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Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration

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ABSTRACT

During chronic kidney disease (CKD), drug metabolism is affected leading to changes in drug disposition. Furthermore, there is a progressive accumulation of uremic retention solutes due to impaired renal clearance. Here, we investigated whether uremic toxins can influence the metabolic functionality of human conditionally immortalized renal proximal tubule epithelial cells (ciPTEC) with the focus on UDP-glucuronosyltransferases (UGTs) and mitochondrial activity. Our results showed that ciPTEC express a wide variety of metabolic enzymes, including UGTs. These enzymes were functionally active as demonstrated by the glucuronidation of 7-hydroxycoumarin (7-OHC; K_m of 12 ± 2 μM and a V_{max} of 76 ± 3 pmol/min/mg) and p-cresol (K_m of 33 ± 13 μM and a V_{max} of 266 ± 25 pmol/min/mg). Furthermore, a wide variety of uremic toxins, including indole-3-acetic acid, indoxyl sulfate, phenylacetic acid and kynurenic acid, reduced 7-OHC glucuronidation with more than 30% as compared with controls ($p<0.05$), whereas UGT1A and UGT2B protein expressions remained unaltered. In addition, our results showed that several uremic toxins inhibited mitochondrial succinate dehydrogenase (*i.e.* complex II) activity with more than 20% as compared with controls ($p<0.05$). Moreover, indole-3-acetic acid decreased the reserve capacity of the electron transport system with 18% ($p<0.03$). In conclusion, this study shows that multiple uremic toxins inhibit UGT activity and mitochondrial activity in ciPTEC, thereby affecting the metabolic capacity of the kidney during CKD. This may have a significant impact on drug and uremic retention solute disposition in CKD patients.

1. Introduction

Renal function is an important aspect in drug clearance and it is widely known that drug disposition is altered in patients with chronic kidney disease (CKD) [1–3]. These changes in pharmacokinetics are partially due to a decreased glomerular filtration and tubular secretion. Another hallmark of CKD is the accumulation of potentially toxic solutes that are normally excreted *via* the urine. These uremic toxins can cause a multitude of pathologies, including renal fibrosis, anemia, bone disorders and cardio-vascular disease [4,5]. Currently, more than 110 uremic toxins are known, divided into three distinct classes based on their physicochemical properties: the small water-soluble compounds, the middle molecules and the protein-bound solutes [5,6]. The latter group of retention solutes are actively secreted by the healthy kidney and are difficult to eliminate using current dialysis strategies [7]. Since protein-bound uremic toxins accumulate during renal failure it could be argued that these compounds affect drug metabolism in CKD patients by interacting with renal enzymes. Many drugs commonly used in the clinic are metabolized by phase II enzymes, which catalyze conjugation reactions, including sulfation, acetylation and glucuronidation [8]. Several

Abbreviations: 7-OHC, 7-hydroxycoumarin; 7-OHCG, 7-hydroxycoumarin glucuronide; AA, antimycin A; ciPTEC, conditionally immortalized human renal proximal tubule epithelial cells; CKD, chronic kidney disease; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; CRF, chronic renal failure; CYP, cytochrome p450; E: ETS, electron transport system; FCCP, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone; FCS, fetal calf serum; GST, glutathione S-transferase; HA, hippuric acid; HEK293, human embryonic kidney cells; HPLC, high-performance liquid chromatography; I3A, indole-3-acetic acid; IS, indoxyl sulfate; KA, kynurenic acid; L, LEAK; M, medium; Mix, uremic toxin mix; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NAD^+, nicotinamide adenine dinucleotide; NAT, N-acetyltransferase; omy, oligomycin A; OAT, organic anion transporter; Ox, oxalate; OXPHOS, oxidative phosphorylation; pC, p-cresol; pCG, p-cresyl glucuronide; pCS, p-cresyl sulfate; PHA, phenylacetic acid; PHG, phenyl glucuronide; PHS, phenyl sulfate; PTEC, proximal tubule cells; Pu, putrescine; QA, quinolinic acid; R, ROUTINE; ROT, rotenone; ROX, residual oxygen consumption; SULT, sulfotransferase; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferases; ZO-1, tight junction protein 1

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studies demonstrated that the pharmacokinetics of drugs solely cleared *via* phase II metabolism is changed in CKD patients. For instance, a decreased glucuronidation of metoclopramide, chloramphenicol, p-aminobenzoic acid, zidovudine and morphine have been reported in patients with chronic renal failure (CRF) [9–14]. Moreover, the acetylation of isoniazid is reduced in CKD patients [15]. However, little information is available about the mechanism underlying the observed decrease in phase II metabolism during renal failure.

UDP-glucuronosyltransferases (UGT) are an important class of phase II enzymes that catalyze the conjugation of glucuronic acid to many xenobiotics, environmental pollutants and endogenous compounds [16,17]. Next to drugs, uremic retention solutes are also prone to glucuronidation, and at least two glucuronides have been identified in uremic biological fluids, p-cresyl glucuronide and indoxyl glucuronide [18–20]. UGTs are expressed in several organs including the liver, gastro-intestinal tract and kidney, and to date 19 human UGT proteins have been identified [21,22]. Due to the relative abundance of the essential cofactor UDP-glucuronic acid (UDPGA), glucuronidation is the most prevalent conjugation reaction and under normal metabolic conditions, the supply of UDPGA is not rate-limiting for this process [23]. Yet, during excessive glycogenesis or under altered redox conditions, UGT activity is impaired [23,24]. After the liver, UGT activity is highest in the kidney, emphasizing the pivotal role of this organ in facilitating xenobiotic clearance *via* glucuronidation [8,25]. Previously, Yu et al. demonstrated that UGT expression and activity were down-regulated in the liver and kidney of 5/6 nephrectomized rats. However, this effect was also observed in control pair-fed rats and was possibly due to a decreased food intake [26]. Thus, the repercussions of CKD on UGTs remain to be elucidated.

In the present study, conditionally immortalized human renal proximal tubule epithelial cells (ciPTEC) were used to investigate the impact of multiple uremic toxins on renal UGT activity. Our results show that ciPTEC express a broad array of drug metabolism enzymes, similar to human kidney. Furthermore, UGT proteins were functionally active in ciPTEC, as demonstrated by 7-hydroxycoumarin (7-OHC) and p-cresol glucuronidation. Uremic toxins inhibited the glucuronidation of 7-OHC without affecting UGT1A and UGT2B protein expression, indicating a reduction in enzyme activity. Moreover, exposure of ciPTEC to uremic toxins caused a reduction in mitochondrial succinate dehydrogenase activity and in the maximum capacity of the oxidative phosphorylation (OXPHOS) system, which could explain the observed inhibitory effect of uremic toxins on glucuronide formation. These results present a novel pathway *via* which uremic retention solutes affect the metabolic capacity of the kidney and are likely involved in altering drug metabolism by glucuronidation in CKD patients.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise. Stock solutions of uremic toxins were prepared as described by Cohen et al., [27] and were stored at −20 °C. Both p-cresyl sulfate and phenyl sulfate were synthesized as a potassium salt as described previously [28]. P-cresyl glucuronide was produced from glucuronyl-trichloroacetimidate and p-cresol using the method previously described by Van der Eycken et al. [29].

2.2. Cell culture

The ciPTEC line was generated as previously described by Wilmer et al. [30]. The cells were cultured in ciPTEC medium containing phenol red free DMEM/F12 medium (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 to 1:6 at 33 °C. For 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.

2.3. Quantitative PCR array

To study the gene expression of drug metabolism enzymes, ciPTEC were cultured and differentiated cells (7 days at 37 °C) were harvested. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations. Subsequently, cDNA was generated using the Omniscript RT-kit (Qiagen) according to the manufacturer's recommendations. Following cDNA-synthesis, RT^2 Profiler PCR arrays (drug metabolism: phase I and phase II enzymes; Qiagen) were performed according to the manufacturer's recommendations, using a CFX96 Real-Time PCR detection system (Bio-rad, Veenendaal, The Netherlands). Quantification of gene expression was performed using the CFX96 system software (Bio-rad) and the web-based PCR array data analysis software (Qiagen). GAPDH was used as housekeeping gene, and relative expression levels were calculated as percentage as compared with GAPDH (100%).

2.4. Western blotting

To study the protein expression of UGT1A and UGT2B, ciPTEC were cultured and exposed to 0–2 mM of different uremic toxins for 48 h. After treatment, cells were harvested using RIPA buffer containing 1% (v/v) Igepal CA630, 0.5% (v/v) Nadeoxycholate, 0.1% (w/v) SDS, 0.01% (w/v) phenylmethane sulphonylfluoride, 3% (v/v) aprotinin and 1 mM Na-orthovanadate. Total protein (50 μg) was separated *via* SDS/PAGE using 10% (w/v) gels and blotted onto nitrocellulose membranes using the iBlot dry blotting system (Invitrogen). Afterwards, the membrane was blocked using Odyssey Blocking Buffer, (1:1 diluted with PBS; LI-COR Biosciences, Lincoln, NE, USA) during 1 h at RT. The membrane was then incubated overnight at 4 °C with rabbit polyclonal UGT1A or UGT2B antibody (1:200; both Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Mouse monoclonal β-actin antibody (1:10,000; Sigma) was simultaneously incubated to serve as a protein loading control. Antibodies were diluted in Odyssey Blocking Buffer containing 0.1% (v/v) Tween-20. Afterwards, the membrane was thoroughly washed three times during 10 min with PBS containing 0.1% (v/v) Tween-20. The secondary antibodies, goat-α-mouse Alexa Fluor 680 (1:20,000; Invitrogen) and goat-α-rabbit IRDye 800 (1:20,000; Rockland, Gilbertsville, PA, USA), were incubated for 1 h at RT in Odyssey Blocking Buffer containing 0.1% (v/v) Tween-20 and 0.01% (w/v) SDS. The membrane was thoroughly washed, as described above, and then scanned using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Intensity of the protein bands was quantified using the Odyssey Application software version 2.1.

2.5. Confocal microscopy

Cellular localization of UGT1A and UGT2B proteins was investigated using confocal microscopy. ciPTEC were seeded on 12-well Corning Costar Transwell Permeable Supports (type 3460, Corning Costar, NY, USA). Before seeding, the supports were coated with 50 μg/ml collagen type IV for 2 h at 37 °C. Subsequently, supports were washed with HBSS buffer (Gibco) and cells were seeded at a density of 1.33×10^5 cells/cm^2. Following maturation, as described above, cells were washed with wash solution (4% (v/v) FCS in HBSS) and fixed for 5 min with 2% (w/v) paraformaldehyde in HBSS. Next, cells were permeabilized for 10 min in HBSS with 0.3% (v/v) Triton and aspecific epitopes were blocked for 30 min with blocking buffer (2% (v/v) FCS, 0.5% (w/v) bovine serum albumin and 0.1% (v/v) Tween-20 in HBSS). Subsequently, the cells were incubated overnight at 4 °C with rabbit polyclonal UGT1A or