Published in final edited form as:

Toxicology. 2020 November ; 444: 152582. doi:10.1016/j.tox.2020.152582.

Microphysiological System Modeling of Ochratoxin A-Associated Nephrotoxicity

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Abstract

Ochratoxin A (OTA) is one of the most abundant mycotoxin contaminants in food stuffs and possesses carcinogenic, nephrotoxic, teratogenic, and immunotoxic properties. Specifically, a major concern is severe nephrotoxicity, which is characterized by degeneration of epithelial cells of the proximal tubules and interstitial fibrosis. However, the mechanism of OTA toxicity, as well as the genetic risk factors contributing to its toxicity in humans has been elusive due to the lack of adequate models that fully recapitulate human kidney function *in vitro*. The present study attempts to evaluate dose-response relationships, identify the contribution of active transport proteins that govern the renal disposition of OTA, and determine the role of metabolism in the bioactivation and detoxification of OTA using a 3D human kidney proximal tubule microphysiological system (kidney MPS). We demonstrated that LC₅₀ values of OTA in kidney MPS culture (0.375 – 1.21 μ M) were in agreement with clinically relevant toxic concentrations of OTA in urine. Surprisingly, no enhancement of kidney injury biomarkers was evident in the effluent of the kidney MPS after OTA exposure despite significant toxicity observed by LIVE/DEAD staining. Instead, these biomarkers decreased in an OTA concentration-dependent manner. Furthermore, the effect of 1-

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Declaration of Conflict of Interest Statement:
There are no conflicts to declare.

aminobenzotriazole (ABT) and 6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX), pan-inhibitors of P450 and glutathione S-transferase (GST) enzymes, respectively, on OTA-induced toxicity in kidney MPS was examined. These studies revealed significant enhancement of OTA-induced toxicity by NBDHEX (3 μ M) treatment, whereas ABT (1 mM) treatment decreased OTA-induced toxicity, suggesting roles for GSTs and P450 enzymes in the detoxification and bioactivation of OTA, respectively. Analysis of transcriptional changes using RNA-sequencing of kidney MPS treated with different concentrations of OTA revealed downregulation of several nuclear factor (erythroid derived-2)-like 2 (NRF2)-regulated genes by OTA treatment, including GSTs. The transcriptional repression of GSTs is likely playing a key role in OTA toxicity via attenuation of glutathione conjugation/detoxification. The sequential molecular events may explain the mechanism of toxicity associated with OTA. Additionally, OTA transport studies using kidney MPS in the presence and absence of probenecid (1 mM) suggested a role for organic anionic membrane transporter(s) in the kidney specific disposition of OTA. Our findings provide a clearer understanding of the mechanism of OTA-induced kidney injury, which may support changes in risk assessment, regulatory agency policies on allowable exposure levels, and determination of the role of genetic factors in populations at risk for OTA nephrotoxicity.

Keywords

ochratoxin A; nephrotoxicity; microphysiological systems; proximal tubule epithelial cells; oxidative stress; GSTs

1. Introduction

Ochratoxin A, a common toxin produced by various *Penicillium* and *Aspergillus* species, is one of the most abundant mycotoxin contaminants in the food chain as it is present in cereal grains, beans, dried fruits, wine, coffee, and tea (Duarte et al. 2010). Not only is OTA nephrotoxic, it is a potential carcinogen (Group 2B) and a neuro- and immunotoxin (Duarte et al. 2011). In addition, OTA may also be associated with chronic kidney disease of unknown etiology (CKDu) based on epidemiological studies that link OTA exposure and development of CKDu in patients with Tunisian Nephropathy (Hassen et al. 2004) and kidney disease in Sri Lanka (Desalegn et al. 2011). However, there is currently no definitive line of evidence from epidemiological studies of OTA exposure to the development of CKDu. Some studies of CKDu-affected populations have observed higher urinary OTA concentrations in cases versus controls, whereas others have not (Domijan et al. 2009; Gilbert et al. 2001; Jonsyn-Ellis 2001; Nikolov et al. 1996; Wafa et al. 1998). As such, the actual significance of OTA exposure in human health remains unclear. In the present study, we utilized microphysiological systems (MPS; organs-on-chips) that recapitulate organ-like kidney function *in vitro* to define dose-response relationships of OTA-induced nephropathy.

In addition to clarification of dose-response relationship of OTA-associated nephrotoxicity, it is necessary to understand its mechanism of toxicity, which remains unclear although several *in vitro* and animal studies have suggested involvement of direct macromolecule synthesis, DNA adduct formation, lipid peroxidation, oxidative damage, inflammatory response and mitochondrial uncoupling (Gekle et al. 2005; Hou et al. 2020; Koszegi and Poor 2016; Tao

et al. 2018). OTA biotransformation is also complex; it undergoes metabolism by both phase I and phase II enzymes to several metabolites including hydroxy ochratoxin A, ochratoxin B, ochratoxin hydroquinone (OTHQ), ochratoxin quinone (OTQ) and its glutathione conjugates (Heussner and Bingle 2015). Most metabolites have been shown to exhibit little to no toxicity compared with OTA, however, OTA undergoes biotransformation generating reactive intermediates OTQ/OTHQ which may be associated with OTA nephrotoxicity through redox cycling and the formation of reactive oxygen species (Calcutt et al. 2001; Dai et al. 2002). However, the contribution of intact OTA or its metabolites to nephrotoxicity is still unknown. Furthermore, given that OTA is negatively charged at physiological pH and the main route of excretion is via renal clearance, renal membrane transporters are likely critical for OTA excretion and may contribute to the development of nephrotoxicity.

To address these challenges, the present study aimed to identify the pathway(s) modulating OTA-induced toxicity and the contribution of membrane transporters to kidney specific disposition/excretion of OTA. We also defined dose-response relationships of OTA by employing a kidney MPS populated with primary human proximal tubule epithelial cells (PTECs) that express functional and appropriately localized phase-I/II enzymes and transporters (Chapron et al. 2020; Weber et al. 2018); a significant advance over immortalized cell lines that rapidly lose biotransformation enzyme expression and transporter polarization. This report is the first evaluation of OTA toxicity utilizing the MPS.

2. Materials and methods

2.1. Isolation of PTECs

Human PTECs were isolated as previously described (Weber et al. 2016), in accordance with a protocol approved by the University of Washington Human Subjects Institutional Review Board (IRB STUDY00001297). Detailed kidney tissue donor information is available in Table S1. Following isolation, cells were expanded in tissue culture flasks to passages 1–2 before use in all experiments. DMEM/F12 media containing ITS-A, 50 nM hydrocortisone, penicillin, streptomycin and amphotericin B were used for PTEC cultures as previously described.

2.2. PTEC seeding and establishment of kidney MPS

The Nortis triple channel MPS devices (Triplex™, Figure 1) (<https://www.nortisbio.com/>) were filled with 6 mg/mL of rat tail collagen I and left overnight at room temperature to allow the collagen I matrix to solidify. The mandrels of the MPS platforms were removed, leaving hollow channels through the collagen I matrix. Cell channels were coated with 5 µg/mL mouse collagen IV solution to facilitate human PTEC adhesion. T-flask cultures of PTECs were trypsinized to obtain single cell suspensions. The cells were counted and resuspended with PTEC culture media at a concentration of 20×10^6 cells/mL and 2–2.5 µL were injected into the collagen IV-coated channels of the MPS. Cells were allowed to attach for 3 hr before starting perfusion at a rate of 1.0 µL/min. PTECs were cultured under flow for at least 6 days to form the tubular cell structures. Prior to initiating experiments, a visual inspection by light microscopy was performed to confirm complete (~100%) coverage of the

PTEC tubule. Only kidney MPS that exhibited continuous media flow characteristics were selected for use in functional experiments.

2.3. Dose-response relationship of OTA in kidney MPS culture

Dose-response relationships in the toxicity of OTA in the kidney MPS were assessed with increasing concentrations of OTA from 0 to 3 μM (Figure S1) or 0 to 100 μM (Figure 2) from three different donors and morphological changes were monitored by microscopy. Experiments were carried out in triplicates for all the conditions tested except for OTA at 100 μM in Bio125 and 0.1 μM in Bio101, where experiments were conducted in duplicate. At the end of the experiment, cytotoxicity was assessed by LIVE/DEAD staining.

2.4. LIVE/DEAD staining to assess cytotoxicity in kidney MPS

A LIVE/DEAD staining viability/cytotoxicity kit and Hoechst 33342 were used to assess the percentage of cytotoxicity according to manufacturer's specifications (Life Technologies). Calcein AM, EthD-1 and Hoechst 33342 were diluted in prewarmed PBS (+/+) at final concentrations of 2, 4 and 50 μM , respectively. Kidney MPS chips were perfused at 5 $\mu\text{L}/\text{min}$ via the luminal port for 20 min and then incubated for 10 min at 37°C. After the staining procedure, chips were imaged using fluorescent microscopy to visualize live (green stained cells) and dead cells (red-stained nuclei) with nucleus marker Hoechst 33342 (blue-stained cells). Viability % was calculated from an equation below:

$$\text{Viability (\%)} = 100 \times \left(1 - \frac{\text{number of Dead cells} + \text{number of lost cells (}\Delta\text{Hoechst 33342)}}{\text{number of original cells (Hoechst 33342)}} \right)$$

, where the number of original cells was calculated from a randomly selected image of kidney tubule cells under a microscope with 100x magnification (Hoechst 33342 stained 175 ± 15 human cells, $n=3$) in an area of $150 \times 925 \mu\text{m}^2$ (width $\times$ length), the number of lost cells was calculated by the subtraction of the number of cells stained by Hoechst 33342 with various treatments from the cell numbers of the treatment-free controls, reflecting the number of cells that have sloughed off. The LC_{50} value was calculated by fitting the data to the following equation using GraphPad Prism v. 7 (GraphPad Software, La Jolla CA, USA).

$$\text{Viability (\%)} = \text{Bottom} + (\text{Top}(100\%) - \text{Bottom}) / (1 + 10^{(\log\text{OTA concentration} - \log\text{LC}_{50})})$$

Where the top was constrained to 100 (%), bottom was the remaining % of viable cells at the plateau of the highest OTA concentration.

2.5. Analysis of kidney injury biomarker in kidney MPS

MSD Kidney Injury Panel 3 kits (Meso Scale Diagnostics, MD) were used to measure biomarkers associated with kidney injury (KIM-1, Clusterin, Osteoactivin, VEGF and α -GST) in kidney MPS effluents exposed to OTA at 0, 1, 10 and 100 μM or polymyxin B (positive control; 50 μM) for 24 and 72 hr following the manufacturer's protocol.

2.6. Isolation of mRNA and RNA sequencing (RNAseq)

PTECs from two different donors, Bio125 and Bio101, cultured within the kidney MPS were isolated after 72 or 168 hr of OTA treatment, respectively. Tested concentrations of OTA were 0, 0.1, 1, 10 and 100 μ M or 0, 0.3, 1, 3, 10, 30 and 100 μ M for Bio101 (n= 2 to 4) and Bio125 (n=1 to 3), respectively. RNA was isolated using the Qiagen RNeasy Micro Kit following the manufacturer's protocol. Total RNA was normalized to 2 ng/ μ L in a total volume of 9 μ L and then transcribed to cDNA in a dedicated PCR clean workstation using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio, Mountain View, CA). Sequencing libraries were constructed from cDNA using the SMARTer ThrUPLEX DNA-Seq kit (Takara Bio). Final libraries were quantified and library insert size distribution was checked using a Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were normalized and pooled prior to treatment to block free adapters in the sequencing reaction. Final pools were normalized to 10 nM prior to sequencing. Barcoded libraries were pooled using liquid handling robotics prior to loading. Massively parallel sequencing-by-synthesis with fluorescently labeled, reversibly terminating nucleotides was carried out on the Illumina NovaSeq sequencer. All RNA sequencing was performed at the University of Washington Northwest Genomics Center (NWGC). Base calls were generated in real-time on the NovaSeq 6000 instrument (RTA 3.1.5). Demultiplexed, unaligned BAM files produced by Picard ExtractIlluminaBarcodes and IlluminaBasecallsToSam were converted to FASTQ format using SamTools bam2fq (v1.4). Sequences were aligned to reference genome GENCODE human release 30 (GRCh38) using STAR (v2.6.1d). Aligned data were read into R and summarized as counts per gene using the GenomicAlignments package. RNAseq data have been deposited to GEO (GSE151776).

Statistical analysis for global transcriptomics was carried out using R (version 4.0.0). Before fitting any models, we first excluded any genes that were expressed at consistently low levels across all samples using filterByExpr function in the edgeR package (v3.30.0) (Chen et al. 2016). Prior to filtering we had 58870 genes and after filtering we had data for 12902 genes. We then performed a trimmed mean of M-values (TMM) normalization (Robinson and Oshlack 2010). To test the treatment difference, we used a GLM framework with quasi-likelihood method in edgeR package (Lun et al. 2016). Rather than using a post hoc fold change filtering criterion, we used the glmTreat function from edgeR, which is analogous to the TREAT approach (McCarthy and Smyth 2009). We selected genes based on a threshold of 1.5 fold-change and a false discovery rate of 5%.

2.7. Synthesis of 6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol (NBDHEX) and functionality testing of synthesized NBDHEX

NBDHEX was synthesized as described previously (Ricci et al. 2005). Briefly, NBD-Cl (1mmol) and 6 mercapto-1-hexanol (2 mmol) were incubated for 6 h in 20 ml of a 1:1 (v/v) mixture of ethanol and 0.1 M potassium phosphate buffer, pH 7.0, at room temperature. The reaction pH was periodically monitored and kept approximately neutral by the addition of small amounts of 1 M NaOH. At the end of the reaction, excess 6-mercapto-1-hexanol was quenched with 3-bromopyruvate (1.5 mmol). The reaction product, NBDHEX, was collected by filtration, washed copiously with ice-cold water, and dried under vacuum. NBDHEX was obtained in greater than 95% purity as judged by ¹H-NMR spectroscopy (Figure S3A). The

functionality of synthesized NBDHEX was tested using MDCK cells, where two fluorogenic substrate probes for GSTs, monochlorobimane (MCB) and fluorescein diacetate (FDA), were used. After preincubation of NBDHEX (10 μ M) for 2 hr, fluorescence intensity associated with metabolism of MCB (40 μ M) or FDA (10 μ M) were monitored by fluorescent microscopy and signals were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) (Figure S3B).

2.8. Effect of ABT and NBDHEX on OTA-induced nephropathy in kidney MPS

1-Aminobenzotriazole (ABT) was purchased from Sigma-Aldrich (St. Louis, MO). Human PTECs cultured in MPS were checked for cell confluency and tubule morphology before OTA treatment in the presence or absence of ABT or NBDHEX. Kidney MPS with 95–100% confluence was used for further treatment. PTECs cultured in MPS were treated with or without OTA (10 μ M) in the presence or absence of ABT (1 mM) or NBDHEX (3 μ M) in PTEC culture media for 72 hr at a flow rate of 1.0 μ L/min. Concentrations of ABT and NBDHEX were selected based on 2D findings that the LC₅₀ values of NBDHEX and ABT against HK2 cells and PTECs were 4.3 μ M and > 0.5 mM, respectively (Figure S4). At the end of treatment, LIVE/DEAD staining was performed to analyze the effect of ABT or NBDHEX on OTA-induced cytotoxicity. Quantitative percent cytotoxicity was calculated as described in the LIVE/DEAD staining section (shown in materials and methods section 2.4).

2.9. Establishment of vascularized proximal tubule MPS used for transport experiments with OTA

Vascularized proximal tubule MPS was established as described previously (Chapron et al. 2020). The inner chamber of Nortis dual-channel MPS platforms (Duplo™, Figure 1) was used for the study. Collagen I matrix filling and the PTECs seeding procedure were performed as described in section 2.2. Following 6 days of culture, the epithelial tubule was established and perfusion to the channel was stopped for a brief period and then equilibrated with EGM-2 media containing 2 % FBS for 2 hr at 1.0 μ L/min. HUVECs (passages 5 to 10) were seeded into the equilibrated channel at a concentration of 20×10^6 cells/mL and 2–2.5 μ L were injected into the empty channel. HUVECs were allowed to attach for 30 min to establish an endothelial cell lined-vessel. Flow to both channels was resumed at 1.0 μ L/min with both channels receiving their respective culture media. The day after HUVEC seeding, the vascularized proximal tubule MPS were ready for drug transport experiments. Prior to the beginning of experiments, a visual inspection under light microscope was performed to confirm complete (100%) coverage of both HUVEC vessel and PTEC tubule, respectively. Only vascularized proximal tubule MPS that exhibited even, non-perturbed media flow characteristics (i.e., comparable volume of output media from HUVEC vessel and PTEC tubule) were selected for use in functional experiments.

2.10. OTA transport in vascularized proximal tubule MPS

Vascularized proximal tubule MPS platforms were perfused with media containing OTA (10 μ M) in the presence or absence of 1 mM of probenecid (Sigma-Aldrich (St. Louis, MO)) for 24 hr at 37 °C. MPS platforms were then washed and imaged using a Nikon Eclipse Ti-S (Tokyo, Japan) and inverted spinning disk microscope in phase contrast and fluorescent modes. Fluorescent signals associated with OTA were quantified using ImageJ software.

2.11. Statistical Analysis

Statistical analysis was conducted with GraphPad Prism v.7. One-way ANOVA followed by Dunnett's test were employed to assess multiple comparisons for the experiments evaluating the effect of ABT or NBDHEX on OTA-induced nephropathy. For other studies, a 2-tailed Student's t-test was employed to assess the difference between two different conditions. A P value of less than 0.05 was considered significant.

3. Results

3.1. Dose-response relationships of ochratoxin A-induced nephropathy in kidney MPS culture

OTA dose-range finding studies were performed using three different human PTEC donors grown in 3D MPS culture. First, OTA in the kidney MPS was examined by titrating OTA concentrations from 0 to 3 μM for 48 hr (Donor 1), which resulted in no significant changes in morphology (Figure S1). Thus, in a subsequent experiment the duration of exposure was extended to 72 hr with OTA concentrations up to 100 μM (Donor 2). This resulted in toxicity, in an OTA-dependent manner, manifested by a high percentage of dead cells (Figure 2A). To determine the effect of chronic OTA dosing, as seen in at-risk populations, we determined the toxicity of OTA to human PTECs dosed up to 100 μM for up to 168 hr (Donor 3). As shown in Figure 2B, this resulted in severe dose-dependent toxicity, as demonstrated by loss of tubular integrity and a high percentage of dead cells in a dose-dependent manner. A series of dose-range finding experiments in MPS revealed that LC_{50} values of OTA were around 1.21 and 0.375 μM under acute and chronic conditions, respectively. A summary of these studies is shown in Table 1.

3.2. Kidney injury biomarker in kidney MPS effluents treated with OTA

Release of kidney specific injury marker, KIM-1, in the kidney MPS effluents was assessed as a potential biomarker of OTA-induced injury at 8, 24, 48, 72 and 168 hr after starting exposure to increasing concentrations of OTA (Figure S2). As a positive control, we included a treatment group dosed with 50 μM polymyxin B (PMB) that has been shown to cause significant release of KIM-1 in MPS effluents (Weber et al. 2018). OTA treatment did not induce KIM-1 release in the effluents, in contrast to PMB treatment. To expand the panel of biomarkers associated with kidney injury, analysis of MPS effluents was carried out using MSD Kidney Injury Panel 3 (Figure 3). Again, no induction of KIM-1 was observed by OTA treatment, nor did we observe enhancement of other kidney injury biomarkers in contrast to the PMB positive control.

3.3. Effect of ABT or NBDHEX on OTA-induced toxicity in kidney MPS

To describe the role of GST-mediated glutathione conjugation and P450-mediated oxidation in OTA disposition and nephrotoxicity, kidney MPS were treated with OTA (10 μM) in the presence and absence of pan-inhibitors of GSTs and P450s, NBDHEX and ABT, respectively. The inhibitor functionality of synthesized NBDHEX was tested using MDCK cells, where two fluorogenic substrate probes for GSTs, MCB and FDA, were used (Fujikawa et al. 2018; Mukanganyama et al. 2011). After preincubation of NBDHEX (10

μM) for 2 hr, fluorescence due to GST-mediated metabolism of MCB (40 μM) and FDA (10 μM) were markedly decreased, confirming that synthesized NBDHEX was acting as expected (Figure S3). Kidney MPS were treated with NBDHEX (3 μM) in the presence of OTA (10 μM), which resulted in marked enhanced toxicity associated with OTA, with a high percentage of dead cells ($98.7 \pm 0.3\%$ with NBDHEX vs $55.0 \pm 6.2\%$ without NBDHEX, $p < 0.01$), whereas ABT (1 mM) treatment led to attenuation of OTA-induced toxicity (percentage of dead cells $25.3 \pm 14.9\%$ with ABT vs $55.0 \pm 6.2\%$ without ABT, $p < 0.05$) (Figure 4A and B). These results demonstrate that P450s and GSTs play a role in the bioactivation and detoxification of OTA, respectively. ABT or NBDHEX treatment alone had minimal toxicity against PTECs (viability of 90.4 and 88.1%, respectively).

3.4. Transcriptional response of PTECs in kidney MPS culture to OTA

Analysis of transcriptional changes was conducted using RNAseq for PTECs in kidney MPS culture from two different donors, Bio101 and Bio125, exposed to OTA. Principal component analysis (PCA) revealed that most samples from the same donor within each treatment tended to cluster together (Figure 5A), with the OTA treatment effect in donor Bio125 separated on PC1, and treatment effect in donor Bio101 separated on PC2. To characterize the transcriptional changes by OTA treatment, global gene expression analysis was conducted using RNAseq comparing 1 μM or 10 μM of OTA treatment group versus control in donor Bio101 (168 hr exposure), which identified 3034 and 3406 differentially expressed genes (DEGs) (fold change >1.5 and FDR of 0.05), respectively. Similarly, we identified 2396 and 3826 DEGs comparing 1 μM or 10 μM of OTA treatment group versus control in donor Bio125 (72 hr exposure) (fold change >1.5 and FDR of 0.05), respectively. Our previous data had suggested that GSTs play a role in the detoxification of OTA. GSTs are known to be regulated by NRF2 (Kensler et al. 2007), therefore, we focused on transcriptional changes of canonical genes in the NRF2-mediated antioxidant response pathway. Of 50 NRF2-regulated antioxidant response genes tested, 22 and 26 genes were differentially expressed between control and OTA treatment groups for Bio101 and Bio125, respectively. Those differentially expressed genes are depicted in the heatmaps presented in Figure 5B. Notably, OTA treatment decreased the NRF2-regulated genes in both donors (20 out of 22 in Bio101 and 17 out of 26 in Bio125), including genes responsible for glutathione synthesis and recycling, xenobiotic metabolism and detoxification, TXN-based anti-oxidant system and genes with antioxidant properties (Figure 5C, Table S2). The differences in response of the two donors may likely be due to the differences of exposure duration (168 hr for Bio101 and 72 hr for Bio125) as we observed differences of KIM-1 concentrations in effluents between 72 hr and 168 hr exposure of OTA (Figure S2), although the possibility remains that individual differences might account for the differences.

3.5. OTA transport in vascularized-human kidney proximal tubule MPS

Given that OTA is a molecule with biophysical properties that necessitate active transport processes for tubular secretion, transport studies were conducted using the vascularized-human kidney proximal tubule MPS model. PTECs were treated with OTA (10 μM) in vascularized-human kidney proximal tubule MPS culture for 24 hr in the presence and absence of an organic anion transporter inhibitor, probenecid (1 mM), and the cellular disposition of OTA was monitored by fluorescence (Figure 6A and B). Intracellular

accumulation of fluorescence (blue) was observed after OTA treatment, and decreased from 100% to either $28.5 \pm 12.7\%$ (donor A, mean $\pm$ SD (n=3)) or to 57.0% (donor B, mean of duplicate) in the presence of probenecid. These data suggest that organic anion transporters are likely to be involved in tubular secretion of OTA.

4. Discussion

The present study aimed to evaluate the dose-response relationships, identify the contribution of active transport processes that govern renal disposition of OTA and delineate the role of P450- and GST-mediated metabolism in OTA-induced toxicity by utilization of human kidney proximal tubule MPS.

OTA dose-range finding studies were performed using three different human PTEC donors grown in 3D MPS culture. PTECs exhibited sensitivity to OTA with LC₅₀ values of 1.21 to 0.375 μ M for 72 hr and 168 hr of OTA exposure, respectively (Figure 2, Table 1). As expected, longer OTA exposure in kidney MPS culture led to lower LC₅₀ values with lower percentage of living cells at the highest OTA concentration (25.6% for 72 hr vs 1.39% for 168 hr). Compared to reported toxic concentrations of OTA under subchronic/chronic exposure, the LC₅₀ value for OTA in the kidney MPS culture was in good agreement (urinary OTA levels found in children from Sierra Leone in urine ranged up to a high of 148 ng/mL (0.37 μ M)) (Jonsyn-Ellis 2001), suggesting that the kidney MPS model reflects chronic toxicity of OTA that was not achievable in 2D culture conditions. It should be noted that although there was concern about possible adsorption of OTA to the PDMS matrix that composes MPS structure given the high logP of OTA (4.74 based on calculation from <http://www.t3db.ca/toxins/T3D3605>), the effect was minimal as 61–63 % of dosed OTA was recovered into MPS effluents at 24 and 168 hr after starting infusion (Table S3).

Contrary to the OTA concentration-dependent cytotoxicity observed in kidney MPS culture, kidney injury biomarker analysis in the effluents of kidney MPS revealed no increase in well-known kidney injury markers, KIM-1, clusterin, osteoactivin, VEGF, and α -GST following OTA treatment regardless of the time period or exposure concentration. Similar observations have been reported by Rached et al that KIM-1, lipocalin-2, and Timp-1 were not significantly affected by OTA treatment at 10 to 30 μ M with NRK-52E cells for 24 and 48 hr (Rached et al. 2008). The reason for this lack of enhancement of kidney injury markers despite severe toxicity observed by LIVE/DEAD staining is unclear. Given that kidney injury markers were significantly increased by PMB treatment, a known nephrotoxicant, minimal changes in those biomarkers by OTA treatment could be attributable to a unique mechanism of toxicity by OTA. Transcriptional analysis of kidney MPS revealed 26 DEGs out of 63 p53 signaling pathways genes comparing the 10 μ M of OTA treatment group versus control (donor Bio101 168 hr exposure; fold change >1.5 and FDR of 0.05) including downregulation of the p53 gene ($\log_2$ FC of -2.33 , FDR <0.05) (data not shown). Given that upstream of the p53 signaling pathway is activation of p38MAPK (Bonney 2017) and inhibition of p38MAPK can lead to the suppression of KIM-1 shedding in 769-P cells (Zhang et al. 2007), our observation of decreased KIM-1 in both transcripts and protein concentration by OTA treatment might be explained by inhibition of phosphorylation of p38MAPK. Similarly, p38MAPK is also known to regulate the expression of clusterin

(Criswell et al. 2005), which was also decreased by OTA treatment in our studies. In addition, it is reported that ERK1/2 regulates KIM-1 expression in murine AKI models and that OTA activates ERK1/2 (Le et al. 2020), it may also be possible that OTA can affect KIM-1 expression through perturbation of ERK signaling pathway. On the other hand, the possibility still remains that the lack of increases in kidney injury markers is attributable to other mechanism of toxicity of OTA such as inhibition of protein synthesis (Creppy et al. 1984; Koszegi and Poor 2016). Further studies are being undertaken to explore and identify biomarker(s) that will enable us to monitor OTA-induced toxicity based on its mechanism of action, including evaluation of global transcriptomics in PTECs in 2D and MPS culture with increasing concentrations of OTA.

In order to examine the role of phase I bioactivation and phase II detoxification pathways for OTA as possible genetic factors contributing to the development of CKDu, we investigated the effects of ABT and NBDHEX, pan-inhibitors of P450 and GST enzymes respectively, on OTA-induced toxicity in kidney MPS. Concentrations of ABT and NBDHEX used in our studies (1 mM and 3 μ M, respectively) were high enough to inhibit P450 and GSTs based on previously reported inhibition data (GSTP1-1:IC₅₀ 0.80 and GSTM2-2:IC₅₀ < 0.01 μ M) (Ricci et al. 2005). When these inhibitors were tested in combination with OTA (10 μ M) in kidney MPS, interestingly, NBDHEX treatment resulted in marked enhancement of OTA-induced toxicity (viability without NBDHEX 45.1 $\pm$ 6.2% vs with NBDHEX 1.33 $\pm$ 0.33%, p < 0.01), whereas ABT treatment led to reduced OTA-induced toxicity (viability 74.7 $\pm$ 14.9%, p < 0.05). In view of the fact that ABT or NBDHEX alone had minimal cytotoxicity, this observation is likely due to the combined effects of ABT or NBDHEX with OTA. Increased OTA-induced toxicity observed with NBDHEX treatment is presumably caused by decreased clearance of the reactive quinone metabolite, OTQ, as the result of GST(s) inhibition by NBDHEX. In contrast, reduction of OTA-induced toxicity by ABT could be explained by decreased formation of OTQ/OTHQ mediated by P450 enzyme(s). These observations are suggestive of important roles for GSTs and P450 enzymes in the detoxification and bioactivation of OTA, respectively. In sum, OTA is metabolized and bioactivated by P450s to form OTHQ/OTQ, which are further detoxified by GST(s) into non-toxic GSH conjugates. Indeed, an association of genetic polymorphisms of GSTP1 in urothelial cells and susceptibility to OTA has been reported (Lebrun et al. 2002; Lebrun et al. 2006), supporting our current observations. Additionally, formation of GSH conjugates in humans was further implicated by the observation of N-acetyl-L-cysteine (NAC) conjugates in human urine samples. These metabolites result from enzymatic cleavage of a GSH conjugate by γ -glutamyltransferase and dipeptidase, which generates the corresponding cysteine conjugate that is then N-acetylated by N-acetyltransferase to yield NAC conjugates (Nelson 1992; Sueck et al. 2020). While the question of whether OTA and/or its metabolites are responsible for OTA-induced nephrotoxicity has been controversial up to date, our studies demonstrate, at least partly, that metabolite(s) of OTA, OTQ/OTHQ, are strongly implicated in toxicity.

To address the mechanism of OTA nephrotoxicity in association with regulation of GSTs, we conducted global transcriptome analysis for PTECs in MPS culture after exposure to OTA (Figure 5). This revealed marked downregulation of NRF2-regulated antioxidant response genes by OTA treatment from a concentration as low as 0.1 μ M (Figure S5) which

is a biologically relevant considering the high logP of OTA in a protein-free culture system. Our observation is also supported by the report that OTA inhibits NRF2 and decreases the production of GST(s) (Cavin et al. 2007; Guilford and Hope 2014; Limonciel and Jennings 2014). Together with our findings on the effects of NBDHEX and ABT on OTA-associated toxicity of PTECs, these data suggest that formation of OTQ/OTHQ through bioactivation of OTA by P450 may cause toxicity directly (covalent DNA adduction) and/or indirectly (oxidative DNA/protein damage). In parallel, responsible enzymes (GSTs) that detoxify OTQ/OTHQ are down regulated by inhibition of NRF2 by OTA, presumably resulting in intracellular accumulation of OTQ/OTHQ, thereby aggravating toxicity. This sequence of molecular events presents a possible mechanistic explanation for OTA toxicity.

Given that OTA is negatively charged at physiological pH and the main route of excretion is renal clearance (Ringot et al. 2006; Studer-Rohr et al. 2000), membrane transporters are predicted to play an essential role in uptake/accumulation/secretion in the kidney. OTA is highly fluorescent with an excitation/emission of 375/440 nm, which enabled us to evaluate cellular transport/accumulation of OTA in vascularized-human kidney proximal tubule MPS by direct fluorescence imaging. After treatment with OTA (10 μ M), fluorescence associated with OTA inside of the PTECs in vascularized-human kidney proximal tubule MPS was detected, which became minimal in the presence of the organic anion transporter(s) (OATs) inhibitor probenecid (1 mM) (Figure 6). This suggests a critical role of probenecid-sensitive active transporter(s) in OTA uptake from the basolateral aspect into kidney epithelial cells, presumably attributable to organic anion transporter(s) (OAT) 1 and/or 3. This is in accordance with the report that OAT transports OTA using a gene-overexpression systems (Anzai et al. 2010; Jung et al. 2001). Since OATs are highly expressed in the kidney, the presence of an active uptake process by OATs may contribute to kidney-specific exposure of OTA, leading to a subsequent increase in intracellular concentration of OTQ/OTHQ, which results in more severe kidney toxicity compared with other organs. In addition to cellular uptake from the blood side, subsequent efflux process(es) may also govern renal disposition of OTA (Anzai et al. 2010; Ringot et al. 2006). Although not included in the present study, use of inhibitors for MRP, Pgp or BCRP may help identify specific efflux transporters involved and identify associated genetic factors governing OTA toxicity.

It should be noted that although the current study provided a successful example, technical limitations in the application of MPS for toxicological assessment still remain. First, limited number of cells per MPS (~5000) makes bioanalytics (e.g. Western blotting, qPCR analysis of multiple gene sets, or direct measurements of intracellular concentration of tested drugs) technically challenging. Thus, we chose RNAseq analyses as the most appropriate means to capture differences in RNA transcripts and employ specific inhibitors as an indirect method of studying biotransformation pathways and presumptive products in the present study. Second, although our current MPS is significant in that it enables flexible experimental designs such as flow rate or collection of large volume of effluents, it lacks in throughput. This limits the number of simultaneous parallel experiments, unlike 2D culture or other MPS with higher throughput. Inevitably, the more complex the system becomes, the lower the throughput becomes. Lastly, there are several limitations in cell sourcing. PTECs are cultured in media devoid of sex hormones, carrier proteins, and sex-matched serum, thus limiting observation of sex-related effects. Additionally, PTECs in our culture do not exhibit

a disease state phenotype based on the protocol used to isolate, propagate and passage cells under culture conditions lacking patient- and disease- specific morbidities. This helps to evaluate toxicity profiles and clarify mechanism(s) of toxicity free of influence from pre-existing conditions of donors; however, when testing effect of sex or disease state of donors on toxicity, it will be necessary to investigate the optimum culture conditions that will recapitulate sex- or disease- specific differences.

In summary, the present study defined dose-response relationships of OTA, clarified the contribution of transport proteins to the disposition of OTA and highlighted the role of P450 and GST enzymes in the bioactivation and detoxification of OTA (Figure 7). LC₅₀ values of OTA obtained in kidney MPS for 7 days were consistent with clinically toxic concentrations. In addition, we hypothesize that OTA metabolites (OTQ/OTHQ) are at least in part responsible for OTA-induced toxicity, due to the role of GST(s) in detoxification. Furthermore, we identified a role for organic anion transporter(s) in the kidney specific disposition of OTA. This study provides a better understanding of the mechanism of OTA-induced kidney injury that will support changes in risk assessment, regulatory agency policies on allowable exposure levels, and determination of genetic risk factors for OTA-induced nephropathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

The authors would like to acknowledge and thank Sengkeo Srinouanprachanh, and the Northwest Genomics Center for their assistance in sample analysis and handling. We would also like to thank Dr. Terrance Kavanagh for his critical reading of this manuscript. We acknowledge assistance from the Functional Genomics, Proteomics and Metabolomics Core Facility of the University of Washington Interdisciplinary Center for Exposures, Diseases, Genes and Environment (UW EDGE Center; P30ES007033 to EJK and JH). Research reported in this publication was supported by the National Center for Advancing Translational Sciences of the NIH under award numbers 5UH3TR000504 and UG3TR002158 (to JH) and an EDGE Center pilot award (P30ES007033) to EJK. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. Additional support was provided by Assistance Agreement 83573801 awarded by the US Environmental Protection Agency (EPA; to EJK). The content has not been formally reviewed by the EPA. The views expressed in this document are solely those of the authors and do not necessarily reflect those of the agency. The EPA does not endorse any products or commercial services mentioned in this publication. This work was also supported by an unrestricted gift from the Northwest Kidney Centers to the Kidney Research Institute.

Abbreviations:

ABT	1-aminobenzotriazole
CKDu	chronic kidney disease of unknown etiology
DEGs	differentially expressed genes
GST	glutathione S-transferase
MPS	microphysiological systems
NBDHEX	6-(7-Nitro-2,1,3-benzoxadiazol-4-ylthio) hexanol

NRF2	nuclear factor (erythroid derived-2)-like 2
OAT	organic anion transporter
OTA	ochratoxin A
OTHQ	ochratoxin hydroquinone
OTQ	ochratoxin quinone
PTECs	proximal tubule epithelial cells
RNAseq	RNA-sequencing

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Figure 1. Establishment of kidney MPS

(A) The Nortis triple channel MPS devices (Triplex™) used for the establishment of renal proximal tubule MPS. PTECs were injected through injection port into MPS filled with type I collagen to form a tubular structure. Media was perfused through the flow path (shown in blue). Each chip has three PTEC tubules. (B) The Nortis dual channel MPS devices (Duplo™) used for the establishment of vascularized proximal tubule MPS. Vascularized proximal tubule MPS was employed for transport studies of OTA. Extracellular matrix space is depicted in pink and media flow is shown in blue. PTECs were seeded and allowed to grow for 6 days, followed by seeding of HUVECs for 24 hr prior to the transport experiment. (C) chemical structure of OTA.

Figure 2. Dose-response relationships of OTA-induced nephropathy in human kidney proximal tubule culture

Upper panel: Representative phase contrast images of human PTECs from two different donors (Bio101 and Bio125) cultured in MPS after OTA treatment up to 72 hr (A) for Bio125 or 168 hr (B) for Bio101 with increasing concentrations. Experiments were carried out in triplicates for all the conditions tested except for OTA at 100 μM in Bio125 and 0.1 μM in Bio101, where experiments were conducted in duplicate, and one representative image is shown. Cell survival was assessed at the end of the experiment after treatment with OTA by LIVE/DEAD staining. Lower panel: LC₅₀ concentrations were obtained by calculation of viability at each OTA concentration. Data represent mean ± SD (n=3) for all the conditions except for OTA at 100 μM in Bio125 and 0.1 μM in Bio101 (mean of duplicate). Scale bar:100 μm.

Figure 3. Biomarker analysis of effluents in kidney MPS culture

MPS effluents were collected (pre-dosing, 24 hr and 72 hr after starting exposure of OTA) and urinary kidney injury biomarkers (A. KIM-1, B. Osteoactivin, C. α-GST, D. Clusterin and E. VEGF) were measured. As a positive control, cells were treated with PMB (50 μM) (shown in red). * $p < 0.05$, significant difference from PMB (50 μM) treatment vs. control.

Figure 4. Effect of ABT and NBDHEX on OTA-induced nephropathy

(A) Representative phase contrast images of human PTECs cultured in MPS after OTA treatment (10 μM) in the presence or absence of ABT (1 mM) or NBDHEX (3 μM) up to 72 hr with increasing concentration. Cell survival was assessed at the end of the experiment after treatment with OTA under various conditions by LIVE/DEAD staining. Scale bar: 100 μm. (B) Quantitative cytotoxicity of images in A. Data represent mean ± SD (n=3) for control and OTA treatment group in the presence or absence of ABT or NBDHEX and mean of duplicate for group with ABT or NBDHEX treatment group in the absence of OTA. * $p < 0.05$, ** $p < 0.01$ significant difference from OTA (10 μM) treatment in the absence of ABT (1 mM) or NBDHEX (3 μM).

Figure 5. Transcriptional response to OTA exposure in PTECs in kidney MPS culture by RNAseq
 (A) PCA plot where color was coded by treatment (control, 1 and 10 μ M OTA) and symbol was coded by donor (Bio101 and Bio125). (B) Heatmap of differentially expressed NRF2-regulated cytoprotective defense genes grouped by treatment (control, 1 and 10 μ M OTA) with two different donors (Bio101; left, Bio125; right). In order to generate a heatmap, a Z-score of the log₂CPM for each of the donors was calculated and adjusted to a mean of zero and a standard deviation of 1. The heatmap is generated using the ComplexHeatmap R package. (C) Transcriptional response visualized for differentially expressed NRF2-regulated cytoprotective defense genes after OTA exposure to PTECs in two different donors (Bio101; left, Bio125; right). Closed and open bars represent OTA exposure at 1 and 10 μ M, respectively.

Figure 6. OTA transport in vascularized-human kidney proximal tubule MPS

(A) Vascularized-human kidney proximal tubule MPS from two different donors were treated with OTA (10 μ M) in the presence or absence of probenecid (1 mM) for 24 hr and cellular accumulation was imaged by fluorescence. Scale bar:100 μ m. (B) Quantification of a cell-associated OTA fluorescent signal (fluorescence inside of the tubules normalized by outside). White (donor A) and gray (donor B) columns represent data from two different donors. Data represents mean $\pm$ SD (n=3) for donor A and mean of duplicate for donor B. *: $p < 0.05$, significant difference from no-inhibitor control.

Figure 7. Proposed metabolic pathway illustrating OTA nephrotoxicity

OTA is metabolized by P450(s) to form ochratoxin hydroquinone (OTHQ) and ochratoxin quinone (OTQ). OTQ, as well as intact OTA, are primarily responsible for nephrotoxicity.

Table 1.

Summary of Toxicity of OTA in 3D and MPS culture

PTEC donor	3D (kidney MPS)		
	Donor1 (Bio38)	Donor2 (Bio125)	Donor3 (Bio101)
Duration of exposure (hr)	48	72	168
LC ₅₀ (μM)	> 3	1.21	0.375
Remaining cells at highest concentration (%)	-	25.6	1.39

Cell viability after PTECs exposed to various concentration of OTA in 3D kidney MPS culture. LC₅₀ values were calculated for two different human donors.