

Calnexin Depletion by Endoplasmic Reticulum Stress During Cholestasis Inhibits the Na⁺-Taurocholate Cotransporting Polypeptide

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Cholestasis-induced accumulation of bile acids in the liver leads to farnesoid X receptor (FXR)-mediated transcriptional down-regulation of the bile acid importer Na⁺-taurocholate cotransporting protein (NTCP) and to induction of endoplasmic reticulum (ER) stress. However, whether ER stress affects bile acid uptake is largely unknown. Here, we investigated the role of ER stress on the regulation and function of the bile acid transporter NTCP. ER stress was induced using thapsigargin or subtilase cytotoxin in human osteosarcoma (U2OS) and human hepatocellular carcinoma (HepG2) cells stably expressing NTCP. Cellular bile acid uptake was determined using radiolabeled taurocholate (TCA). NTCP plasma membrane expression was determined by cell surface biotinylation. Mice received a single injection of thapsigargin, and effects of ER stress on NTCP messenger RNA (mRNA) and protein were measured by reverse-transcription polymerase chain reaction (RT-PCR) and western blot analysis. Effects of cholestasis on NTCP and ER stress were assessed in response to 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) feeding or bile duct ligation in FXR^{-/-} mice after 7 or 3 days, respectively. Novel NTCP-interacting proteins were identified by mass spectrometry (MS), interaction verified, and assessed by co-immunoprecipitation and TCA uptake for functional relevance in relation to ER stress. ER stress induction strongly reduced NTCP protein expression, plasma membrane abundance, and NTCP-mediated bile acid uptake. This was not controlled by FXR or through a single unfolded protein response (UPR) pathway but mainly depended on the interaction of NTCP with calnexin, an ER chaperone. In mice, expression of both NTCP and calnexin was reduced by thapsigargin or cholestasis-induced ER stress. Calnexin down-regulation *in vitro* recapitulated the effect of ER stress on NTCP. **Conclusion:** ER stress-induced down-regulation of calnexin provides an additional mechanism to dampen NTCP-mediated bile acid uptake and protect hepatocytes against bile acid overload during cholestasis. (*Hepatology Communications* 2018;2:1550-1566).

The enterohepatic circulation of bile acids is regulated by several transporters in the intestine and liver to maintain bile acid balance. In humans, the majority of bile acids from the portal blood are re-absorbed in a Na⁺-dependent manner at the basolateral membrane of the hepatocytes

by NTCP encoded by the solute carrier family 10A member 1 (SLC10A) gene.⁽¹⁾ Therefore, NTCP is a key component in the regulation of intracellular and systemic bile acid concentration. In addition, NTCP was recently identified as a receptor for hepatitis B virus (HBV) and hepatitis D virus.⁽²⁾

Abbreviations: ATP1A1, adenosine triphosphatase Na⁺/K⁺ transporting subunit alpha 1; BIP, binding immunoglobulin protein; BSEP, bile salt export pump; CANX, calnexin; CHOP, DNA-damage inducible transcript 3; DDC, 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine; DMSO, dimethyl sulfoxide; EIF2α, eukaryotic translation initiation factor 2 α; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; FLAG, flagellar protein; FXR, farnesoid X receptor; GFP, green fluorescent protein; GRP78, glucose-regulated protein 78 kDa; HepG2 cells, hepatocellular carcinoma cells; hNTCP, human Na⁺-taurocholate cotransporting polypeptide; IRE1, inositol-requiring 1; mRNA, messenger RNA; MS, mass spectrometry; MyrB, myrcludex B; NB, no biotin; NTCP, Na⁺-taurocholate cotransporting polypeptide; P, cells without NTCP; PERK, protein kinase R-like endoplasmic reticulum kinase; PNGase F, peptide-N-glycosidase F; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; sh, short hairpin; shCtrl, scramble short hairpin RNA; SHP, small heterodimer partner; SubAB, subtilase cytotoxin; TCA, taurocholate; Tg, thapsigargin; U2OS cells, osteosarcoma cells; UPR, unfolded protein response; WT, wild type; XBP1s, X-box binding protein 1, spliced form.

Cholestasis, or disruption of bile acid flow, due to obstruction of the biliary tract or genetic defects can result in an accumulation of bile acids in the liver, resulting in severe hepatocellular toxicity and inflammation.^(3,4) In response to toxic bile acid accumulation, several pathways are induced in hepatocytes, among which protective mechanisms, such as the transcriptional down-regulation of bile acid uptake transporters, increase in bile acid efflux, and decrease of bile acid synthesis.⁽⁵⁻⁷⁾ Limiting bile acid entry into hepatocytes appears to be a crucial mechanism to prevent cell injury and reduce the deleterious effect of intracellular bile acid accumulation. The role of NTCP in bile acid uptake designates this transporter as an important target in this process. In this regard, NTCP amount has been shown to be reduced in cholestasis, leading to a decreased influx of bile acids into the cells.⁽⁵⁾ In cholestasis, the activation of the nuclear receptor for bile acids (FXR) and subsequent induction of the small heterodimer partner (*SHP*) leads to the down-regulation of *NTCP* at a transcriptional level.^(8,9) Although this FXR/*SHP* pathway is a key mechanism in cholestasis, little is known about

the regulation of NTCP at the protein level in this condition.

Cholestasis also triggers stress response pathways, such as the ER stress response. ER stress triggers the UPR, which is primarily involved in the homeostatic control of protein folding, regulation of protein synthesis, and degradation, but it can also trigger apoptosis if the stress remains unresolved.⁽¹⁰⁾ The potentially protective role of ER stress in reducing bile acid accumulation through its control on protein homeostasis has not yet been investigated.

The aim of the present study was to assess the effect of ER stress on NTCP protein expression during cholestasis and to identify the regulatory mechanisms. We herein show that ER stress *in vitro* and *in vivo* down-regulates NTCP and that this takes place at the protein level with a decreased amount of NTCP at the plasma membrane leading to a reduction of bile acids uptake *in vitro*. We herein identify calnexin as an FXR-independent regulator of NTCP expression both *in vitro* and in mouse models of ER stress and cholestasis. Calnexin-depleted cells show increased susceptibility to ER stress that is paralleled by reduced

Received April 17, 2018; accepted September 3, 2018.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1262/supinfo.

Supported by the Netherlands Organization for Scientific Research (VIDI 91713319 to S.F.J.vdG.) and the European Research Council (starting grant 337479 to S.F.J.vdG.).

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DOI 10.1002/hep4.1262

Potential conflict of interest: Nothing to report.

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calreticulin levels. Therefore, this study designates a new layer of regulation to dampen NTCP-mediated bile acid uptake during cholestasis.

Materials and Methods

CELL CULTURE AND CHEMICAL TREATMENT OF CELLS

HepG2 and U2OS cells stably expressing HA-tagged human (h)NTCP (HepG2 HA-hNTCP, U2OS HA-hNTCP, respectively) were cultured as described.^(11,12) The parental HepG2 cells were purchased from ATCC. These cells were confirmed to be mycoplasma negative. Treatment started when the cells reached 80% confluence. Cells were treated for 18 hours with medium containing 125 nM thapsigargin or with subtilase cytotoxin (SubAB) at 100 ng/mL (U2OS cells) or 200 ng/mL (HepG2 cells) or with control medium containing 0.06% volume dimethyl sulfoxide (DMSO) or 200 ng/mL protease-dead SubAA272B.

ANIMAL STUDIES

Two-month-old male wild-type (WT) C57BI6/J mice were purchased from Envigo (Venray, the Netherlands). Cholestasis was induced by feeding the mice a chow diet (D12450B1, OpenSource Diets; Research Diets, Inc.), supplemented with 0.1% DDC (Sigma) for 7 days.^(13,14) In FXR^{-/-} mice and WT counterparts, cholestasis was induced by 3 days of bile duct ligation.⁽¹⁵⁾ For ER stress induction, adult mice received a single intraperitoneal injection of thapsigargin dissolved in DMSO (1 mg/kg) or DMSO alone 8 hours or 24 hours before being killed. Livers were snap frozen in liquid nitrogen, and membranes were isolated as described.⁽¹⁶⁾ The study design and all protocols for animal care and handling were approved by the respective local animal care and use committees.

LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY SAMPLE PREPARATION

Parental or HA-hNTCP-expressing HepG2 cells were grown in a 150-mm culture dish until 80% confluence. After washing with phosphate-buffered saline, cells were lysed in sucrose lysis buffer

(150 mM NaCl, 50 mM Tris [pH 7.4], 5 mM ethylene diamine tetraacetic acid, 10% (weight/volume) sucrose, 1% Nonidet P40) supplemented with protein inhibitors. Three culture dishes were pooled to generate one sample. Equal protein amounts were incubated with monoclonal anti-HA antibody immobilized on agarose beads (Sigma 9568) for 16 hours at 4°C. Precipitated proteins were denatured with 8 M urea in 1 M ammonium bicarbonate reduced with tris(2-carboxyethyl)phosphine (10 mM) at room temperature for 30 minutes, after which the cysteines were alkylated with chloroacetamide (40 mM end concentration) for 30 minutes. After 4-fold dilution with ammonium bicarbonate, proteins were on-bead digested overnight at room temperature with 150 ng of trypsin/LysC (Promega), after which peptides were bound to an in-house-made c18 material stage tip washed with buffer A (0.1% formic acid) and stored at 4°C until liquid chromatography (LC)/tandem mass spectrometry (MS/MS) analysis.

Detailed materials and methods can be found in the Supporting Materials.

Results

ER STRESS AFFECTS NTCP FUNCTION AND PLASMA MEMBRANE EXPRESSION *IN VITRO*

To assess the effect of ER stress on NTCP, HepG2 and U2OS cells stably expressing HA-tagged hNTCP were treated with the sarco/ER Ca²⁺ adenosine triphosphatase inhibitor thapsigargin. Following thapsigargin treatment, cells showed a significant increase in the splicing of X-box binding protein 1 (*XBP1s*) mRNA (Fig. 1A,B) and in mRNA levels of binding immunoglobulin protein/glucose-regulated protein 78 kDa (*BIP/GRP78*) (Supporting Fig. S1A,B), indicating an activation of the UPR. The bile acid uptake function of NTCP, measured by TCA uptake, was significantly reduced with induction of ER stress in both HepG2 (Fig. 1C) and U2OS cells (Fig. 1D). To induce ER stress without the potential Ca²⁺-mediated off-target effects of thapsigargin, cells were treated with SubAB, a proteolytic bacterial toxin that specifically cleaves BIP/GRP78.⁽¹⁷⁾ Similarly to

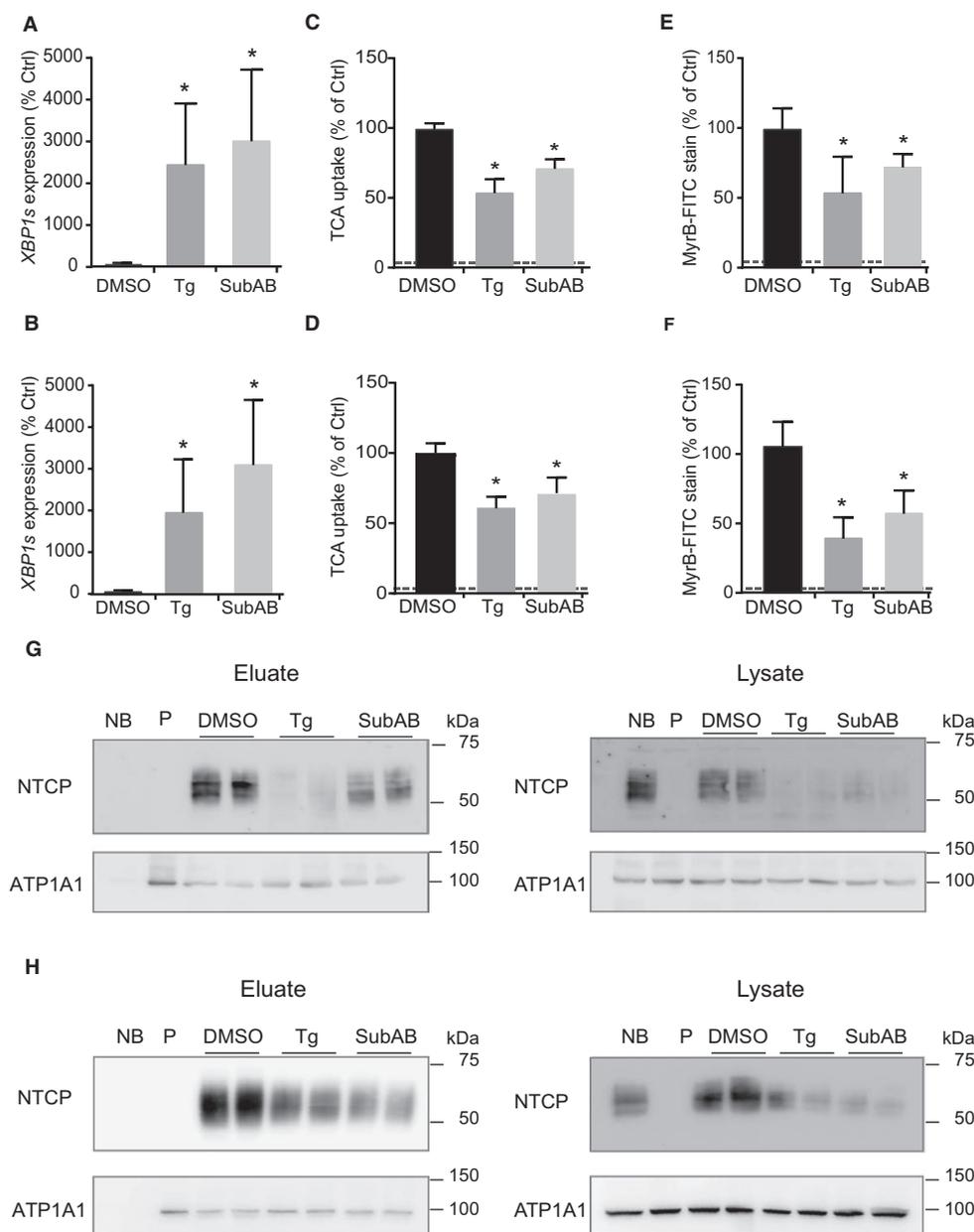


FIG. 1. ER stress down-regulates NTCP *in vitro*. (A,B) *XBP1s* mRNA expression analyzed by qRT-PCR and shown as a percentage of DMSO-treated cells in (A) HepG2 HA-hNTCP cells or (B) U2OS HA-hNTCP cells following thapsigargin or SubAB treatment as indicated; $n = 5$ independent experiments performed in quadruplicate. (C,D) TCA uptake assay following treatment with thapsigargin or SubAB expressed as a percentage of DMSO-treated cells in (C) HepG2 HA-hNTCP cells or (D) U2OS HA-hNTCP cells; $n = 3$ independent experiments performed in quadruplicate. The dotted line represents the level of uptake in cells lacking NTCP. (E-H) NTCP expression at the plasma membrane after ER stress induction by thapsigargin or SubAB either semiquantified by (E,F) myrcludex B-FITC intensity or (G,H) shown by cell surface biotinylation in (E,G) HepG2 HA-hNTCP cells or (F,H) U2OS HA-hNTCP cells. The dotted line (E,F) represents the level of myrcludex B-FITC detected in cells lacking NTCP. Background fluorescence was subtracted before normalization, and the net fluorescence was expressed relative to DMSO-treated cells. (G,H) Eluate is the plasma membrane fraction, lysate is the total proteins, molecular mass is indicated in kDa on the right side. Western blots are representative of at least three independent experiments. * $P < 0.05$, compared to DMSO control; analysis of variance followed by Bonferroni's multiple comparison test. Abbreviation: Ctrl, control.

thapsigargin treatments, induction of ER stress by SubAB in HepG2 (Fig. 1A; Supporting Fig. S1A) and U2OS cells (Fig. 1B; Supporting Fig. S1B) led to a reduction of TCA uptake by NTCP (Fig. 1C,D). Subsequently, we examined the plasma membrane abundance of NTCP using fluorescein isothiocyanate (FITC)-labeled myrcludex B. Myrcludex B is a myristoylated peptide based on the pre-S1 domain of the HBV-L protein that specifically interacts with NTCP at position 157-165.^(12,18-20) As myrcludex B-FITC is unable to diffuse into cells, it can be used to specifically label plasma membrane-resident NTCP, and we found that the myrcludex B-FITC signal was decreased after ER stress induction by either thapsigargin or SubAB compared to DMSO-treated cells (Fig. 1E,F). This demonstrates a reduced amount of NTCP at the plasma membrane, and this was confirmed by cell surface biotinylation (Fig. 1G,H, left panels). In addition, the total level of NTCP was reduced as indicated by the protein level in the total cell fraction, which was not correlated with a decrease in *NTCP* mRNA (Fig. 1G,H, right panels; Supporting Fig. S3A,B). Cell viability (Supporting Fig. S2), metabolic activity (Supporting Fig. S2A,D), cell density (Supporting Fig. S2B,C,E,F), and caspase3/7 activity (Supporting Fig. S2G-L) remained unchanged after or during thapsigargin and SubAB treatment in both U2OS and HepG2 cell lines, indicating that cells were viable for the duration of the treatment. Altogether, these results suggest that ER stress leads to a reduction of NTCP protein levels, provoking a depletion of this protein from the plasma membrane and decreased uptake of bile acids.

ER STRESS AFFECTS NTCP FUNCTION AND PLASMA MEMBRANE EXPRESSION *IN VIVO*

As cholestasis has been shown to exhibit reduced protein and mRNA level of NTCP/*Ntcp* together with activation of the UPR,^(5,21) we assessed whether we could recapitulate these features in a mouse model of cholestasis. Mice were fed a normal or DDC-diet for 7 days, which leads to biliary luminal porphyrin plugs and cholestasis in this model.⁽¹³⁾ Mice fed a DDC diet had a reduction of NTCP at both the protein and mRNA level when compared to their control-fed counterparts (Fig. 2A; Supporting Fig. S3C), an increase in splicing of *Xbp1* and in DNA-damage

inducible transcript 3 (*Chop*) mRNA, and an increase in BIP/GRP78 protein (Fig. 2B; Supporting Fig. S1C,G). These results recapitulated previous findings in cholestasis models.⁽²¹⁻²⁴⁾ To discriminate whether these changes were solely regulated by FXR, we assessed the effect of bile duct ligation-induced cholestasis in FXR^{-/-} mice⁽¹⁵⁾ (Fig. 2C-E). As in WT mice, protein levels of NTCP were significantly reduced in FXR^{-/-} mice 3 days after bile duct ligation compared to sham-operated controls (Fig. 2C). This decrease in NTCP protein was correlated with an increase in the ER stress markers BIP/GRP78 (Fig. 2D) and spliced *Xbp1* (Fig. 2E). Together, these results suggest that NTCP is regulated at the protein level in absence of FXR through a mechanism that may involve induction of ER stress.

To dissect effects of ER stress from other cholestasis-induced pathways, mice received a single injection of thapsigargin (1 mg/mL) or vehicle solution. In the liver, treatment with thapsigargin resulted in ER stress after 24 hours as indicated by an increased BIP/GRP78 protein level (Fig. 3A), the splicing of *Xbp1* mRNA (Supporting Fig. S1E), and an increase in *Chop* mRNA (Supporting Fig. S1F); its effects were less noticeable after 8 hours (Supporting Fig. S1D). We therefore further assessed the effect of thapsigargin 24 hours after injection; in this condition, NTCP protein level was reduced (Fig 3B). In contrast to what was observed *in vitro* (Supporting Fig. S3A,B), *Ntcp* mRNA levels were decreased in mice subjected to thapsigargin treatment at 24 hours (Supporting Fig. S3D), pointing to the existence of transcriptional regulation in complement of protein regulation. Regulation through activation of the FXR-SHP pathway was excluded as neither *Fxr* nor *Shp* or Abcb11/bile salt export pump (*Bsep*) activation was observed in the thapsigargin-treated mice (Fig. 3C-E). Hence, ER stress induction reduced NTCP levels in the liver and could complement the already known transcriptional pathway that regulates NTCP in cholestasis.

ER STRESS-DEPENDENT NTCP REDUCTION IS NOT REGULATED BY A UNIQUE UPR BRANCH

We further assessed the role of the UPR pathways in ER stress-induced reduction of the NTCP protein. The UPR consists of three branches: inositol-requiring 1 (IRE1), protein kinase R-like ER kinase (PERK),

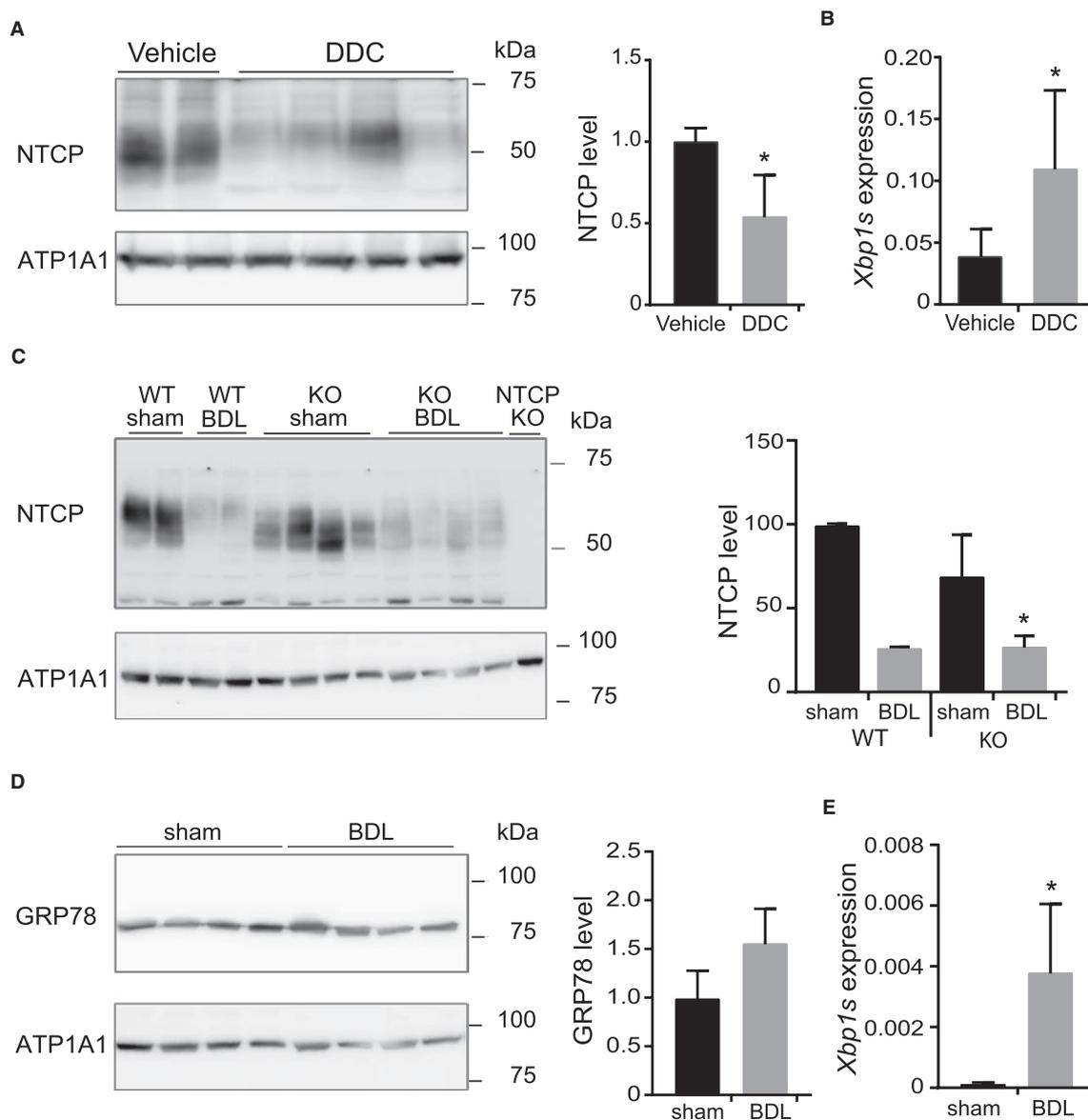


FIG. 2. Cholestasis leads to FXR-independent NTCP down-regulation *in vivo*. (A) Level of NTCP protein in mouse liver extracts analyzed and quantified by western blot after 7 days of DDC diet ($n = 4$) or chow diet ($n = 2$). (B) mRNA expression of *Xbp1* splicing in mice fed a control or DDC diet, analyzed by qRT-PCR; $n = 8$ per condition. (C,D) Level of NTCP or BIP/GRP78 protein in mouse liver extracts analyzed and quantified by western blot 3 days after bile duct ligation or sham operation in WT ($n = 2$ per condition) and/or FXR KO ($n = 4$ per condition). (E) *Xbp1*-spliced mRNA expression in the liver of FXR KO mice 3 days after bile duct ligation or sham operation analyzed by qRT-PCR; $n = 4$. Data are presented as mean \pm SD. (A,B) $*P < 0.05$, compared to untreated condition; Student *t* test. (C-E) $*P < 0.05$ compared to FXR KO sham operated; Mann-Whitney U test. Abbreviations: BDL, bile duct ligation; KO, knockout.

and activating transcription factor 6; together, this regulates complex cellular responses, including RNA decay, translation inhibition, proteasomal degradation, and autophagy.⁽²⁵⁾ These responses, leading to protein regulation, were investigated for their contribution in NTCP expression control.

We first investigated the XBP1-dependent branch of the IRE1 pathway. *XBP1* mRNA is spliced in response to IRE1 activation, and this active XBP1s form functions as a transcription factor to up-regulate numerous genes increasing the folding capacity of the ER. *XBP1s* was down-regulated using short hairpin (sh)

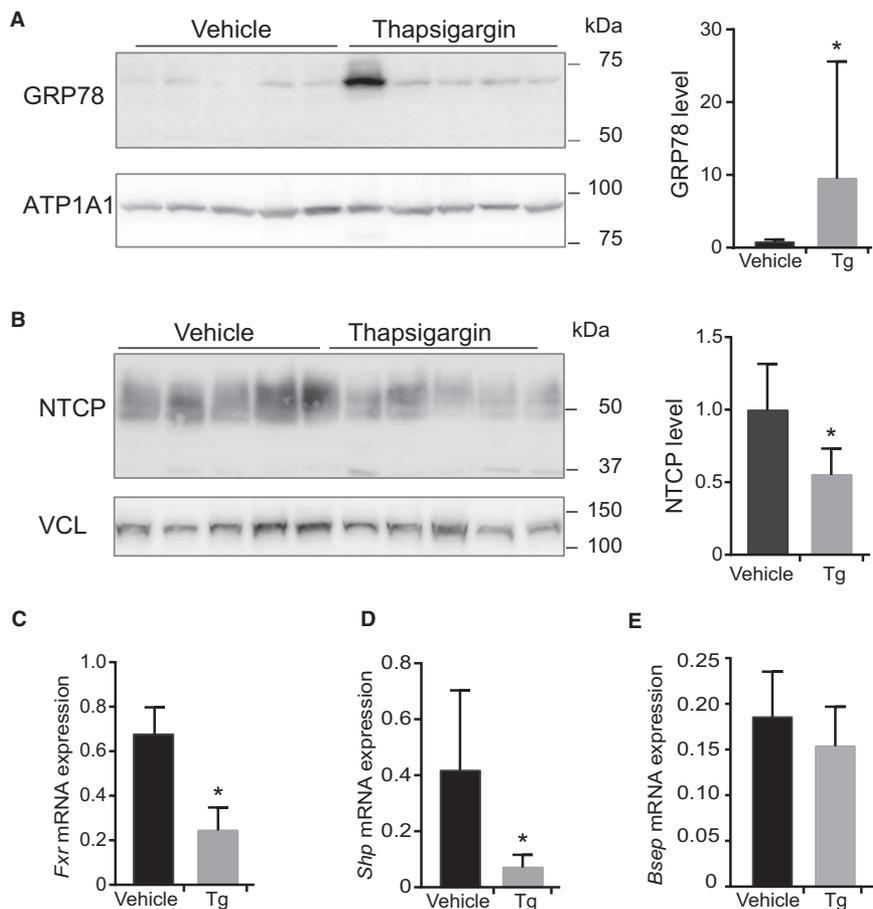


FIG. 3. ER stress leads to NTCP down-regulation *in vivo*. (A) Level of BIP/GRP78 or (B) NTCP protein in mouse liver extracts analyzed and quantified by western blot 24 hours after treatment with thapsigargin or vehicle ($n = 5$ per condition). (C) *Fxr*, (D) *Shp*, and (E) *Bsep* mRNA expression in vehicle or thapsigargin-treated mice analyzed by qRT-PCR; $n = 7$. Data are presented as mean \pm SD. * $P < 0.05$, compared to untreated condition; Student t test.

RNA in U2OS cells expressing HA-hNTCP. Notably, the increase of *XBP1s* after thapsigargin treatment was lost in cells expressing shXBP1 (Supporting Fig. S4A). The down-regulation of *XBP1s* was effective as reported by the decreased induction of the XBP1s target gene DNAJ homolog subfamily C member 3 (*P58^{IPK}*) (Supporting Fig. S4B), while the PERK pathway was unaffected as indicated by similar changes in *CHOP* mRNA after ER stress induction (Supporting Fig. S4C). The uptake function of NTCP was significantly reduced following induction of ER stress in both control and XBP1-depleted cells (Supporting Fig. S4D). In addition, to assess whether it would recapitulate the effects of thapsigargin treatment, we overexpressed XBP1 in intestinal LS174T cells expressing HA-hNTCP. Induction of XBP1 by doxycycline

treatment led to an increase in *XBP1s* mRNA and its target gene *P58^{IPK}* similar to that observed after thapsigargin treatment, while *CHOP* levels remained low (Supporting Fig. S4E-G). However, induction of XBP1s could not recapitulate the effect of thapsigargin treatment on TCA uptake by NTCP (Supporting Fig. S4H). We then assessed the role of the PERK pathway in NTCP down-regulation. PERK phosphorylates eukaryotic translation initiation factor 2 α (EIF2 α), which leads to the attenuation of translation initiation and limits the protein-folding load on the ER.⁽¹⁰⁾ To assess the role of PERK-mediated EIF2 α phosphorylation, we overexpressed A1, a segment of the phosphatase growth arrest and DNA damage 34 that dephosphorylates EIF2 α and interferes with PERK activation.⁽²⁶⁾ Overexpression of A1 did not

prevent the thapsigargin-dependent reduction of bile acid uptake by NTCP (Supporting Fig. S4I) despite a dampening of EIF2 α phosphorylation (Supporting Fig. S4J,K). Therefore, neither the IRE1-XBP1 nor the PERK pathway alone seemed sufficient to mediate the thapsigargin effect on NTCP protein; this was potentially due to redundancy between the UPR pathways.

CALNEXIN INTERACTS WITH NTCP

We subsequently aimed to identify proteins that could contribute to the ER stress-dependent regulation of hepatic bile acid uptake by direct interaction with NTCP. To this end, lysates of HA-tagged NTCP-expressing HepG2 cells and parental HepG2 cells were subjected to immunoprecipitation with anti-HA. Label-free quantitative LC-MS/MS analysis identified the proteins that were enriched in the NTCP-containing fraction (Fig. 4A). SLC10A1 (NTCP) was identified as a significantly enriched protein, confirming the validity of the assay. Moreover, calnexin was significantly increased in the NTCP-expressing cells, identifying a possible interaction partner for NTCP. The presence of this interaction was confirmed by co-immunoprecipitation using U2OS stably expressing HA-hNTCP and transiently transfected with calnexin-FLAG. Calnexin-FLAG coprecipitated with HA-hNTCP (Fig. 4B). Omission of HA-hNTCP prevented precipitation of calnexin, demonstrating the specificity of the co-immunoprecipitation. Similarly, NTCP coprecipitated with calnexin in the reciprocal approach using anti-FLAG antibody-coated beads, validating the interaction of calnexin with NTCP (Fig. 4C).

CALNEXIN REGULATES NTCP EXPRESSION AND FUNCTION

Calnexin regulates the quality control of glycosylated protein folding in the ER together with calreticulin.⁽²⁷⁾ We and others have shown that NTCP is an N-linked glycoprotein with two glycosylation sites.^(12,28,29) These glycosyl moieties regulate NTCP trafficking and localization at the plasma membrane.⁽¹²⁾ ER stress did not alter NTCP glycosylation as seen by comparing the molecular weight of NTCP in both treated and untreated cells, while the

NTCP abundance was lowered (Fig. 5A). Treatment with peptide-N-glycosidase F (PNGase F), an amidase that removes N-glycosylation, resulted in a similar shift in molecular weight in all samples (Fig. 5B). To investigate whether calnexin is involved in reducing NTCP protein expression during ER stress, we assessed the effect of ER stress on calnexin level in U2OS and HepG2 cells expressing HA-hNTCP. Following ER stress induction by either thapsigargin or SubAB, the mRNA level of calnexin was increased in both HepG2 (Supporting Fig. S3E) and U2OS cells (Supporting Fig. S3F). In contrast, the protein level of calnexin was reduced and correlated with a reduction in NTCP protein level (Fig. 5C). We could reproduce these findings in mice 24 hours after a single injection of thapsigargin (Fig. 5D) or fed a DDC diet (Fig. 5E). Together, these results suggest that cholestasis-associated ER stress leads to reduced calnexin protein levels accompanied by down-regulation of NTCP, without affecting NTCP glycosylation status.

We further assessed the role of calnexin on NTCP regulation more directly using U2OS stably expressing HA-hNTCP in which calnexin was knocked down by shRNA (Supporting Fig. S3G,H). Calnexin shRNA expression led to an apparent complete depletion of the protein (Fig. 6A) and was correlated with a reduction in NTCP protein level (Fig. 6A) but not *NTCP* mRNA (Supporting Fig. S3I,J), similar to what was observed in thapsigargin-treated cells (Fig. 5A; Supporting Fig. S3A,B). Levels of spliced *XBP1*, *BIP*, and *CHOP* mRNA (Supporting Fig. S3K-M) were similar in control and calnexin knocked-down cells, indicating that the reduction of NTCP was not caused by ER stress induction. Treatment with PNGase F showed that NTCP is still glycosylated in the calnexin-depleted cells (Fig 6B). This decrease in protein amount was associated with a reduction in TCA uptake in U2OS and HepG2 cells expressing both HA-hNTCP and calnexin-targeting shRNA (Fig. 6C,D). The reduced uptake could suggest a depletion of NTCP from the plasma membrane as observed in thapsigargin-treated cells. Localization of NTCP at the membrane was assessed by myrcludex B-FITC interaction and cell surface biotinylation in cells co-expressing HA-hNTCP and either a control shRNA or calnexin-targeting shRNA. Both the myrcludex B signal (Fig. 6E,F) and surface-biotinylated fraction of NTCP (Fig. 6G) were reduced in calnexin-depleted cells, demonstrating a reduction of NTCP

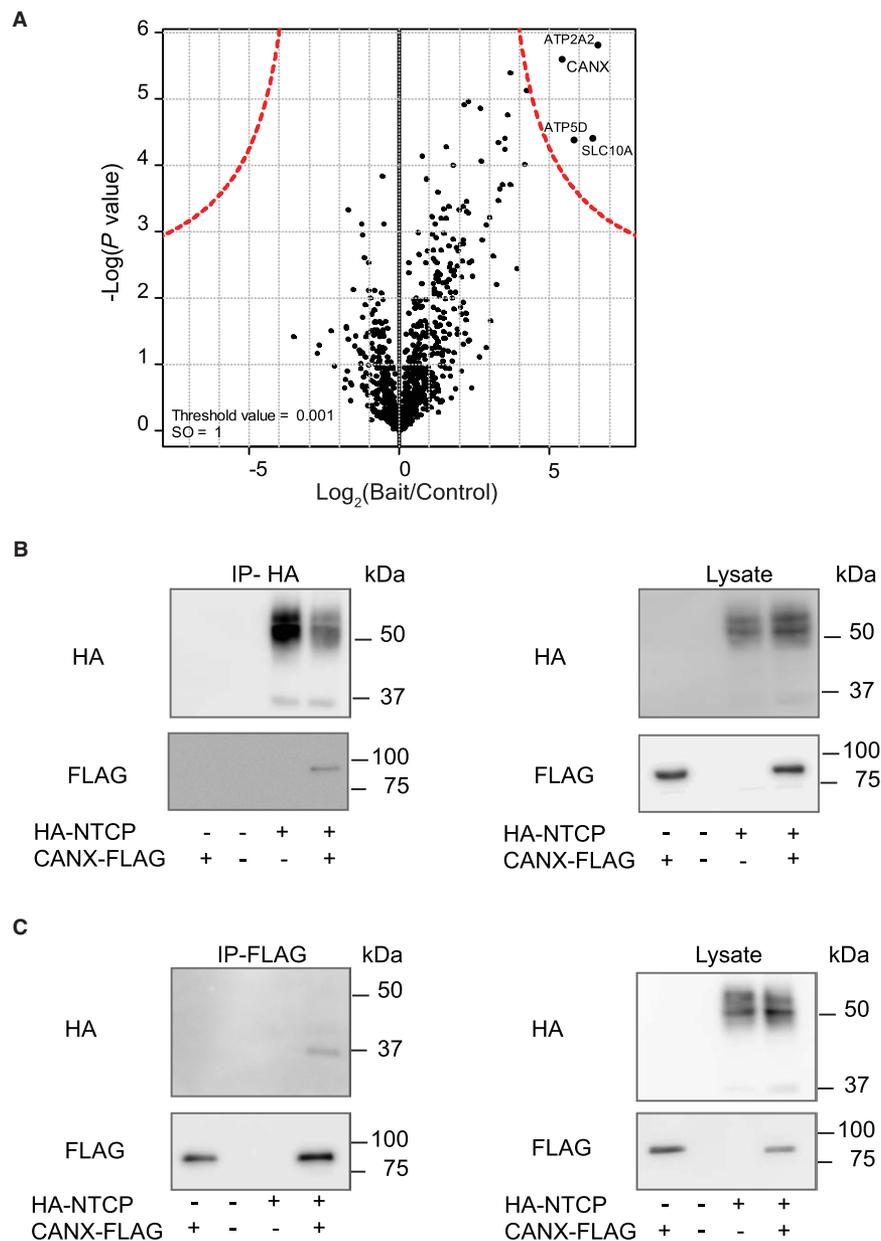


FIG. 4. Calnexin interacts with NTCP. (A) Volcano plot of the label-free quantitative MS data plotting the logarithmic difference in protein levels in the immunoprecipitated fraction of NTCP-expressing HepG2 cells and the parental HepG2 cells versus negative logarithmic P values of the t test performed from quadruplicates. The dotted curve indicates a false discovery rate of 0.001; $n = 4$ independent precipitated samples of NTCP-expressing HepG2 or parental HepG2 cells. (B) Immunoprecipitation of HA-hNTCP or (C) calnexin-FLAG in U2OS cells with or without stable expression of NTCP or parental U2OS and transiently transfected with calnexin-FLAG or a control vector, which shows co-immunoprecipitation of (B) calnexin-FLAG or (C) HA-hNTCP. Lysate is the total proteins. Western blots are representative of three independent experiments. Abbreviation: IP, immunoprecipitated.

at the plasma membrane. These results indicate that calnexin depletion can recapitulate the key features of thapsigargin-induced NTCP regulation.

We then investigated whether calnexin is responsible for the reduction of NTCP after ER stress. For this purpose, we transiently expressed green

fluorescent protein (GFP) or calnexin-FLAG in U2OS HA-hNTCP cells, which were then subjected to SubAB treatment (Fig. 7A,B). Similar to our initial observations (Fig. 1C,D and 1G,H, right panels), the total level of NTCP and TCA uptake was significantly reduced following induction of ER stress in

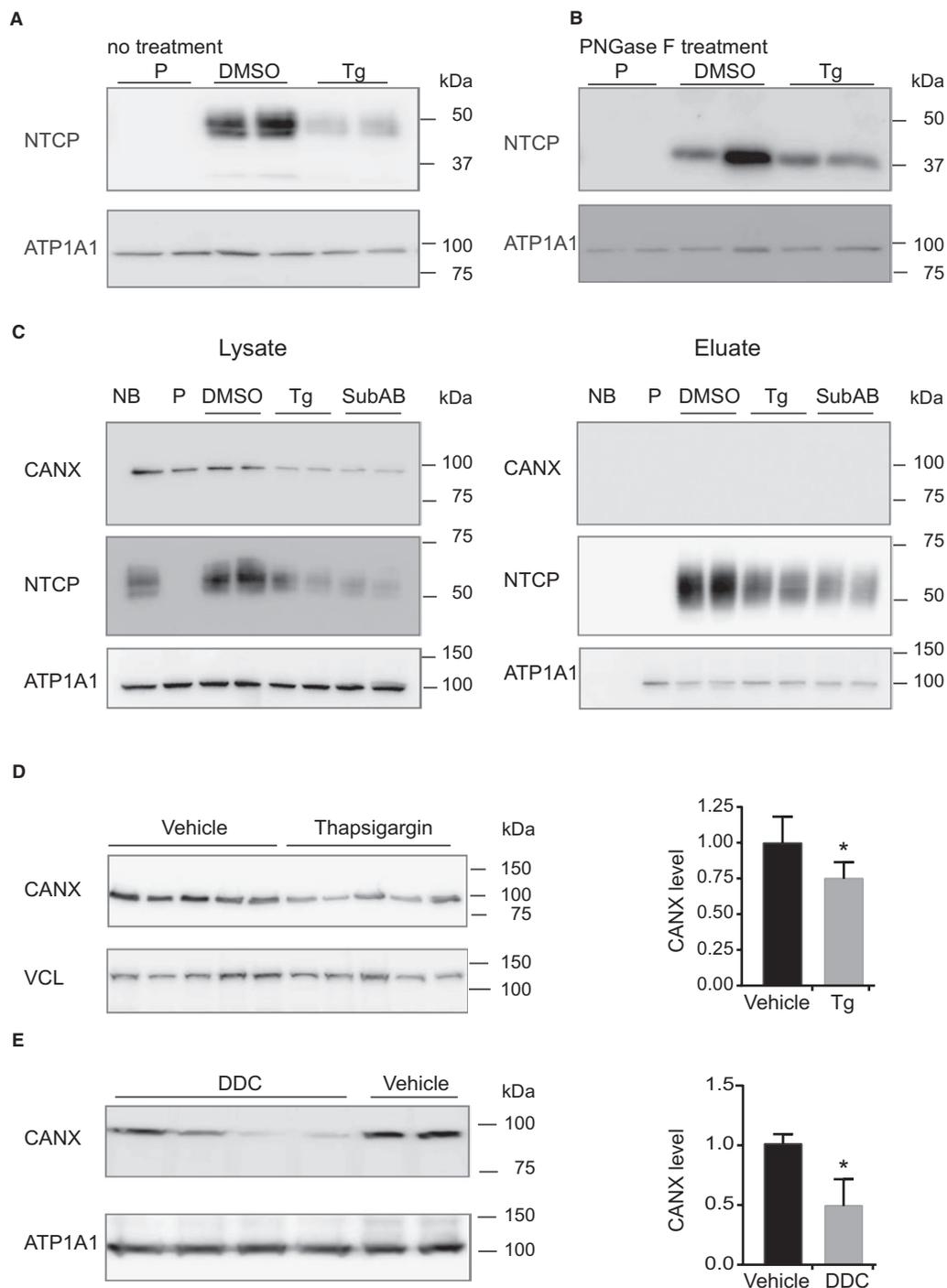


FIG. 5. ER stress lowers calnexin levels *in vitro* and *in vivo*. (A,B) Western blots showing NTCP as a glycosylated protein with or without ER stress induction as indicated by its change in molecular weight between (A) native condition or (B) after removal of all glycosylations (PNGase F treatment) in U2OS HA-hNTCP cells. Western blots are representative of two independent experiments. (C) Cell surface biotinylation showing the total (lysate) and plasma membrane (eluate) protein levels of calnexin and NTCP after ER stress induction by either thapsigargin or SubAB in U2OS HA-hNTCP cells. Western blots are representative of three independent experiments. (D,E). Level of CANX protein in liver membrane extracts of mice analyzed and quantified by western blot after a single injection of (D) thapsigargin ($n = 7$ per condition) or (E) after feeding with a DDC diet ($n = 4$) or chow diet ($n = 2$). Data are presented as mean \pm SD. * $P < 0.05$, compared to untreated condition; Student t test. Abbreviation: VCL, vehicle.

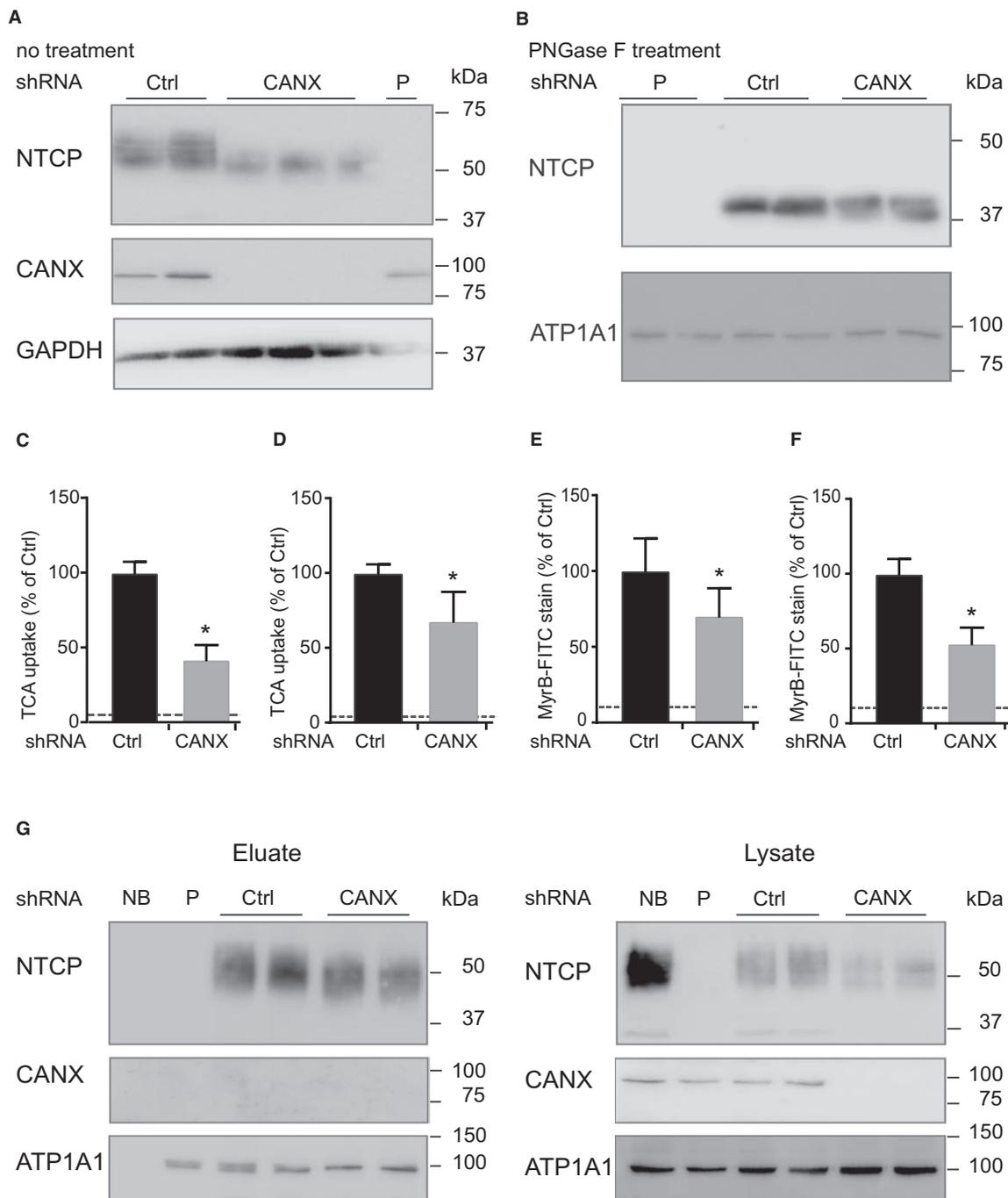


FIG. 6. Knockdown of calnexin reduces NTCP expression and function *in vitro*. (A,B,D,F,G) U2OS HA-hNTCP or (C,E) HepG2 HA-hNTCP cells expressing either scramble shRNA or shRNA targeting calnexin are analyzed for their effect on NTCP function and expression. (A) Glycosylation changes of NTCP analyzed by western blot in an unmodified condition or (B) after removal of all glycosylation. Blots are representative of two independent experiments. (C,D) TCA uptake presented as percentage of control cells. (E-G) Level of NTCP present at the plasma membrane as measured by (E,F) NTCP-myrccludex B-FITC staining or (G) by cell surface biotinylation. The blots shown are representative of three independent experiments and show the plasma membrane (eluate) or total (lysate) protein fractions. (C-F) $n = 3$ independent experiments performed in quadruplicate. Data are presented as mean \pm SD. * $P < 0.05$, compared to control condition; Student t test. Abbreviations: Ctrl, scramble shRNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cells expressing GFP (Fig. 7A,B). Both NTCP protein level and uptake function after ER stress were rescued following expression of FLAG-calnexin (Fig. 7A,B). This effect was correlated with an increase in calnexin amount compared with GFP-expressing cells treated with SubAB (Fig. 7A), indicating that depletion of calnexin is responsible for NTCP down-regulation after ER stress. Altogether, these results indicate that down-regulation of calnexin mediates the effect of ER stress on NTCP at the protein level.

ER STRESS INDUCTION FURTHER REDUCES NTCP FUNCTION IN CALNEXIN-DEPLETED CELLS

We investigated whether calnexin depletion underlies the NTCP reduction following ER stress. Calnexin depletion led to a reduction in TCA uptake similar to thapsigargin treatment in HepG2 (Fig. 8A) and U2OS (Fig. 8B) cells. However, treatment of cells lacking calnexin with thapsigargin resulted in a moderate but significant additional decrease in TCA uptake (Fig. 8A,B). This result correlated with a decreased level of NTCP

at the plasma membrane (Fig. 8C-E). In U2OS HA-hNTCP, myrcludex B-FITC binding was similarly reduced in thapsigargin- and SubAB-treated scramble shRNA (shCtrl) cells and in untreated calnexin-depleted cells (Fig. 8C). However, induction of ER stress by thapsigargin or SubAB in cells already depleted of calnexin led to a further decrease in myrcludex B-FITC binding (Fig. 8D). The additional decrease in NTCP plasma membrane reduction was verified by cell surface biotinylation (Fig. 8E). These results confirmed that the decreased TCA uptake was due to a depletion of NTCP from the plasma membrane. Therefore, it seems that in the absence of calnexin, NTCP is more sensitive to the effect of ER stress.

CALRETICULIN COULD COMPENSATE FOR CALNEXIN DECREASE AFTER ER STRESS INDUCTION

Because calnexin exerts its role in ER resident quality control of newly synthesized glycoproteins together with calreticulin,⁽³⁰⁾ we investigated whether

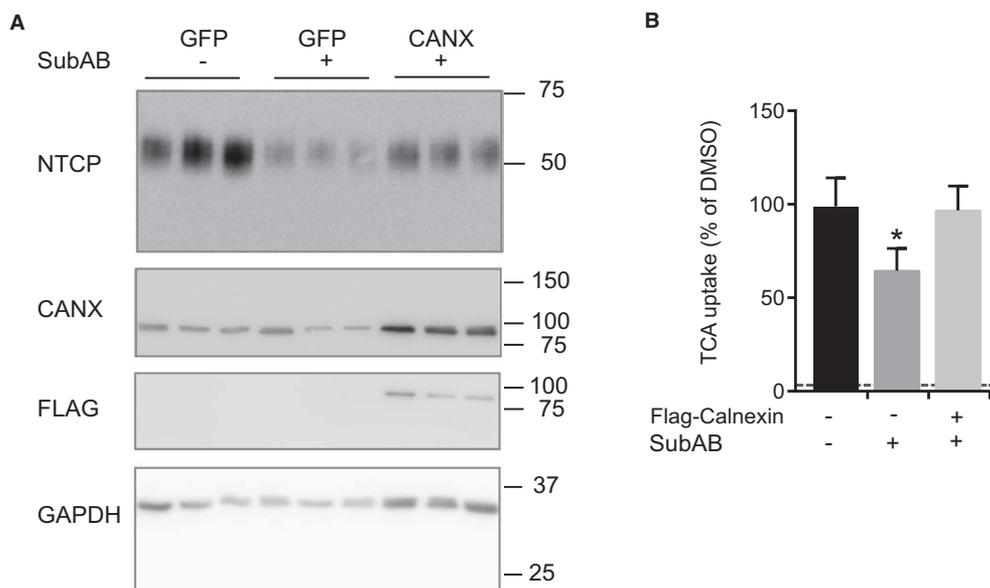


FIG. 7. Calnexin overexpression rescues NTCP down-regulation induced by ER stress. ER stress induction by SubAB in U2OS HA-hNTCP cells expressing either FLAG-calnexin or GFP. (A) Level of NTCP, endogenous calnexin, and overexpressed FLAG-calnexin protein after ER stress induction analyzed by western blot. The blot shown is representative of three independent experiments. (B) TCA uptake assay following treatment with SubAB expressed as percentage of GFP-expressing mock-treated cells; $n = 3$ independent experiments performed in quadruplicate. The dotted line represents the level of uptake in cells lacking NTCP. Data are presented as mean \pm SD. * $P < 0.05$, compared to control condition; analysis of variance followed by Bonferroni's multiple comparison test. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

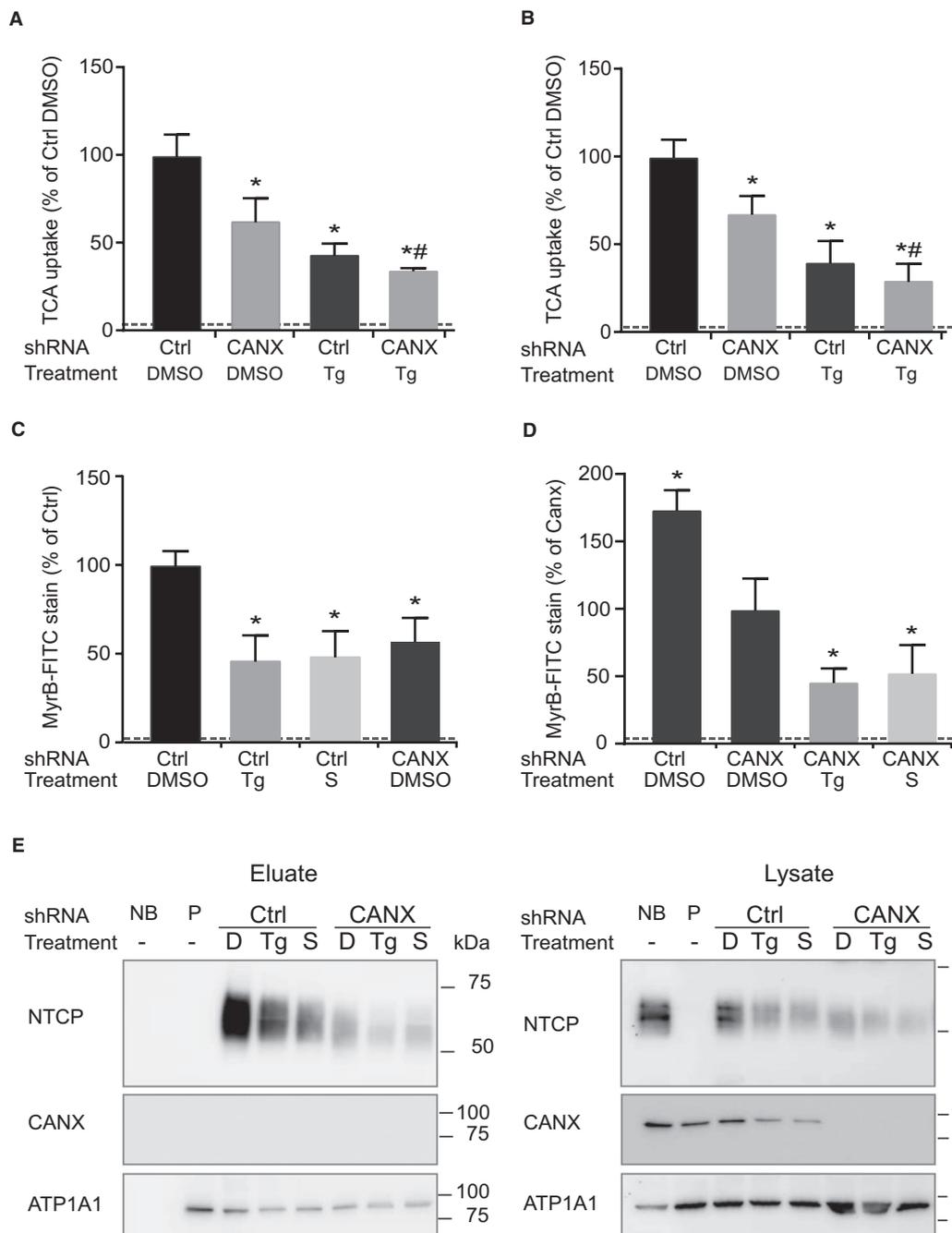


FIG. 8. Additional effect of ER stress in cells lacking calnexin. (A-E) ER stress induction by either thapsigargin or SubAB in (A) HepG2 HA-hNTCP or (B-E) in U2OS HA-hNTCP cells expressing either scramble shRNAs or shRNAs targeting calnexin. (A,B) TCA uptake presented as percentage of DMSO-treated cells. Dotted lines indicate the level of uptake in cells lacking NTCP; $n = 3$ independent experiments performed in quadruplicate. Data are presented as mean \pm SD. * $P < 0.05$, compared to untreated cells expressing control shRNA; ** $P < 0.05$, compared to untreated sh-calnexin cells; Student t test. (C,D) Level of NTCP present at the plasma membrane measured by NTCP myrcludex B-FITC interaction as percentage of DMSO-treated cells. Dotted line indicates the level of myrcludex B-FITC detected in cells lacking NTCP; $n = 3$ independent experiments each performed in at least quadruplicate. (E) Cell surface biotinylation showing the plasma membrane (eluate) and total (lysate) protein levels of NTCP and calnexin. Controls without NTCP or biotin confirm the reliability of the assay. Western blots are representative of three independent experiments. Abbreviation: Ctrl, scramble shRNA.

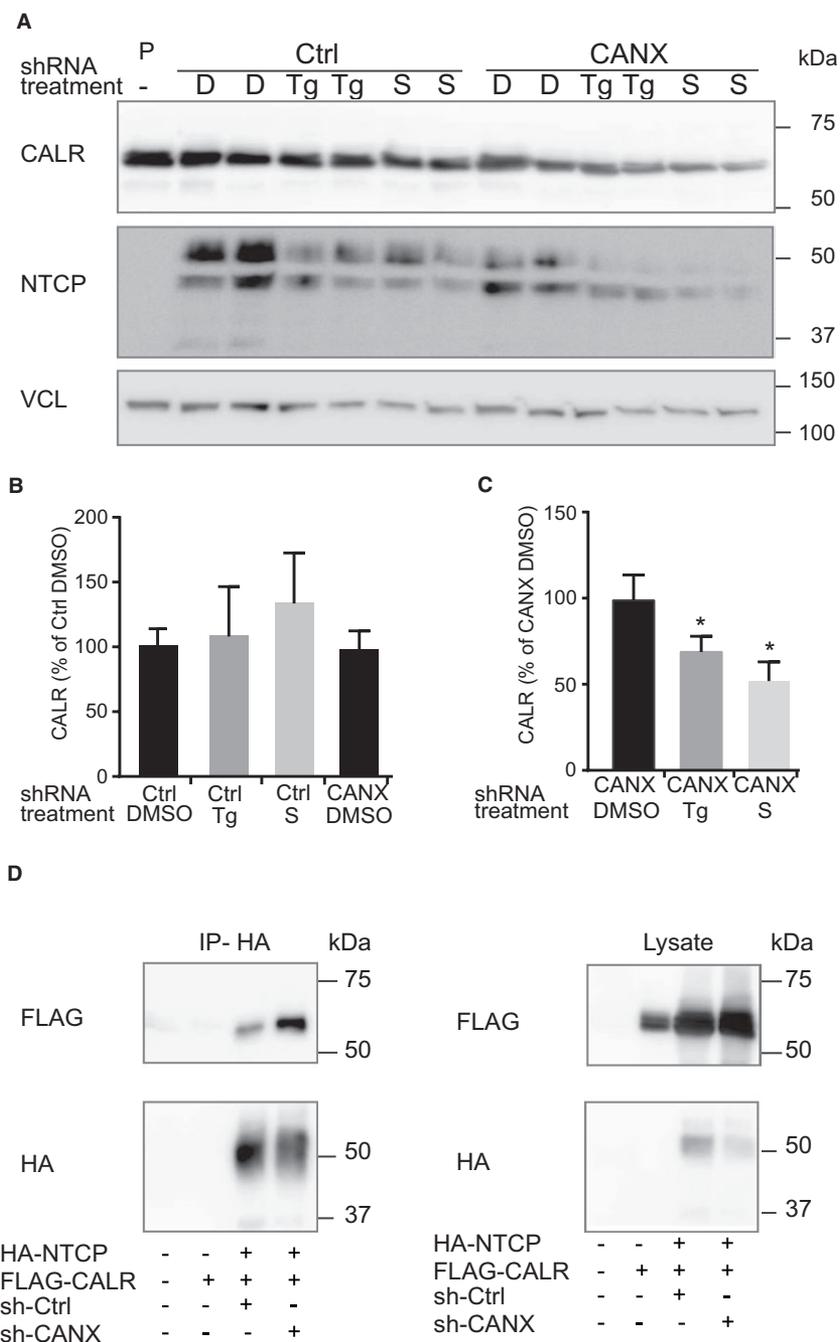


FIG. 9. Induction of ER stress in cells lacking calnexin leads to a reduced amount of calreticulin. (A) Western blot and (B,C) quantifications showing calreticulin level after ER stress induction in U2OS cells expressing either scramble shRNAs or shRNAs targeting calnexin. Calreticulin protein levels are expressed relative to (B) untreated shCtrl cells or (C) as percentage of untreated calnexin-depleted cells. (D) Immunoprecipitation of HA-hNTCP in U2OS cells expressing either scramble shRNAs or shRNAs targeting calnexin and transiently transfected with either FLAG-calreticulin or a control vector, which shows co-immunoprecipitation of FLAG-calreticulin. Western blot is representative of three experiments; Data are presented as mean \pm SD. * $P < 0.05$, compared to untreated cells expressing calnexin shRNA; analysis of variance followed by Bonferroni's multiple comparison test. Abbreviations: CALR, calreticulin; Ctrl, scramble shRNA; D, DMSO; IP, immunoprecipitated; S, SubAB; VCL, vehicle.

the latter has a role in the additional effect of ER stress on NTCP in the absence of calnexin. Protein levels of calreticulin remained unchanged following induction of ER stress by either thapsigargin or SubAB (Fig. 9A,B). Similarly, depletion of calnexin did not affect calreticulin levels under basal conditions (Fig. 9A,B). However, in cells already depleted of calnexin, induction of ER stress with thapsigargin or SubAB led to a reduction of calreticulin (Fig. 9A,C), which was correlated with the additional decrease in NTCP protein (Fig. 9A). Subsequently, we investigated whether calreticulin could interact with NTCP by co-immunoprecipitation in U2OS cells stably expressing HA-hNTCP and either an shCtrl or a calnexin-targeting shRNA. These cells were transiently transfected with FLAG-calreticulin or a GFP construct. Calreticulin coprecipitated with HA-hNTCP using an anti-HA antibody (Fig. 9D). Omission of HA-NTCP prevented the precipitation of FLAG-calreticulin, demonstrating the specificity of the co-immunoprecipitation. Therefore, we concluded that calreticulin was able to physically interact with NTCP. Furthermore, depletion of calnexin resulted in a ~3-fold increase in the FLAG-calreticulin signal in the NTCP-associated fraction.

Discussion

Our findings show that ER stress induction leads to calnexin down-regulation *in vitro* and *in vivo* but also that calnexin depletion leads to reduced NTCP protein levels and decreased bile acid uptake *in vitro*. Furthermore, we show that cholestasis in mice correlates with a reduction of calnexin and an induction of ER stress, providing a possible rationale for FXR-independent NTCP protein down-regulation in bile duct-ligated mice.

We therefore propose that this mechanism contributes to the hepatoprotective repression of hepatic bile acid uptake in cholestatic conditions, complementing FXR-dependent transcriptional down-regulation of *NTCP* mRNA.^(24,31) We recently demonstrated that pharmacologic inhibition of NTCP by myrcludex B injection reduced the level of liver damage in specific mouse models of cholestasis, illustrating the relevance of such an additional posttranscriptional fine tuning of NTCP regulation.⁽¹⁴⁾ The control of NTCP by ER stress-induced calnexin down-regulation could act as a naturally occurring (patho)physiologic mechanism

to dampen hepatic bile acid uptake that can be further improved by pharmacologic treatments.

A potential additional level of complexity arose from a recent study showing that FXR can activate the IRE/XBP1 pathway.⁽³²⁾ That study notably suggests a role for FXR in XBP1 activation in the early stages of cholestasis, using a mouse bile duct ligation model. Those results indicate a potential role for FXR in the ER stress regulation of NTCP that we observed in our models. In our FXR^{-/-} model, an increase in *Xbp1* splicing was still detected 3 days after bile duct ligation. Both experimental settings were similar, apart from a sex and moderate timing difference, which could suggest that FXR regulation of *Xbp1* is highly time dependent and is involved in the very early stages of cholestasis. Another possibility would be that, *in vivo*, a compensatory mechanism could regulate *Xbp1* and overall induction of ER stress in the absence of FXR. Unfortunately, the authors did not assess the level of NTCP in these early stages, and our other mouse model using the DDC diet was analyzed at a more advanced stage, impeding comparison between these two models. Additionally, in our cell models, the sole activation of XBP1 could not recapitulate the effects of ER stress induction on NTCP level and function. Therefore, it is unlikely that the FXR-XBP1 pathway is responsible for the down-regulation of NTCP under sustained ER stress or in more advanced stages of cholestasis but could instead be involved in regulation processes at early stages. We could not identify a unique UPR pathway responsible for the regulation of NTCP, likely due to compensatory mechanisms. However, we could demonstrate that calnexin interacts with NTCP and that a decreased calnexin level leads to the reduction of NTCP protein in human cells. In addition, we found that the lowering of calnexin protein level under ER stress could be partially compensated by the second ER resident lectin chaperone calreticulin.

Two recent studies indicated that ER stress can regulate bile acid transporters at the transcriptional level, independently of the classical FXR pathway.^(33,34) Both studies showed that chronic ER stress reduces *Bsep*, *Ntcp*, and *Fxr* mRNA levels in mice. These results were supported by previous observations demonstrating that ER stress leads to down-regulation of hepatocyte nuclear factor 4 α independently of SHP and FXR.^(35,36) Here, we could confirm these findings in cholestatic mice while providing evidence for an additional level of regulation of NTCP at the protein level.

Indeed, the results suggest that ER stress can regulate NTCP protein by lowering the chaperone calnexin, possibly affecting NTCP folding within the ER.

Surprisingly, we found that calnexin protein levels are reduced in response to ER stress. Similarly, calreticulin levels are sensitive to ER stress but only in the absence of calnexin. Reduction of glycoprotein chaperones in a situation where protein folding is already challenged seems counterintuitive. In multiple cell types, calnexin level was not changed after chemically induced ER stress, and current view implies that following ER stress induction, calnexin is modified and relocalized in a subcompartment of the ER, thus increasing its chaperone function.^(27,37) To our knowledge, only three other studies could link ER stress with decreased lectin chaperone levels.⁽³⁸⁻⁴⁰⁾ Numata et al.⁽³⁸⁾ observed that overexpression of a mutant form of proteolipid protein 1 led to induction of ER stress and down-regulation of calreticulin. As we observed for calnexin, the chaperone was only affected at the protein level, while its mRNA level was increased. Using a different approach, Kuang and coworkers⁽³⁹⁾ showed that blocking the proteasome, and therefore potentially affecting the stress level of the ER, led to reduced intracellular protein levels of calreticulin. In these two studies, calnexin was not affected, and neither of these groups assessed the effect of additional stress when calreticulin level was reduced. The earlier work from Zuppini et al.⁽⁴⁰⁾ assessed the effect of thapsigargin on T-lymphoblastoid leukemia cell lines with a calnexin deficiency. They showed that thapsigargin treatment led to a proportionally larger decrease in calreticulin in cells lacking calnexin compared to WT cells, similarly to what we observed in cells expressing sh-calnexin. However, unlike us, they observed an initial down-regulation of calreticulin in WT cells after thapsigargin treatment. It is possible that the basal level of calnexin in different cell types, as suggested by the study of Delom and coworkers,⁽³⁷⁾ together with the basal ER activity level of these cells can lead to various answers and adaptability following ER stress.

The reduction of calnexin following ER stress induction leading to a decrease of NTCP activity contributes to dampened hepatic bile acid uptake under cholestatic conditions. A recent study suggests that this process might also play a role in noncholestatic liver disease, although the functional consequence is unclear. Using a large transcriptome analysis in human liver,

Clarke et al.⁽⁴¹⁾ found that numerous genes involved in protein processing and biosynthesis of N-glycosylated proteins, including calnexin and calreticulin, were down-regulated during nonalcoholic fatty liver disease progression. This broad reduction in the effectors of the glycosylation pathway was correlated with a decrease in NTCP at the protein level. As multiple studies show that nonalcoholic steatohepatitis (NASH) correlates with ER stress induction (reviewed in Zhang et al.⁽⁴²⁾), this suggests that ER stress-induced calnexin reduction and subsequent NTCP protein down-regulation can occur in both cholestasis and NASH.

Acknowledgment: We thank D. Slijepcevic, R.L.P. Roscam Abbing, J.M. Donkers, L.C.J.M. Haazen, and C.C. Croes for their contribution to the DDC cholestasis mouse model.

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