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Mitigation of cell apoptosis induced by ochratoxin A (OTA) is possibly through organic cation transport 2 (OCT2) knockout

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ABSTRACT

Ochratoxin A (OTA) is a secondary metabolite of fungi such as *Aspergillus ochraceus, A. niger* and *A. carbonarius, Penicillium verrucosum*, and various other *Penicillium, Petromyces,* and *Neopetromyces* species. Various foods can be contaminated with OTA, potentially causing several toxic effects such as nephrotoxicity, hepatotoxicity and neurotoxicity. Typically, OTA is excreted by organic anion transporters (OATs). There is no research indicating organic cation transporters (OCTs) are involved in OTA nephrotoxicity.

In our study, NRK-52E cells and rats were treated with OTA. OTA changed the expression of OCT1, OCT2 and OCT3 in NRK-52E cells and rat kidneys. TEA alleviated OTA-induced cell death, apoptosis, and DNA damage, and increased ROS. The OCT2 knockout cell line was constructed by the CRISPR/Cas 9 system. OCT2 knockout did not change the gene expression of OCT1, OAT1 and OAT3. OCT2 knockout alleviated the increase of Caspase 3 and CDK1 induced by OTA, leading to a reduction of apoptosis. In addition, OCT2 overexpression increased cell toxicity and expression of Caspase 3. In short, our findings indicate that OCT2 knockout possibly mitigate OTA-induced apoptosis by preventing the increase of Caspase 3 and CDK1.

1. Introduction

Ochratoxin A (OTA) is a secondary metabolite of fungi such as *Aspergillus ochraceus, A. niger* and *A. carbonarius, Penicillium verrucosum*, and various other *Penicillium, Petromyces,* and *Neopetromyces* species. Food and beverages such as pork, poultry, dairy, chocolate, wine, beer, coffee, grape juice, dried fruit, and spices, can be contaminated with OTA (Hope and Hope, 2012). OTA has adverse effects on both human and animal health, and was classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC) in 1993 (IARC, 1993). Its most serious effects can include nephrotoxicity, hepatotoxicity, neurotoxicity, teratogenicity and immunotoxicity in various animal species. In humans, the main target organ of OTA is the kidney, and OTA is capable of leading to nephrotoxicity for all mammals. Research suggests that OTA is a possible cause of Balkan nephropathy (Pfohl-Leszkowicz and Manderville, 2007). However, it is not clear how OTA enters into the kidney to induce nephrotoxicity.

In the classic transport system for OTA, OTA is excreted mainly by organic anion transporters (OATs), which are located at the plasma membrane of renal proximal tubule cells. They play a critical role in the renal excretion and detoxification of a wide variety of compounds including drugs, toxins, hormones, and neurotransmitter metabolites, which are often organic anions (OAs). Previous studies have shown that hOAT1, as well as hOAT3, mediate a high-affinity transport of OTA on the basolateral side of the proximal tubule (Jung et al., 2001; Zhang et al., 2004). hOAT4 mediates the high-affinity transport of OTA on the apical side of the proximal tubule (Babu et al., 2002). Murine Oat5-expressing *Xenopus laevis* oocytes could increase the accumulation of OTA (Youngblood and Sweet, 2004). Rat Oat8 mediated the uptake of

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OTA (Yokoyama et al., 2008).

There have been no reports showing the relationship of organic cation transporters (OCTs) to OTA nephrotoxicity. However, some compounds can serve as substrates of both organic anion and cation transporters (Zhang et al., 2004). In one study, both hOAT1-4 and hOCT1-2 were shown to mediate the uptake of aflatoxin B1 (Tachampa et al., 2008). Furthermore, OCT2 (gene: *Slc22a2*) was expressed primarily in the cortical pars recta (S2) and medullary pars recta (S3) of the proximal tubules in the kidney, being localized basolaterally. The location in the kidneys is precisely that in which nephrotoxicity was induced by OTA. Therefore, research suggests that OCT2 is involved in OTA nephrotoxicity. OCTs (OCT1, 2, 3) mediate the transport of a broad range of structurally diverse organic cations. Research also demonstrates that hOCT1 and hOCT2 mediate the transport of some anionic compounds (Jonker and Schinkel, 2004).

In this study we investigated whether rat (r)OCT2 was involved in OTA nephrotoxicity. For this purpose, we used cell lines engineered to specifically knock out rOCT2 and overexpress rOCT2 in order to assess OTA-induced cell toxicity. Deepening insight into the role of OCT2 could further illuminate OTA toxicity, as well as facilitate treatment on OTA toxicity in the kidney.

2. Materials and methods

2.1. Cell culture

Rat proximal tubule cells (NRK-52E) cells were bought from the Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China). NRK-52E cells were grown in DMEM supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Cells were passaged every 3–4 days. At approximately 70% confluence, cells were washed with PBS and then treated with OTA for 24 h.

2.2. Ethics statement

Animal studies were conducted in accordance with the ethical guidelines for the care and use of laboratory animals set forth by the Supervision & Testing Center for Genetically Modified Organisms Food Safety, Ministry of Agriculture (Beijing, China) (license number SYXK (Beijing) 2010–0036). All procedures were approved by the Animal Care ethics Committee of China Agricultural University (permission number: 120020).

2.3. Animal treatment

Rats were housed three per cage in a temperature controlled room (22 ± 2 °C) with a relative humidity of 40–70% and a 12 h light-dark cycle. Feed and sterilized water were consumed *ad libitum*. Rats (six per group) were randomly assigned to the control group (C), low-dose group (L), or high-dose group (H), and gavaged with OTA at 0, 70 or 210 μg/kg bw, for 4 (BC, BL, BH groups) and 13 weeks (CC, CL, CH groups) respectively. OTA was dissolved in corn oil (Aladin, China). After the rats were sacrificed, the kidneys were frozen immediately in liquid nitrogen and kept at −80 °C for further studies.

2.4. Cell viability assay

Cell viability was assessed by WST-8 staining with the Cell Counting Kit-8 (CCK-8) (Beyotime, China) according to the manufacturer's instructions. 8000 cells were seeded in 96-well plates and treated with 0, 2.5, 5, 10, 15, 20, 25, 30 or 50 μM OTA. After incubation for 24 h, 10 μL WST-8 dye was added to each well, and the cells were incubated at 37 °C for 1 h. The absorbance was read at 450 nm using a Varioskan Flash microplate reader (Thermo Scientific, USA). For OCT2-KO cells, they were treated with 10, 20, 50 μM OTA with or without tetraethylammonium chloride (TEA, inhibitor of OCTs) for 24 h.

Table 1
Gene-specific primers used in quantitative real-time PCR.

Primer name	Sequence (5′to3′)	Reference
GADPH	Forward: ATGGGAAGCTGGTCATCAAC Reverse: GTGGTTCACACCCATCACAA	(Ryan et al., 2010)
OCT1	Forward: TGGCCGTAAGCTCTGTCTCT Reverse: TCAAGGTATAGCCGGACACC	(Schneider et al., 2011)
OCT2	Forward: GCCTCCTGATCCTGGCTG Reverse: GGTGTCAGGTTCTGAAGAGAG	(Thevenod et al., 2013)
OCT3	Forward: ATATCCTGTTTCGGCGTTGG Reverse: TTTCCAAACACCCCTTGCAG	(Nakanishi et al., 2011)

2.5. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was isolated from the kidney and NRK-52E cells using the modified TRIzol method (Simms et al., 1993). The integrity of the purified RNA was tested on a 1% agarose gel and quantified spectrophotometrically at 260 and 280 nm. First-strand cDNA was synthesized from 2 μg of RNA using oligo (dT) as the primers. The qRT-PCR reaction was performed using the RealMasterMix (SYBR green I) (Tiangen, China) in a Bio-Rad CFX96 Real-time PCR System (Bio-Rad, USA). The thermal cycling program was set as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Following the amplification, a melting curve analysis was conducted by heating from 65 °C to 95 °C with increments of 0.5 °C for 5 s to determine the specificity of the PCR reactions. The fluorescence signal was collected at the end of the elongation step for each cycle. The internal control for mRNA normalization was *GAPDH*. The primer sequences are shown in Table 1. Relative gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Six rats were used per group, and each reaction was performed in triplicates.

2.6. Western blotting

Following OTA treatment, NRK-52E cells were lysed on ice in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1.5 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and complete protease inhibitor cocktail (sodium orthovanadate, sodium fluoride, EDTA, leupeptin) (Beyotime, PRC) supplemented with 1 mM PMSF. Cells were then homogenized using a 1 mL syringe. For animals, 0.05 g of kidney tissue was ground into powder by liquid nitrogen. Samples were added RIPA lysis buffer and shaken for 15 min. They were then centrifuged at 13,000 g for 10 min at 4 °C, as described by Shen (Shen et al., 2013). The supernatant was collected and quantified using BCA Protein Assay Kit (Cwbiotech, PRC). Equal amounts of protein were resolved on a precast 12.5% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane at 80 V for 2 h. The membrane was then blocked in Tris-buffered saline containing 0.1% Tween 20 and 1% BSA (TBST). After incubating with primary and AP-labeled goat anti-rabbit or goat anti-mouse secondary antibodies (Beyotime, China), specific bands were detected using BCIP/NBT (Merck-Calbiochem). The primary antibodies were OCT1 (1:1000) (Santa Cruz Biotechnology, USA), OCT2 (1:1000) (Sigma, USA), OCT3 (1:200) (Santa Cruz Biotechnology, USA), Caspase 3 (1: 1000) (Cell Signaling Technology, USA), CDK1 (1:1000) (Abcam, UK), GAPDH (1:2000) (Abcam, UK). The relative intensity of each band was digitized using ImageJ 1.4.3.

2.7. Apoptosis assay

Cells were treated with 20 μM or 50 μM OTA, with or without TEA, for 24 h. Cells were exposed to TEA for 15 min first, followed by incubation with OTA and TEA. Cells were then collected by trypsinization