Research Article

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# *Nor*Ursodeoxycholic acid ameliorates cholemic nephropathy in bile duct ligated mice

Elisabeth Krones[1], Kathrin Eller[2], Marion J. Pollheimer[3], Silvia Racedo[1], Alexander H. Kirsch[2], Bianca Frauscher[2], Annika Wahlström[4], Marcus Ståhlman[4], Michael Trauner[5], Florian Grahammer[6], Tobias B. Huber[6,7,8], Karin Wagner[9], Alexander R. Rosenkranz[2], Hanns-Ulrich Marschall[4], Peter Fickert[1,*]

[1]*Research Unit for Experimental and Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Medical University of Graz, Austria;* [2]*Clinical Division of Nephrology, Department of Internal Medicine, Medical University of Graz, Austria;* [3]*Department of Pathology, Medical University of Graz, Austria;* [4]*Sahlgrenska Academy, Institute of Medicine, Department of Molecular and Clinical Medicine, University of Gothenburg, Sweden;* [5]*Hans Popper Laboratory of Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Austria;* [6]*Department of Medicine IV, Medical Center and Faculty of Medicine, University of Freiburg, Germany;* [7]*BIOSS Center for Biological Signaling Studies, Albert-Ludwigs-University Freiburg, Freiburg, Germany;* [8]*FRIAS – Freiburg Institute for Advanced Studies and ZBSA – Center for Systems Biology, Albert-Ludwigs-University, Freiburg, Germany;* [9]*Core Facility Molecular Biology, Center for Medical Research, Medical University Graz, Graz, Austria*

**Background & Aims**: Severe cholestasis may cause cholemic nephropathy that can be modeled in common bile duct ligated (CBDL) mice. We aimed to explore the therapeutic efficacy and mechanisms of *nor*ursodeoxycholic acid (*nor*UDCA) in cholemic nephropathy.

**Methods**: In 8-week CBDL mice fed with *nor*UDCA (prior or post CBDL) or chow we evaluated serum urea levels, urine cytology and urinary neutrophil gelatinase associated lipocalin (uNGAL), kidney and liver tissue quantification of fibrosis by hydroxyproline content and gene chip expression looking at key genes of inflammation and fibrosis. Moreover, we comprehensively analysed bile acid profiles in liver, kidney, serum and urine samples.

**Results**: *Nor*UDCA-fed CBDL mice had significantly lower serum urea and uNGAL levels and less severe cholemic nephropathy as demonstrated by normal urine cytology, significantly reduced tubulointerstitial nephritis, and renal fibrosis as compared to controls. *Nor*UDCA underwent extensive metabolism to produce even more hydrophilic compounds that were significantly enriched in kidneys.

**Conclusion**: *Nor*UDCA ameliorates cholemic nephropathy due to the formation of highly hydrophilic metabolites enriched in kidney. Consequently, *nor*UDCA may represent a medical treatment for cholemic nephropathy.

**Lay summary**: The term cholemic nephropathy describes renal dysfunction together with characteristic morphological alterations of the kidney in obstructive cholestasis that can be mimicked by ligation of the common bile duct in mice. Feeding the hydrophilic bile acid *nor*UDCA to bile duct ligated mice leads to a significant amelioration of the renal phenotype due to the formation of highly hydrophilic metabolites enriched in the kidney and may therefore represent a medical treatment for cholemic nephropathy.



## Introduction

Impairment of renal function in liver disease represents a severe life-threatening event and may be related to numerous causes including hepatorenal syndrome (HRS) [1–4]. Patients with cirrhosis with concomitant infections and those with severe jaundice represent a high-risk group with dismal prognosis [5–8]. Notably, infection and cholestasis commonly co-exist as inflammation-induced cholestasis [9], and infection also represents a major trigger for acute on chronic liver failure (ACLF) [10].

Specific kidney alterations due to cholestasis are known as cholemic nephropathy, also referred to as bile cast nephropathy [11–13]. These umbrella terms cover impaired renal function in patients with jaundice with tubular epithelial damage predominantly at the level of distal nephron segments together with characteristic intratubular bile cast formation [13–15].

Aiming to explore novel treatment strategies, we had recently modeled cholemic nephropathy in long-term common bile duct ligated (CBDL) mice [16]. We showed that compensatory renal instead of biliary excretion of bile acids leads to: (i) tubular epithelial injury, cast formation, basement membrane defects; (ii) leakage of urine into the kidney parenchyma; (iii) induction of a reactive phenotype of tubular epithelial cells resulting in interstitial nephritis, and finally (iv) kidney fibrosis.

Keywords: Acute kidney injury; Bile acids; Bile acid therapy; Bile cast nephropathy; Kidney fibrosis; Liver cirrhosis; Renal failure.

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* Corresponding author. Address: Research Unit for Experimental and Molecular Hepatology, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. Tel.: +43 316 385 81792; fax: +43 316 385 17108.

*E-mail address:* peter.fickert@medunigraz.at (P. Fickert).

Modified bile acid derivatives open exciting novel therapeutic opportunities for liver disorders and beyond [17,18]. *Nor*ursodeoxycholic acid (*nor*UDCA) is a side-chain shortened derivative of UDCA [19,20] and was recently tested in a phase II clinical trial in patients with primary sclerosing cholangitis. In mouse models of cholestasis, *Nor*UDCA increases bile acid-independent, bicarbonate-dependent bile flow and induces alternative secretory routes for potentially toxic endogenous bile acids by ameliorating their metabolism and basolateral hepatocellular export [20–22]. Importantly, *nor*UDCA also protects the kidney by preventing collecting duct injury after 3 days of CBDL in mice [16]. Consequently, *nor*UDCA may represent an attractive substance for the treatment of cholemic nephropathy, especially in advanced cholestatic liver disease. Since currently there is no medical treatment available for cholemic nephropathy, we performed a longitudinal study in a well-characterized mouse model in order to unravel the therapeutic potential of *nor*UDCA and its mechanism(s) of action.

## Materials and methods

*Animal experiments, serum biochemical analysis and histology*

All experimental protocols were approved by the local authorities (BMWF-66.010/0045-II/10b/2010, BMWF-66.010/0012-II/3b/2014 and BMWFW-66.010/0129-WF/V/3b/2016) according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the U.S. National Academy of Science (National Institutes of Health publication 86–23, revised 1985). Experiments were performed with 8–10 week-old male C57BL/6 mice (25 to 30 g body weight), housed with a 12:12 h light:dark cycle and permitted *ad libitum* consumption of water and food. CBDL was performed as described previously [23].

The therapeutic efficacy of *nor*UDCA in cholemic nephropathy was studied using two experimental arms referred to as: *norUDCA* for prevention and *norUDCA* for rescue. *Nor*UDCA was a generous gift from Dr. Falk Pharmaceuticals (Freiburg, Germany). *Nor*UDCA for prevention (5 *vs.* 5 mice): To determine whether *nor*UDCA prevents cholemic nephropathy in long-term CBDL mice, a *nor*UDCA-enriched diet (0.125% w/v, corresponding to 200 mg/kg/day for a mouse of 25 g body weight eating about 4 g daily) or a standard mouse diet (Sniff, Soest, Germany) were started 5 days prior to CBDL and were continued until harvesting 8 weeks thereafter. One day prior to CBDL and the day before harvesting mice were housed in metabolic cages for 12 h for urine sampling. Urinary output was determined 5 days after diets were started and 3 days following CBDL (pooled urine samples of 4 *vs.* 4 mice; 2 mice per metabolic cage). *Nor*UDCA for rescue (5 *vs.* 5 mice): To determine whether *nor*UDCA rescues the renal phenotype in CBDL mice, a *nor*UDCA-enriched diet (0.125% w/v) or a standard mouse diet was started 3 days after CBDL and was continued until harvesting after 8 weeks. Day 3 after CBDL was chosen based on the serum bile acids' peak and the fact that the first morphological kidney alterations are observed at that time point [16]. In both experimental groups, blood was collected during harvesting and serum biochemical analysis (alanine aminotransferase, ALT; alkaline phosphatase, AP; bilirubin and urea) was performed on a Cobas 501 analyser (Roche Diagnostics, Mannheim, Germany). For morphological analysis, livers and kidneys were fixed in 4% neutral buffered formaldehyde solution for 24 h, embedded in paraffin and further processed for Haematoxylin and Eosin (H&E), periodic acid–Schiff (PAS), Sirius Red (SR), and acid fuchsine–Orange G (SFOG) staining (2 µm thick sections) as previously described [16]. Quantification of kidney fibrosis was determined by measurement of renal hydroxyproline concentration by a calorimetric method as described previously [16].

For transcriptional profiling using microarray technology, we compared sham-operated (SOP) mice and 3-week CBDL mice that were either fed 0.125% *nor*UDCA-enriched or standard mouse diets (*nor*UDCA-enriched diets were started 5 days prior to CBDL and were continued until harvesting 3 weeks thereafter; 3 mice per experimental group were studied).

To assess whether *nor*UDCA improves the renal phenotype in alternative nephropathy models, *nor*UDCA-fed mice were: (i) subjected to ischemia/reperfusion (I/R) injury or (ii) to unilateral ureteral obstruction (UUO). To assess whether *nor*UDCA improves the renal phenotype in a model of lipopolysaccharide (LPS)-induced acute kidney injury, chow- and *nor*UDCA-fed mice were intraperitoneally injected with LPS (5 µg/kg BW) or vehicle and harvested after 12 h (5 mice per group). Detailed experimental procedures are described in Supplementary material and methods.

*Cytological urinalysis*

Cytological urinalysis as an indirect marker for tubular epithelial damage was performed as described in detail in the Supplementary materials and methods.

*Immunohistochemistry, immunofluorescence and Western blot*

Immunohistochemistry for vascular cell adhesion molecule-1 (Vcam-1) and macrophage and dendritic cell marker F4/80 was performed as previously described [16]. Antibodies used for these and further experiments (see below) are summarized in Table S1.

Immunofluorescence for active caspase 3 and proliferation marker Ki67 was performed as described in detail in the Supplementary materials and methods.

Western blot for vascular cell adhesion molecule-1 (Vcam-1). Detailed experimental procedures are described in the Supplementary material and methods.

*Determination of urinary neutrophil gelatinase associated lipocalin (uNGAL)*

Based on previous studies in humans we hypothesized that urinary (u)NGAL measurements were suitable to monitor tubular epithelial injury and therapeutic effects of *nor*UDCA in our mouse model [24]. NGAL levels were determined in urine samples collected at time of harvesting using a commercially available ELISA kit (Lipocalin-2/NGAL DuoSet Mouse, R&D Systems, Abingdon, UK) or Western blot using a commercially available antibody (Table S1). Detailed experimental procedures are described in Supplementary material and methods.

*Bile acid profiling by UPLC-MS/MS*

Profiles of liver, kidney, serum and urine bile acids on an Applied Biosystems AB SCIEX QTRAP 5,500 platform as published recently [25]. Detailed experimental procedures are described in Supplementary material and methods.

*Transcriptional profiling using microarray technology and microarray data analysis*

To detect genes that are transcriptionally influenced by our experimental set up (SOP, CBDL with and without feeding of *nor*UDCA), the highly standardized Affymetrix Microarray platform was chosen. Detailed experimental procedures are described in Supplementary material and methods.

Determination of renal messenger RNA using quantitative real-time reverse-transcription polymerase chain reaction analysis was performed as described in Supplementary materials and methods.

*Statistical analysis*

Data from animal experiments (*nor*UDCA for prevention, *nor*UDCA for rescue) are reported as arithmetic means with standard deviation of 4 (for measurement of urine output) to 5 animals in each group. Depending on normal distribution of data, statistical analysis using IBM SPSS statistics 23 included either Student's *t* test or analysis of variance with Bonferroni post- hoc testing when three or more groups were compared. A $p$ value $\leq 0.05$ was considered significant. Details on statistical analysis of data from liver, kidney, serum and urine bile acid profiling and transcriptional profiling are given within the respective sections (legend to Table S5 and description of detailed experimental procedures for transcriptional profiling in Supplementary material and methods).

For further details regarding the materials used, please refer to the CTAT table.

## Results

*NorUDCA protects long-term CBDL mice from cholemic nephropathy*

Both experimental arms that were used to study the therapeutic efficacy of *nor*UDCA in cholemic nephropathy (*nor*UDCA for prevention and *nor*UDCA for rescue, Fig. 1A) led to significant