Effects of bile salts on hepatic lipoprotein production

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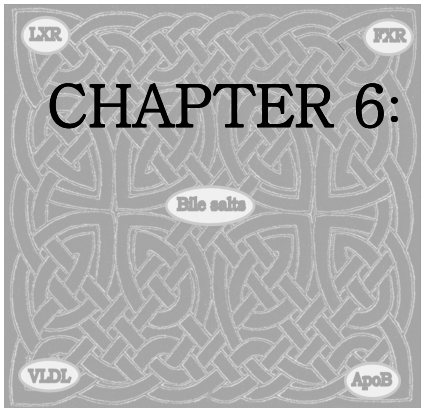

CHAPTER 6: Inhibition of apolipoprotein B secretion by taurocholate is independent of lipoprotein assembly in rat hepatoma McA-RH7777 cells

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ABSTRACT

Bile salts inhibit the secretion of apolipoprotein B (apoB) and triacylglycerol (TG) in primary rat and human hepatocytes. We investigated whether assembly of apoB into a lipoprotein particle is required for this inhibitory action of bile salts. The sodium/taurocholate co-transporting polypeptide (Ntcp) was co-expressed in McArdle-RH7777 (McA-RH7777) cells stably expressing the full-length human apoB100 (h-apoB100, secreted as triacylglycerol-rich lipoprotein particles) or carboxyl-truncated human apoB18 (h-apoB18, secreted in lipid-free form). The amounts of Ntcp produced in the doubly-transfected cell lines (h-apoB/r-Ntcp) was similar to those found in cultured rat hepatocytes and effectively accumulated taurocholic acid (TC). TC incubation decreased the secretion of endogenous rat apoB100 (-50%) and h-apoB18 (-35%), but did not affect secretion of rat apoA-I. Pulse-chase experiments (^{35}S-methionine) indicated that the impaired secretion of radio labeled h-apoB18 and h-apoB100 was associated with accelerated intracellular degradation of h-apoB18 and h-apoB100. The proteasomal inhibitor ALLN partially prevented TC-induced intracellular apoB degradation but did not affect the amount of either h-apoB18 or h-apoB100 secreted into the medium, indicating that inhibition of apoB secretion by TC is not due to enhanced proteasomal degradation. We conclude that the inhibitor effect of TC on apoB secretion is independent of apoB lipidation during VLDL assembly. These data suggest that bile salts inhibit apoB secretion via a mechanism dependent on the N-terminal end of apoB.

INTRODUCTION

Apolipoprotein B is the key structural protein required for the assembly of Very-Low-Density Lipoproteins (VLDL) [1]. VLDL mainly originate from the liver and are secreted into the blood as particles with a core of neutral lipids (triacylglycerol (TG), cholesteryl ester (CE)) surrounded by a monolayer containing cholesterol, phospholipids (PL) and apolipoproteins (apoB, apoE, apoC). Each VLDL particle contains a single apoB molecule and, therefore, secretion of apoB molecules corresponds with the number of VLDL particles produced [2]. In liver and intestinal cells, the expression of the *APOB* gene and the synthesis rate of apoB protein are rather constant under experimental conditions associated with widely varying apoB secretion rates [3]. Thus, the rate of apoB secretion is mainly controlled at a post-translational level, e.g., by control of intracellular degradation [4-8]. A number of studies suggest that apoB can be degraded either in the cytosol (co-translational degradation [9]) or in the secretory pathway (ER/Golgi lumen [10]). Co-translational degradation of apoB mainly occurs via proteasomal pathways when the translocation of the nascent apoB polypeptide into the ER is interrupted [11,12]. Upon continuing translation, a part of apoB becomes exposed to the cytosol which facilitates interactions with chaperones [17,18] and components of the ubiquitin-proteasomal pathway [15,16], leading to proteasomal degradation [19, 20]. ER-associated degradation of apoB occurs inside the ER and Golgi compartments by ER-resident proteases [21-23].

Previously, we demonstrated that bile salts inhibit VLDL secretion both in vitro in human, rat and murine hepatocytes [24,25] and in vivo in mice [26], but do not affect the secretion of apoA-I or albumin. In primary rat and mouse hepatocytes, incubation with TC inhibited the secretion of both TG and apoB moieties of VLDL. Both the lipid constituents and the apoB protein are essential for the assembly of VLDL. We aimed to determine whether the inhibition of apoB secretion by bile salts occurs at the stage of apoB lipidation during lipoprotein assembly. We used McArdle rat hepatoma cell lines stably transfected with either carboxyl-truncated human apoB18 (h-apoB18; N-terminal 18% of apoB100), or full-length human apoB100 (h-apoB100). h-ApoB18 is secreted from these cells at a density above 1.23 g/ml [27], i.e., at the density of non-lipoprotein secretory proteins. Herscovitz *et al*. demonstrated that ~98% of apoB17 is secreted as lipid-poor protein whereas only ~ 2% of apoB17 is associated with lipids [28]. It is therefore reasonable to assume that secretion of h-apoB18 does not require lipidation with neutral lipids such as TG. In contrast, h-apoB100 is only secreted in the form of buoyant particles secreted with

a density of d ≤ 1.006 g/ml [27]. Theoretically, bile salts could impair VLDL secretion by interfering with lipidation of apoB (assembly of TG-rich VLDL) or, alternatively, by a mechanism independent from the lipidation. If bile salts would inhibit apoB secretion through interference with lipoprotein assembly, bile salts would be expected to inhibit the secretion of h-apoB100 but not that of h-apoB18 in McA-RH7777 cells. Alternatively, if the effect of bile salts on apoB secretion would be independent from apoB lipidation, the secretion of the essentially lipid-free h-apoB18 may also be inhibited.

Bile salt uptake into the hepatocyte is essential for inhibition of VLDL secretion by bile salts [24,25]. McA-RH7777 cells, however, do not have the capacity to take up bile salts [29]. To allow assessment of the effect of bile salts on the secretion of the human apoB peptides, bile salt transport was reconstituted by stable transfection with the rat liver bile salt transporter, i.e., sodium/taurocholate co-transporting polypeptide (r-Ntcp) [30-32]. The results of this study indicate that taurocholate does not only inhibit the secretion of lipidated h-apoB100, but also that of virtually lipid-free h-apoB18 in rat hepatoma cells, establishing a novel mechanism by which bile salts interfere with metabolism of TG-rich lipoproteins.

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), geneticin, hygromycin, penicillin, streptavidin, natural horse serum (NhoS), Hank's buffered saline solution (HBSS) were all obtained from Corning Costar (Cambridge, MA, USA). 60 mm cell culture dishes were from Nunc Brand Products (Roskilde, Denmark) or Greiner Bio-one B.V. (Alphen a/d Rijn, the Netherlands). Fugene transfection reagent and ALLN were obtained from Roche Diagnostics (Mannheim, Germany). Taurocholic acid (TC), oleic acid, fumed silica, methionine and trichloroacetic acid (TCA) were all from Sigma Chemical Co. (St. Louis, MO, USA). SDS polyacrylamide 4-15% gradient gels were obtained from BioRad (Herculus, CA, USA). The monoclonal mouse anti-human 1D1 was obtained from dr. Y. Marcel (Ottawa Heart Institute, Ottawa, Ontario, Canada) [33]. Polyclonal sheep anti-human apoB antibodies were purchased from Roche Diagnostics (Mannheim, Germany) and Biodesign International (Saco, ME, USA) and the polyclonal

rabbit anti-human apoA-I was obtained from Calbiochem (San Diego, CA, USA). Hyperbond nitrocellulose membrane, ECL Western blotting kit, hyperfilm MP and Amplify were purchased from Amersham Pharmacia Biotech (Roosendaal, The Netherlands) as well as the peroxidase-conjugated donkey anti-sheep, rabbit anti-mouse, goat anti-rabbit IgG antibodies. Radiochemicals (Expre^{35}S^{35}S labeling mix, [^{3}H]-taurocholic acid) were from New England Nuclear (Boston, MA, USA).

Cell culture of McA-RH7777 cells

McA-RH7777 cells stably expressing truncated human apoBs (h-apoB18, h-apoB100) were cultured as previously described [27].

Transfection of McA-RH7777 cells with rat Ntcp

McA-RH7777 cell lines stably expressing h-apoB18 (McA-B18), h-apoB100 (McA-B100) or the empty vector pNeor (McA-Neo), were transfected with the rat bile salt transporter Ntcp (pCMV5-Ntcp expression vector) [29], and cotransfected with pHygro^{R} (confers hygromycin resistance) using FuGene transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. After 24 h the medium was replaced by medium containing 500 µg/ml hygromycin to select for stable transfectants. Ntcp concentration in the McA-RH7777 clones was determined in cell lysates by Western blotting and compared to that in freshly isolated hepatocytes by determining the relative intensity of the ntcp protein bands using a CCD camera of Image Masters VDS system (Amersham Pharmacia Biotech). Hereafter, the Ntcp-transfected cell lines were cultured in DMEM containing 200 µg/ml hygromycin.

Ntcp protein expression in McA-RH7777 clones

The surviving colonies were plated into 6 well plates and harvested at a confluency of 70% by scraping the cells into 2 ml of HBSS. The cells were pelleted by short centrifugation (2 min., 13.000 rpm, 4°C), resuspended in 200 µl SDS sample buffer and incubated for 15 min. at 75°C. The McA-RH7777 cell suspensions and freshly isolated rat hepatocyte cell suspensions (as a control for Ntcp expression) were applied to a SDS polyacrylamide 4-15% gradient gel, normalized for total cellular protein concentration. Ntcp was separated from other proteins by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with rabbit anti-rat Ntcp K4, kindly provided by dr. B. Stieger, Zürich, Switzerland [31]

and the peroxidase-conjugated goat anti-rabbit IgG, respectively, and visualized by ECL detection according to the manufacturer's instructions. The Ntcp protein concentration in the McA-RH7777 clones was compared to that in freshly isolated hepatocytes by determining the relative intensity of the Ntcp protein bands using a CCD camera of Image Masters VDS system (Amersham Pharmacia Biotech).

Ntcp activity assays

Transport activity of the transfected r-Ntcp was determined by measuring steady state intracellular TC concentrations in the cell lines McA-neo(Ntcp), McA-B18(Ntcp) and McA-B100(Ntcp) after a 24-h incubation with 200 μM TC. At the start of the experiment, the standard cell culture medium was removed from the cells and replaced by 1 ml DMEM containing 10% (v/v) FCS, 10% (v/v) NHoS, pen/strep, geneticin, hygromycin, 0.5 μCi [^{3}H]-TC (3.47 Ci/mmol) supplemented with unlabeled TC (final TC concentration 200 μM). Cells were incubated in 5% CO_2 at 37°C for 24 h. The assay was terminated by aspirating the medium and washing the cells three times with cold HBSS containing unlabeled TC (TC concentration 1 mM). Cells were scraped into 1 ml HBSS and lysed by pushing the cell suspension several times through a syringe. An aliquot of the cell suspension was used for total cellular protein measurement [34] and another aliquot of the cell suspension was used for determination of radioactivity by liquid scintillation counting.

Secretion of human apoB18, endogenous apoB100 and apoA-1 in McA-RH7777 cells stably transfected with rat Ntcp

McA-RH7777 cells stably expressing h-apoB18 and r-Ntcp (McA-B18(Ntcp)) were plated into 6-well plates 2 days prior to the start of the experiment. At a cell confluency of approximately 50% the medium was replaced by 1 ml DMEM per well containing 10% FCS, 10% NHoS, pen/strep, geneticin, hygromycin, 0.75 mM oleate, 25 μM glycerol with or without 200 μM TC. After 24 h incubation the medium was collected and centrifuged briefly (3 minutes, 13000 rpm, room temperature) to remove debris. The cells were washed 3 times with HBSS, scraped in 2 ml of HBSS and cellular protein mass was determined [34]. Apolipoproteins (human apoB18, endogenous rat apoB100 (r-apoB100), endogenous rat apoA1 (r-apoA1) in the medium were concentrated with fumed silica [35]. r-ApoB100 and h-apoB18 were separated from other proteins by SDS-PAGE using 4-15% gradient gels and transferred to a nitrocellulose membrane. For detection of h-apoB18, the

nitrocellulose membrane was incubated with the primary monoclonal antibody 1D1 (anti-human apoB) [33] and the secondary peroxidase-conjugated rabbit-anti-mouse IgG. r-ApoB100 was detected by incubating the nitrocellulose membrane with sheep anti-human apoB and peroxidase-conjugated donkey anti sheep IgG, respectively. r-ApoA-I was determined by incubating the nitrocellulose membrane with polyclonal rabbit anti-human apoA-I and secondary peroxidase-conjugated goat anti rabbit IgG. All protein bands were visualized by ECL detection as described above.

In vitro measurement of VLDL-TG secretion by wild type and apoE-deficient mice

Isolation and culture of mouse hepatocytes was performed as described previously using wild type C57BL6/J mice and apoE-deficient mice ($apoe^{(-/-)}$). Hepatocytes were plated in 35 mm 6-well plastic dishes (Costar Corp., Cambridge, MA, USA), precoated with collagen (Serva Feinbiochemica, Heidelberg, Germany) at a density of 1.0×10^6 cells/well in 2 ml of William's E medium containing insulin, fetal calf serum (FCS), dexamethasone and penicillin/streptomycin. After overnight incubation the medium was removed and hepatocytes were washed with hormone-free and FCS-free (HF-SF) William's E medium and subsequently incubated with HF-SF William's E medium for 4 hours. After four hours the medium was removed and replaced by 1 ml HF-SF William's E medium per well with or without 200 μM taurocholate also containing 25 μM [^{3}H]-glycerol (4.4 μCi/well) and 0.75 mM oleate (C18:1) complexed with bovine serum albumin (free fatty acid free). After 3 hours incubation the medium was collected and centrifuged to remove debris. Hepatocytes were washed with ice-cold Hank's balanced salt solution (HBSS) and scraped into 2 ml of HBSS for lipid extraction. Medium lipids secreted by mouse hepatocytes were extracted as described previously and TG was separated form other lipids by thin-layer chromatography (TLC) with hexane/diethyl ether/acetic acid (80/20/1) as developing solvent. After iodine staining, the spots containing ^{3}H-TG (as a measure for VLDL-TG secretion) were scraped into vials and assayed for radioactivity by scintillation counting.

Measurements of steady state mRNA expression levels by Real Time PCR

McA-B18(Ntcp) cells were incubated in DMEM containing 10% FCS, 10% NHoS, pen/strep, geneticin, hygromycin and 200 μM TC for 24 h. Total RNA from McA-B18(Ntcp) cells was isolated by the SV Total RNA Isolation System according to the manufacturer's instructions. Isolated total RNA was converted to single stranded cDNA by a reverse transcription procedure with M-Mulv-RT according to manufacturer's protocol. cDNA levels were measured by real-time PCR using the ABI prism 770 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the PCR amplification studies, an amount of cDNA corresponding to 10 ng of total RNA was amplified using the qPCR core kit essentially according to the manufacturer's protocol and optimized for amplification of the particular gene using the appropriate forward and reverse primers (GIBCO, Paisly, UK) and a template specific 3'-TAMRA, 5'-6-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). In the same experiments, calibration curves were run on serial dilutions of a 4-fold concentrated cDNA solution as used in the assay, resulting in a series containing 4x, 2x, 1x, 0.5x, 0.25x, 0.125x and 0.063x of the cDNA present in the assay incubation. Both assay and calibration incubations were performed simultaneously. During amplification, breakdown of the probe released the fluorescent 6-FAM-dye, resulting in increase in fluorescence. The fluorescence data were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, USA). All quantified expression levels were within the linear part of the calibration curves and were calculated using these curves. Following primer sets were used: *β-Actin* forward primer: AGC CAT GTA CGT AGC CAT CCA, reverse primer: TCT CCG GAG TCC ATC ACA ATG, probe: TGT CCC TGT ATG CCT CTG GTC GTA CCA C, genbank accession NM_031144, *apoa1* forward primer: CCC AGT CCC AAT GGG ACA, reverse primer: CAG GAG ATT CAG GTT CAG CTG TT, probe: CAA ACT GGG ACA CAT AGT CTC TGC CGC T, genbank accession NM_009692, *Fxr* forward primer: CGC TGA GAT GCT GAT GTC TTG, reverse primer: CCT TCA CTG CAC ATC CCA GAT, probe: TGA CCA CAA GTT CAC CCC GCT CCT, genbank accession U18374.

Pulse-chase experiments of McA-B18(Ntcp) and McA-B100(Ntcp)

Two days prior to the start of the experiment McA-B18(Ntcp) cells or McA-B100(Ntcp) cells were plated on 60 mm culture dishes and cultured as described above. At the start of the experiment, the medium was replaced by DMEM containing 10% FCS, 10% NHoS, pen/strep, geneticin, hygromycin, 0.75 mM oleate, 25 µM glycerol with or without 200 µM TC. After 20 h, the medium was removed and the cells were washed three times with HBSS. The cells were pulsed with methionine- and cysteine-free DMEM with or without 200 µM TC containing 150 µCi/dish of Express-^{35}S-labeling mix (mainly ^{35}S-methionine) for 2 h. After the 2h-pulse, radioactivity was chased for defined periods of time up to 4 h in DMEM with or without 200 µM TC containing 400 µM unlabeled methionine. In some experiments, the proteasomal inhibitor ALLN was added to the pulse and chase medium in a concentration of 40 µg/ml. The chase medium was collected and the cells were washed several times with HBSS. The cells were lysed and scraped into vials in hot (75°C) 1% SDS-RIPA solution. ApoB was immunoprecipitated from the medium samples and from a sample of the cell lysates with a polyclonal sheep-anti-human apoB antibody conform Wang *et al*. [36]. After immunoprecipitation the apoB proteins were separated by SDS-PAGE on 4-15% gradient gels. The gel was impregnated with Amplify for 15-30 minutes and dried. Either autoradiography of the gel was performed at -80°C for 4-6 days and the relative intensity of the bands was determined using a CCD camera of Image Masters VDS system (Amersham Pharmacia Biotech) or the gel was silver-stained after which the apoB bands were excised from the gel and digested with 20% hydrogen peroxide [37] and assayed for radioactivity by liquid scintillation counting.

Measurement of total protein synthesis

Total protein synthesis and secretion rate of newly synthesized proteins were determined by precipitating total proteins in cell lysates (50 µl) and chase medium samples (50 µl) with 10% trichloroacetic acid (TCA). Precipitated cellular and secreted proteins were centrifuged (3 min., 13000 rpm, room temperature) and assayed for radioactivity by scintillation counting.

Statistics

All values represent means ± standard deviation for the number of experiments indicated. Statistical analysis of groups was assessed by Mann Whitney U test. Level of significance was set at $p<0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, USA).

RESULTS

Reconstitution of bile salt uptake in McArdle RH-7777 expressing truncated forms of human apoB

The cDNA encoding the rat bile salt transporter Ntcp under control of the CMV5 promoter was stably transfected in different McA-RH7777 cell lines. After approximately 5 cell-division cycles, Ntcp protein could be detected by Western blotting. Ntcp protein mass in the transfected McArdle cell lines was compared to Ntcp protein mass in freshly isolated rat hepatocytes, normalized for total cellular protein concentration (**Figure 1A**).

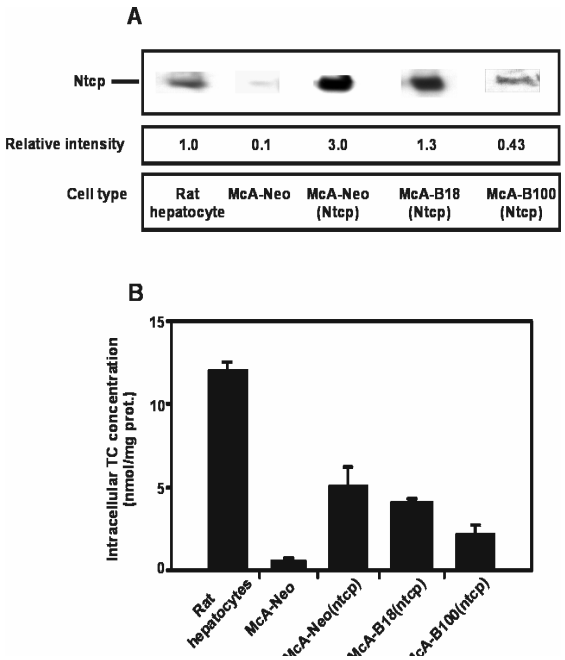

Figure 1. Stable transfection of McA-RH7777 with the rat bile salt transporter ntcp. A. McA-RH7777 cell lines stably expressing h-apoB18 (McA-B18), h-apoB100 (McA-B100) or the empty vector pNeor (McA-Neo) transfected with the rat bile salt transporter Ntcp. Ntcp concentration in the McA-RH7777 clones was determined in cell lysates by Western blotting. **B.** Intracellular steady state TC concentrations in McA-Neo(Ntcp), McA-B18(Ntcp), McA-B100(Ntcp) and McA-Neo (negative control) cells, incubated with 200 μM ^{3}H-taurocholic acid for 24 h. Values represent the mean intracellular TC concentration ± SD in nmol TC per mg total cellular protein (n=6 separate experiments per cell line).

In McA-B18(Ntcp), Ntcp protein concentration was similar to that in freshly isolated hepatocytes and higher than in McA-B100(Ntcp) cells. Functionality of the expressed Ntcp

transporter in the transfected McArdle lines was tested by measuring intracellular steady state TC concentration after 24 h incubation with TC-containing medium (**Figure 1B**). McA-neo(Ntcp), McA-B18(Ntcp) and McA-B100(Ntcp) did accumulate TC, although each to a lesser extent than primary hepatocytes (5.1, 4.1 and 2.1 nmol TC/mg cellular protein vs. 12.3 nmol TC/mg cellular protein in rat hepatocytes, respectively).

Effect of TC on secretion of endogenous r-apoB100, r-apoA1 and h-apoB18

McA-B18(Ntcp) cells secreted carboxyl-truncated human apoB18 and endogenous rat apoB100 and apoB48 (**Figure 2**). As shown in **Figure 2**, TC reduced the secretion of r-apoB100 (-34% compared to control, n= 3 experiments, values in duplo, $p<0.05$) as well as of h-apoB18 (-34% compared to control, n=3 experiments, values in duplo, $p<0.05$,). The effects of TC on apoB secretion appeared specific, since apoA-I secretion was not affected. TC incubation also did not influence r-apoA-I mRNA expression levels in McA-B18(Ntcp) cells (data not shown).

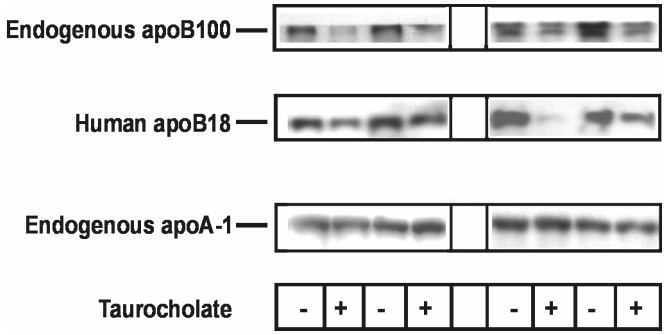

Figure 2. **Apolipoprotein secretion by McA-B18(Ntcp) after incubation with TC for 24h.** McA-B18(Ntcp) cells were incubated with control medium or medium containing 200 μM TC for 24 h. Secreted h-apoB18, r-apoB100 and r-apoAI in the medium was determined from control or TC-incubated cells, normalized for total cellular protein, by SDS PAGE followed by Western blotting. The apolipoprotein blots are representative for the results from three independent experiments

Intracellular degradation of truncated h-apoB18 upon TC treatment

To establish whether TC-impaired secretion of apoB was associated with increased intracellular degradation of apoB, we performed pulse-chase studies in McA-RH7777 cells stably expressing the truncated h-apoB18 (**Figure 3**).

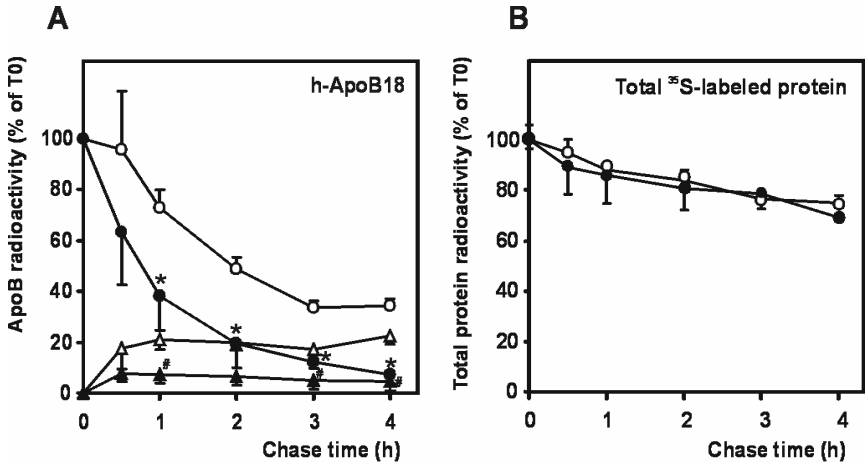

Figure 3. ^{35}S-**Pulse-chase experiments in control and TC-incubated McA-B18(Ntcp) cells. (A)** After an 18-h incubation with or without 200 μM TC, McA-B18(Ntcp) cells were labelled with ^{35}S methionine for 2 h in methionine-free DMEM. Hereafter, cells were chased for up to 4h in methionine-containing DMEM medium and apoB from control and TC-incubated cells was immunoprecipitated from medium and cell lysates. ApoB proteins were separated by SDS-PAGE on 4-15% gradient gels and assayed for radioactivity. Radioactivity in secreted and cellular apoB is represented as mean % of radioactivity present in cellular apoB after the 2h pulse period (T0) ± SD from 4 identical, separate experiments. (○ intracellular h-apoB18, control; ● intracellular h-apoB18, TC-incubation; △ secreted h-apoB18, control; ▲ secreted h-apoB18, TC incubation; * $p<0.05$, significantly different from intracellular control; # $p<0.05$, significantly different from medium control). **(B)** Total labelled cellular protein was determined at the indicated time points during the chase period by precipitating total proteins in the cell lysates. Radioactivity in the protein pellets was determined by liquid scintillation counting and is represented as mean % of radioactivity present in total cellular protein after the 2 h pulse (T0) ± SD from 4 identical, separate experiments. (○ total labeled protein, control; ● total labeled protein, TC incubation).

Under control conditions, radioactivity in cellular h-apoB18 decreased as a function of time with a concomitant increase in the amount of radioactive h-apoB18 recovered from the medium. h-ApoB18 secretion accounted only partly for the loss of cellular h-apoB18, the remainder reflecting intracellular degradation. In TC-treated cells, a smaller fraction of radioactive h-apoB18 present at the start of chase (t=0) was secreted into the medium than observed for control cells after 4h (4 ± 4% vs. 23 ± 0.3%, $p<0.05$, respectively). The decreased secretion of h-apoB18 into the medium was associated with an enhanced intracellular h-apoB18 degradation in TC-treated cells after 4h (88 ± 5% vs. 43 ± 3 %, $p<0.05$). Similar to the results obtained for h-apoB18, an enhanced intracellular degradation of endogenous r-apoB100 was also detected in TC-treated McA-B18(Ntcp) cells (data not shown). To confirm the specific effect of TC on apoB secretion and

degradation, total radioactive cellular protein was measured in control cells and TC-treated cells (**Figure 3B**). The decrease of radioactivity in total cellular protein was similar in TC-treated and control McA-B18(Ntcp) cells, again indicating the specificity of the TC effect on apoB degradation.

Intracellular degradation of full-length h-apoB100 upon TC treatment

Experiments as described above for h-apoB18 were also performed with McA-h-apoB100 cells (**Figure 4**). In TC-treated and control cells cellular labeled h-apoB100 disappeared as a function of time with a concomitant increase in radioactive secreted h-apoB100. Part of the decrease in radioactivity in intracellular h-apoB100 could be recovered in secreted h-apoB100 (control: 22 ± 6%, TC treatment: 10 ± 1%, $p<0.05$). Intracellular degradation of h-apoB100 was enhanced in TC-treated cells compared with controls (53 ± 9% vs. 29 ± 5% (**Figure 4A**), 59 ± 2 vs. 43 ± 2 (**Figure 5B**), both $p<0.05$, respectively). The appearance of labeled proteins in medium (data not shown) and simultaneous disappearance of total labeled cellular protein (**Figure 4B)** was similar in TC-treated and control cells.

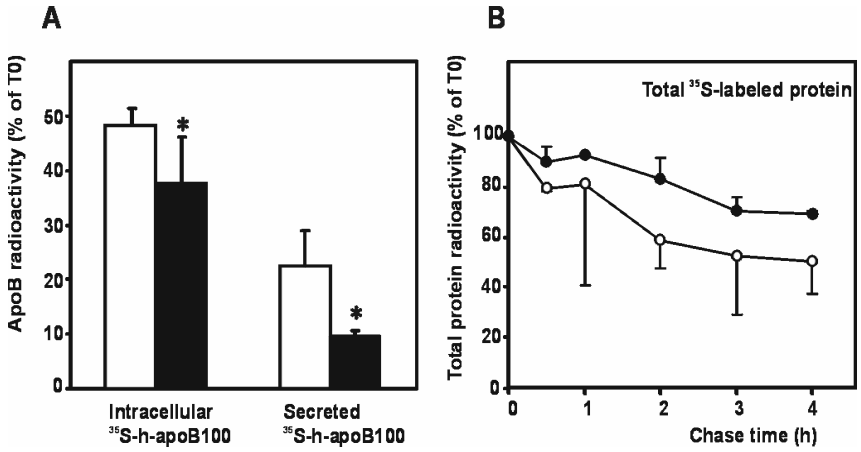

Figure 4. ^{35}S-**Pulse-chase experiments in control and TC-incubated McAB100(Ntcp) cells.** After an 18-h incubation with or without 200 μM TC, McA-B100(Ntcp) cells were labelled with ^{35}S methionine for 2 h in methionine-free DMEM. Hereafter, cells were chased for up to 4h in methionine-containing DMEM medium with or without 200 μM TC. **(A).** ApoB from control and TC-incubated cells was immunoprecipitated from medium and cell lysates. ApoB proteins were separated by SDS-PAGE and assayed for radioactivity. Radioactivity in secreted and cellular apoB is represented as mean % ± SD of radioactivity present in cellular apoB after the 2h pulse period (T0) obtained from 3 identical, separate experiments. Open bars: h-apoB100, control; closed bars: h-apoB100, TC-incubation; * $p<0.05$, significantly different from control. **(B)** Total labelled cellular protein was determined at the indicated time points during the chase period by precipitating total proteins in the cell lysates. Radioactivity in the protein pellets is represented as mean % ± SD of radioactivity present in total cellular protein after the 2 h pulse (T0) from 3 identical, separate experiments. (○ total labeled protein, control; ● total labeled protein, TC incubation).

Inhibition of intracellular proteasomal degradation of h-apoB18 and h-apoB100 does not prevent TC-induced impairment of apoB secretion

We next investigated whether enhanced intracellular degradation of apoB in TC-treated cells is a primary cause of its decreased secretion, or rather reflects a secondary effect. The cystein protease inhibitor ALLN (Calpain inhibitor I), which blocks proteasomal degradation of apoB, was added to McA-B18(Ntcp) or to McA-B100(Ntcp) during the pulse chase experiments as described above (**Figure 5**).

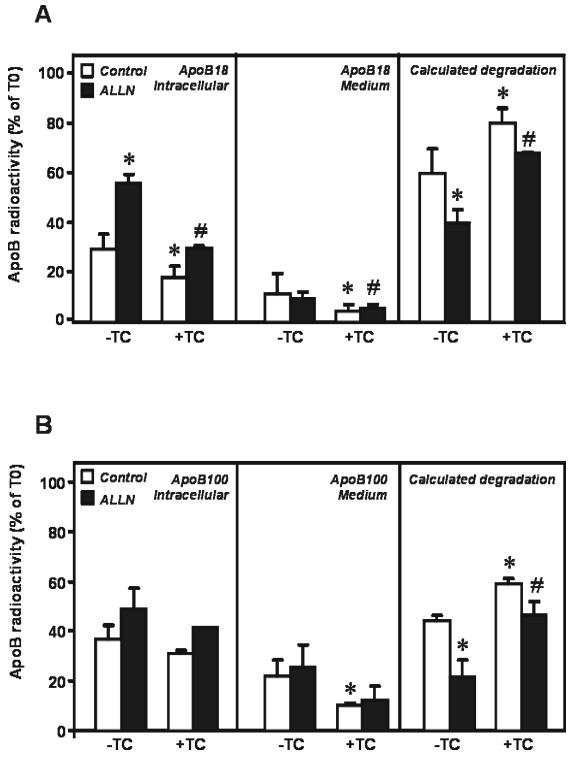

Figure 5. ^{35}S-Pulse-chase experiments in control and TC-incubated McA-B18(Ntcp) and McA-B100(Ntcp) cells in the presence or absence of the proteasomal inhibitor ALLN. After 18-h pre-incubation with or without 200 μM TC, **McA-B18(Ntcp)** cells (top panel, (**A**)) were labelled with ^{35}S methionine for 2 h in methionine-free DMEM containing 40 μg/ml ALLN or not with or without 200 μM TC. Hereafter, cells were chased for 3 h in methionine-containing DMEM medium with or without 40 μg/ml ALLN with or without 200 μM TC. ApoB was immunoprecipitated from medium and cell lysates. ApoB proteins were separated by SDS-PAGE and assayed for radioactivity. **McA-B100(Ntcp)** cells (bottom panel, (**B**)) were subjected to the same above mentioned experimental conditions. Radioactivity in secreted and cellular apoB after the 3-h chase is represented as mean % ± SD of radioactivity present in cellular apoB after the 2h pulse period (T0) from 3 identical, separate experiments for each cell line. Open bars: control (without ALLN); closed bars: with ALLN; * $p<0.05$, significantly different from control (without ALLN); # $p<0.05$, significantly different from control with ALLN.

At 3 h after the pulse, in control cells (absence of ALLN and TC), 11 ± 8% of radiolabelled h-apoB18 present at time= 0 h was secreted and 29 ± 6% was present as intracellular h-apoB18. Addition of ALLN increased the amount of intracellular labelled h-apoB18 at t=3

h in control cells (29 ± 6% vs. 56 ± 3%, respectively, $p<0.05$), but did not affect h-apoB18 secretion in control cells (11 ± 8% vs. 10 ± 3%, control vs. ALLN). In TC-incubated cells, 4 ± 2% of labelled h-apoB18 was detected in the medium and 18 ± 5% intracellular. Addition of ALLN increased intracellular labelled h-apoB18 in TC-incubated cells (from 18 ± 5% to 29 ± 6%, $p<0.05$), but the amount was still lower than observed in ALLN-incubated control cells (29 ± 6% vs. 56 ± 3%, $p<0.05$). Addition of ALLN to TC-incubated cells did not affect h-apoB18 secretion by TC-treated cells (4 ± 2% vs. 6 ± 1%, TC vs. TC+ALLN, not significant).

Similar results were obtained when McA-B100(Ntcp) cells were incubated with ALLN, TC or ALLN+TC. **Figure 5b** shows that addition of ALLN to control or TC-treated McA-B100(Ntcp) cells did not affect h-apoB100 secretion (control 22 ± 6% vs. ALLN 25 ± 9%, TC 10 ± 1% vs. TC+ALLN 11 ± 6%). Incubation with ALLN resulted in increased intracellular accumulation of labeled h-apoB100 in control and TC-treated cells (control: 37 ± 6% vs. ALLN: 49 ± 8%, TC: 31 ± 1% vs. TC+ALLN: 42 ± 0.1%, respectively). Inhibition of proteasomal degradation of apoB did not promote apoB secretion in TC-treated cells, which supports the notion that the increased intracellular degradation of apoB upon TC treatment is not the cause, but the result of inhibition of apoB secretion.

Effect of TC on secretion of VLDL-TG by primary apoE-deficient hepatocytes

Previous studies from our lab demonstrated that apolipoprotein E participates in the regulation of VLDL-TG secretion by the liver. In this study we also investigated whether bile salts inhibit VLDL secretion by affecting apoE. As shown in **Figure 6**, basal VLDL-TG secretion by *apoe*$^{(-/-)}$ hepatocytes was ~2-fold decreased compared to basal VLDL-TG secretion by wild type hepatocytes, which was in agreement with results from Mensenkamp *et al*. Incubation with taurocholate for 3 hours inhibited VLDL-TG secretion to a similar extent in both wild type hepatocytes and *apoe*$^{(-/-)}$ hepatocytes. These data demonstrate that the inhibtor effect of bile salts on VLDL secretion is independent of apoE.

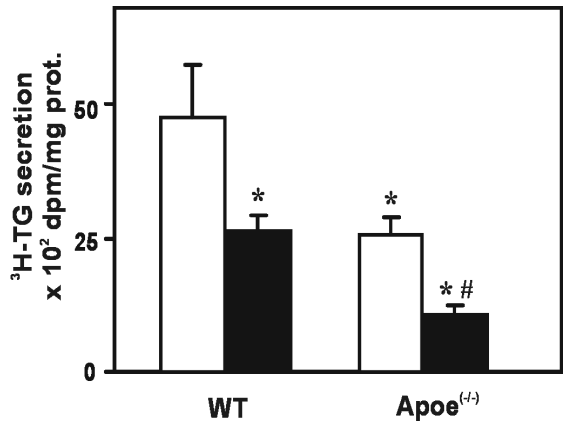

Figure 6. VLDL-TG secretion by wild type and apoE-deficient hepatocytes after TC incubation for 3 hours. Primary mouse hepatocytes were labeled with [^{3}H]-glycerol (25 μM) for 3 h in the presence or absence of 200 μM TC. Lipid was extracted from the medium and ^{3}H-TG was separated by thin layer chromatography (TLC). ^{3}H-VLDL-TG secretion is presented as dpm per mg cellular protein. ^{3}H-VLDL-TG values are means ± SD from 2 independent experiments, each carried out in triplo. (□: control,■: TC).* $p<0.05$.

DISCUSSION

Previously we demonstrated that bile salts inhibit the secretion of VLDL-TG and of apoB in human, rat and mouse hepatocytes *in vitro*, and in mice *in vivo* [24-26]. In the present study we investigated whether bile salts inhibit VLDL secretion by interfering with the assembly of apolipoprotein B into a TG-rich VLDL particle or via a mechanism independent of intracellular lipoprotein assembly. Results indicate that BS do not only inhibit the secretion of apoB assembled into a lipid-rich lipoprotein, but also inhibit that of a truncated apoB peptide that is secreted in lipid-free form. The intracellular assembly of apoB into TG-rich VLDL is apparently not required for the inhibitory action of bile salts. Present results also indicate that the inhibition of apoB secretion by bile salts is independent of enhanced proteasomal degradation of apoB.

Based on previous studies involving VLDL formation and assembly, we chose to use rat hepatoma McA-RH7777 cell lines, which stably expressed carboxyl-truncated human apoB18 or full length human apoB100 [27,39-42]. In contrast to the human hepatoma cell line HepG2, McA-RH7777 cells secrete human-apoB100-containing lipoproteins with a density resembling that of normal human VLDL ($d \leq 1.006$ g/ml) and LDL ($d \leq 1.019$ g/ml), indicating an intact machinery for the assembly of buoyant lipoproteins [43,44]. h-ApoB100 requires lipidation with neutral lipids for its secretion into the medium of McArdle RH7777 cells. Human apoB18 is secreted by the McA-RH7777 cells in the

density range of at least 1.23 g/ml, indicating that h-apoB18 is not secreted as a lipid-rich buoyant lipoprotein particle, but in (virtually) lipid-free form [27]. It has been suggested that h-apoB18 can be lipidated to a small extent with phospholipids [27]. However, Vermeulen *et al*. showed that h-apoB18 secretion was not dependent of intracellular phospholipid availability and synthesis [45].

Morphological changes have been described in Ntcp-transfected McA-RH7777 after incubation with 100 μM TC [48], which might affect VLDL secretion, but we did not observe morphological changes after a 24-h incubation with 200 μM TC, possibly related to the lower uptake capacity for TC in the present, doubly-transfected Ntcp-transfected cells (data not shown).

In the McA-B18(Ntcp) cell line, secretion of endogenous r-apoB100 was inhibited by TC. The 36% inhibition of endogenous r-apoB100 resembles TC-mediated inhibition of apoB secretion from hepatocytes (human, rat, murine) reported in our earlier studies [24-26]. TC also inhibited the secretion of h-apoB18. Rusinol *et al*. [39] also described inhibition of apoB18 secretion by McA-RH7777 cells after incubation with the phospholipid PMME. Increased PMME enrichment in the cell membrane resulted in decreased apoB secretion independent of assembly of apoB into a lipoprotein particle. The secretion of apoA-I was unaffected after TC treatment, indicating specific inhibition of apoB secretion by TC. Recently, Claudel *et al*. showed that bile salts negatively regulate mRNA concentrations of human apoA-I via activation of the bile salt receptor FXR in human primary hepatocytes and in HepG2 cells [38]. However, neither apoA-I protein secretion nor apoA-I mRNA expression (data not shown) was affected in McA-B18(Ntcp) cells after TC treatment. FXR mRNA expression in McA-RH7777 cells appeared very low and no differences between control and TC-treatment were detected (data not shown). Low FXR expression might explain the unaffected apoA-I expression upon TC incubation.

In hepatocytes, the apoB protein is constitutively synthesized in excess of requirement for VLDL assembly [3]. Excess apoB undergoes intracellular degradation. Increase of intracellular degradation has been identified as the mechanism by which for example ω-3 fatty acids inhibit apoB secretion [49,50]. Therefore, we examined whether TC-impaired secretion of apoB was associated with increased intracellular degradation of apoB. The secretion of both truncated h-apoB18 and full-length h-apoB100 was inhibited, i.e., by 67% and 60%, respectively, with a concomitant increase in intracellular degradation of both h-apoB18 and h-apoB100. In HepG2 cells, proteasomal degradation

constitutes quantitatively the most important route for intracellular apoB degradation [4,9,11,19]. In rat hepatoma McA-RH7777 cells, however, the major part of intracellular degradation occurred not via proteasomal degradation after the first stage of apoB translocation into the ER [51], which indicates that apoB degradation in McA-RH7777 cells predominantly occurred in endoplasmic reticulum and Golgi. Indeed in this study, ALLN inhibited intracellular proteasomal degradation of apoB only by 20-40%, indicating that the remainder is not proteasome-dependent. Inhibition of proteasomal degradation of apoB in TC treated cells did not lead to increased apoB secretion and prevented the degradation of apoB only partially (**Figure 5**). During inhibition of proteasomal degradation by ALLN apoB degradation was still increased upon incubation with TC (h-apoB18: 65% vs. 34%, respectively, $p < 0.05$, h-apoB100: 47% vs. 26%, respectively, $p<0.05$). Our data demonstrate that the increased intracellular degradation of apoB upon TC treatment is the result of inhibition of apoB secretion.

Our data indicate that the inhibitory action of TC on apoB secretion does not involve a disturbed lipidation of apoB, i.e. a disturbed assembly of VLDL. An effect mediated on the activity of microsomal triglyceride transfer protein (MTP), the rate-limiting enzyme involved in lipidation of apoB with neutral lipids, does not seem likely, since in one of our previous studies [26], we showed that the activity or mRNA concentration of MTP is not affected by bile salts. We also tested the involvement of apolipoprotein E in TC-inhibited apoB secretion, because apolipoprotein E is known to participate in regulation of VLDL secretion [52]. *In vitro* studies using primary cultures of murine apoE-deficient and wild type hepatocytes showed that TC inhibited VLDL-TG secretion rate to a similar extent in both wild type and apoE-deficient hepatocytes (**Figure 6**).

The data presented in this study suggest that bile salts inhibit apoB secretion via a mechanism dependent on the N-terminal end of apoB. The N-terminal part of the apoB protein has a few distinctive characteristics, which could be a site for interference by bile salts, such as disulfide bonds or N-glycosylation sites. The first 21% of the apoB protein contains 7 disulfide bonds [53,54]. Disruption of disulfide bonds resulted in strongly inhibited apoB secretion and increased intracellular degradation, regardless of the length of apoB [55], which we also observed in TC-incubated McA-B18(Ntcp) and McA-B100(Ntcp), although the TC effect was less pronounced. TC might indirectly disturb disulfide bond formation during folding of the nascent apoB peptide or at a later stage in the secretory pathway. The N-terminal side of apoB also contains some sites for glycosylation, which takes place in ER and Golgi [56]. Specific disruption of N-

glycosylation sites within h-apoB17, h-apoB37 and h-apoB50 led to decreased secretion of all truncated apoB's with a concomitant increase in degradation [57], as was observed in TC-incubated McA-B18(Ntcp) and McA-B100(Ntcp) cells. Intracellular degradation of apoB was enhanced by TC even when proteasomal degradation was inhibited by ALLN. This indicates that TC does not affect apoB at the cytosolic side of the ER, but merely affects secretion of apoB at a site after translocation of apoB into the ER lumen, i.e., at the level of ER lumen or Golgi. ApoB has been described to interact with the luminal membrane of the ER during translocation and directly after translocation presumably for stabilization of the protein [58]. Changes in ER membrane phospholipid composition lead to instability of apoB, thereby accelerating its intracellular degradation [39]. Rusinol *et al*. changed PL membrane composition by enriching McA-RH7777 cells with phosphatidylmonomethylethanolamine (PMME) [39]. This resulted in decreased secretion of h-apoB18 and other apoB's of various lengths, similar to what we observed in TC-treated cells. Also an increased intracellular degradation specific for apoB was observed, which could not be prevented by proteasomal inhibitor ALLN. Therefore, TC-induced alterations in ER/ Golgi membrane might affect stability of apoB still resident in the ER and Golgi.

Bile salts have been shown to interact with several signal transduction pathways via activation of protein kinases C, JNK, ERK and PI3 kinase [59-61]. PI3 kinase has also been implicated in the insulin-mediated inhibition of apoB secretion [13,14,62]. Studies in rat hepatocytes and McA-RH7777 cells demonstrate that inhibition of PI3 kinase abolished the inhibitory effect of insulin on apoB secretion [13,63]. Bile salts may inhibit secretion of apoB via a similar signal transduction route as has been proposed for insulin. Activation of PI3 kinase via bile salts can trigger alterations in intracellular calcium fluxes by releasing calcium stores from ER and Golgi. Degradation of specific proteins inside the ER has been reported to be regulated via changes in redox potential of ER and Golgi by depletion of calcium stores [14,62-66].

In this study we show that the inhibitory action of bile salts on apoB secretion is not at the stage of assembly of apoB into TG-rich VLDL.

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