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## LIVER FAILURE AND GROWTH

# The organic solute transporters alpha and beta are induced by hypoxia in human hepatocytes

Carlos A. Schaffner<sup>1</sup>, Jessica Mwinyi<sup>1</sup>, Zhibo Gai<sup>1</sup>, Wolfgang E. Thasler<sup>2</sup>, Jyrki J. Eloranta<sup>1</sup> and Gerd A. Kullak-Ublick<sup>1</sup>

<sup>1</sup> Department of Clinical Pharmacology and Toxicology, University Hospital Zurich, Zurich, Switzerland

<sup>2</sup> Department of Surgery, Klinikum Grosshadern, Munich, Germany

## Keywords

bile acid transport – chronic renal failure  
– gene regulation – ligand – nephrectomy  
– nuclear receptor – organic anion transport  
– rat liver

## Abbreviations

CDCA, chenodeoxycholic acid; CRF, chronic renal failure; EMSA, electrophoretic mobility shift assay; HRE, hypoxia responsive element; OST $\alpha$ / $\beta$ , organic solute transporter  $\alpha$ / $\beta$ ; PCR, polymerase chain reaction; PHH, primary human hepatocytes; VEGFa, vascular endothelial growth factor A.

## Correspondence

Gerd A. Kullak-Ublick, Department of Clinical Pharmacology and Toxicology, University Hospital Zurich, Rämistrasse 100, 8091 Zurich, Switzerland  
Tel: +41 44 255 2068  
Fax: +41 44 255 4411  
e-mail: gerd.kullak@usz.ch

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Bile acids undergo extensive enterohepatic cycling. After being synthesized in the liver, bile acids are excreted into bile and the intestine, subsequently re-absorbed into the portal venous blood and finally transported back to the liver. The enterohepatic circulation of bile acids is maintained by bile acid uptake transporters and excretion pumps, located at the basolateral and apical membranes of intestinal, hepatic and renal cells, which are regulated by changes in the intracellular concentration of bile acids (1).

## Abstract

**Background & Aims:** The organic solute transporters alpha and beta (OST $\alpha$ -OST $\beta$ ) form a heterodimeric transporter located at the basolateral membrane of intestinal epithelial cells and hepatocytes. Liver injury caused by ischaemia-reperfusion, cancer, inflammation or cholestasis can induce a state of hypoxia in hepatocytes. Here, we studied the effect of hypoxia on the expression of OST $\alpha$ -OST $\beta$ . **Methods:** OST $\alpha$ -OST $\beta$  expression was measured in Huh7 cells and primary human hepatocytes (PHH) exposed to chenodeoxycholic acid (CDCA), hypoxia or both. OST $\alpha$ -OST $\beta$  promoter activity was analysed in luciferase reporter gene assays. Binding of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) to the OST $\alpha$ -OST $\beta$  gene promoters was studied in electrophoretic mobility shift assays (EMSA). **Results:** Expression of OST $\alpha$  and OST $\beta$  increased in PHH under conditions of hypoxia. Exposure of Huh7 cells or PHH to CDCA (50  $\mu$ M) enhanced the effect of hypoxia on OST $\alpha$  mRNA levels. In luciferase assays and EMSA, the inducing effect of low oxygen could be assigned to HIF-1 $\alpha$ , which binds to hypoxia responsive elements (HRE) in the OST $\alpha$  and OST $\beta$  gene promoters. Site-directed mutagenesis of either the predicted HRE or the bile acid responsive FXR binding site abolished inducibility of the OST $\alpha$  promoter, indicating that both elements need to be intact for induction by hypoxia and CDCA. In a rat model of chronic renal failure, the known increase in hepatic OST $\alpha$  expression was associated with an increase in HIF-1 $\alpha$  protein levels. **Conclusion:** OST $\alpha$ -OST $\beta$  expression is induced by hypoxia. FXR and HIF-1 $\alpha$  bind in close proximity to the OST $\alpha$  gene promoter and produce synergistic effects on OST $\alpha$  expression.

Any reduction in the regular availability of oxygen in human tissues leads to hypoxia. Various conditions can cause hypoxia or induce hypoxia modulators, such as ischaemia, ischaemic reperfusion injury, cancer and inflammation. In addition, cholestasis induces hypoxia in the liver (2–4) and low oxygen levels regulate bile salt transporters. However the exact mechanism by which hypoxia modulates the expression of these transport proteins has not been elucidated. Furthermore, no

inducing effects on the expression of transport proteins have so far been shown in the literature. An important part of the cellular response to the lack of oxygen is mediated by hypoxia-inducible factor 1 (HIF-1). HIF-1 consists of a hypoxia regulated subunit  $\alpha$  (HIF-1 $\alpha$ ) and a constitutively expressed subunit  $\beta$  (HIF-1 $\beta$ ), also known as the aryl hydrocarbon receptor nuclear translocator protein (ARNT). Under normoxic conditions HIF-1 $\alpha$  is hydroxylated and consequently ubiquitinated, so that the half-life of HIF-1 $\alpha$  is heavily reduced by proteasomal degradation. Under low oxygen conditions, the oxygen dependent hydroxylase activity is reduced and HIF-1 $\alpha$  is less prone to degradation (5, 6). Accumulated HIF-1 $\alpha$  dimerises with ARNT, translocates to the nucleus and binds to the hypoxia responsive element (HRE) in the promoter region of a given target gene. With very little variation, the core HRE is composed of the sequence ACGTG (7).

The heterodimeric organic solute transporter  $\alpha/\beta$  (OST $\alpha/\beta$ ), encoded by the genes *SLC51A* and *SLC51B*, respectively, is a bile acid transporter expressed in the liver, intestine and kidney. In hepatocytes the OST heterodimeric transporters are located in the basolateral membrane, where they are assumed to mediate basolateral efflux of organic anions including bile acids, thereby potentially protecting hepatocytes from intracellular accumulation. In enterocytes OSTs are located in the basolateral membrane, where they are responsible for the efflux of bile acids into the portal venous blood (8, 9).

Cholic acid and chenodeoxycholic acid (CDCA) are the two primary bile acids in humans (10). The nuclear farnesoid X receptor (FXR), also known as the bile acid receptor (BAR), has been identified as an important mediator of CDCA-dependent regulation of gene expression (11, 12). CDCA is an agonistic ligand of FXR and leads to the regulation of a whole battery of genes involved in bile acid metabolism and transport in both the liver and intestine. We previously showed that CDCA transactivates the OST $\alpha$  and OST $\beta$  genes via an FXR dependent pathway. (9).

In this study, we investigated *in vitro* to what extent hypoxic conditions affect the expression of OST $\alpha/\beta$  in the liver using hepatocyte-derived cell lines as well as a chronic renal failure (CRF) rat model. We characterize the transcriptional pathway that mediates hypoxic effects on OST $\alpha/\beta$  gene expression. By exposing cells to elevated bile acid concentrations and hypoxic conditions in parallel, we also simulate the combined effects of hypoxia and cholestasis on the transcriptional regulation of OST $\alpha/\beta$ .

## Material and methods

### Chemicals

Chenodeoxycholic acid (CDCA) and dimethylsulphoxide (DMSO) were purchased from Sigma Aldrich and Invitrogen respectively. Maintenance and transfection

media as well as supplements for cell culture were obtained from Invitrogen (Basel, Switzerland) (OptiMEM, Penicillin/Streptomycin), PAA Laboratories (Les Mureaux, France) [fetal bovine serum (FBS), RPMI 1640 medium] and Lonza (Walkersville, MD, USA) (Hepatocyte maintenance medium with ultraglutamine). Short interfering RNAs (siRNAs) and the TransIT-TKO transfection reagent used in knock-down experiments were purchased from Dharmacon (Lafayette, CO, USA). The Fugene HD reagent used in transfection experiments for luciferase gene reporter assays was obtained from Roche Diagnostics (Basel, Switzerland). Antibodies used in Western blot and EMSA analysis were obtained from Abcam (Cambridge, UK) ( $\beta$ -actin antibody Ab8227), Santa-Cruz Biotechnology Inc. (Dallas, TX, USA) (anti-HIF-1 $\alpha$  antibody sc13515, antimouse Ost $\alpha$  antibody sc10078), Merck Millipore (Darmstadt, Germany) (anti-HIF-1 $\alpha$  antibody ABE279) and Pierce/Thermo Fisher Scientific (Wohlen, Switzerland) (horseradish peroxidase-conjugated goat antimouse antibody #32460). MultiScribe Reverse Transcriptase and TaqMan Gene Expression Assays used for real-time PCR-based expression analysis were purchased from Applied Biosystems (Rotkreuz, Switzerland). Acrylamide 40%/Bisacrylamide 2% used for SDS-Page gel preparation was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

### Cell culture

Huh7 cells (American Type Culture Collection; Molsheim, France) were cultured in RPMI 1640 medium supplemented with 10% FBS plus 100 U/ml penicillin and 100  $\mu$ g/streptomycin. The isolation and culture of PHH has been described in detail (13). Upon arrival, primary human hepatocytes (PHH) were kept for 5 h in hepatocyte maintenance medium with ultraglutamine before any further treatment. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> at either regular atmospheric or 0.2% oxygen partial pressure (Ruskin Invivo 400 system; Ruskin Technology Ltd, Bridgend, UK).

### RNA isolation and real-time PCR

Huh7 cells were seeded in twelve-well plates and, when 80% confluent, subjected to normoxia or hypoxia as well as to treatment with 50  $\mu$ M CDCA or empty vehicle (DMSO). Total RNA was isolated using TRIzol reagent. Two micrograms of each RNA sample were reverse transcribed by random priming (Reverse Transcription System). Two microlitres of cDNA were used in each real-time PCR reaction. Real-time PCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems) using the TaqMan Gene Expression Assays Hs00380895\_m1, Hs00418306\_m1 and Hs99999070\_m1 for human OST $\alpha$ , OST $\beta$  and Vascular endothelial growth factor A (VEGF $\alpha$ , used as an hypoxia marker) respectively.

Constitutively expressed  $\beta$ -actin mRNA was measured as an internal standard for sample normalization (4310881E). Relative mRNA levels were calculated using the comparative threshold cycle ( $\Delta$ CT) method. Each PCR was performed in triplicate and all experiments were repeated three times.

#### Transfection with siRNA

Huh7 cells were transfected in twelve-well plates at 80% confluence. Per transfection, 5  $\mu$ l of TransIT-TKO transfection reagent were mixed with 120  $\mu$ l of serum-free OptiMEM followed by the addition of either the SMARTpool siRNA targeting HIF-1 $\alpha$  or FXR or siControl non-targeting siRNA no. 1 to a final concentration of 25 nM.

After an incubation at room temperature for 10 min, the siRNA mixtures were added to 600  $\mu$ l RPMI 1640 medium supplemented with 10% FBS in each well. Six hours after transfection, cells were subjected to CDCA/hypoxia treatment for 18 h. Cellular RNAs were extracted using TRIzol reagent and subjected to real-time PCR as described above.

#### In silico analysis of the OST gene promoter region

The first 5000 bp upstream of the transcription start site within the OST $\alpha$  and OST $\beta$  5' flanking regions were scanned for putative HRE. The analyses were based on the reference sequences NT\_029928 and NW\_925884 (NCBI database) respectively. The Genomatix MatInspector program (<http://www.genomatix.de>) was used to perform the scan.

#### Reporter gene constructs and expression vectors

Promoter fragments of the human OST $\alpha$  and OST $\beta$  genes were PCR amplified using primers described previously (9), generating amplicons of partial sequences corresponding to the reference sequences listed in the NCBI database (NT\_029928 and NW\_925884). The PCR products were cloned into the luciferase (Luc) vector pGL3basic to generate the OST $\alpha$  (−1475/+161) and

OST $\beta$  (−4748/+29) reporter constructs. Point mutations were introduced into putative transcription factor binding sites using the QuikChange site-directed mutagenesis kit; the oligonucleotides are listed in Table 1. The sequences of all constructs were confirmed by DNA sequencing (Microsynth, Balgach, Switzerland). The mammalian expression plasmids pCMX-FXR and pCMX-RXR were kindly provided by Dr D. Mangelsdorf (University of Texas Southwestern Medical Center).

#### Electromobility shift assay (EMSA)

Oligonucleotides used in EMSA analysis (Table 1) were designed to have a 5'-AGCT overhang at both ends when annealed, allowing radioactive labelling by fill-in reactions. Twenty-five nanograms of annealed oligonucleotides were labelled in a 10  $\mu$ l reaction mix containing 25 units MultiScribe™ Reverse Transcriptase and corresponding reaction buffer, as well as 0.25 mM dGTP/dCTP/dTTP plus 20  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP. Unincorporated nucleotides were removed using Microspin G-25 columns. HIF-1 $\alpha$  protein was synthesized using the TNT rabbit reticulocyte lysate-coupled *in vitro* transcription/translation system. One microlitre of synthesized protein was added to each reaction. Approximately 50 000 cpm (0.5–1.5 ng) of the radioactive probe was added per reaction. Protein-DNA complexes were formed in binding buffer [10 mM Tris HCl (pH 8.0), 40 mM KCl, 0.05% (vol/vol) Igepal CA-630, 6% (vol/vol) glycerol, 1 mM DTT and 50 ng/ml poly(dI-dC)-poly(dI-dC)] in a total volume of 20  $\mu$ l for 10 min at 30°C. In super-shift experiments, 1  $\mu$ g of HIF-1 $\alpha$  sc13515 antibody was added to the reaction 30 min before adding the radioactively labelled oligonucleotide and incubated at 4°C. In competition experiments, a 10-, 50- or 250-fold molar excess of the competing oligonucleotides was added immediately before adding the radioactively labelled probe. Samples were loaded onto pre-electrophoresed 5% (acrylamide/bis 30:1) native acrylamide gels and run at 200 V in 0.5  $\times$  44 mM Tris-borate, 1 mM EDTA (TBE) for 1.5 h. Gels were fixed in 10% (vol/vol) acetic acid for

**Table 1.** Sequences of oligonucleotides used for EMSAs and site-directed mutagenesis. The 5' overhangs are marked in blue, the core hypoxia responsive elements are underlined and the introduced mutations are marked in red. The reverse complementary oligonucleotides are not shown

Oligonucleotide	Sequence (5'–3')	Purpose
VEGFA HRE consensus	AGCTCACAGTGCATACGTGGGCTCCAACA	EMSA
HRE hOST- $\alpha$ (−1345)	AGCTGACCTGGCGAACGTGCCTGCGGAGA	EMSA
HRE hOST- $\alpha$ (−1345) mut	AGCTGACCTGGCGAACCTCCCTGCGGAGA	EMSA
HRE hOST $\beta$ (−202) HRE1	AGCTTCCAGGCTCCACGTGCTCCTGGGTT	EMSA
HRE hOST $\beta$ (−3961) HRE2	AGCTACCCCGGCTAACGTGGTGAACCCC	EMSA
HRE hOST $\beta$ (−4103) HRE3 mut	AGCTCAAACCTGGATACCTCCTTTGATAGA	EMSA
hOST $\alpha$ (−1478/+90)luc HRE mut	AATGACCTGGCGAACCTCCCTGCGGAGATGC	hOST $\alpha$ (−1478/+90)luc HRE mutagenesis
hOST $\beta$ (−4738/+30)luc HRE mut	GAAGCAAACCTGGATACCTCCTTTGATAGAAATAG	hOST $\beta$ (−4738/+30)luc HRE mutagenesis

10 min, rinsed with water, dried onto Whatman DE81 paper under vacuum, and exposed to Kodak BioMax MR-1 (Milian AG, Nesselbach/Niederwil, Switzerland) films at  $-80^{\circ}\text{C}$ .

#### Transient transfections and reporter assays

Huh7 cells were seeded into 48-well plates at a density of 25 000 cells/well and transfected with 400 ng of the indicated Luciferase reporter constructs or the empty vector pGL3 basic and 100 ng of pHRG-TK reporter plasmid to normalize for transfection efficiency. The Fugene HD reagent was used at a ratio of 3  $\mu\text{l}$  per 1  $\mu\text{g}$  DNA. Six hours after transfection, cells were treated with either 50  $\mu\text{M}$  CDCA or vehicle (DMSO) and subjected to either normoxic or hypoxic conditions (0.2% oxygen) for 18 h. Cells were lysed and luciferase activities determined using the Luciferase Assay System and a GloMax Multi luminometer (Promega, Madison, WI, USA). Transfection experiments were performed three times. Gene reporter activities obtained for the empty pGL3basic under each treatment condition and for the wild-type construct in the normoxic control, respectively, were set to one, and fold activities were calculated relative to these activity levels.

#### Preparation of protein extracts

For the preparation of whole cell protein extracts, cells grown in six-well plates were washed with ice-cold phosphate buffered saline (PBS) and lysed by a 5-min incubation in 150  $\mu\text{l}$  of ice-cold lysis buffer supplemented with complete protease inhibitors [50 mM Tris\_HCl (pH 8.0), 150 mM NaCl, 1% (vol/vol) Igepal CA-630, 0.5% (wt/vol) Na-deoxycholate, 1 mM EDTA, 0.1% (wt/vol) SDS and 10% (vol/vol) glycerol]. For the preparation of proteins from CRF to sham-operated rat livers, the hepatic tissue was homogenized in RIPA buffer at  $4^{\circ}\text{C}$  (Polytron, Kinematica, Lucerne, Switzerland) and centrifuged. In both cases, protein concentrations were determined with the bicinchoninic acid (BCA) Protein assay (Thermo scientific). Samples were stored at  $-20^{\circ}\text{C}$  until further use.

#### Immunoblot analysis

Thirty micrograms of Huh7, 15  $\mu\text{g}$  of PHH or 20  $\mu\text{g}$  of liver tissue derived whole protein extract were used for Western blot analysis. The samples were separated on 12% SDS-polyacrylamide gels and electroblotted onto Amersham hybond ECL nitrocellulose membranes. Membranes were blocked overnight (cell protein extracts) or for 1 h (liver derived protein extracts) in 5% (wt/vol) non-fat milk in PBS-Tween 20 [0.1% (vol/vol) Tween 20 in PBS; PBS-T] blocking solution. The membranes were probed with antibodies against HIF-1 $\alpha$  (ABE279 diluted 1:1000 in blocking solution), an antibody raised in rabbit against an

OST $\alpha$  peptide (obtained from the laboratory of N. Ballatori, diluted 1:500 in blocking solution), or an antibody against mOst $\alpha$  cross-reactive with hOST $\alpha$  (sc10078 diluted 1:800 in blocking solution). All membranes were incubated overnight at  $4^{\circ}\text{C}$ . The next day, the membranes were washed three times with blocking solution and incubated with secondary horseradish peroxidase-conjugated goat antimouse antibody at a concentration of 10 ng/ml for 1 h. Blots were washed three times with blocking solution, twice with PBS-T, and analysed using the SuperSignal West Femto Maximum Sensitivity Substrate and the Fusion FX luminescence detector system (Vilber Lourmat, Marne-La-Vallée, France). To ensure equal loading of the protein samples, blots were stripped with Restore Plus Western Blot Stripping Solution, reblocked, and reprobed for constitutively expressed  $\beta$ -actin antigen.  $\beta$ -actin probing and detection were performed as described above except that the  $\beta$ -actin antibody was used at a concentration of 200 ng/ml and horseradish peroxidase-conjugated goat antirabbit antibody was used as secondary antibody at a concentration of 10 ng/ml. The relative intensities of the  $\beta$ -actin bands were used to normalize the results for OST $\alpha$  expression. The experiments were repeated with at least three independent batches of PHH obtained from different donors and Huh7 cells at different passages.

#### Animal models

Liver tissue for immunoblot analysis was obtained from male Sprague-Dawley rats that had undergone either 5/6 nephrectomy (chronic renal failure group, CRF) or sham surgery (Sham), as described previously (14). Rats were killed 8 weeks after surgery. The livers were removed immediately, snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent homogenization.

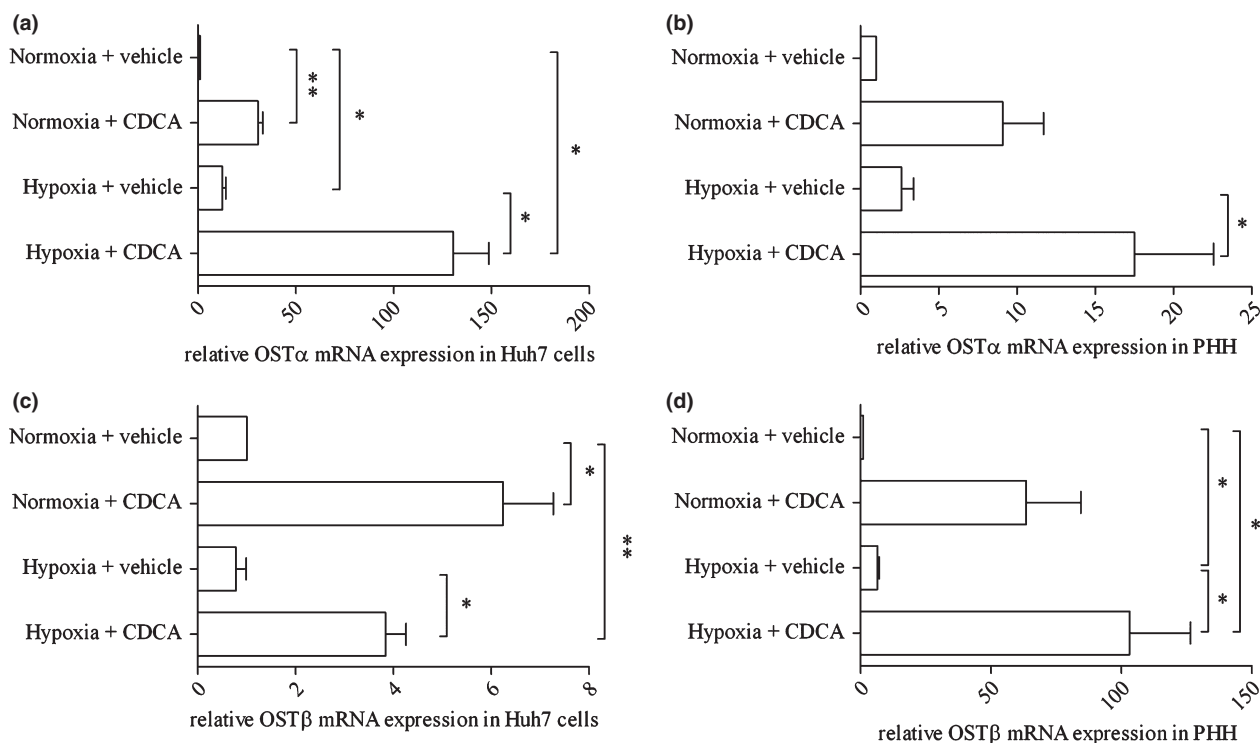
#### Statistical analysis

Quantitative data obtained from luciferase gene reporter assays and real-time TaqMan PCR are shown as mean values  $\pm 1$  SD. Average results were statistically evaluated using the one sample *t*-test or the unpaired *t*-test respectively. A *P*-value of  $<0.05$  was considered to be significant. Statistical analysis was performed using GRAPHPAD PRISM 5.

## Results

### OST $\alpha$ -OST $\beta$ mRNA levels are increased by exposing cells to CDCA or hypoxia

The relative mRNA expression of OST $\alpha/\beta$  was determined in Huh7 cells and PHH using TaqMan real-time PCR. As shown in Figure 1, mRNA levels of OST $\alpha$  and OST $\beta$  were significantly increased in Huh7 cells upon treatment with the FXR agonist CDCA. OST $\alpha$  mRNA



**Fig. 1.** OST $\alpha$ -OST $\beta$  mRNA levels are increased after 18 h of treatment with CDCA or hypoxia. After 18 h of treatment with 50  $\mu$ M CDCA in DMSO and exposure to either normoxia or hypoxia, cells were harvested and mRNA levels were determined by TaqMan real-time PCR. Figures a–d show average results of at least three independent experiments, \* $P$  < 0.05, \*\* $P$  < 0.01. (a) Relative OST $\alpha$  mRNA determined in Huh7 cells. All treatment combinations show significant upregulation of OST $\alpha$  mRNA. (b) Relative OST $\alpha$  mRNA determined in PHH. A significant upregulation of OST $\alpha$  mRNA is seen under combined treatment conditions. (c) and (d) Significant upregulation of OST $\beta$  mRNA is seen only with CDCA in both Huh7 cells (c) and PHH (d). In PHH, a significant increase in OST $\beta$  mRNA was induced by hypoxia (d).

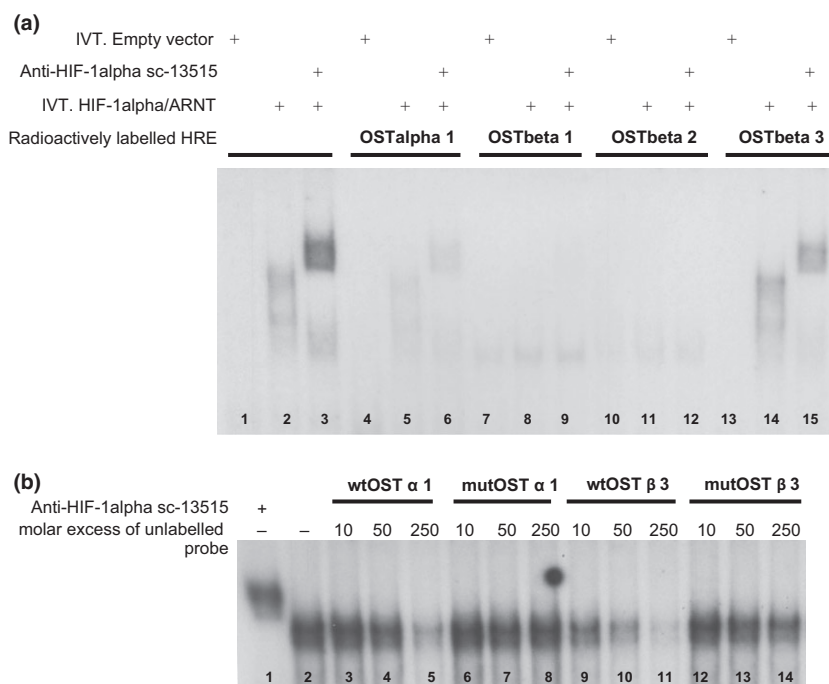
levels were also increased in Huh7 cells exposed to 0.2% oxygen pressure (hypoxia) for 18 h. Interestingly, a greater than additive effect on OST $\alpha$  mRNA levels in Huh7 cells and PHH could be observed when cells were exposed simultaneously to 50  $\mu$ M CDCA and 0.2% oxygen for 18 h (Fig. 1a and b). OST $\beta$  mRNA was induced in both Huh7 cells and PHH by CDCA (Fig. 1c), but only in PHH by hypoxia (Fig. 1d). In PHH, a small but non-significant additive effect on OST $\beta$  mRNA expression was observed under a combined regimen of 50  $\mu$ M CDCA and 0.2% oxygen for 18 h (Fig. 1d).

#### HIF-1 $\alpha$ binds to hypoxia response elements in the OST $\alpha$ and OST $\beta$ promoters

A search for putative HRE within the 5'-flanking region of OST $\alpha$  revealed only one element at position –1345 upstream of the predicted transcription start (HRE1). Interestingly, the predicted HRE appears to be located in close proximity to a functionally relevant FXR binding site within the OST $\alpha$  promoter (9). Only five base pairs separate these two binding elements. In the case of OST $\beta$ , three different putative HRE were

identified, at –202 (HRE1), –3961 (HRE2) and –4103 (HRE3) base pairs upstream of the predicted transcription start.

The *in vitro* binding of HIF-1 $\alpha$  to the newly identified, putative HREs within the OST $\alpha$  and OST $\beta$  promoters was assessed by electrophoretic mobility shift assay (EMSA). Oligonucleotides carrying a functional VEGFa HRE (15) were used as a positive control in all experiments. To confirm specific binding of HIF-1 $\alpha$ , super-shift experiments were performed using a HIF-1 $\alpha$  specific antibody. As demonstrated in Figure 2a, the predicted OST $\alpha$  HRE was able to bind HIF-1 $\alpha$ . In the case of OST $\beta$  the binding intensity varied among the three sites. The binding to the putative HRE at position –4103 upstream of the transcription start (short HRE3) appeared to be the strongest. Therefore, this binding site was selected to further elucidate its functional relevance. The capacity of the wild-type or mutated annealed oligonucleotides to compete with VEGFa HRE for HIF-1 $\alpha$  binding was shown by competition experiments (Fig. 2b). Three different molar excesses of each of the different annealed oligonucleotides were applied to otherwise identical binding reactions of the reference



**Fig. 2.** HIF-1 $\alpha$  binds to putative hypoxia responsive elements in OST $\alpha$  and OST $\beta$  promoters *in vitro*. EMSA was performed to determine the binding of HIF-1 $\alpha$  to predicted putative HREs within the OST $\alpha$  and OST $\beta$  promoters. (a) Radiolabeled annealed oligonucleotides containing four different putative HREs were incubated with either a mix of *in vitro* translated (IVT) HIF-1 $\alpha$  and ARNT or a negative control (*in vitro* translated empty vector backbone) and run on a native polyacrylamide gel. Super-shift reactions were performed by addition of anti-HIF-1 $\alpha$  antibody to the binding reactions. (a) Positive shift and super-shift reactions were observed for the positive control using labelled oligonucleotides containing the VEGFa HRE (lanes 2 and 3), for the HRE within the OST $\alpha$  promoter (lanes 5 and 6) as well as for HRE3 in the OST $\beta$  promoter (lanes 14 and 15). (b) Shows a competition EMSA. Three different molar excesses of unlabelled wild-type (wt) or mutated (mut) HRE-containing oligonucleotides were added to a mix of *in vitro* translated HIF-1 $\alpha$  and ARNT together with radiolabeled VEGFa HRE oligonucleotides. In lane 1, the supershift was induced with the anti-HIF-1 $\alpha$  antibody. In lane 2, no competing unlabelled oligonucleotide was added. Wild-type HRE oligonucleotides were able to compete off the binding of HIF-1 $\alpha$  and ARNT in a dose dependent manner. Oligonucleotides containing the HREs in mutated form were not able to compete successfully (lanes 6–8 and 12–14).

VEGFa oligonucleotide. When the core elements of the HRE in OST $\alpha$  and the HRE3 in OST $\beta$  were mutated, their capacity to compete for binding to HIF-1 $\alpha$  was strongly impaired when compared to the wild-type.

#### The inducing effect of hypoxia and CDCA on OST $\alpha$ expression is dependent on the transcription factors HIF-1 $\alpha$ and FXR

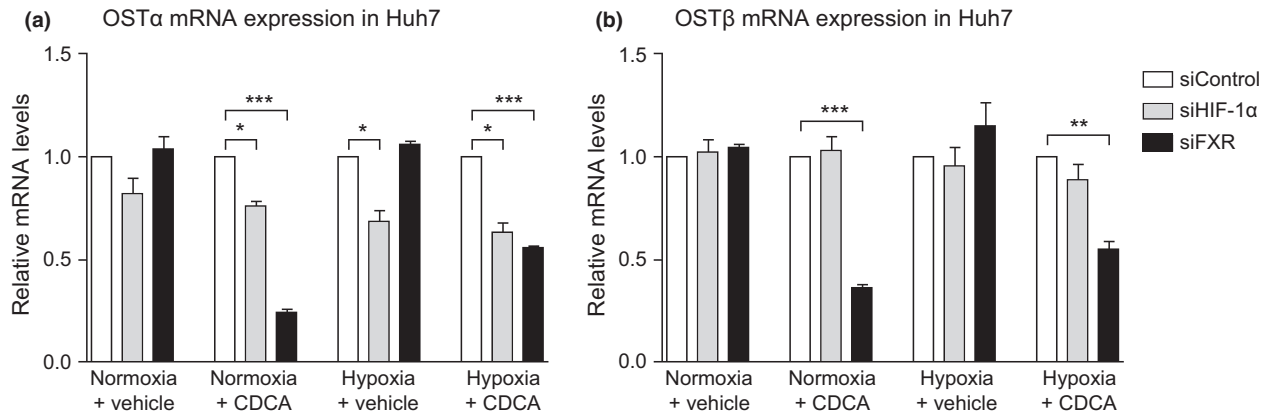
The increase in OST $\alpha$  mRNA levels induced by hypoxia or CDCA was significantly reduced when Huh7 cells were transfected with siRNAs targeting HIF-1 $\alpha$  (Fig. 3a). A comparable effect was seen when transfecting cells with siRNA targeting FXR, inducing a reduction in the response to CDCA treatment and suggesting a functionally important role for HIF-1 $\alpha$  and FXR in OST $\alpha$  regulation (Fig. 3a). In contrast, an siRNA-dependent knock-down of HIF-1 $\alpha$  did not show any significant effect on the expression of OST $\beta$  mRNA when the cells were subjected to hypoxia (Fig. 3b). The increase in OST $\beta$  mRNA levels upon CDCA treatment was reduced when siRNA against FXR was transfected, confirming

the known functional role for FXR in regulating OST $\beta$  gene transcription (Fig. 3b).

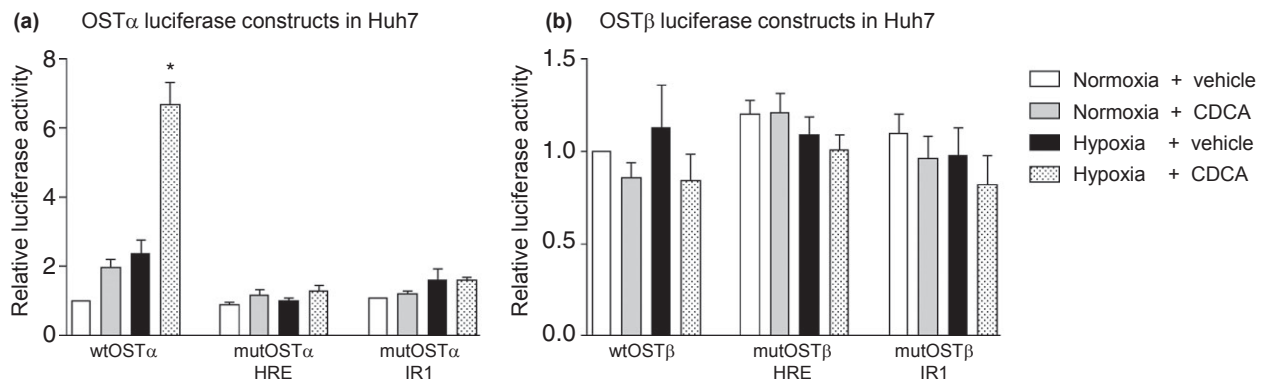
#### The hypoxia-induced upregulation of OST $\alpha$ expression is mediated by the newly identified HRE

Luciferase gene reporter constructs carrying subcloned OST $\alpha$  and OST $\beta$  promoter fragments were used in dual luciferase assays. When the wild-type OST $\alpha$  promoter construct was transfected into Huh7 cells, a significant increase in luciferase activity was observed treating cells with both 50  $\mu$ M CDCA and 0.2% oxygen. When the treatment conditions were applied separately, only a slight, non-significant increase in luciferase activity was observed (Fig. 4a). In contrast, no inducing effects of the different treatment conditions on luciferase activity were observed when transfecting the OST $\beta$  promoter construct (Fig. 4b).

The increased luciferase activity induced by a combined treatment with CDCA and hypoxia was lost when the core element of the putative HRE within the OST $\alpha$  promoter construct was artificially mutated from



**Fig. 3.** The upregulation of OST $\alpha$  mRNA by hypoxia and CDCA is dependent on HIF-1 $\alpha$  and FXR. The figure shows the relative OST mRNA levels in Huh7 after transfection with siRNA targeting HIF-1 $\alpha$  or FXR. The results of each treatment condition (CDCA, hypoxia, or both) are shown relative to cells transfected with a random scrambled siRNA (siControl). The figure shows average results of at least three independent experiments, with \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . (a) Knock-down of HIF-1 $\alpha$  significantly reduces the induction of OST $\alpha$  mRNA in cells treated with CDCA or hypoxia, either alone or in combination. As expected, a knock-down of FXR abrogates the inducing effect of CDCA on OST $\alpha$  mRNA expression. (b) While knock-down of FXR significantly reduces the inducing effect of CDCA on OST $\beta$  mRNA expression, HIF-1 $\alpha$  knock-down does not influence the induction of OST $\beta$  mRNA in cells treated with CDCA under either normoxic or hypoxic conditions.



**Fig. 4.** Hypoxia induces luciferase activity in promoter constructs of OST $\alpha$ , an effect that is dependent on the newly identified hypoxia responsive element. Dual luciferase assays with OST $\alpha$  and OST $\beta$  promoter constructs transfected into Huh7 cells are represented as relative luciferase activity, normalized to the pcDNA3.1 empty vector backbone. Plasmids containing either the wild-type (wt) promoter or promoters with HRE or IR1 in mutated form (mut) were transfected into cells that were treated for 18 h with 50  $\mu$ M CDCA or DMSO and exposed to either normoxia or hypoxia. (a) A significant increase (\* $P < 0.05$ ) in promoter activity of wt OST $\alpha$  was seen in cells treated with hypoxia and CDCA together. A non-significant trend was observed for single treatments. The inducing effects were markedly reduced when either the HRE or the IR1 were mutated. (b) Luciferase activity of the OST $\beta$  promoter construct was not significantly changed by any of the treatment conditions applied. Mutation of either the HRE or IR1 site did not significantly affect the basal activity.

ACGTG to ACCTC. A similar observation was made when the functionally relevant FXR binding site described previously (9) was mutated (Fig. 4a). The mutation of the HRE or FXR binding site in the OST $\beta$  promoter construct did not reduce the basal luciferase activity (Fig. 4b).

#### Hypoxia treatment increases OST $\alpha$ protein levels in Huh7 cells and PHH

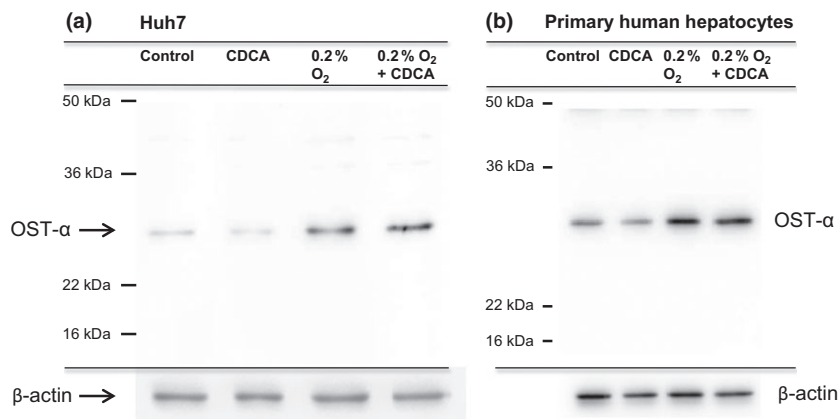
To determine the regulation of OST $\alpha$  at the protein level, Western blot analysis was performed. The anti-OST $\alpha$  antibodies sc100078 as well as a non-commercial

antibody generated in the laboratory of Ballatori and coworkers (data not shown) were used to detect the target protein. Exposure to 0.2% oxygen for 18 h clearly induced the expression of OST $\alpha$  three-fold at the protein level in Huh7 cells (Fig. 5a) and two-fold in PHH (Fig. 5b).

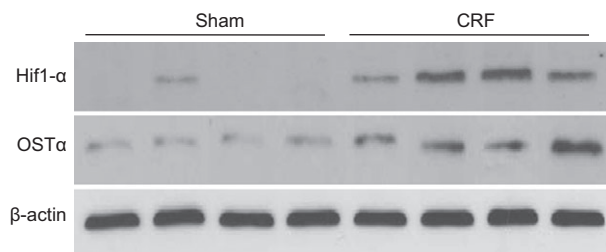
#### Rats suffering from chronic renal failure show concordantly upregulated HIF-1 $\alpha$ and OST $\alpha$ protein in liver tissue

We previously showed a marked increase in OST $\alpha$  expression in liver tissue of rats that had undergone 5/6





**Fig. 5.** Hypoxia treatment for 18 h increases OST $\alpha$  protein levels in Huh7 cells and PHH. Cells were treated with 50  $\mu$ M CDCA or DMSO alone and were either exposed to hypoxia or kept under normoxic conditions for 18 h. Total protein from whole Huh7 cell extracts (30  $\mu$ g) (a) or 15  $\mu$ g from whole cell extracts isolated from PHH (b) were run on an SDS-PAGE gel and immunoblotted. An anti-OST $\alpha$  antibody was used to probe the membrane, followed by stripping and probing with  $\beta$ -actin antibody. Exposure of the cells to hypoxia-induced protein expression in both Huh7 (three-fold) and PHH (two-fold).



**Fig. 6.** The hepatic expression of HIF-1 $\alpha$  and OST $\alpha$  is significantly induced in rats suffering from chronic renal failure. Four sham-operated rats and four CRF rats that had undergone 5/6 nephrectomy were compared with regard to the expression level of HIF-1 $\alpha$  and OST $\alpha$  in hepatic tissue 8 weeks after the procedure. Western blot analysis shows a significant increase in both HIF-1 $\alpha$  and OST $\alpha$  under CRF conditions compared to sham-operated controls. Actin was probed for assay normalization.

nephrectomy to simulate CRF conditions (14). Because the mechanism of hepatic OST $\alpha$  induction in the CRF model was not resolved, we hypothesized that an increase in HIF-1 $\alpha$  expression could be involved. We therefore analysed the expression of OST $\alpha$  and HIF-1 $\alpha$  protein in CRF rats compared to sham-operated controls. As shown in Figure 6, rats suffering from CRF showed markedly increased OST $\alpha$  and HIF-1 $\alpha$  protein levels as compared to controls.

## Discussion

Tissue hypoxia in the liver can be caused by several forms of liver injury (2–4). In cholestasis the toxic effect of bile acids within hepatocytes is prevented by adaptive responses such as induction of bile acid efflux transporters. This study shows that both hypoxia and elevated bile acid levels have the potential to modulate the expression of bile acid transporters via transcription

factor mediated pathways, as demonstrated here for the heterodimeric transport protein OST $\alpha$ -OST $\beta$ . To determine whether hypoxic conditions affect expression at the transcriptional level, relative mRNA levels were measured in both the hepatoma-derived cell-line Huh7 and PHH treated with hypoxia or CDCA, separately or in combination. The inducing effect of CDCA on OST $\alpha$ -OST $\beta$  expression was reported previously (9). Here, we show that hypoxia alone is able to increase OST $\alpha$  and OST $\beta$  mRNA. In Huh7 cells, the combination of CDCA treatment and hypoxia appeared to induce a greater than additive elevation of OST $\alpha$  mRNA levels, suggesting a possible synergistic effect. In all experiments in which relative OST $\alpha$  and OST $\beta$  mRNA levels were measured, the induction of VEGFa mRNA was confirmed as a positive control for the effect of hypoxia on gene expression. CDCA alone had no effect on relative VEGFa mRNA levels (data not shown). The inducing effect of hypoxia on OST $\alpha$  was also evident at the protein level in both Huh7 cells and PHH after 18 h. This was confirmed in the same samples using a second OST $\alpha$  antibody provided by the laboratory of N. Ballatori (data not shown). Activation of OST $\beta$  gene transcription by CDCA was also observed, as described previously (9, 16). A significant inducing effect of hypoxia on OST $\beta$  was found in PHH but not Huh7 cells (Fig. 1c and d).

The binding of HIF-1 $\alpha$  to the HRE in the OST promoter regions was analysed *in vitro* by EMSA as shown in Figure 2. HIF-1 $\alpha$  binds to the HRE identified in the OST $\alpha$  promoter and preferentially to one of the three HRE detected in the OST $\beta$  promoter (Fig. 2a). When the core of the HRE was mutated HIF-1 $\alpha$  binding was significantly reduced in both OST $\alpha$  and OST $\beta$ , as shown by the competition EMSA in Figure 2b. Using siRNA to knock-down HIF-1 $\alpha$  and FXR, the inducing effects of

CDCA and hypoxia on OST $\alpha$  regulation could be disrupted (Fig. 3a). In contrast to OST $\alpha$ , no relevant effect of anti-HIF-1 $\alpha$  siRNA on OST $\beta$  expression was observed, suggesting that the overall magnitude of OST $\beta$  regulation by HIF-1 $\alpha$  may be less than for OST $\alpha$ .

Dual luciferase assays using wild-type and mutated OST $\alpha$  promoter constructs confirmed the importance of the predicted HRE for the regulation of OST $\alpha$  gene expression by hypoxia (Fig. 4a). Interestingly, the induction of the OST $\alpha$  promoter by CDCA was reduced when an OST $\alpha$  promoter construct carrying the mutated HRE site was employed. This observation could be explained by the close proximity of the HRE sequence and the FXR binding site. Mutation of the HRE could affect the functionality of the FXR binding site. In the case of the OST $\beta$  promoter, no relevant effect of CDCA and hypoxia on luciferase reporter activity was seen (Fig. 4b), again suggesting that HIF-1 $\alpha$  may not activate OST $\beta$  gene transcription to the degree that OST $\alpha$  gene transcription is induced. The absence of an effect of CDCA on the promoter activity of OST $\alpha$  and OST $\beta$  in dual luciferase assays (Fig. 4) does not contradict previous results (9), as the present set-up relied on shorter incubation times and on the activity of endogenously expressed transcription factors only.

Dimerization of OST $\alpha$  with OST $\beta$  is thought to be essential for transport function (17–19). Despite the functional synergism, several reports suggest that OST $\alpha$  and OST $\beta$  appear to be regulated differently. This was concluded from the divergent effects that transcription factors exert on the two promoters, or from the fact that certain transcriptional effects appear to regulate only one of the subunits. (20–23). It has been shown that OST $\beta$  is important for both the trafficking of OST $\alpha$  to the plasma membrane as well as for its function (24). However, the lack of co-immunoprecipitation of the mature, glycosylated form of OST $\alpha$  upon immunoprecipitation of OST $\beta$  suggests that the primary interaction may occur early in the biosynthetic pathway and may be transient (16). This would support the mutual independence of the subunits in substrate transport and gives rise to the theory that different dimerization partners exist for the OST subunits. Another indication of the possibility of alternative binding partners or conformations of OST $\alpha$  is the existence of different splicing variants that have been deposited in the available databases. The exact role of these splice variants has not been investigated.

OST $\alpha$ -OST $\beta$  is considered to be an efflux system for bile acids across the basolateral membrane of hepatocytes that allows extrusion of bile acids into sinusoidal blood. This could protect hepatocytes from the intracellular accumulation of potentially toxic bile acids, e.g. during cholestatic liver injury or under conditions of hypoxia. Hypoxia caused by arterial liver ischaemia has previously been shown to decrease expression of the Na<sup>+</sup>-dependent bile acid uptake transporter Ntcp and the bile salt export pump Bsep, which could lead to cho-

lestasis (25). Induction of OST $\alpha$  in this setting could represent a rescue mechanism for reducing the intracellular bile acid load when the canalicular efflux pump shows reduced expression.

Chronic renal failure is associated with hypoxia in the kidney. In this study, we show that the previously observed increase in OST $\alpha$  expression in the liver tissue of rats suffering from CRF (14) could be attributable to the increased expression of HIF-1 $\alpha$  (Fig. 6). It is assumed that chronic hypoxia occurring during CRF is a multifactorial event caused by damage of renal arterioles, a distortion of peritubular capillaries and imminent interstitial fibrosis. Hypoxia itself is able to induce strong profibrogenic effects leading, among other pathways, via induction of HIF-1 $\alpha$  dependent signalling to further destruction of peritubular capillaries, fibrotic remodelling of tubular cells and thus, to the further progression of kidney failure (26–28). Induction of HIF-1 $\alpha$  in the liver suggests that hypoxic stimuli occurring during CRF modulate HIF-1 $\alpha$  dependent signalling pathways in other tissues as well.

In conclusion, the expression of OST $\alpha$  and OST $\beta$  is significantly induced by bile acids and hypoxia. While the CDCA-dependent induction of OST $\alpha$ -OST $\beta$  expression is known to be mediated by FXR, the hypoxia effect is mediated by the transcription factor HIF-1 $\alpha$  through newly detected, functionally relevant HIF-1 $\alpha$  responsive elements within the OST $\alpha$  and OST $\beta$  gene promoters. In the case of the OST $\alpha$  promoter, FXR and HIF-1 $\alpha$  bind in close proximity and putatively interact to produce synergistic effects on OST $\alpha$  expression.

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