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Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology

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ABSTRACT

The uremic solutes *p*-cresyl sulfate (pCS) and *p*-cresyl glucuronide (pCG) accumulate in patients with chronic kidney disease (CKD), and might contribute to disease progression. Moreover, retention of these solutes may directly be related to renal tubular function. Here, we investigated the role of the efflux transporters Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP) in pCS and pCG excretion, and studied the impact of both solutes on the phenotype of human conditionally immortalized renal proximal tubule epithelial cells (ciPTEC).

Our results show that p-cresol metabolites accumulate during CKD, with a shift from sulfation to glucuronidation upon progression. Moreover, pCS inhibited the activity of MRP4 by 40% and BCRP by 25%, whereas pCG only reduced MRP4 activity by 75%. Moreover, BCRP-mediated transport of both solutes was demonstrated. Exposure of ciPTEC to pCG caused epithelial-to-mesenchymal transition, indicated by increased expression of vimentin and Bcl-2, and diminished E-cadherin. This was associated with altered expression of key tubular transporters.

In conclusion, BCRP is likely involved in the renal excretion of both solutes, and pCG promotes phenotypical changes in ciPTEC, supporting the notion that uremic toxins may be involved in CKD progression by negatively affecting renal tubule cell phenotype and functionality.

1. Introduction

A hallmark of chronic kidney disease (CKD) is the retention and accumulation of a wide variety of potential toxic metabolites (Duranton et al., 2012; Vanholder et al., 2003) associated with the plethora of pathologies observed in uremic patients, including renal fibrosis and cardio-vascular disease (Jourde-Chiche et al., 2009; Vanholder et al., 2008). In the healthy population, these so-called uremic retentions solutes are cleared by the kidney via glomerular filtration and transporter-mediated tubular excretion. In early uremic toxin research, *p*-cresol, a phenol derived from tyrosine metabolism, was widely studied and a broad array of pathophysiological effects observed in CKD patients were

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; ciPTEC, conditionally immortalized renal proximal tubule epithelial cells; CKD, chronic kidney disease; CMFDA, 5-chloromethylfluorescein diacetate; EMT, epithelial-to-mesenchymal transition; E1S, estrone-sulfate; ESRD, end-stage renal disease; GSMF, glutathione methylfluorescein; hOAT1/3, human organic anion transporters 1/3; HPLC, high-performance liquid chromatography; LC/MS-MS, liquid chromatography/tandem mass spectrometry; MRP4, multidrug resistance protein 4; MTX, methotrexate; pCG, p-cresyl glucoronide; pCS, p-cresyl sulfate; SULT, sulfotransferase.

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contributed to this compound, including endothelial and immunological dysfunction (Liabeuf et al., 2013; Vanholder et al., 2011). However, years ago, it was demonstrated by several groups that it is not *p*-cresol itself but the metabolites, *p*-cresyl sulfate (pCS) and *p*-cresyl glucuronide (pCG), that are retained during CKD (de Loor et al., 2005; Martinez et al., 2005; Schepers et al., 2007). *p*-cresol is formed in the gut during protein fermentation and is subsequently conjugated to either sulfate or glucuronic acid in the intestinal wall resulting in the formation of pCS or pCG, respectively (Schepers et al., 2010). In a study by Aronov et al. (2011), it was elegantly demonstrated that the colon indeed plays a key role in the production of pCS. In addition, our group recently showed renal *p*-cresol metabolism and subsequent pCG formation in human proximal tubular cells (Mutsaers et al., 2013). Thus, the origin of pCS and pCG is reasonably well understood; however the molecular transport mechanisms involved in physiological urinary excretion of both *p*-cresol metabolites are not fully elucidated.

Whereas recent evidence shows that the human organic anion transporters 1 and 3 (hOAT1 and hOAT3) are involved in the uptake of pCS (Watanabe et al., 2013), both solutes have not yet been described as substrates for any particular efflux transporter. Two pumps that likely contribute to the renal excretion of the *p*-cresol metabolites are multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). Both pumps are transmembrane proteins from the ATP-binding cassette (ABC) transporters superfamily and are expressed in several tissues with a barrier function, including liver, intestine, brain and kidney. Both transporters are known to extrude a wide variety of drugs and endogenous compounds against steep concentration gradients (Giacomini et al., 2010; Masereeuw and Russel, 2010). Previously, we reported that several uremic toxins, such as kynurenic acid, hippuric acid and indoxyl sulfate, inhibited substrate-specific transport by MRP4 and BCRP at clinically relevant concentrations (Mutsaers et al., 2011a). Since pCS and pCG share structural characteristics with the previously studied uremic solutes, we hypothesized that both pumps are also involved in the transport of pCS and pCG into the proximal tubule lumen.

Since it was reported that pCS accumulates in CKD patients, it has been widely used as a model solute to elucidate the capacity of different dialysis modalities to remove protein-bound uremic toxins from the circulation in CKD patients (Meijers et al., 2009). In addition, several studies have shown that pCS can contribute to the pathophysiology of CKD. For instance, pCS can increase endothelial micro-particles shedding by endothelial cells *in vitro*, indicating pCS involvement in endothelial dysfunction (Meijers et al., 2009), it can contribute to inflammation (Meert et al., 2012), it has pro-apoptotic effects (Poveda et al., 2014), suppresses the expression of the renoprotective antiaging gene Klotho both *in vitro* and *in vivo* (Sun et al., 2012), and causes insulin resistance and metabolic disturbances associated with CKD in mice by activating the ERK1/2 pathway (Koppe et al., 2013). Furthermore, pCS is often studied in conjunction with indoxyl sulfate, a protein-bound uremic toxin derived from tryptophan, and it was recently reported by Kim et al. (2012) that indoxyl sulfate induces epithelial-to-mesenchymal transition (EMT) in proximal tubular cells. Conversely, little is known about the biological activity of pCG and the nephrotoxic potential of both *p*-cresol conjugates.

This study aimed to elucidate the renal handling of pCS and pCG by investigating the interaction between both solutes and the renal efflux transporters MRP4 and BCRP. Moreover, the human renal proximal tubule cell line (ciPTEC) was used to investigate the potential contribution of both *p*-cresol metabolites to CKD progression related to tubular changes.

2. Materials and methods

2.1. Ethics statement and patient sample selection

The Radboud University Medical Centre ethical committee on research involving human subjects approved this study, and written informed consent was obtained from each patient and each healthy volunteer. Stored serum samples from chronic renal failure (CRF) patients were obtained from a random subset of the MASTERPLAN study population (van Zuilen et al., 2012). Patient characteristics were obtained from the case report forms. Samples for hemodialysis patients were collected at the start of the dialysis session as a part of routine care and other lab parameters were obtained from electronic patient records.

2.2. Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, the Netherlands) unless stated otherwise. Both pCS and pCG were kindly provided by Profs. R. Vanholder and G. Glorieux (University Hospital Ghent, Belgium). pCS was synthesized as a potassium salt as described previously (Feigenbaum and Neuberg, 1941). pCG was produced from glucuronyl-trichloroacetimidate and p-cresol as previously described (Van der Eycken et al., 2000). Since pCS and pCG were obtained as potassium and an ammonium salt, respectively, KCl and NH_4Cl solutions were used as control. [3′,5′,7′-^{3}H(*n*)]-methotrexate disodium salt ([^{3}H]-MTX) was purchased from Moravek (Brea, USA) and [6′,7′-^{3}H(*n*)]-estrone-sulfate ammonium salt ([^{3}H]-E1S) was obtained from Perkin Elmer (Groningen, the Netherlands).

2.3. High-performance liquid chromatography (HPLC)

Blood samples were obtained from 79 CRF patients during regular check-up, 12 patients with end-stage renal disease (ESRD) before hemodialysis and 4 healthy controls. Clinical characteristics of study subjects are listed in Table 1. None of the subjects had been fasting at the time of blood sampling. Blood was collected in an EDTA Vacutainer and was immediately centrifuged at 3000*g* for 10 min. Subsequently, plasma was collected and stored at −20 °C. Before chromatography an aliquot of plasma was diluted in H_2O (1:1) and deproteinized with perchloric acid (final concentration 3.3% (v/v)). Next, samples were centrifuged at 12,000*g* for 3 min and 50 μl of the supernatant was injected into the HPLC-system (Spectra-Physics Analytical, Spectrasystem SCM400). For the detection of pCS and pCG, chromatography was performed on a C18 HPLC column (GraceSmart RP 18 5u 150 × 4.6 mm) with eluent A (95% (v/v) 50 mM KH_2PO_4 (pH 3.0) and 5% (v/v) acetonitrile) and eluent B (50 mM KH_2PO_4 (pH 3.0), methanol and acetonitrile in a 1:1:1 ratio) using the following gradient: 0–15 min, 100–20% eluent A; 15–16 min, 20–100% eluent A; 16–21 min, 100% eluent A. The flow rate was 1 ml/min and the *p*-cresol conjugates were detected at a wavelength of 220 nm. Standards of the compounds were also run in order to quantify the amount of metabolites found in the samples. Acquired data were processed with PC1000 software (Spectrasystem).

2.4. Transduction of Human Embryonic Kidney cells and membrane vesicle preparation

Human embryonic kidney (HEK293; purchased at American Type Culture Collection, Manassas, VA) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen life sciences, Breda, the Netherlands) containing 10% (v/v) fetal calf serum (MP Biomedicals, Uden, the Netherlands) at 37 °C in a 5% (v/v) CO_2

Table 1
Characteristics of study subjects.

	CRF	ESRD[a]	Control
Number	79	12	4
Age (years)	59 ± 14	55 ± 15	40 ± 12
Women (%)	29	22	50
Urea (mmol/l)	12 ± 4	21 ± 7	ND
Creatinine (μmol/l)	171 ± 58	774 ± 242	ND
eGFR (ml/min/1.73 m^2)[b]	37 ± 14	NA	ND
Dialysis strategy	NA	11 HD, 1 CAPD	NA

Values are shown as mean ± SD. CRF, chronic renal failure; ESRD, end-stage renal disease; ND, not determined; NA, not applicable; HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis.

[a] Clinical data of 3 subjects was undisclosed, 8 samples were whole blood.

[b] eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation (www.nkdep.nih.gov).

atmosphere. To functionally overexpress MRP4 and BCRP, cells were transduced with baculoviruses of human MRP4, BCRP or enhanced yellow fluorescent protein (eYFP; as a negative control), generated via the Bac-to-Bac system (Invitrogen) as previously described (El-Sheikh et al., 2007). Subsequently, cell membranes were isolated and resuspended in isotonic buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4, adjusted with HEPES). Membrane vesicle were prepared via ultrafiltration as described previously (El-Sheikh et al., 2007). Afterwards, vesicles were frozen in liquid nitrogen and stored at −80 °C.

2.5. Membrane vesicle transport and cellular uptake assays

A well-established rapid filtration technique (Dankers et al., 2012; El-Sheikh et al., 2007; Mutsaers et al., 2011a; Wittgen et al., 2011), was used to study the uptake of [^{3}H]-MTX and [^{3}H]-E1S into MRP4 or BCRP membrane vesicles, and the impact of both solutes on this process. In short, 25 μl of TS buffer containing 4 mM ATP, 10 mM $MgCl_2$ and radiolabeled substrate was added to 5 μl of the membrane vesicles (1.5 mg/ml). The transport assay was performed in the absence or presence of various concentrations of pCS or pCG. Transport was started by incubating the mixture at 37 °C for 1 min (BCRP) or 10 min (MRP4), as established (El-Sheikh et al., 2007; Imai et al., 2003). Uptake was stopped by placing the samples on ice and the addition of 150 μl ice cold TS buffer. Subsequently, the samples were transferred to a 96 well filter plate (Millipore, Etten-leur, the Netherlands) pre-incubated with TS buffer and filtered using a Multiscreen HTS-Vacuum Manifold filtration device (Millipore). Afterwards, 2 ml of scintillation liquid was added to each filter and radioactivity was determined using liquid scintillation counting. As negative controls ATP was substituted for AMP and eYFP-membrane vesicles were used. Each experiment was performed in triplicate.

The direct uptake of pCS and pCG by MRP4 and BCRP expressing vesicles was studied by incubating vesicles with pCS or pCG for 10 min at 37 °C. After filtration, perchloric acid (PCA) 3.3% was used as solvent to extract the vesicles content. The concentration of pCS and pCG was subsequently determined by liquid chromatography/tandem mass spectrometry (LC/MS-MS). To this end, 10 μl of extraction was injected into the LC/MS-MS system (Thermo Fischer Scientific, Breda, the Netherlands). Separation was performed at a flow rate of 150 μl/min with eluent A (5 mM ammonium formate + 0.01% (v/v) trifluoroacetic acid) and eluent B (50% acetonitrile), the eluted solutes were directly passed through a TSQ Vantage tandem mass spectrometer (Thermo Fischer Scientific, Breda, the Netherlands), with ion spray voltage of 4 kV, source temperature of 350 °C and collision gas pressure of 1.5 bar. pCS and pCG and the internal standard 1-methyl-tryptophan were quantified. A calibration curve of pCS and pCG was made to quantify the amount in the samples and the results were corrected using the internal standard. The same method was used to determine the intracellular accumulation of pCS and pCG in ciPTEC following treatment with either 1 or 2 mM of the solutes for 48 h, in the absence or presence of the BCRP inhibitor KO143 (10 μM). Acquired data were processed with Thermo Xcaliber software (Thermo Fischer Scientific, Breda, the Netherlands). Net ATP-dependent uptake was also determined, as described above.

2.6. Proximal tubule cell culture

The ciPTEC line was generated as previously described by Wilmer et al. (2010). The cells were cultured in phenol red free DMEM/F12 (Gibco/Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) fetal calf serum (FCS; MP Biomedicals, Uden, the Netherlands), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), epithelial growth factor (10 ng/ml), and tri-iodothyronine (40 pg/ml) at 33 °C in a 5% (v/v) CO_2 atmosphere. Propagation of cells was maintained by subculturing the cells at a dilution of 1:3–1:6 at 33 °C. For all experiments, cells were cultured at 33 °C to 40% confluency, followed by maturation for 7 days at 37 °C. Experiments were performed on the cells between passages 30 and 40.

2.7. Flow cytometry

Protein expression of vimentin, E-cadherin and snail1 was analyzed by flow cytometry. Briefly, ciPTEC were treated for 48 h with either pCS or pCG (0–2 mM). Cells were harvested using trypsin–EDTA and fixated with 4% paraformaldehyde/0.1% saponin. All staining were performed using a 1:100 antibody dilution and incubated for 30 min at room temperature. Primary antibodies used were: mouse-α-human Vimentin-PE; rat-α-human E-cadherin; mouse-α-human snail1 (Abcam, Cambridge, UK). And as secondary antibodies we used: Alexa488 goat-α-rat for E-cadherin, and Alexa488 goat-α-mouse for snail1 (Life Technologies, Carlsbad, USA). Samples were acquired with a BD FACSCalibur (Becton Dickinson, Breda, the Netherlands). Analysis was performed using Flow Jo software (TreeStar, Ashland, USA), gating on viable cells.

2.8. Quantitative PCR

For gene expression, total RNA was isolated from ciPTEC exposed to pCS or pCG (0–2 mM) for 48 h using an RNeasy Mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturers recommendations. Subsequently, cDNA was generated using the Omniscript RT-kit (Qiagen). Following cDNA-synthesis, quantitative PCR was performed using a CFX96 Real-Time PCR detection system (Bio-rad, Veenendaal, the Netherlands). GAPDH was used as housekeeping gene and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method. The primer-probe sets were obtained from Applied Biosystems: GAPDH, hs99999905_m1; OATP4C1, hs00698884_m1; Bcl-2, hs00608023_m1; snail1, hs00195591_m1; BCRP, hs00184979_m1; MRP4, hs00195260_m1; KIM-1, hs03054855_g1; Vanin-1, hs01546812_m1.

2.9. Fluorescent microscopy

Morphological changes of ciPTEC after exposure to solutes were assessed using both standard bright field and fluorescent microscopy, using the fluorescent cytoplasm marker carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Life Technologies). Samples were incubated with CFDA-SE (10 μM) for 10 min prior to fixation with 4% paraformaldehyde. Images were acquired via a Leica DMI6000B-automated high-content microscope (Leica, Wetzlar, Germany).

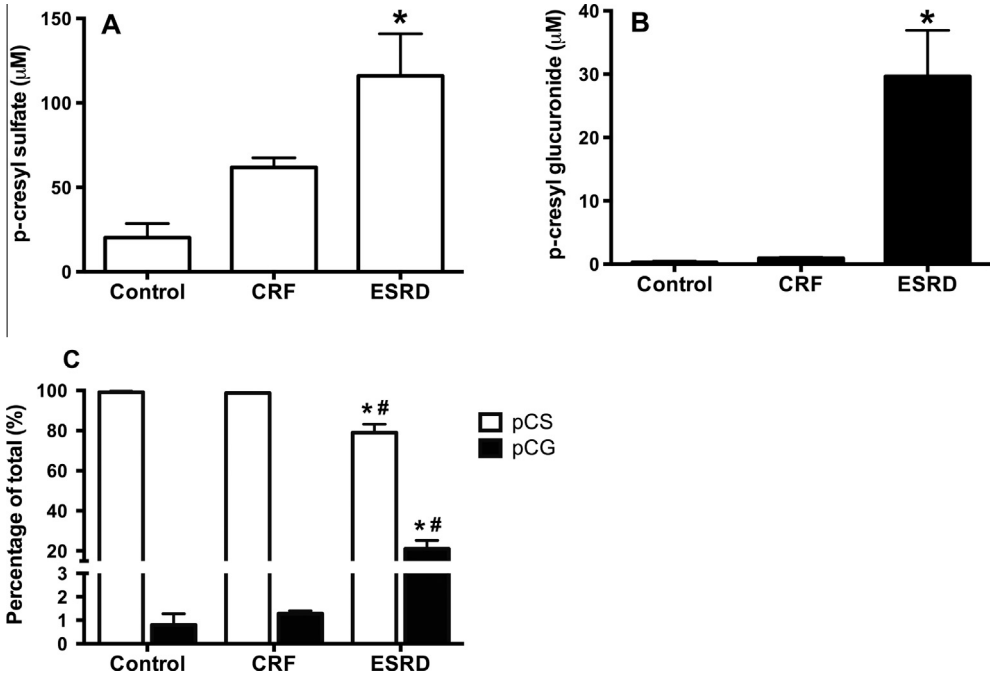

Fig. 1. Accumulation of p-cresol metabolites during CKD. HPLC was used to measure the total plasma concentrations of (A) pCS and (B) pCG. Plasma samples were obtained from healthy volunteers ($n = 4$) and patients with CRF ($n = 79$) or ESRD ($n = 12$). Standards of the compounds were also analyzed in order to quantify the amount of toxins found in the samples. Acquired HPLC data were processed with PC1000 software (Spectrasystem). (C) Percentage pCS and pCS of total metabolites. Statistical analysis was performed via one-way ANOVA followed by the Dunnett's Multiple Comparison Test for each toxin. Results are presented as mean ± SEM. $^{*}p < 0.05$ compared to Control. $^{\#}p < 0.05$ compared to CRF.

2.10. Functional assays for BCRP and MRP activity

The function of BCRP and MRP4 was evaluated using fluorescent substrates that accumulate intracellular upon inhibition of pump activity. Hoechst33342 was used to evaluate BCRP activity and glutathione methylfluorescein (GSMF), a bi-product of 5-Chloromethylfluorescein Diacetate (CMFDA; Life Technologies), to assess MRP4 activity. To investigate ciPTEC efflux activity, cells were seeded in 96 well plates and, after maturation, incubated for 30 min with 1.25 μM of the fluorescent substrates in Krebs buffer in absence or presence of a concentration range of a specific inhibitor for either BCRP (KO143) or MRP's (MK571). Hoechst33342 fluorescence was measured directly after incubation, whereas GSMF fluorescence was monitored after an additional 30 min at 37 °C. To investigate pCS and pCG effect on transport activity, cells were seeded as described and treated for 48 h with either pCS or pCG (0–2 mM) prior to incubation with either Hoechst33342 or CMFDA (1.25 μM) for 30 min at 37 °C. All fluorescent assays were preformed in triplicate and measures preformed in a Victor 3 Multilabel plate reader (Perkin Elmer, Waltham, USA).

2.11. Statistics

Statistics were performed using GraphPad Prism 5.02 via one-way analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's Multiple Comparison Test, or by an unpaired Student's *t*-test. Differences between groups were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Accumulation of pCS and pCG and changes in p-cresol metabolism in CKD patients

Fig. 1 illustrates that mean pCS levels markedly increased from 20 μM in healthy controls to 62 μM in non-dialysis CRF patients and to 116 μM in dialysis patients. The concentration of pCG increased from 0.3 μM (control) to 1 μM (CRF) and 30 μM (ESRD). In addition, HPLC revealed that the *p*-cresol metabolite fraction shifted from sulfation to glucuronidation with a pCG percentage of 1.3% in CRF patients and 21% in ESRD patients (Fig. 1C). Note that with the PCA extraction method used, the compounds are stable in the extract for up to 16 h, where after degradation into p-cresol was observed (data not shown).

3.2. Apical transporters involved in pCS and pCG clearance

Next, we aimed to elucidate the transporters involved in the renal clearance of both *p*-cresol metabolites. Using membrane vesicles, the highest concentration tested (1 mM) of pCS inhibited MRP4-mediated [^{3}H]-MTX uptake with 40% and BCRP-mediated [^{3}H]-E1S uptake with 25% (Fig. 2A and B). In contrast, pCG reduced [^{3}H]-MTX uptake by MRP4, in a concentration-dependent manner by 18% at 1 μM and 75% at 1 mM (Fig. 2A). To identify whether pCG and pCS are substrates for MRP4 and BCRP, accumulation of the solutes in transporter-expressing vesicles was investigated. As shown in Fig. 2C, we did not observe uptake of pCS in MRP4 expressing vesicles, whereas pCG appeared to be transported, although not significantly ($p = 0.07$). Conversely, direct uptake of both pCS and pCG in BCRP vesicles was clearly demonstrated (Fig. 2D). Lastly, we set out to confirm these results in a more physiological model. Both pCS and pCG are taken up by ciPTEC as demonstrated by their accumulation in ciPTEC after exposure to either 1 or 2 mM of the solutes for 48 h (Fig. 2E). Intracellular levels of pCS were determined to be 204 ± 65 and 344 ± 1 11 ng/ml, respectively, while pCG levels were 239 ± 50 and 440 ± 174 ng/ml, respectively, indicating a dose-dependent uptake of both metabolites. Moreover, inhibition of BCRP activity by KO143 increased the intracellular levels of pCS (Fig. 2E), further supporting the notion that this transporter is involved in renal pCS handling. In contrast, suppressing BCRP functionality did not alter intracellular pCG concentrations (Fig. 2E), possibly due to MRP4-mediated transport of

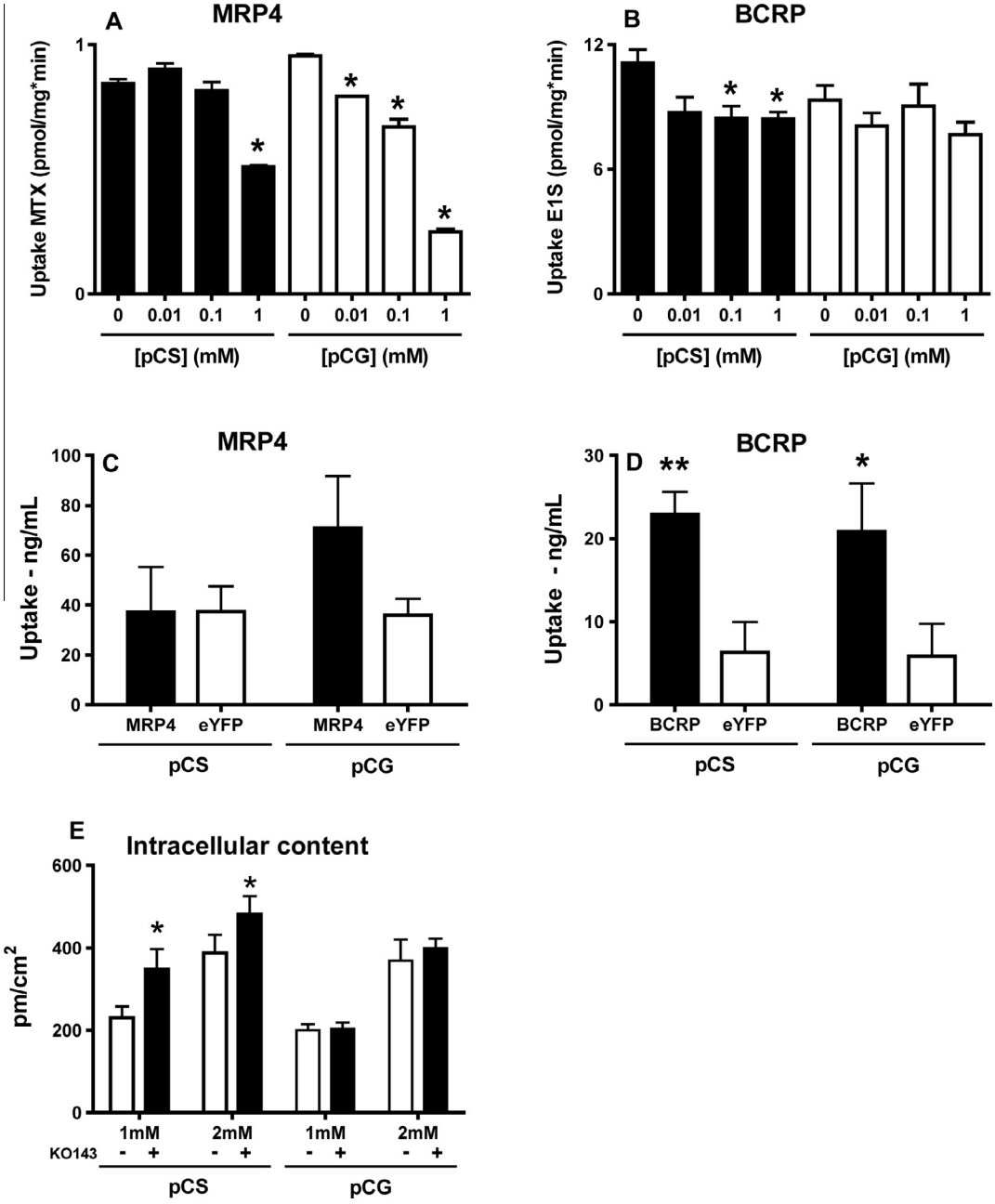

Fig. 2. Uremic toxins are substrates and/or inhibitors of MRP4- and BCRP-mediated transport. A rapid filtration technique was used to study (A) MRP4-mediated [^{3}H]-MTX uptake or (B) BCRP-mediated [^{3}H]-E1S uptake into membrane vesicles in the presence of various concentrations of p-cresol metabolites. Radioactivity was determined using liquid scintillation counting. Results are presented as mean ± SEM of one representative experiment performed in triplicate. Statistical analysis was performed via one-way ANOVA followed by the Dunnett's Multiple Comparison Test for each toxin. Uptake of pCS and pCG into (C) MRP4- or (D) BCRP-overexpressing vesicles as determined by LC/MS-MS. (E) Intracellular content of pCS and pCG in pmol/cm^2 ciPTEC after 48 h exposure, in the absence or presence of the BCRP inhibitor KO143, as determined by LC/MS-MS. Results are presented as mean ± SEM of three independent experiments performed at least in duplicate Statistical analysis was performed by an unpaired Student's *t*-test for each toxin. $^*p < 0.05$ and $^{**}p < 0.01$ compared to eYFP transduced cells.

pCG. Nor did the MRP4 inhibitor, MK571, have any effect (data not shown). Taken together, these findings suggest that both *p*-cresol metabolites are actively transported by BCRP, and pCG appears to be both a substrate and a potent inhibitor of MRP4.

3.3. Induction of EMT by pCG

The toxicity of pCS has been widely investigated (Wu et al., 2012), however, little is known about the biological activity of pCG. Previously, our group demonstrated that pCS and pCG (1 and 2 mM) mitigated metabolic activity in ciPTEC, without affecting cell viability (Mutsaers et al., 2013). Here, we studied the impact of both solutes on ciPTEC phenotype emphasizing on EMT, a known consequence of pCS exposure (Vanholder et al., 2014). Fig. 3 shows that pCG concentration-dependently increased the expression of the mesenchymal marker vimentin (Fig. 3A/D), while decreasing the expression of the epithelial marker E-cadherin, although this effect was not significant (Fig. 3B/E). Snail is an important transcription factor involved in EMT and snail protein levels were unaltered following pCG exposure (Fig. 3C/F),

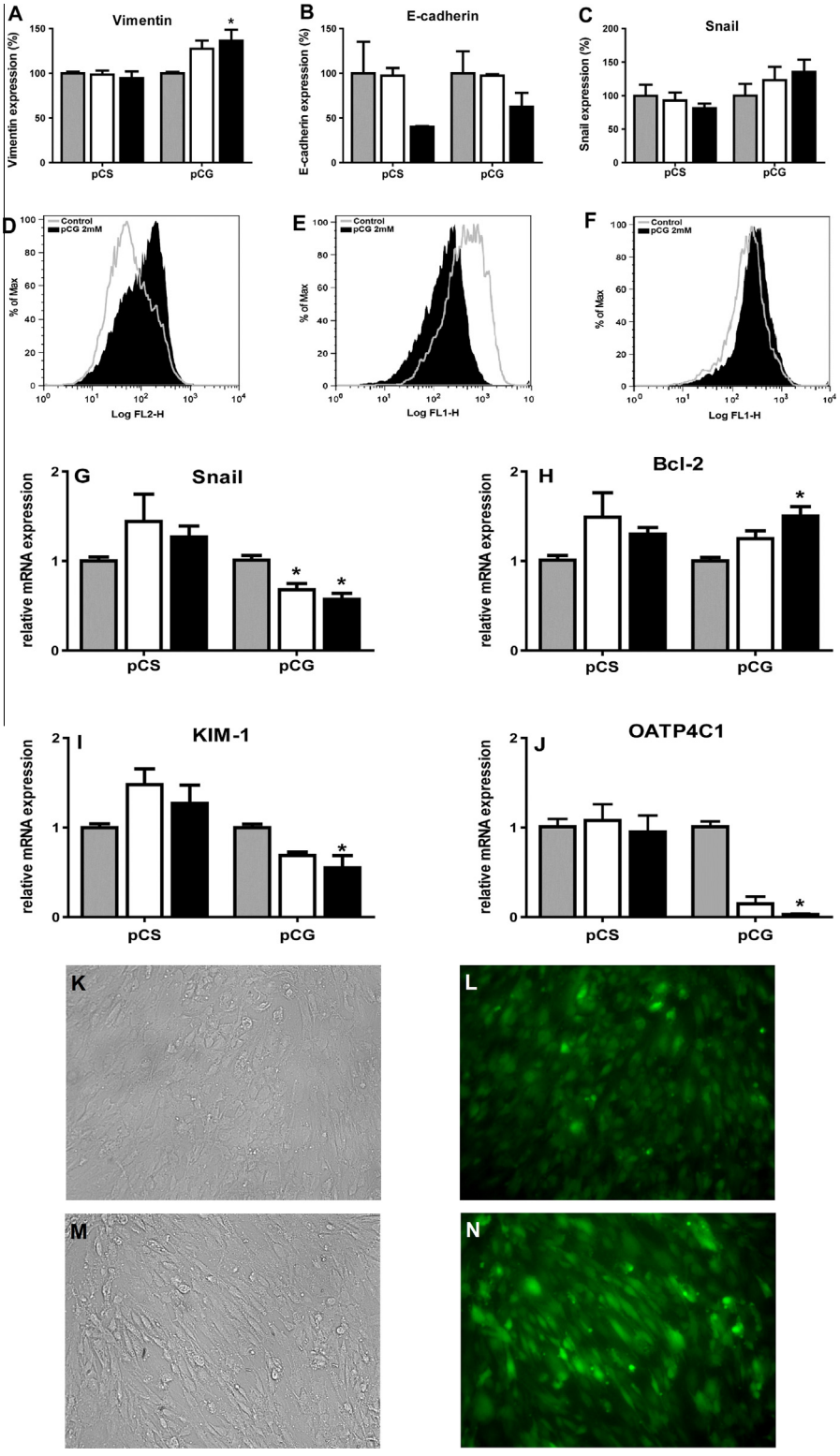

Fig. 3. Induction of EMT by pCG in ciPTEC. Cells were exposed for 48 h to salt control solution (grey bar), 1 mM (white bars) or 2 mM (black bars) of pCS or pCG. Following treatment, cells were harvested and stained with either (A, D) mouse-α-human Vimentin-PE, (B, E) rat-α-human E-cadherin or (C, F) mouse-α-human Snail. Representative histograms show the salt control (grey line) and the treatment with pCG 2 mM (black) for (D) Vimentin, (E) E-cadherin and (F) Snail. Quantification of staining was done with a BD FACSCalibur flow cytometer using channel FL-2 or FL1 and analyzed with FlowJo software gating on viable cells. Furthermore, gene expression of (G) Snail, (H) Bcl-2, (I) KIM-1 and (J) OATP4C1 was studied using qPCR. GAPDH was used as reference gene and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Representative images of cells incubated with either control salt solution (K, L) and 2 mM pCG (M, N), Images were acquired by bright field and fluorescent microscopy, cytoplasm was stained with CFDA-SE. Statistical analysis was performed via one-way ANOVA followed by the Bonferonni's Multiple Comparison Test for each toxin. Results are presented as mean ± SEM of at least three independent experiments performed in duplicate. $*$ indicates $p < 0.05$ compared with control.

whereas, gene expression was significantly down-regulated (Fig. 3G). Moreover, expression of the anti-apoptotic gene Bcl-2 was induced by pCG (Fig. 3H), in concordance with transitioning epithelial cells being resistant to apoptosis (Zeisberg and Neilson, 2009). Next, it was investigated whether pCS and pCG induced cell injury by studying the expression of two early markers of tubular damage: kidney injury molecule-1 (KIM-1) and vanin-1 (Hosohata et al., 2012; Huo et al., 2010). Fig. 3I shows that exposure to pCG resulted in a slight reduction in KIM-1 expression while pCS had no effect. Furthermore, vanin-1 levels remained unchanged by either pCS or pCG (data not shown). These findings suggest that both solutes did not induce tubular cell damage, in line with the observed induction of EMT. In confirmation, treatment with pCG resulted in transformation of the typical epithelial cobblestone morphology into a more prolonged, fibroblast-like, morphology (Fig. 3K–N).

Since pCG exposure led to loss of proximal tubular characteristics, gene expression of key transporters was investigated. Following treatment with pCG, the influx transporter potentially involved in p-cresol metabolite handling (Mutsaers et al., 2011b), organic anion transporting polypeptide (OATP4C1), was significantly down-regulated (Fig. 3J), whereas expression of BCRP increased more than 2-fold (Fig. 4A), consistent with effects previously reported upon renal stress or injury (Masereeuw and Russel, 2012). In addition, no changes in MRP4 mRNA levels were observed, as demonstrated in Fig. 4B. Together with the observed induction of EMT, these results suggest that pCG exposure causes cell stress whereas pCS does not exert such biological activity in ciPTEC.

3.4. Exposure to pCG results in a functional up-regulation of BCRP

To investigate further the functional consequences of cell stress induced by pCG, the transport activity of BCRP and MRP4 was studied. Fig. 4C shows an increase in fluorescence when cells were exposed to Hoechst33342 in presence of the BCRP inhibitor KO143, indicating inhibition of efflux. Similarly, exposing cells to CMFDA (precursor of GSMF) in presence of the MRP blocker MK571 also resulted in augmented fluorescence (Fig. 4D). Thus, fluorescent substrates are viable tools to monitor BCRP and MRP4 efflux activity in ciPTEC. Following exposure to pCG (2 mM, 48 h) Hoechst33342 retention was approximately reduced by 50%, which corresponded to a 2-fold increase in BCRP activity (Fig. 4E). This effect is consistent with the observed increase in BCRP gene expression (Fig. 4A). Moreover, no significant differences were observed in GSMF fluorescence (Fig 4F), also correlating with the lack of effect of pCG and pCS on MRP4 expression (Fig. 4B). These findings further support the hypothesis that pCG promotes cell stress.

4. Discussion

CKD is characterized by the accumulation of a diverse range of metabolites, which are normally cleared by the healthy kidney. Yet, it remains unclear which solutes are the true culprits in CKD progression and contribute to the myriad of comorbidities associated with the disease. The results of the present study demonstrate that the uremic retention solutes, pCS and pCG, accumulate in chronic kidney disease patients and pCG exposure causes phenotypical changes in renal proximal tubule cells. Furthermore, both pCS and pCG seem to depend on BCRP activity for their urinary secretion. It is well known that BCRP and MRP4 are of key importance for clearing a wide range of drugs and metabolites into the urine, and under uremic conditions their function may be affected, causing toxins to accumulate (Mutsaers et al., 2011a). We also describe how exposure to pCG up-regulates BCRP activity, which may indicate an inducible response mechanism to cope with increased toxin levels that otherwise could lead to cellular stress. These results underline the importance of ABC transporters in the excretion of uremic toxins by proximal tubule cells and demonstrate how inactivity of these transporters may contribute to tubular damage and CKD progression by promoting pCS and pCG accumulation (Masereeuw et al., 2014). Moreover, the present findings provide the first support that pCG exposure results in EMT and cell stress, as was demonstrated by increased vimentin and Bcl-2 expression as well as decreased E-cadherin expression, accompanied by a change in morphology. Furthermore, we observed reduced levels of snail1 mRNA, whereas protein levels where unaffected by pCG. These results are in line with reports showing that in human kidney (HK-2) cells undergoing cyclosporine A-induced EMT, snail gene expression decreased while protein expression increased due to a reduced rate of protein degradation (Berzal et al., 2012). Furthermore, pCG caused a reduction in the expression of KIM-1, a marker associated with acute injury due to an inflammatory response (Wilkinson et al., 2014), supporting the notion that pCG promotes EMT rather than inducing nephrotoxicity.

In contrast to the marked impact of pCG on ciPTEC phenotype, no changes were observed following exposure to pCS. Which is in stark disparity with previous results obtained in leucocytes (Meert et al., 2012). This suggests that the toxicity of both *p*-cresol metabolites is cell type-specific. Also, in previous studies, the models used to investigate the impact of pCS on human renal cells, such as HK-2 cells, have limited physiological resemblance to proximal tubules (Jenkinson et al., 2012). Moreover, other reports showing that pCS evokes EMT in proximal tubules use murine models (Sun et al., 2013). Still, it should be noted that the pCS and pCG concentrations used were rather high as compared to plasma concentrations. This was done because the ciPTEC used in the present study do not express the influx transporters OAT1 and OAT3 (Jansen et al., 2014) that play a role in the uptake of both solutes (Masereeuw et al., 2014; Mutsaers et al., 2011b), yet we did clearly observe intracellular accumulation of both solutes in the cells. Furthermore, we believe that pCS and pCG mainly exert toxicity via an intracellular mechanism and until now the intracellular concentrations of both solutes during uremia are unknown. Nevertheless, we demonstrate that pCG might promote CKD progression by inducing phenotypical changes in human proximal tubule cells.

The paradigm of *p*-cresol as a uremic toxin of major importance was an artifact from strong acidification of plasma samples for deproteinization resulting in hydrolysis of both pCS and pCG (Vanholder et al., 2011). In this study, PCA extraction was used as a method for protein removal, still the pCS levels determined are similar to the concentrations reported by several groups using different deproteinization methods including methanol extraction, enzymatic degradation or heating (de Loor et al., 2005; Martinez et al., 2005; Meert et al., 2012; Meijers et al., 2009). Reports on pCG concentrations in CKD patients are scarce and considerably diverse. Our results corroborate the findings by Meert et al. (2012), suggesting that pCG levels are much higher than previously reported. The observed change in glucuronidation might be caused by secondary factors, such as exposure to drugs or smoking, and warrants further clinical investigation beyond the scope of the present study. Taken together, our results indicate that acidification can be successfully used as a deproteinization method when measuring plasma levels of pCS and pCG; yet one has to take into account that the *p*-cresol conjugates are prone to hydrolysis.

In addition to endogenous metabolites, it is widely known that also drug disposition is altered in CKD patients (Dreisbach and Lertora, 2008; Sun et al., 2006). This includes the kinetics of drugs

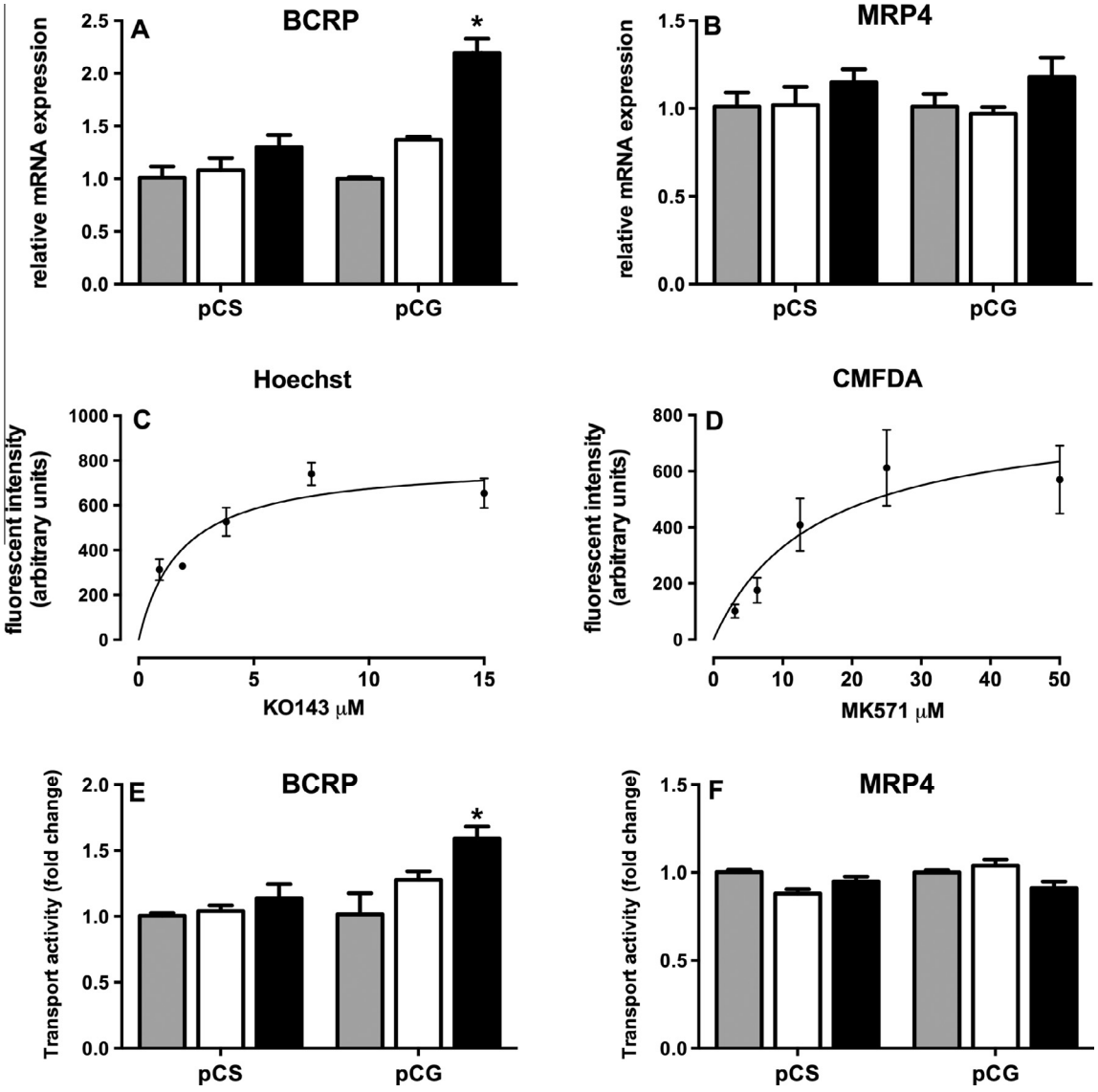

Fig. 4. Impact of pCS and pCG on expression and function of apical transporters. To determine the impact of pCS and pCG, cells were exposed for 48 h to salt control solution (grey bar), 1 mM (white bars) or 2 mM (black bars) of pCS or pCG. Following treatment, ciPTEC were harvested and total mRNA was isolated. Afterwards, cDNA was synthesized and (A) BCRP and (B) MRP4 expression was studied using qPCR. GAPDH was used as housekeeping gene and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. (C) To determine BCRP functional activity in ciPTEC, cells were incubated with Hoechst33342 (1.25 μM) in combination with a gradient of the inhibitor KO143, (D) for MRP activity cells were incubated with CMFDA (1.25 μM) and GSMF accumulation was measured in combination with a MK571 inhibitor gradient. Results are expressed in fluorescent intensity, compensated for background (arbitrary fluorescent units). Changes in transport activity of BCRP and MRP following pCS and pCG treatment were determined by incubating the cells, after the treatment, with (E) 1.25 μM Hoechst33342 and (F) 1.25 μM CMFDA. Results are expressed in activity fold compared to control (i.e. no toxin exposure). Statistical analysis was performed via one-way ANOVA followed by the Bonferonni's Multiple Comparison Test for each toxin. Values are shown as mean ± SEM of minimally two independent experiments performed in triplicate.

solely cleared via phase II metabolism (Kim et al., 1993; Osborne et al., 1993; Verbeeck, 1982), whose enzymes catalyze conjugation reactions such as glucuronidation, acetylation and sulfation. Recently, using ciPTEC, our group demonstrated that uremic toxins could diminish renal UDP-glucuronosyl transferase functionality, probably through interference with mitochondrial succinate dehydrogenase activity and by reducing the reserve capacity of the energy-generating oxidative phosphorylation system (Mutsaers et al., 2013). Simard et al. (2008), showed that exposing rat hepatocytes to uremic serum resultes in a decreased expression of N-acetyltransferases (NAT)1 and NAT2. Expression of both enzymes was also lower in the liver of CKD rats, which was accompanied by a reduction in NAT2-mediated acetylation of *p*-aminobenzoic acid (Simard et al., 2008). However, little is known about sulfotransferase (SULT) expression and functionality in patients with renal failure. Together with the observed shift in *p*-cresol metabolite profile in this study, these findings provide the first evidence that SULT-mediated *p*-cresol metabolism is saturated or reduced in dialysis patients. Of interest, Sugimura et al. (2002) reported that SULT1C2 gene expression was reduced in an acquired polycystic kidney disease model in rats. Still, the link between CKD and SULTs requires further investigation and may contribute to our understanding of the pathophysiology of CKD.

In conclusion, in this study the retention of pCS and pCG in CKD patients is reported and we provide the first evidence for the interference of both solutes with MRP4 and BCRP activity, with potential consequences for renal solute clearance. Moreover, we demonstrated active transport of pCS and pCG by BCRP.

Furthermore, our results demonstrated that pCS does not exert nephrotoxic effects, whereas pCG induced cell stress and EMT, as well as changes in transporter expression in ciPTEC. These findings offer novel insights in the diverse contributions of both *p*-cresol conjugates to CKD pathophysiology.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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