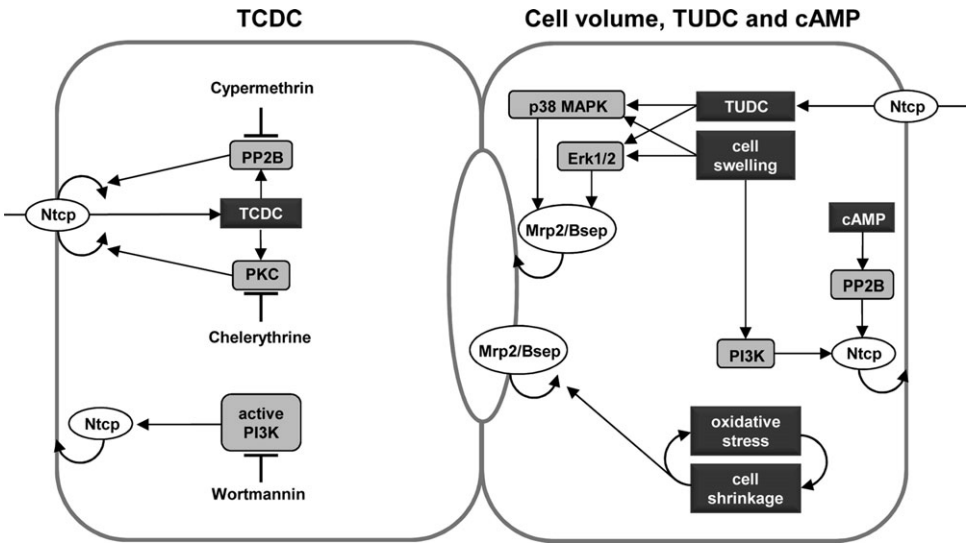

Fig. 8. Short-term regulation of bile salt transporters in rat liver. Schematic illustration of signaling pathways involved in the short-term regulation of Ntcp and the canalicular transporters Bsep and Mrp2. TUDC and cell swelling induces choleresis by Erk1/2 or p38MAPK-mediated insertion of Bsep/Mrp2 into the canalicular membrane.[25,48,49] Furthermore, cell swelling causes Ntcp exocytosis by way of PI3K.[20] cAMP stimulates protein phosphatase 2B-mediated dephosphorylation of NTCP and leads to an increased retention of Ntcp at the plasma membrane.[44] In contrast, NADPH-dependent ROS formation (oxidative stress) triggers cell shrinkage and cholestasis by endocytosis of Bsep/Mrp2.[5,31,53] In this study we show that TCDC induce PKC- and PP2B-mediated inhibition of sinusoidal TC uptake, most likely by internalization of Ntcp. This is counteracted by the PI3K.

Here, in perfused rat liver inhibition of PI3K by wortmannin significantly decreased TC* uptake after TC as well as TCDC stimulation. Net TC*-uptake was decreased from 63 ± 5% to 25 ± 6% from total input in TC-stimulated livers. Likewise, in TCDC perfused livers the cholestatic effect of TCDC was enhanced and net TC* uptake was decreased from 33 ± 10% to 10 ± 8% (Figs. 6A, 7A; Supporting Fig. 9). In bile, wortmannin decreased the amount of TC* from 57 ± 7% to 21 ± 5% TC* from total input in TC perfused livers, and from 30 ± 8% to 7 ± 4% in TCDC perfused livers (Fig. 7B; Supporting Fig. 9). Taken together, deactivation of PI3K is not the mechanism that mediates TCDC-induced bile salt retention.

TCDC-Dependent Effect Is Partly Mediated by PKC and PP2B. Activation of protein kinase C (PKC) isoforms by phorbolesters is known to induce cholestasis[21] and bile salts were shown to activate PKCs.[22,23] The PKC activator phorbol-12-myristate-13-acetate (PMA) caused a significant increase of TC* in the effluent from 33.9 ± 6.3% to 80.3 ± 2.3% of total input. This effect was concentration-dependent (data not shown). When chelerythrine was perfused 20 minutes before and during TCDC perfusion, the effect of TCDC on TC*-uptake was significantly decreased from 66 ± 9% to 51 ± 8% TC* from total input (Fig. 6B,7A; Supporting Fig. 9). Furthermore, PP2B is known to dephosphorylate Ntcp at Serine[226], which is involved in Ntcp exocytosis.[24] Here, the PP2B inhibitor cypermethrin decreased the amount of TC* in the effluent in TCDC stimulated livers from 66 ± 9% to 54 ± 8% TC* from total input (Fig. 7A; Supporting Fig. 9).

TCDC-Dependent Effect on Net TC Uptake Is Insensitive To the Inhibitors Apocynin, SB202190, Okadaic Acid, and PD98059. Hydrophobic bile salts can trigger hepatocyte shrinkage by NADPH oxidase-dependent formation of reactive oxygen species (ROS).[5] However, apocynin, an NADPH oxidase inhibitor, had no modulating effect on TC* recovery in the effluent in either TC- or TCDC-stimulated livers. Cell swelling or TUDC activate p38MAPKinases, which regulate insertion of the bile salt export pump Bsep into the canalicular membrane.[25] Furthermore, hepatocyte swelling stimulated rapid Ntcp insertion into the basolateral membrane and increases TC uptake in hepatocytes.[20] In perfusion experiments p38MAPKinase inhibition with SB202190 had an additive effect on TCDC-dependent inhibition of TC* secretion into bile. SB202190 significantly decreased the amount of TC* in bile in TCDC-perfused livers from 31 ± 7% to 13 ± 7% total TC* input. The effect observed in the effluent was statistically not significant. Phorbolesters activate the MAP kinase kinase MEK1 by way of PKC.[26] To test whether MEK1 is a downstream effector of TCDC, livers were perfused with the MEK1 inhibitor PD98059. An effect on TC* recovery in the effluent or secretion into bile was not observed.

The inhibitor okadaic acid, an inhibitor of PP1/2A, which inhibits cAMP-induced increases in cytosolic Ca^{+} and Ntcp translocation to the plasma membrane[24] was also tested. It was shown that okadaic acid does not significantly alter TCDC-mediated decreases in TC* uptake (Fig. 7; Supporting Figs. 9, 10).

Discussion

This article describes a novel mechanism of regulation of bile salt transport *in vivo*, which involves endocytosis of Ntcp in hepatocytes in response to CDC and its conjugates. Maintenance of bile salt homeostasis is important because bile salts exert many biological effects in liver and extrahepatic organs.[27] Long-term adaptation of bile salt transporters involves changes at the messenger RNA (mRNA) and protein levels,[28] whereas covalent modifications (e.g., phosphorylation),[29] substrate availability,[30] or rapid endo- and exocytosis of transporter-containing vesicles[19,31] represent modalities of short-term regulation. Current concepts assume mechanisms that would limit intracellular accumulation of potentially toxic bile salts under cholestatic conditions.[17,28,32] This includes down-regulation of Ntcp[33] or OATPs[34] at the basolateral membrane in order to limit bile salt uptake, or up-regulation of Mrp4,[34] Mrp3,[35] and OSTα/β,[12] which may enhance bile salt secretion back into the systemic circulation. Bile salt inflow to the liver may increase considerably under pathophysiological conditions, but may also reach physiological concentrations of up to 150 μmol/L (in rat).[36] TCDC was shown to rapidly decrease bile salt uptake[37]; however, the underlying mechanism has not yet been characterized.

In our study, TCDC (and other hydrophobic bile salts) induced Ntcp endocytosis in Ntcp-expressing HepG2 cells as shown by a recently established flow cytometric method[17] (Fig. 3; Supporting Fig. 2). In line with these findings, TCDC induced a redistribution of Ntcp-immunoreactivity *in vivo* (Supporting Fig. 3C,D) as shown by a new automated image analysis technique. TCDC, but not TC, decreased Ntcp at the plasma membrane and increased cytoplasmic Ntcp, as assessed by the numerical descriptor x, which compares plasma membrane to cytoplasmic fluorescence intensities (Fig. 1A). Furthermore, TCDC stimulation results in a significant increase of colocalization of Ntcp and EEA1, indicating Ntcp endocytosis in early endosomes (Supporting Fig. 4). In contrast, TCDC perfusion had no effect on Bsep fluorescence intensity distribution (Supporting Fig. 5).[38] Subsequent functional analysis in perfused rat liver revealed that CDC and its taurin- and glycin-conjugates induce a significant reduction of net bile salt uptake within minutes (Fig. 5; Supporting Fig. 6). Based on perfusion experiments in dog liver it was suggested that TCDC inhibited TC-uptake by substrate competition.[39] In Huh7 cells expressing human NTCP, TLC competitively inhibits TC uptake,[40] whereas in rat hepatocytes a noncompetitive inhibition of bile salt uptake by TLC has been described.[41] In our setup, simple substrate competition was ruled out, because net bile salt uptake was also reduced by TCDC-stimulation when TC and ^{3}[H]-TC were replaced by TCDC and ^{3}[H]-TCDC, respectively (Supporting Fig. 8). Rat Ntcp has a K_M-value of about 5 μM for TCDC.[42] TCDC concentrations >5 μM approach maximum transport velocity and lead to a rapid increase in intracellular TCDC concentrations. Consequently, reduction of V_{max} by an internalization of transport molecules may decrease uptake of TCDC at physiological concentrations.[20] This mechanism may be especially relevant for CDC derivatives, which have unfavorable effects on cell survival.[5]

Apart from this, sensitivity of TCDC-induced cholestasis toward specific inhibitors points to the involvement of distinct signaling pathways stimulated by TCDC. As demonstrated recently in HepG2 cells transfected with rat Ntcp, activation of PKC induces a rapid endocytosis of Ntcp.[17]

Up-regulation of Ntcp by insertion of Ntcp into the plasma membrane is mediated by phosphoinositide-3-kinase (PI3K) signaling pathway and protein phosphatase 2B (PP2B)-mediated dephosphorylation of Ntcp.[43-45] In line with this, inhibition of PI3K by wortmannin significantly decreased bile salt uptake independent of TC- or TCDC-stimulation (Figs. 6A, 7A; Supporting Fig. 9A-C), indicating that PI3K is constitutively active under these conditions.

Inhibition of PKC by chelerythrine significantly modulates TC net uptake in perfused rat livers in TCDC-, but not in TC-stimulated livers (Figs. 6B, 7A; Supporting Fig. 9D-F), which is in line with an involvement of PKC-isoforms in bile secretion.[21,26,46,47] The identification of the responsible isoform needs further investigation.

TUDC or cell swelling is mediated by dual activation of p38MAPK and Erk1/2, which regulate insertion of Bsep into the canalicular membrane and result in choleresis.[25,48,49] Upstream signaling involves integrin sensing, focal adhesion kinases (FAK), and Src kinase activation.[50] In contrast, in isolated rat hepatocytes, inhibition of MEK1, an upstream effector of Erk1/2, failed to affect cell swelling-induced translocation of Ntcp to the plasma membrane.[20] Here, PD98059 or the p38MAPK inhibitor SB202190 had no significant

effect on TCDC-dependent reduction of TC uptake, indicating that Erk1/2 and p38MAPK are not downstream effectors of TCDC. Likewise, the involvement of ROS seems unlikely.

It was shown in isolated rat hepatocytes that PP2B is involved in cAMP-induced exocytosis of Ntcp,[44] whereas PP2A indirectly influenced cAMP-induced translocation of Ntcp.[51] In our setup cypermethrin (an inhibitor of PP2B) but not okadaic acid (an inhibitor of PP2A) significantly inhibited TCDC-induced decrease in net uptake of TC (Fig. 7; Supporting Fig. 9,G-L). Hence, TCDC-dependent regulation of Ntcp activity partly involves PP2B but not PP2A.

A potential role of the membrane-bound bile acid receptor TGR5 cannot be excluded. However, this receptor is not expressed in hepatocytes and is therefore unlikely to mediate the effects of TCDC on the hepatocyte level. TGR5 is expressed in Kupffer cells, which only become responsive to bile acids by way of TGR5 when activated by lipopolysaccharides.[52]

Figure 8 summarizes our findings and known signaling pathways involved in short-term regulation of bile salt transport. Increased portal TCDC concentrations stimulate endocytosis of Ntcp, which may rapidly adjust bile salt uptake along the acinus to protect hepatocytes from toxic bile salt concentrations.

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References

1. Oude Elferink RP, Groen AK. Genetic defects in hepatobiliary transport. Biochim Biophys Acta 2002;1586:129-145.
2. Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, et al. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature 2006;439:484-489.
3. Graf D, Kohlmann C, Haselow K, Gehrmann T, Bode JG, Häussinger D. Bile acids inhibit interleukin-6 signaling via gp130 receptor-dependent and -independent pathways in rat liver. Hepatology 2006;44: 1206-1217.
4. Keitel V, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, et al. The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology 2007;45:695-704.
5. Becker S, Reinehr R, Graf D, vom DS, Häussinger D. Hydrophobic bile salts induce hepatocyte shrinkage via NADPH oxidase activation. Cell Physiol Biochem 2007;19:89-98.
6. Stieger B, Hagenbuch B, Landmann L, Hoechli M, Schröder A, Meier PJ. In situ localisation of the hepatocytic Na + /taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-1787.
7. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. Biochim Biophys Acta 2003;1609:1-18.
8. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998;273: 10046-10050.
9. Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, et al. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 2001;1511:7-16.
10. Hirohashi T, Suzuki H, Takikawa H, Sugiyama Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). J Biol Chem 2000;275:2905-2910.
11. Rius M, Nies AT, Hummel-Eisenbeiss J, Jedlitschky G, Keppler D. Cotransport of reduced glutathione with bile salts by MRP4 (ABCC4) localized to the basolateral hepatocyte membrane. Hepatology 2003; 38:374-384.
12. Ballatori N, Li N, Fang F, Boyer JL, Christian WV, Hammond CL. OST alpha-OST beta: a key membrane transporter of bile acids and conjugated steroids. Front Biosci 2009;14:2829-2844.
13. Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ, et al. The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. Gastroenterology 2001;121:140-147.
14. Dranoff JA, McClure M, Burgstahler AD, Denson LA, Crawford AR, Crawford JM, et al. Short-term regulation of bile acid uptake by microfilament-dependent translocation of rat ntcp to the plasma membrane. Hepatology 1999;30:223-229.
15. Schonhoff CM, Thankey K, Webster CR, Wakabayashi Y, Wolkoff AW, Anwer MS. Rab4 facilitates cyclic adenosine monophosphate-stimulated bile acid uptake and Na+-taurocholate cotransporting polypeptide translocation. Hepatology 2008;48:1665-1670.
16. Mukhopadhyay S, Ananthanarayanan M, Stieger B, Meier PJ, Suchy FJ, Anwer MS. Sodium taurocholate cotransporting polypeptide is a serine, threonine phosphoprotein and is dephosphorylated by cyclic adenosine monophosphate. Hepatology 1998;28:1629-1636.
17. Stross C, Helmer A, Weissenberger K, Görg B, Keitel V, Häussinger D, et al. Protein kinase C induces endocytosis of the sodium taurocholate cotransporting polypeptide. Am J Physiol Gastrointest Liver Physiol 2010;299:G320-G328.
18. Noe J, Stieger B, Meier PJ. Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology 2002;123:1659-1666.
19. Kubitz R, Wettstein M, Warskulat U, Häussinger D. Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. Gastroenterology 1999;116:401-410.
20. Webster CR, Blanch CJ, Phillips J, Anwer MS. Cell swelling-induced translocation of rat liver Na(+)/taurocholate cotransport polypeptide is mediated via the phosphoinositide 3-kinase signaling pathway. J Biol Chem 2000;275:29754-29760.
21. Kubitz R, Saha N, Kühlkamp T, Dutta S, vom Dahl S, Wettstein M, et al. Ca2+-dependent protein kinase C isoforms induce cholestasis in rat liver. J Biol Chem 2004;279:10323-10330.
22. Bouscarel B, Gettys TW, Fromm H, Dubner H. Ursodeoxycholic acid inhibits glucagon-induced cAMP formation in hamster hepatocytes: a role for PKC. Am J Physiol 1995;268:G300-G310.
23. Beuers U, Throckmorton DC, Anderson MS, Isales CM, Thasler W, Kullak-Ublick GA, et al. Tauroursodeoxycholic acid activates protein kinase C in isolated rat hepatocytes. Gastroenterology 1996;110: 1553-1563.
24. Anwer MS, Gillin H, Mukhopadhyay S, Balasubramaniyan N, Suchy FJ, Ananthanarayanan M. Dephosphorylation of Ser-226 facilitates plasma membrane retention of Ntcp. J Biol Chem 2005;280: 33687-33692.
25. Kurz AK, Graf D, Schmitt M, vom Dahl S, Häussinger D. Tauroursodesoxycholate-induced choleresis involves p38(MAPK) activation and translocation of the bile salt export pump in rats. Gastroenterology 2001;121:407-419.
26. Kubitz R, Huth C, Schmitt M, Horbach A, Kullak-Ublick GA, Häussinger D. Protein kinase C-dependent distribution of the Multidrug Resistance Protein 2 from the canalicular to the basolateral membrane in human HepG2 cells. Hepatology 2001;34:340-350.
27. Hylemon PB, Zhou H, Pandak WM, Ren S, Gil G, Dent P. Bile acids as regulatory molecules. J Lipid Res 2009;50:1509-1520.
28. Dawson PA, Lan T, Rao A. Bile acid transporters. J Lipid Res 2009; 50:2340-2357.

29. Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62: 385-427.
30. Häussinger D, Schmitt M, Weiergräber O, Kubitz R. Short-term regulation of canalicular transport. Semin Liver Dis 2000;20:307-321.
31. Schmitt M, Kubitz R, Lizun S, Wettstein M, Häussinger D. Regulation of the dynamic localization of the rat Bsep gene-encoded bile salt export pump by anisoosmolarity. HEPATOLOGY 2001;33:509-518.
32. Wolters H, Elzinga BM, Baller JF, Boverhof R, Schwarz M, Stieger B, et al. Effects of bile salt flux variations on the expression of hepatic bile salt transporters in vivo in mice. J Hepatol 2002;37:556-563.
33. Gartung C, Ananthanarayanan M, Rahman MA, Schuele S, Nundy S, Soroka CJ, et al. Down-regulation of expression and function of the rat liver Na+/bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
34. Keitel V, Burdelski M, Warskulat U, Kühlkamp T, Keppler D, Häussinger D, et al. Expression and localization of hepatobiliary transport proteins in progressive familial intrahepatic cholestasis. HEPATOLOGY 2005;41:1160-1172.
35. Donner MG, Keppler D. Up-regulation of basolateral multidrug resistance protein 3 (Mrp3) in cholestatic rat liver. HEPATOLOGY 2001;34:351-359.
36. Baumgartner U, Miyai K, Hardison WG. Modulation of hepatic biotransformation and biliary excretion of bile acid by age and sinusoidal bile acid load. Am J Physiol 1987;252:G114-G119.
37. Drew R, Priestly BG. Choleretic and cholestatic effects of infused bile salts in the rat. Experientia 1979;35:809-811.
38. Domanova O, Borbe S, Mühlfeld S, Becker M, Kubitz R, Häussinger D, et al. Toponomics method for the automated quantification of membrane protein translocation. BMC Bioinformatics 2011;12:370.
39. Glasinovic JC, Dumont M, Duval M, Erlinger S. Hepatocellular uptake of bile acids in the dog: evidence for a common carrier-mediated transport system. An indicator dilution study. Gastroenterology 1975;69:973-981.
40. Schonhoff CM, Yamazaki A, Hohenester S, Webster CR, Bouscarel B, Anwer MS. PKC{epsilon}-dependent and -independent effects of taurolithocholate on PI3K/PKB pathway and taurocholate uptake in HuH-NTCP cell line. Am J Physiol Gastrointest Liver Physiol 2009;297:G1259-1267.
41. Schwenk M, Schwarz LR, Greim H. Taurolithocholate inhibits taurocholate uptake by isolated hepatocytes at low concentrations. Naunyn Schmiedebergs Arch Pharmacol 1977;298:175-179.
42. Schroeder A, Eckhardt U, Stieger B, Tynes R, Schteingart CD, Hofmann AF, et al. Substrate specificity of the rat liver Na(+)-bile salt cotransporter in Xenopus laevis oocytes and in CHO cells. Am J Physiol 1998;274:G370-G375.
43. Webster CR, Srinivasulu U, Ananthanarayanan M, Suchy FJ, Anwer MS. Protein kinase B/Akt mediates cAMP- and cell swelling-stimulated Na+/taurocholate cotransport and Ntcp translocation. J Biol Chem 2002;277:28578-28583.
44. Webster CR, Blanch C, Anwer MS. Role of PP2B in cAMP-induced dephosphorylation and translocation of NTCP. Am J Physiol Gastrointest Liver Physiol 2002;283:G44-G50.
45. Anwer MS. Cellular regulation of hepatic bile acid transport in health and cholestasis. HEPATOLOGY 2004;39:581-590.
46. Beuers U, Probst I, Soroka C, Boyer JL, Kullak-Ublick GA, Paumgartner G. Modulation of protein kinase C by taurolithocholic acid in isolated rat hepatocytes. HEPATOLOGY 1999;29:477-482.
47. Schonhoff CM, Gillin H, Webster CR, Anwer MS. Protein kinase Cdelta mediates cyclic adenosine monophosphate-stimulated translocation of sodium taurocholate cotransporting polypeptide and multidrug resistant associated protein 2 in rat hepatocytes. HEPATOLOGY 2008;47: 1309-1316.
48. Schliess F, Kurz AK, vom Dahl S, Häussinger D. Mitogen-activated protein kinases mediate the stimulation of bile acid secretion by tauroursodeoxycholate in rat liver. Gastroenterology 1997;113:1306-1314.
49. Noe B, Schliess F, Wettstein M, Heinrich S, Häussinger D. Regulation of taurocholate excretion by a hypo-osmolarity-activated signal transduction pathway in rat liver. Gastroenterology 1996;110:858-865.
50. Häussinger D, Kurz AK, Wettstein M, Graf D, vom Dahl S, Schliess F. Involvement of integrins and Src in tauroursodeoxycholate-induced and swelling-induced choleresis. Gastroenterology 2003;124:1476-1487.
51. Mukhopadhyay S, Webster CR, Anwer MS. Role of protein phosphatases in cyclic AMP-mediated stimulation of hepatic Na+/taurocholate cotransport. J Biol Chem 1998;273:30039-30045.
52. Keitel V, Donner M, Winandy S, Kubitz R, Häussinger D. Expression and function of the bile acid receptor TGR5 in Kupffer cells. Biochem Biophys Res Commun 2008;372:78-84.
53. Cantore M, Reinehr R, Sommerfeld A, Becker M, Haussinger D. The Src family kinase Fyn mediates hyperosmolarity-induced Mrp2 and Bsep retrieval from canalicular membrane. J Biol Chem 2011;286: 45014-45029.