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Ochratoxin A Secretion in Primary Cultures of Rabbit Renal Proximal Tubule Cells

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Abstract. Primary cultures of rabbit renal proximal tubule cells grown under improved culture conditions were used to study the transepithelial transport of the nephrotoxic mycotoxin ochratoxin A. The basal-to-apical transepithelial flux, *i.e.*, secretion, of this fluorescence organic acid was measured in primary cultures of rabbit renal proximal tubule cells. The basal-to-apical flux of ochratoxin A increased with time and reached a steady state after 12 h. On the other hand, the apical-to-basal flux, *i.e.*, reabsorption, of ochratoxin A was minimal over time. The secretory flux of ochratoxin A was as much as eightfold greater than the reabsorptive flux, indicating that net secretion is the primary mechanism for ochratoxin A clearance by the proximal tubule. The kinetic analysis of ochratoxin A flux revealed secretion to be a saturable and very high-affinity process with an apparent K_{50} of 0.33 ± 0.21 mM. A saturating concentration of the prototypical organic anion substrate *para*-aminohippurate (PAH) reduced ochratoxin A secretion by approximately 75%. The kinetic analysis of PAH inhibition of ochratoxin A secretion revealed an IC_{50} of 195 mM, which is similar to the IC_{50} for PAH inhibition of peritubular ochratoxin A uptake in tubule suspensions and the K_m values for peritubular PAH uptake. The organic anions probenecid, octanoate, and α-ketoglutarate reduced ochratoxin A excretion to the same degree as PAH, whereas the amino acid phenylalanine had a minimal effect on ochratoxin A secretion. Thus, collectively, these observations indicate that the secretion of ochratoxin A in primary cultures of rabbit renal proximal tubules is limited to the organic anion secretory pathway. The high affinity measured for the basal-to-apical flux of ochratoxin A suggests that at concentrations typical of naturally occurring exposures, transepithelial secretion by the organic anion transport pathway represents a significant avenue for excretion of this mycotoxin by the renal proximal tubule.

Contamination of cereals and grains with *Aspergillus* and *Penicillium* fungi has resulted in the production of the mycotoxin ochratoxin A (OTA) (1,2). This contamination with OTA has been associated with the induction of Balkan nephropathy in humans and porcine nephropathy in domestic swine, as well as carcinogenesis (2–4). The proximal tubule of the kidney is a primary site targeted in OTA-induced nephrotoxicity (5). Secretion of organic anions is one mechanism used by the kidney to remove endogenous metabolites, drugs, and potentially harmful xenobiotics from the systemic circulation (6–8). This pathway has broad substrate specificity, and, hence, interacts with a wide variety of substrates. Although OTA also was shown to be a substrate for the organic anion transporter in opossum kidney (OK) cell cultures (9), apical transport pathways were found to have a markedly greater affinity for this mycotoxin than the basolateral PAH transporter. *In vivo* studies of OTA nephrotoxicity to rats showed that probenecid pretreatment potentiated OTA toxicity (10), which led to the proposal that renal reabsorption was more important in OTA pathogenesis. Thus, reabsorptive, rather than secretory, pathways are suggested to be more important in OTA accumulation and toxicity.

Recently, our laboratory demonstrated that the basolateral membrane organic anion transport pathway is involved in OTA accumulation by the renal cell (11). The affinity for peritubular OTA transport measured in suspensions of rabbit renal proximal tubules (RPT) is among the highest reported (7), and is substantially higher than the affinities for basolateral OTA transport measured in OK cells and canine renal basolateral membrane vesicles (9,12). However, organic anion transport in the renal proximal tubule cell is a secretory process, involving uptake across the basolateral membrane and exit across the apical membrane. In the canine kidney, OTA has been shown to interact with the organic anion transporter in both basolateral and brush border membrane vesicles (12), which suggests that OTA should be secreted. Although OTA uptake by the peritubular organic anion transport pathway in the intact rabbit RPT has been demonstrated (11,13), the role of organic anion secretion in the elimination of this mycotoxin by the rabbit RPT cell has not been clearly established.

The objectives of this study were to determine whether the nephrotoxic mycotoxin OTA is secreted by the RPT and to examine the role the organic anion transport system plays in OTA secretion using primary cultures of rabbit RPT cells. In

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contrast to standard tissue culture systems, these cultures are grown under improved culture conditions that result in the retention of more *in vivo*-like RPT cell oxidative metabolism, as reported previously by Nowak and Schnellmann (14–16). Our results show that organic anion secretion is the primary pathway involved in OTA elimination by primary cultures of rabbit RPT cells grown under these improved culture conditions and that renal reabsorption plays essentially a minimal role in OTA accumulation.

Materials and Methods

Animals and Materials

New Zealand White rabbits of either gender were purchased from Big D Rabbitry (Dade City, FL). OTA, probenecid, *para*-aminohippurate (PAH), octanoic acid (octanoate), piroxicam, DME-F12 culture media, transferrin, hormones, deferoxamine and L-lactic acid were purchased from Sigma Chemical Co. (St. Louis, MO). L-Ascorbic acid-2-phosphate magnesium salt (AscP) was obtained from Wako Bioproducts (Richmond, VA). Collagenase was purchased from Worthington Biochemical (Freehold, NJ). All other chemicals were purchased from standard sources as reported previously and were of the highest quality available (14–17).

Isolation of Proximal Tubules and Culture Conditions

Isolation and purification of rabbit RPT were based on the *in vitro* perfusion with iron oxide as described previously (18). Renal proximal tubules were plated on 24-mm (4.7-cm^2 surface area) Transwell-Clear polyester membranes inside a Transwell cell culture chamber (Costar, Cambridge, MA) at 1 mg protein/insert, and 1.5 and 2.5 ml of medium was added above and below the insert, respectively. Cultures were grown in a serum-free 50:50 mixture of Dulbecco's modified Eagle's essential medium and Ham's F-12 nutrient mix without phenol red, supplemented with 15 mM $NaHCO_3$, 15 mM Hepes, and 10 mM lactate (pH 7.4, 290 mosmol/kg H_2O). Human transferrin (5 mg/ml), selenium (5 ng/ml), hydrocortisone (50 nM), bovine insulin (10 nM), and L-AscP (1 mM) were added to the medium immediately before media change. Cells were cultured in a humidified incubator under a 95% air/5% CO_2 atmosphere at 37°C and were constantly swirled on an orbital shaker (80 to 85 rpm) to improve oxygenation of the media (14,15). Culture media were changed 48 h after seeding and every day from then on with serum/antibiotic-free culture medium. Renal proximal tubular cells grown to confluence (days 4 to 5*)* were used for studies of OTA transport. For transport studies, culture medium was removed and replaced with an incubation medium containing (in mM): 110 NaCl, 25 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 1.8 $CaCl_2$, 10 sodium acetate, 8.3 D-glucose, 5 alanine, 0.9 glycine, 1.5 lactate, 1 malate, and 1 sodium citrate (pH 7.4, 295 mosmol/kg). Because neither the transepithelial fluxes of OTA nor its fluorescence were affected by the presence of L-AscP, this agent was added to cultures during all transport measurements.

Measurement of Inulin Diffusion and Transepithelial Resistance

The movement of FITC-conjugated inulin (FL-I) diffusion across the monolayer was measured to evaluate monolayer permeability. Culture medium was removed, and incubation medium containing 100 mM (final concentration) FL-I was added to the basal or apical compartments and incubation medium alone was added to the opposite compartment. Cultures were incubated at 37°C as described above, and at timed intervals up to 4 h, aliquots (600 ml) were removed from both compartments and FL-I fluorescence (excitation nm = 490; emission nm = 520) was determined using a Hitachi F2000 fluorescence spectrophotometer (Naperville, IL). Some fluorescence measurements were performed using a Molecular Devices F_{max} fluorescence microplate reader (Sunnyvale, CA), which only required 100-ml sample aliquots to measure fluorescence. Differences in calculated inulin diffusion were minimal between these fluorometers.

Before use for permeability or transport studies, monolayer integrity was evaluated by measuring transepithelial electrical resistance with an EVOM epithelial voltohmmeter equipped with an Endohm tissue resistance measurement chamber electrode (World Precision Instruments, Sarasota, FL).

Measurement of Monolayer Viability

To determine monolayer viability, oxygen consumption by primary RPT cell cultures was measured by gently detaching confluent monolayers from the inserts with a cell scraper. Cells were suspended in culture medium, and oxygen consumption was measured with a Clark-type electrode as described previously (15). Final concentrations of nystatin were 0.5 mg/ml.

Measurement of Transepithelial OTA Transport

The transepithelial transport of OTA was studied in confluent cultures (days 4 to 5) as assessed by measurement of transepithelial resistance. For transport studies, 2.5 ml of an incubation medium containing OTA (5 mM) in the presence or absence of inhibitors of organic anion transport was added to the basolateral side or apical side, and OTA-free incubation buffer was added to the *trans*-compartment. Cultures were incubated in a humidified incubator under a 95% air/5% CO_2 atmosphere at 37°C and were constantly swirled on an orbital shaker (80 to 85 rpm). At timed intervals up to 4 h, 600-ml aliquots of the OTA-free incubation medium were removed from the *trans*-compartment and transferred to a Hitachi F2000 for measurement of OTA fluorescence (ex 375 nm, em 440 nm).

The kinetics of transepithelial OTA secretion was measured in cultures incubated with increasing concentrations of OTA from 0.1 to 10 mM. After 2 h, a 100-ml aliquot of medium was removed from the apical and basolateral compartments, and OTA fluorescence was measured using the F_{max} fluorescence microplate reader. Cultures were incubated for 2 h in the presence of 5 mM OTA and increasing concentrations of PAH (0 to 5000 mM) to examine the kinetics of inhibition of OTA secretion by this prototypical organic anion substrate. The fluorescence of OTA accumulated in the apical compartment was measured as described.

Calculations of OTA Clearance

To compare transepithelial organic anion transport and fluorescein-inulin diffusion, the transepithelial flux of these agents was expressed as clearance units. The clearance is defined as the volume of medium that is totally cleared of the test substrate at a time *t*, normalized to the surface area (4.7 cm^2) of the permeable membrane. The basal-to-apical and apical-to-basal transepithelial fluxes of Fl-I, OTA, fluorescein were calculated and expressed as clearance by the following equations:

$$C_{b-a} = \frac{(F_a)(V_a)}{F_b} \quad (1)$$

where C_{b-a} is the clearance from the basal-to-apical side, F_a are arbitrary fluorescence units/ml measured in the apical compartment, V_a is the volume of incubation medium, typically 1.5 ml, in the apical compartment, and F_b are arbitrary fluorescence units/ml measured in the basal compartment.

$$C_{a-b} = \frac{(F_b)(V_b)}{F_a} \quad (2)$$

where C_{a-b} is the clearance from the apical-to-basal side, F_b are arbitrary fluorescence units/ml measured in the basal compartment, V_b is the volume of incubation medium, typically 2.5 ml, in the basal compartment and F_a are arbitrary fluorescence units/ml measured in the apical compartment. The clearance data were normalized to the surface area of the Transwell culture membrane (4.7 cm^2), and the results were expressed as ml/cm^2. All data, except those data expressing the kinetics of OTA secretion and inhibition of OTA secretion by PAH, were corrected for paracellular leakage by subtracting inulin diffusion from total OTA transport measured in separate cultures at each examined time point.

Quantification of Fluorescence

To quantify OTA or fluorescein accumulation in the apical compartment for kinetic measurements, the concentration of OTA or fluorescein (not corrected for paracellular leakage of fluorescein-inulin) was calculated by comparing fluorescence intensity to a calibration curve generated when incubation buffer was spiked with different known concentrations of the fluorescence substrate. OTA and fluorescein fluorescence intensity were linearly correlated to OTA concentration over a concentration range up to 10 mM OTA and 50 mM fluorescein. Apical OTA or fluorescein accumulation is expressed as $nmol/cm^2$ per 2 h.

Statistical Analyses

Data are presented as mean ± SEM. Each preparation of primary cultures from a single rabbit represented a separate experiment. Data from three to five separate experiments were compared for statistical significance using ANOVA and a *post hoc* test with Fisher's protected least significant difference. $P < 0.05$ was considered significant.

Results

Oxygen Consumption and Monolayer Integrity/ Confluence

At days 4 to 5 of growth, when primary cultures had reached visible confluence as determined by phase contrast microscopy, transepithelial electrical resistance was measured to examine monolayer integrity. Intact monolayers, as viewed by phase contrast, displayed an average resistance of approximately 110 $\Omega \cdot cm^2$ before performing transport measurements.

The functional integrity and impermeability of the monolayers were examined by measuring cellular oxygen consumption and inulin diffusion. Cultures grown in DME-F12 medium supplemented with lactate, AscP, and shaking have been demonstrated to have improved physiologic functions such as oxygen consumption (14,15). The basal and nystatin-stimulated oxygen consumption by primary cultures grown on Transwell membranes is 23 ± 0.88 and 31 ± 2.0 nmol $O_2 \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively, which is similar to the 17 ± 0.6 and 25 ± 0.8 nmol $O_2 \cdot mg\ protein^{-1} \cdot min^{-1}$ measured in freshly isolated proximal tubule suspensions. The value for basal oxygen consumption in primary cultures also is similar to the values measured by Nowak and Schnellmann (14,15) for primary cultures of rabbit renal proximal tubule cells grown on impermeable membranes (plastic). The paracellular flux of Fl-I from the basal-to-apical compartments was minimal for as long as 6 h between days 4 and 8 of culture (Figure 2). The clearance of Fl-I was ≤2 and 3 $\mu l/cm^2$ after 1 and 6 h, respectively. The corresponding apical-to-basal ratios of Fl-I (as arbitrary fluorescence units/ml) were 0.01 after 1 and 6 h as well. Although not shown, the apical-to basolateral clearance of Fl-I was similar to that measured for basal-to-apical clearance as shown in Figure 1.

Transepithelial Flux of OTA in Primary Cultures

The flux of OTA from the basal to the apical compartments increased with time and was linear for 2 h (Figure 2). The basal-to-apical transepithelial flux of OTA continued to increase over 4 h (Figure 2) and reached a steady state after approximately 12 h (data not shown). The addition of 2 mM probenecid or 2.5 mM PAH reduced the transepithelial flux of OTA by approximately 80% after 2 and 4 h of incubation. The apical-to-basal transepithelial flux of OTA was minimal during the 4-h period and was similar to the basal-to-apical flux of OTA measured in the presence of probenecid and PAH (Figure 2). The organic anions probenecid and PAH also reduced the basal-to-apical transepithelial flux of the fluorescence organic anion fluorescein (a substrate for the PAH pathway; [30]) by approximately 80%, which was similar to the inhibition produced with OTA (Figure 3).

Kinetics of the Transepithelial Flux of OTA in Primary Cultures

To evaluate the kinetics of the basal-to-apical transepithelial flux OTA, the concentration-dependent basal-to-apical trans-

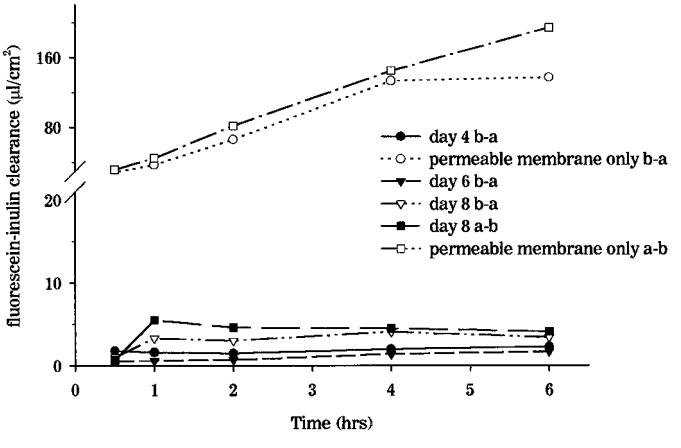

Figure 1. Basal-to-apical and apical-to-basal fluorescein-inulin flux by monolayers of primary cultures of rabbit renal proximal tubule (RPT) cells grown on Costar Transwell permeable membranes under improved culture conditions as described in Materials and Methods. The concentration of fluorescein-inulin added to the basal compartment was 100 mM. Values are the means ± SEM for a representative experiment.

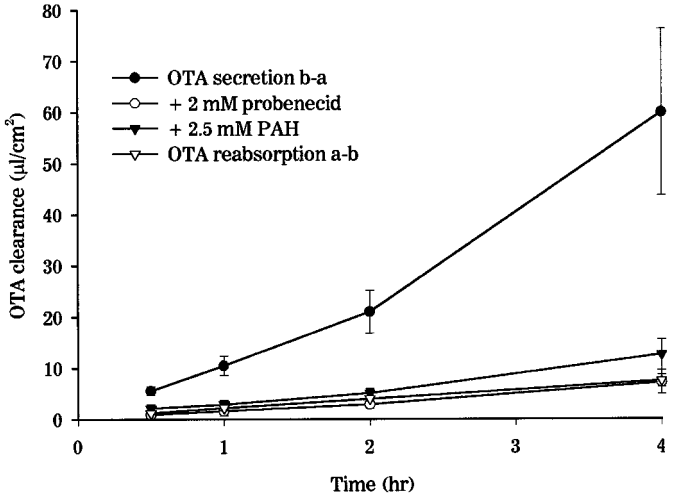

Figure 2. Time course of the transepithelial flux of ochratoxin A (OTA) in the presence and absence of *para*-aminohippurate (PAH) and probenecid by 4- to 5-d-old monolayers of primary cultures of rabbit RPT cells. Each clearance value was corrected for the paracellular diffusion of fluorescein-inulin. Data are expressed as mean ± SEM ($n = 3$).

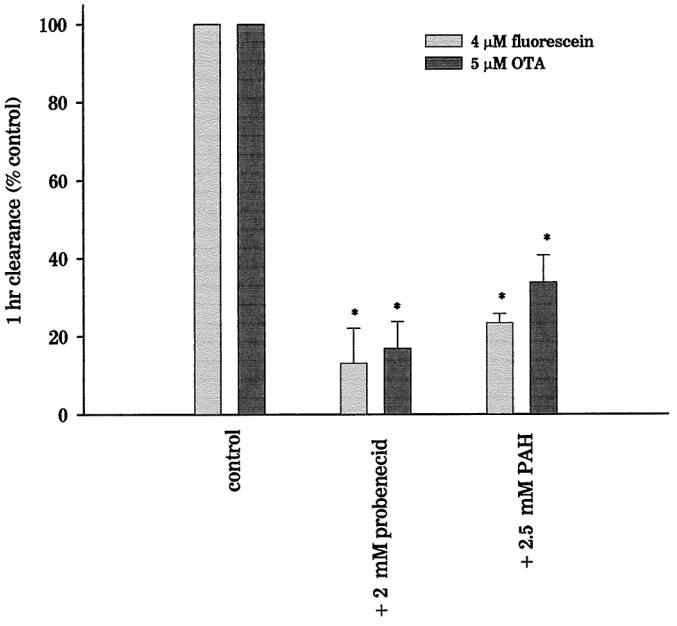

Figure 3. Cis-inhibition of fluorescein and OTA basal-to-apical flux after 1 h by probenecid and PAH in 4- to 5-d-old monolayers of primary cultures of rabbit RPT cells. Clearance values were corrected for the paracellular diffusion of fluorescein-inulin. Data are expressed as mean ± SEM ($n = 3$).

epithelial flux of OTA was measured in monolayers by adding increasing concentrations of OTA to the basal medium and measuring the fluorescence that accumulated in the apical compartment. Because transepithelial OTA flux was linear for more than 2 h, a 2-h incubation was selected for use in these studies to ensure sufficient flux of fluorescence material to provide an adequate signal at the lowest concentrations of substrate studied. Kinetic data are expressed as the total basal-to-apical flux of the measured substrate (OTA or fluorescein where appropriate, including diffusional and/or paracellular flux) expressed as nmol/cm^2. As shown in Figure 4, the basal-to-apical transepithelial flux of OTA was a saturable process that can be adequately described by an equation of the same form as that of Michaelis-Menten. This equation included a saturable term and a second, first-order term:

$$J = \frac{J_{max}[OTA]}{K_{50} + [OTA]} + D[OTA] \quad (3)$$

where J is the rate of OTA flux across the monolayer from an extracellular concentration of [*OTA*] in the basal medium. The kinetic parameter J_{max} is defined as the total capacity of the proximal tubule epithelium to effect a net transepithelial flux of OTA. The parameters K_{50} and D are defined, respectively, as the concentration of OTA in the basal medium at 1/2 J_{max}, and a coefficient describing the nonsaturable transepithelial flux of OTA (paracellular flux, passive diffusion, and/or nonspecific binding). The average values for the J_{max} and K_{50} generated from three separate experiments were 0.8 ± 0.004 nmol/cm^2 per 2 h and 0.33 ± 0.21 μM, respectively.

The kinetics of OTA basal-to-apical transepithelial flux were compared with the values measured for the fluorescence organic anion fluorescein (Figure 5), which is also a substrate for the organic anion/PAH transport pathway (19). The basal-to-apical transepithelial flux of fluorescein was also a saturable process adequately described by equation 3. From three separate experiments, the J_{max} and K_{50} for fluorescein flux generated were 1.0 ± 0.7 nmol/cm^2 per 2 h and 23.7 ± 11.9 μM, respectively.

Increasing concentrations of the organic anion substrate PAH progressively reduced the basal-to-apical transepithelial

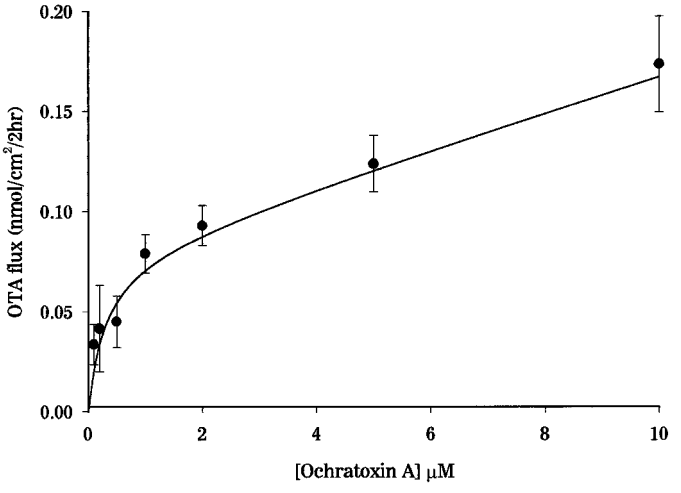

Figure 4. Concentration-dependent basal-to-apical flux of OTA by primary cultures of rabbit RPT cells. Monolayers were incubated for 2 h in the presence of increasing concentrations of OTA added to the basal compartment. Each point was corrected for the paracellular diffusion of fluorescein-inulin. Data are expressed as mean ± SEM for duplicate measurements from three separate kinetic experiments.

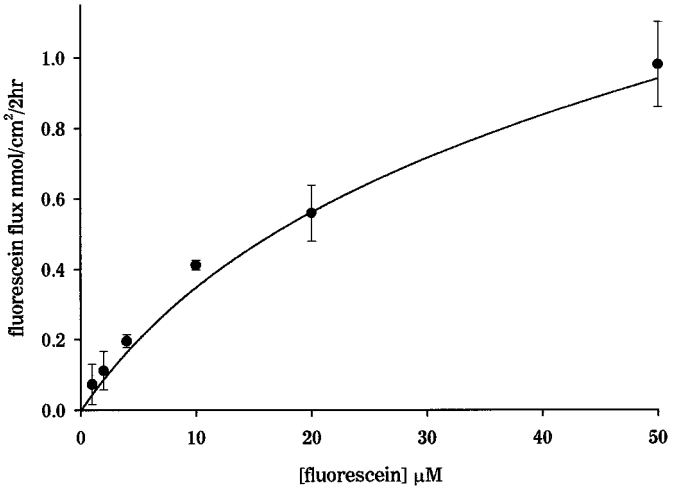

Figure 5. Concentration-dependent basal-to-apical flux of fluorescein by primary cultures of rabbit RPT cells. Monolayers were incubated for 2 h in the presence of increasing concentrations of fluorescein added to the basal compartment. Each point was corrected for the paracellular diffusion of fluorescein-inulin. Data are expressed as mean ± SEM for duplicate measurements from three separate kinetic experiments.

flux of OTA (Figure 6). The highest concentration of PAH, however, failed to completely block the transepithelial flux of OTA, which is consistent with the presence of some paracellular flux for OTA. The data represent the total basal-to-apical flux of the OTA (including diffusional and/or paracellular flux) expressed as arbitrary fluorescence units. This relationship can be adequately described by the kinetics of competitive inhibition, and results in an apparent inhibitor constant, which, at the OTA concentration used, is an overestimate of the true inhibitory interaction between these substrates (11). The IC_{50} generated for PAH inhibition of the transepithelial flux of OTA from this analysis was 195 ± 112 μM, a value similar to the K_t for peritubular PAH transport in intact proximal tubules, which is consistent with an interaction of PAH with OTA secretion at the peritubular membrane.

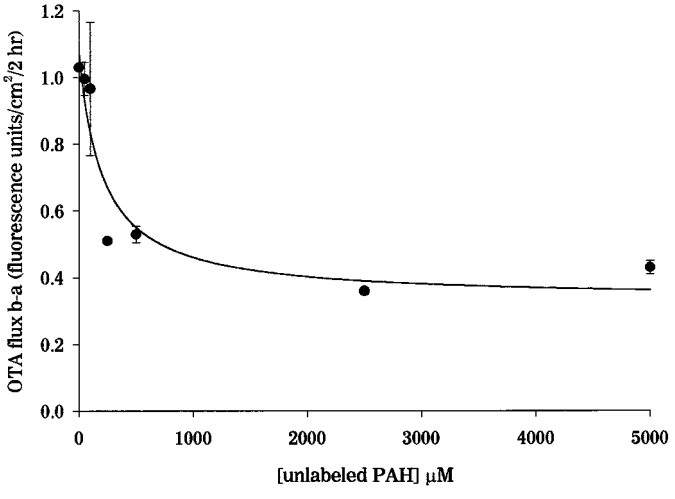

Figure 6. Cis-inhibition of the basal-to-apical flux of OTA by increasing concentrations of PAH in primary cultures of rabbit RPT cells. Monolayers were incubated for 2 h in the presence of 5 mM OTA with increasing concentrations of OTA added to the basal compartment. Each point was corrected for the paracellular diffusion of fluorescein-inulin. Data are expressed as mean ± SEM from a representative experiment.

Inhibition of OTA Transepithelial Flux

In freshly isolated rabbit RPT suspensions, the organic anions probenecid and octanoate produced approximately 50% more inhibition of the basolateral uptake of OTA compared with PAH, which suggested that OTA may be transported by a probenecid- and octanoate-sensitive, but PAH-insensitive pathway (11). In contrast to tubule suspensions, OTA transport across the basolateral membrane of single S2 segments from the rabbit kidney involved only the PAH-sensitive organic anion pathway (12). Consistent with the observation in single tubules, and as seen in Figure 4, both probenecid and PAH inhibit the basal-to-apical transepithelial flux of OTA to the same extent, which suggests that OTA is secreted solely by the organic anion transport pathway. In contrast, the neutral amino acid phenylalanine, which also has no effect on peritubular OTA uptake into tubule suspensions (11), had no significant effect on the basal-to-apical transepithelial flux of OTA (Figure 7). However, inhibition of the transepithelial flux of OTA in the presence of the organic anions α-ketoglutarate and octanoate was not different from that seen with probenecid and PAH (Figure 7). The net apical-to-basal OTA transepithelial flux was approximately 80% less than the net basal-to-apical flux (Figure 7), and the amino acid phenylalanine had no effect on the apical-to-basal flux of OTA (data not shown). Collectively, the inhibition of OTA by various organic anion substrates supports the observation that OTA secretion is mediated solely by the organic anion transporter.

Discussion

Organic anion secretion is one mechanism used by the kidney to remove potentially harmful xenobiotics from the systemic circulation (6). The nephrotoxic mycotoxin OTA has been shown to be a substrate for the organic anion transporter in various systems (9,11). However, apical pathways for transport were found to have a greater affinity for OTA in OK cell cultures (9). To this end, the transepithelial secretion of OTA in an *in vitro* model and a clear role for the organic anion pathway in this transepithelial secretion, *i.e.* basal-to-apical-flux, have been demonstrated using primary cultures of rabbit RPT cells.

Primary cultures of rabbit RPT cells grown on permeable membranes were used as the *in vitro* model to study transepithelial flux of OTA. Compared with cultures grown on noncoated Millicell HA membranes (20,21), primary cultures of rabbit RPT cells grown on collagen IV-coated membranes reached confluence after 5 d and maintained tight monolayers for up to 15 d in culture (22). However, in the current study, monolayers grown under improved culture conditions on noncoated Transwell membranes with 1 mM AscP added to the media reached confluence within 4 to 5 d, had improved

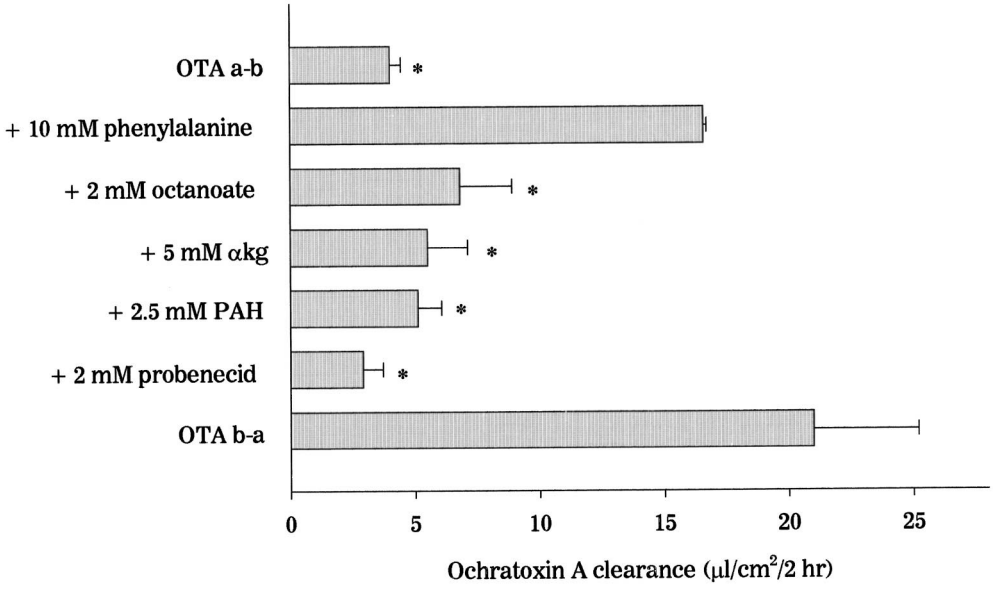

Figure 7. Cis-inhibition by organic anions of the basal-to-apical flux of OTA in monolayers of primary cultures of rabbit RPT cells. Monolayers were incubated for 2 h in the presence of 5 mM OTA with the organic anion added to the basal compartment. Each bar represents the mean ± SEM ($n = 3$ to 5). An asterisk indicates values significantly different from control.

aerobic metabolism, and maintained tight monolayers for up to 8 d as evidenced by the low paracellular flux of Fl-I. On the other hand, growth of cultures without L-AscP resulted in increased paracellular diffusion of OTA and a marked decrease in substrate inhibition of OTA fluxes (data not shown).

The apical-to-basal ratio of 0.01 for fluorescein-inulin diffusion across primary cultures after 6 h was significantly less than the ratio of 0.06 for inulin diffusion previously reported after 4 h in primary cultures of rabbit RPT cells grown on collagen type IV (22). Also, even in 10- to 12-d-old primary cultures of rabbit RPT cells grown under the presently described conditions, 5 mM PAH reduced the transepithelial flux of OTA after 2 h by approximately 70% (data not shown). Thus, these cultures maintain tight junctions and active transepithelial transport function for extended periods without the use of a collagen substrate. In contrast, Ford *et al.* (20) and Palmoski *et al.* (21) reported that primary cultures of rabbit RPT cells seeded at 4 mg/membrane, a density approximately fourfold higher than that used in this study, and grown on 30-mm Millicell-HA filters without collagen required 10 to 12 d to reach confluence. The basal-to-apical transepithelial flux of PAH in these cultures was reduced only 55% during a 20-min coincubation with probenecid (21). Thus, in contrast to previous studies, the cultures grown under the conditions described in the present study reached confluence more quickly and maintained carrier-mediated organic anion transport for extended periods.

The growth of primary cultures of rabbit RPT cells on membranes coated with collagen type IV was documented to improve their physiologic function (22) compared with cultures characterized by Ford *et al.* (20) and Palmoski *et al.* (21). However, the use of standard high glucose medium for cell growth and maintenance resulted in increased lactate production by the monolayers (22), whereas the culture conditions used in the present study have been well documented to stimulate more *in vivo*-like aerobic metabolism and lactate consumption, rather than production (14–16). Since RPT cells *in vivo* also consume lactate as a respiratory substrate (23), the improved culture model represents a system that appears to maintain a significantly higher level of physiologic function than previously shown. Thus, the current culture system provides a method for the study of the transepithelial transport of substrates in which physiologic function more closely resembles the *in vivo* RPT cell.

The transepithelial basal-to-apical flux of OTA was up to eightfold greater than the apical-to-basal flux, and is consistent with the net secretion of this mycotoxin by primary cultures of rabbit RPT cells. Thus, in contrast to OTA transport in OK cells where luminal reabsorptive pathways were suggested to be predominant in cellular accumulation (9), in rabbit RPT cells transepithelial secretion appears to be a predominant mechanism for OTA excretion. However, in the microperfused rat nephron, approximately 70% and 20% of the OTA infused appeared to be reabsorbed by the proximal and distal segments, respectively (24). The reabsorption of OTA in the rat nephron was mediated in part by the H^+-dipeptide cotransport and nonionic diffusion (24). Similar mechanisms were demonstrated to account for OTA reabsorption using the distal tubule cell lines MDCK-C7 and MDCK-C11 (25). The proximal tubule and distal tubule reabsorption of OTA by the rat kidney has been proposed to contribute to the long T1/2 for OTA *in vivo* (24). However, OTA reabsorption by primary cultures of rabbit RPT cells was minimal compared with secretion and, hence, does not appear to contribute significantly to OTA accumulation. The differences observed between these studies also might be due to species differences, model differences, or

differences in the experimental conditions. Notably, the lack of proximal tubule cell reabsorption of OTA in the rabbit kidney does not preclude the possibility of significant distal tubule reabsorption of OTA.

The secretion of OTA was substantially reduced by the prototypical organic anion substrate PAH. Interestingly, the IC_{50} value measured for PAH inhibition of OTA secretion in primary cultures was 195 μM and was similar to the measured K_t for basolateral PAH transport measured in suspensions of rabbit RPT (11) and isolated rabbit proximal tubule segments (19,26,27). The similarities in these values support the contention that the basolateral membrane PAH transport pathway mediates the entry step in the secretion of OTA. However, probenecid inhibition of OTA uptake across the basolateral membrane in suspensions of rabbit renal proximal tubules was approximately 40 to 50% greater than the inhibition produced by saturating concentrations of PAH. Thus, the basolateral transport of OTA appears to be mediated by both a PAH-sensitive as well as a PAH-insensitive pathway in this model (11). Similarly, the *in vivo* basolateral transport of OTA in the nonfiltering toad kidney involves two separate pathways, the organic anion and the neutral amino acid carrier (28). In contrast, basolateral transport of OTA in single rabbit renal S2 segments and in renal basolateral membrane vesicles from canine cortex is functionally limited to the organic anion transporter, since PAH and probenecid both completely inhibited OTA uptake (12,13). Consistent with these observations, probenecid or PAH addition to the basal compartment reduced OTA secretion in primary cultures of rabbit RPT cells by approximately 75 to 80%. The organic anions α-ketoglutarate and octanoate, which inhibit PAH and fluorescein transport across the basolateral membrane (19,29), also decreased OTA secretion by approximately 70 to 75%. In contrast, the amino acid phenylalanine, which significantly reduced the luminal uptake of OTA in OK cells (9) and reduced transepithelial OTA secretion in the nonfiltering toad kidney by 75% (28), had a minimal effect. Thus, the secretion of OTA by primary cultures of rabbit RPT cells also appears to be limited to the organic anion, *i.e.*, PAH secretory pathway.

Our previous work has demonstrated that peritubular OTA uptake, the first step in secretion, is a high-affinity process with a K_t of 1.4 μM (11,13). Consistent with this observation, OTA secretion was found to be a saturable process with an affinity of approximately 300 nM. The affinity measured for secretion was substantially lower than that measured for peritubular uptake in tubule suspensions and single S2 segments. This affinity for OTA secretion in primary cultures of rabbit RPT cells is similar to the affinity of 630 nM measured *in situ* for OTA secretion in the nonfiltering toad kidney (28). On the other hand, the affinity for secretion of the fluorescence organic anion fluorescein in primary cultures, although a high-affinity process with a K_{50} of 23 μM, still is substantially lower than that measured for OTA. Similarly, the affinity for PAH secretion by OK cell cultures is as much as 400-fold lower than for OTA (30). Thus, OTA appears to be the highest-affinity substrate handled by the organic anion secretory pathway.

The approximately 99% plasma protein binding for OTA (31,32), which is greater than the 80% plasma protein binding for fluorescein (33), coupled with the reported proximal and distal tubule reabsorption of OTA (24), may account for the prolonged excretion of OTA in the rat kidney. However, the current study demonstrates that in the absence of plasma protein binding, the *in vitro* removal of OTA from the basolateral compartment in primary cultures of rabbit RPT cells appeared to be greater than fluorescein due to the significantly higher affinity of the organic anion transporter for OTA transport.

In summary, the net secretion of OTA by the PAH pathway is the predominant excretory mechanism for this mycotoxin by rabbit RPT cells in primary culture. Thus, from the low micromolar and nanomolar concentrations representative of renal exposures to xenobiotic agents, the secretion of OTA represents a substantial avenue for removal of this mycotoxin from the systemic circulation.

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References

1. Pavlovic M, Plestina R, Krogh P: Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. *Acta Pathol Scand* 87: 243–249, 1979
2. Tapia OO, Seawright AA: Experimental ochratoxicosis A in pigs. *Aust Vet J* 61: 219–222, 1984
3. Krogh P, Hasselager E: Studies on fungal nephrotoxicity. *R Vet Agric Col Yearbook* 198–214, 1968
4. Kuiper-Goodman T, Scott PM: Risk assessment of the mycotoxin ochratoxin A. *Biomed Environ Sci* 2: 179–248, 1989
5. Suzuki S, Kozuka Y, Satoh T, Yamazaki M: Studies on the nephrotoxicity of ochratoxin A in rats. *Toxicol Appl Pharmacol* 34: 479–490, 1975
6. Pritchard JB, Miller DS: Mechanisms mediating renal secretion of organic anions and cations. *Physiol Rev* 73: 765–796, 1993
7. Ullrich KJ: Specificity of transporters for "organic anions" and "organic cations" in the kidney. *Biochim Biophys Acta* 1197: 45–62, 1994
8. Ullrich KJ: Renal transporters for organic anions and cations: Structural requirements for substrates. *J Membr Biol* 158: 95–107, 1997
9. Gekle M, Silbernagl S, Mildenberger S, Freudinger R: Effect on dome formation and uptake of ochratoxin A in proximal tubule-derived opossum kidney cell monolayers. *Cell Physiol Biochem* 3: 68–77, 1993
10. Stein AF, Phillips TD, Kubena LF, Harvey RB: Renal tubular secretion and reabsorption as factors in ochratoxicosis: Effects of probenecid on nephrotoxicity. *J Toxicol Environ Health* 16: 593–605, 1985
11. Groves CE, Morales MN, Wright SH: Peritubular transport of ochratoxin A in rabbit renal proximal tubules. *J Pharmacol Exp Ther* 284: 943–948, 1998
12. Sokol PP, Ripich G, Holohan PD, Ross CR: Mechanism of ochratoxin A transport in kidney. *J Pharmacol Exp Ther* 246: 460–465, 1988
13. Welborn JR, Groves CE, Wright SH: Peritubular transport of ochratoxin A by single rabbit renal proximal tubules. *J Am Soc Nephrol* 9: 1973–1982, 1998

14. Nowak G, Schnellmann RG: Improved culture conditions stimulate gluconeogenesis in primary cultures of renal proximal tubule cells. *Am J Physiol* 268: C1053–C1061, 1995
15. Nowak G, Schnellmann RG: L-Ascorbic acid regulates growth and metabolism of renal cells: Improvements in cell culture. *Am J Physiol* 271: C2072–C2080, 1996
16. Nowak G, Schnellmann RG: Autocrine production and TGF-β1-mediated effects on metabolism and viability in renal cells. *Am J Physiol* 271: F689–F697, 1996
17. Groves CE, Evans KK, Dantzler WH, Wright SH: Peritubular organic cation transport in isolated rabbit proximal tubules. *Am J Physiol* 266: F450–F458, 1994
18. Rodeheaver DP, Aleo MD, Schnellmann RG: Differences in enzymatic and mechanical isolated rabbit renal proximal tubules: Comparison in long-term incubation. *In Vitro Cell Dev Biol* 26: 898–904, 1990
19. Sullivan LP, Grantham JJ: Specificity of basolateral organic anion exchanger in proximal tubule for cellular and extracellular solutes. *J Am Soc Nephrol* 2: 1192–1200, 1992
20. Ford SM, Williams PD, Grassl S, Holohan PD: Transepithelial acidification by cultures of rabbit proximal tubules grown on filters. *Am J Physiol* 259: C103–C109, 1990
21. Palmoski MJ, Masters BA, Flint OP, Ford SM, Oleson FB: Characterization of rabbit primary proximal tubule kidney cell cultures grown on Millicel-HA membrane filters. *Toxicol In Vitro* 6: 557–567, 1992
22. Genestie I, Morin JP, Vannier B, Lorenzon G: Polarity and transport properties of rabbit kidney proximal tubule cells on collagen IV-coated porous membranes. *Am J Physiol* 269: F22–F30, 1995
23. Krebs HA, Bennett DAH, DeGasquet P, Gascuyne T, Yoshida T: Renal gluconeogenesis. *Biochem J* 86: 22–27, 1963
24. Zingerle M, Silbernagl S, Gekle M: Reabsorption of the nephrotoxin ochratoxin A along the rat nephron *in vivo*. *J Pharmacol Exp Ther* 280: 220–224, 1997
25. Schwerdt G, Gekle M, Freudinger R, Mildenberger S, Silbernagl S: Apical-to-basolateral transport of ochratoxin A by two subtypes of Madin-Darby canine kidney cells. *Biochim Biophys Acta* 1324: 191–199, 1997
26. Dantzler WH, Evans KK, Wright SH: Kinetics of interactions of para-aminohippurate, probenecid, cysteine conjugates and *N*-acetyl cysteine conjugates with basolateral organic anion transporter in isolated rabbit renal proximal tubules. *J Pharmacol Exp Ther* 272: 663–672, 1995
27. Shimomura A, Chonko AM, Grantham JJ: Basis for heterogeneity of *para*-aminohippurate secretion in rabbit proximal tubules. *Am J Physiol* 240: F430–F436, 1981
28. Bahnemann E, Kerling HP, Ensminger S, Schwerdt G, Silbernagl S, Gekle M: Renal transepithelial secretion of ochratoxin A in the non-filtering toad kidney. *Toxicology* 120: 11–17, 1997
29. Chatsudthipong V, Dantzler WH: PAH/α-KG countertransport stimulates PAH uptake and net secretion in isolated rabbit renal tubules. *Am J Physiol* 263: F384–F391, 1992
30. Hori R, Okamura M, Takayama A, Hirozane K, Takano M: Transport of organic anion in the OK kidney epithelial cell line. *Am J Physiol* 264: F975–F980, 1993
31. Chu FS: Interaction of ochratoxin A with bovine serum albumin. *Arch Biochem Biophys* 147: 359–366, 1971
32. Chu FS: A comparative study of the interaction of ochratoxins with bovine serum albumin. *Biochem Pharmacol* 23: 1105–1113, 1974
33. Ammer U, Natochin Y, Ullrich KJ: Tissue concentration and urinary excretion pattern of sulfofluorescein by the rat kidney. *J Am Soc Nephrol* 3: 1474–1487, 1993