Peritubular Transport of Ochratoxin A in Rabbit Renal Proximal Tubules

CARLOTTA E. GROVES, MARK MORALES and STEPHEN H. WRIGHT
Department of Physiological Sciences (C.E.G., M.M.), College of Veterinary Medicine, University of Florida, Gainesville, Florida; and Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona
Accepted for publication November 7, 1997 This paper is available online at http://www.jpet.org

ABSTRACT
The transport of the nephrotoxic mycotoxin ochratoxin A across the renal peritubular membrane was examined in suspensions of rabbit renal proximal tubules. Ochratoxin A transport across the peritubular membrane was a high-affinity, low-capacity carrier-mediated process with a $J_{\text{max}}$ value of 0.12 ± 0.4 nmol/mg of protein/min and a $K_{\text{m}}$ value of 1.4 ± 0.1 μM. The apparent Michaelis constants for inhibition of [3H]para-aminomhippurate (PAH) uptake by ochratoxin A inhibition was 1.5 μM, which is similar to the $K_{\text{m}}$ value for ochratoxin A uptake in tubule suspensions and suggests that ochratoxin A could be a substrate for the organic anion pathway. The capacity and affinity for peritubular ochratoxin A transport were 40-fold lower and >100-fold greater, respectively, than those measured for the peritubular uptake of [3H]PAH in tubule suspensions. A concentration of 2.5 mM PAH, which reduced the uptake of [3H]PAH by 90%, reduced ochratoxin A uptake by only 40% to 50%, whereas probenecid concentrations of 0.6 to 2 mM reduced ochratoxin A accumulation in tubule suspensions up to ~80% to 90%. This probenecid-sensitive, PAH-insensitive uptake of ochratoxin A suggested that at least one mediated pathway other than the organic anion transporter was involved in the peritubular uptake of this mycotoxin. A 2 mM concentration of the fatty acid octanoate and 1.5 mM concentration of the nonsteroidal anti-inflammatory agent piroxicam were as effective as probenecid in blocking ochratoxin A uptake. The apparent $K_{\text{m}}$ values for inhibition of ochratoxin A uptake by probenecid, piroxicam and octanoate were 30.5 ± 7.9, 23.2 ± 10.4 and 81.5 ± 8.7 μM, respectively. The ability of octanoic acid to inhibit ochratoxin A transport to the same extent as probenecid and a greater extent than PAH suggests that a separate fatty acid transport pathway may be involved in the accumulation of ochratoxin A by suspensions of rabbit renal proximal tubules.

Balkan nephropathy in humans and domestic animals has been associated with the ingestion of cereals such as corn, wheat, barley and sorghum contaminated with the mycotoxin ochratoxin A, a metabolite of Penicillium and Aspergillus fungi (Pavlovic et al., 1979; Tapia and Seawright, 1984). The proximal tubule is a primary intrarenal target for the nephrotoxicity produced by ochratoxin A. Hence, the accumulation of ochratoxin A by proximal tubule cells may be involved in the nephrotoxicity produced by this mycotoxin. The accumulation of PAH by rat renal slices was impaired in rats pretreated with ochratoxin A in vivo (Suzuki et al., 1975). This inhibition of PAH uptake by ochratoxin A was shown to be noncompetitive in nature, which suggests that the organic anion transport pathway may not be responsible for ochratoxin A accumulation into renal cells (Suzuki et al., 1975). However, the organic anion transport inhibitor probenecid decreased the in vivo renal clearance of ochratoxin A in both sham and partially nephrectomized rats, which suggests an interaction of ochratoxin A with the organic anion transport system (Stein et al., 1985). Furthermore, the transport of [3H]PAH by rabbit renal BLMV and brush border membrane vesicles is cis-inhibited and trans-stimulated by ochratoxin A (Sokol et al., 1988), observations that led to the conclusion that ochratoxin A is a substrate for the organic anion transport system. Thus, the uptake and accumulation of ochratoxin A by the organic anion transport system of proximal tubule cells may play a role in ochratoxin A toxicity.

In the aforementioned studies, researchers examined the effect of ochratoxin A on the uptake of PAH rather than the transport of ochratoxin A itself. Ochratoxin A has been shown to act as a competitive inhibitor of the enzymes L-phenylalanine hydroxylase and L-phenylalanine-tRNA synthetase (Creppy et al., 1983; Moroi et al., 1985). Indeed, ochratoxin A accumulation across the brush border membrane of cultured OK cells was blocked by phenylalanine (Gekle et al., 1993). Thus, ochratoxin A could share one or more phenylalanine (amino acid) carriers and use these as avenues for entrance into proximal cells.

The objective of the present study was to characterize directly the pathway or pathways by which ochratoxin A enters proximal renal cells across the peritubular membrane. Because ochratoxin A is a highly fluorescent molecule, the...
accumulation of fluorescence can be used as a method to directly measure the kinetic characteristics of ochratoxin A transport (Gekle et al., 1993). Using a fluorometric assay to monitor accumulation of ochratoxin A into suspensions of rabbit renal proximal tubules, we determined that peritubular transport of ochratoxin A does involve, but may not be limited to, the organic anion transport pathway.

**Methods**

**Materials.** Ochratoxin A, probenecid, PAH, octanoic acid and piroxicam were purchased from Sigma Chemical (St. Louis, MO). [3H]PAH was purchased from DuPont-New England Nuclear (Boston, MA). All other chemicals were purchased from standard sources as reported previously (Groves et al., 1994).

**Isolation of tubule suspensions.** Suspensions of rabbit renal proximal tubules were isolated and purified from New England White rabbits (1.3–1.5 kg; Myrtle's Rabbity, Thompson, TN) by either an enzymatic (collagenase) procedure that is based on the method of Vinay et al. (1981) as modified by Groves et al. (1994) or a mechanical/enzymatic separation based on the method of Brendel and Meezan (1975) as modified by Rodeheaver et al. (1990) and Groves and Schnellmann (1996). The final tubule pellet was resuspended at a protein concentration of 1 mg/ml in an incubation medium with increasing concentrations of ochratoxin A. Therefore, a 0.5-ml aliquot of tubule suspension was then transferred to a 15-ml tube containing 0.5 ml of incubation medium with 25 nM [3H]PAH and varying concentrations of unlabeled PAH, ochratoxin A or probenecid. After 1 min, 5 ml of ice-cold incubation medium was added to stop uptake, and the tubules were pelleted. The rinse was repeated, the final pellet was dissolved in 1 N NaOH and aliquots were taken for counting of radioactivity.

**Statistics.** Data are presented as mean ± S.E.M. Each preparation of tubules from a single rabbit represented a separate experiment. Data from three or four separate experiments were compared for statistical significance using analysis of variance and a post hoc test with Bonferroni’s correction and a value of P < .05 taken as significant.

**Results**

**Time-dependent uptake of ochratoxin A in tubule suspensions.** The accumulation of 10 μM ochratoxin A was approximately linear for 60 sec (fig. 1). Extrapolation of the relationship between time and uptake revealed a positive intercept. The presence of a positive intercept suggested that at least a fraction of apparent ochratoxin A accumulation involved either rapid binding or a small amount of “carry over” of ochratoxin A in the incubation buffer that was not rinsed away during the rinsing procedure used in our transport assay. However, the component of ochratoxin A uptake that increased progressively over the first 60 sec of incubation was completely blocked by the presence of 2 mM probenecid.

**Quantification of ochratoxin A fluorescence.** To quantify ochratoxin A uptake, the intracellular concentration of ochratoxin A was calculated by comparing fluorescence intensity with a calibration curve generated from blank control tubule pellets (0.5 mg of lysed tubule protein; no previous ochratoxin A exposure) spiked with different concentrations of ochratoxin A. Ochratoxin A fluorescence intensity was linearly correlated to ochratoxin A concentration over a range of 1 to 70 nM ochratoxin A. Ochratoxin A fluorescence also is linear through concentrations at least as high as 25 μM. The intracellular ochratoxin A concentrations, calculated from the calibration curve, were normalized to tubule protein (pmol/mg of protein).

The initial rate of ochratoxin A transport into rabbit renal proximal tubule suspensions in the presence and absence of 2 mM probenecid. The concentration of ochratoxin A was 10 μM. Each point is the mean ± S.E.M. of triplicate measurements from a representative experiment.

![Fig. 1. Initial rate of ochratoxin A transport into rabbit renal proximal tubule suspensions in the presence and absence of 2 mM probenecid. The concentration of ochratoxin A was 10 μM. Each point is the mean ± S.E.M. of triplicate measurements from a representative experiment.](image-url)
neicid, suggesting that time-dependent accumulation of ochratoxin A involved carrier-mediated transport. Accumulation of ochratoxin A approached a steady state within 5 min, and this uptake was also effectively inhibited by the presence of 2 mM probenecid and incubation at 1°C (fig. 2), further supporting the conclusion that peritubular uptake of ochratoxin A was dominated by activity of a carrier-mediated process.

**Kinetics of ochratoxin A uptake in tubule suspensions.** The kinetics of peritubular ochratoxin A uptake were examined to evaluate the physiological characteristics of the transport of this mycotoxin. A 2-min incubation was selected for use in these studies to ensure sufficient accumulation of fluorescent material to provide an adequate signal at the lowest concentrations of substrate studied. The relationship between increasing ochratoxin A concentration and the rate of peritubular ochratoxin A uptake into proximal tubules (fig. 3) was adequately described by an equation that included a saturable (Michaelis-Menten) term and a second, first-order term:

\[
J = \frac{J_{\text{max}} [\text{ochratoxin A}]}{K_m + [\text{ochratoxin A}]} + D[\text{ochratoxin A}]
\]  
(1)

where \( J \) is the rate of ochratoxin A uptake into tubule suspensions from an extracellular concentration of [ochratoxin A], and the kinetic parameters \( J_{\text{max}}, K_m \) and \( D \) are the maximal rate of ochratoxin A transport, the concentration of ochratoxin A at \( \frac{J_{\text{max}}}{2} \) and a coefficient describing the non-saturable accumulation of ochratoxin A (passive diffusion, nonspecific binding and/or experimental carry-over), respectively. The average values for \( J_{\text{max}} \) and \( K_m \) generated from three separate experiments were 0.12 ± 0.04 nmol/mg of protein/min and 1.4 ± 0.1 \( \mu \)M, respectively.

**Inhibition of tubular ochratoxin A uptake by various organic anion inhibitors.** The inhibition of ochratoxin A transport by probenecid suggested that peritubular ochratoxin A uptake involved the organic anion transport pathway. The specificity of probenecid for the classic organic anion transporter has, however, come under question in recent years (e.g., Hsyu et al., 1988). Therefore, we compared the kinetics of inhibition of ochratoxin A transport produced by probenecid with that produced by PAH, the prototypic substrate for the peritubular organic anion transporter (see Ullrich, 1994; referred to hereafter as the PAH transporter). Increasing concentrations of probenecid did produce what appeared to be a monotonic inhibition of ochratoxin A transport, with the highest concentration tested blocking \( \approx 80\% \) of total tubular accumulation of ochratoxin A. Increasing concentrations of PAH also inhibited ochratoxin A uptake, but 50% to 60% of the ochratoxin A accumulation that was blocked by probenecid was not blocked by PAH (fig. 4). The inhibition of ochratoxin A transport produced by both compounds was adequately described by the kinetics of competitive inhibition:

\[
J = \frac{J_{\text{max}}[\text{ochratoxin A}]}{K_m \left( 1 + \frac{[\text{I}]}{K_i} \right) + [\text{ochratoxin A}]} + D[\text{ochratoxin A}]
\]  
(2)

where [ochratoxin A], \( J_{\text{max}}, D \) and \( K_i \) are as previously defined; [I] is the concentration of inhibitor and \( K_i \) is the Michaelis constant of the inhibitor. The data presented in figure 4 resulted in calculation of \( K_i \) values of 149.1 ± 79.9 and 30.5 ± 7.9 \( \mu \)M for PAH and probenecid, respectively, using the analytical method described by Malo and Berteloot (1991) as modified by Groves et al. (1994). As seen in figure 5, probenecid was an equally potent inhibitor of \( ^{3} \text{H} \)PAH uptake.

**Inhibition of PAH uptake in tubule suspensions.** In contrast to the effect on ochratoxin A uptake, saturating concentrations of unlabeled PAH inhibited the peritubular uptake of \( ^{3} \text{H} \)PAH to the same extent as probenecid. As shown in figures 5 and 6, both PAH and probenecid at concentrations of 2.5 and 0.6 mM, respectively, reduced the uptake of \( ^{3} \text{H} \)PAH by \( \approx 90\% \). Increasing concentrations of unlabeled PAH and probenecid progressively reduced the uptake of \( ^{3} \text{H} \)PAH (fig. 5). The highest concentrations of these substrates failed to completely block the uptake of \( ^{3} \text{H} \)PAH, which is consistent with the presence of passive

---

Fig. 2. Time-dependent accumulation of ochratoxin A (5 \( \mu \)M) into rabbit renal proximal tubule suspensions at 37°C in the absence and presence of 2 mM probenecid and in an ice bath (\( \approx 1°C \) average temperature). Each point is the mean \( \pm \) S.E.M. of triplicate measurements from three or four separate tubule preparations.

Fig. 3. Effect of increasing ochratoxin A concentration on the rate of ochratoxin A uptake into rabbit renal proximal tubule suspensions. Initial rates were estimated from 2-min incubations. Each point is the mean \( \pm \) S.E.M. of triplicate measurements from three separate tubule preparations. Line fit to data was calculated from Eq. 1, and kinetic parameters were derived using a nonlinear regression algorithm (Enzfit, Biosoft).
diffusion and/or nonspecific binding. Inhibition of \[^{3}H\]PAH uptake by unlabeled PAH was described with the kinetics of competitive inhibition using the isotope dilution procedure introduced by Malo and Berteloot (1991) and as described by Groves et al. (1995). This procedure can be further modified as previously described by Groves et al. (1995) to determine the apparent \(K_i\) value for probenecid inhibition of \[^{3}H\]PAH uptake. The data presented in figure 5 resulted in calculation of \(K_m\) and \(K_i\) values of 165 ± 28 and 35 ± 7.9 \(\mu M\) for peritubular PAH uptake and probenecid inhibition of PAH uptake, respectively, using the analytical method described above. The \(K_i\) value of 149 \(\mu M\) for PAH inhibition of ochratoxin A uptake is similar to the measured \(K_m\) value of 165 \(\mu M\) for peritubular PAH transport, supporting the conclusion that the interaction between PAH and ochratoxin A represents competition for a common transport pathway (i.e., the PAH transporter). Further supporting the conclusion that ochratoxin A and PAH compete for a common pathway was the observation that ochratoxin A inhibited peritubular uptake of \[^{3}H\]PAH with an apparent \(K_i\) value of 1.5 \(\mu M\) (fig. 5, inset), matching closely the apparent \(K_m\) value of 1.4 \(\mu M\) for ochratoxin A accumulation noted above (fig. 3).

As shown in figure 4, probenecid produced the maximal inhibition of ochratoxin A uptake, whereas PAH blocked 40% to 50% of mediated uptake. Although 2.5 mM PAH reduced ochratoxin A uptake by only 50%, this same concentration of unlabeled PAH reduced the uptake of \[^{3}H\]PAH by 90% (fig. 6). However, probenecid at a concentration as low as 0.6 mM produced a maximal inhibition of both \[^{3}H\]PAH and ochratoxin A uptake (fig. 6). In the light of the inhibitory effectiveness of probenecid, the observation that PAH appeared to block only 40% to 50% of the mediated transport of ochratoxin A was surprising (fig. 4). This observation suggested that ochratoxin A uptake into proximal cells may involve interaction with at least one other mediated pathway in addition to the organic anion (i.e., PAH) transporter. In an effort to determine the identity of a second pathway by which ochratoxin A enters proximal renal cells, we examined the inhibitory effect on ochratoxin A uptake of a battery of other compounds (fig. 7). Lactate and phenylalanine, at concentrations of 10 mM, and urate at a concentration of 2.5 mM exerted no significant inhibition of ochratoxin A transport. In contrast, a 1.5 mM concentration of piroxicam, a nonsteroidal anti-inflammatory agent, and the fatty acid octanoate at a concentration of 2 mM were as effective as 2 mM probenecid at blocking ochratoxin A uptake. Figure 8 shows the kinetic profile of the inhibition of ochratoxin A transport caused by piroxicam and octanoic acid. The apparent \(K_i\) values for inhibition of ochratoxin A transport for piroxicam and octanoic acid were 23.2 ± 10.4 and 81.5 ± 8.7 \(\mu M\), respectively.
Discussion

The ingestion of feed contaminated with the mycotoxin ochratoxin A has been reported to produce nephrotoxicity in humans and domestic animals (Krogh and Hasselager, 1968; Tapia and Seawright, 1984; Kuiper-Goodman and Scott, 1989). Treatment of proximal tubule suspensions isolated from rat and rabbit kidneys with this mycotoxin also results in tubular cell injury and death (Aleo et al., 1991; Rodeheaver and Schnellmann, 1993). Thus, the ability of renal cells to accumulate this nephrotoxin may be paramount to the production of toxicity. Ochratoxin A has been shown to trans-stimulate and competitively inhibit PAH transport in BLMV isolated from rabbit renal cortex (Sokol et al., 1988), leading to the suggestion that this mycotoxin is a substrate for the organic anion pathway. Actual transport of ochratoxin A was not, however, examined. The present results showed that suspensions of rabbit renal proximal tubules (which have collapsed lumens, limiting transport to the peritubular membrane) (Groves and Wright, 1995) accumulated ochratoxin A. This uptake increased with time and was reduced by >80% by probenecid, as well as by ice-cold temperatures, observations that support the conclusion that peritubular accumulation of ochratoxin A involves carrier-mediated transport.

Although the maximal capacity for ochratoxin A transport in tubule suspensions ($J_{max}$) of ochratoxin A = 0.12 nmol/mg/min) was ~40-fold lower than that measured for PAH transport in tubule suspensions ($J_{max}$ of PAH = 5.3 nmol/mg/min), the $K_m$ value for ochratoxin A uptake of 1.4 mM in tubule suspensions suggests that the affinity of the peritubular transport pathway for ochratoxin A is >100-fold greater than that for PAH transport (i.e., $K_m$ = 165 mM). Thus, despite the very low capacity for transport, the very high affinity for ochratoxin A transport makes the peritubular transport of this toxicant a significant avenue for entrance into proximal cells. In contrast to tubule suspensions, an IC$_{50}$ value of 32 mM for ochratoxin A inhibition of PAH transport was measured in rabbit renal cortical BLMV (Sokol et al., 1988). This IC$_{50}$ value in BLMV was similar to the $K_m$ value for peritubular ochratoxin A transport of 27 mM measured in OK cell cultures (Gekle et al., 1993). The basis for the difference in the apparent affinity of the peritubular membrane-mediated pathway or pathways for ochratoxin A uptake in these different preparations is not clear. Some evidence suggests that peritubular PAH transport can be an electrogenic process (Makhuli et al., 1995), involving the mediated exchange of an exchangeable dicarboxylic acid (e.g., a-ketoglutarate; see Pritchard and Miller, 1993). However, the kinetic studies of the interaction of ochratoxin A with PAH in BLMV, performed in the absence of a source of exchangeable substrate and of the inside-negative electrical potential differences present in the intact cell, likely may misrepresent the quantitative interaction of these substrates occurring in intact cells. Similarly, the characteristics of peritubular ochratoxin A transport in cultured renal cells may also be expected to be both quantitatively and qualitatively different from those found in the native proximal tubule. Indeed, in OK cells, luminal reabsorption of ochratoxin A was proposed to be a more important route for the accumulation of this nephrotoxin by OK cell cultures, because the affinity for luminal, ochratoxin A transport was markedly greater than that of the peritubular side in this model (Gekle et al., 1993). The present set of observations suggests that peritubular transport of ochratoxin A into intact rabbit renal tubules possesses characteristics that make this membrane a significant site for tubular accumulation of this toxicant.

Accumulation by the PAH transport pathway was the sole pathway identified to play a role in the peritubular uptake of ochratoxin A by OK cell cultures and rabbit renal BLMV (Sokol et al., 1988; Gekle et al., 1993). However, the profiles of inhibitory interactions for ochratoxin A transport in tubule suspensions indicated that more than one pathway could be involved in its accumulation across the peritubular membrane of tubule suspensions. Although probenecid inhibited ~90% of total peritubular ochratoxin A accumulation, a concentration of PAH, which blocked the uptake of $^3$HPAH by 90% and should have blocked a similar fraction of the ochratoxin A accessing the organic anion transporter, reduced ochratoxin A uptake by only 40% to 50%. Thus, a component...
of peritubular ochratoxin A uptake by suspended rabbit renal proximal tubules appeared to be sensitive to probenecid inhibition but insensitive to inhibition by PAH. In an attempt to identify an interaction between ochratoxin A and other transport pathways, we examined the effect of various organic ions on peritubular ochratoxin A transport. Ochratoxin A is a structural analog of phenylalanine and interaction of organic ions on peritubular ochratoxin A transport. Ochratoxin A might have accessed a phenylalanine transporter as the second pathway for uptake across the peritubular membrane. However, a 10 mM concentration of phenylalanine and 2.5 mM concentration of urate had no inhibitory effect on ochratoxin A transport. Similarly, 10 mM lactate had no effect on ochratoxin A transport. In contrast, the fatty acid octanoic acid and the nonsteroidal anti-inflammatory agent piroxicam both produced an inhibition of ochratoxin A uptake that was similar to that produced by probenecid.

Octanoic acid has been shown to inhibit peritubular organic anion transport in single S2 segments (Sullivan et al., 1992). Similarly, because preloading of rat renal BLMV with very high concentrations (10 mM) of probenecid or PAH trans-stimulated octanoic acid transport, this fatty acid appears to be a substrate for the organic anion transporter (Trimble, 1989). Thus, a portion of octanoic acid inhibition of ochratoxin A uptake involved inhibition of the PAH pathway. However, octanoate, like probenecid, produced a greater degree of inhibition of ochratoxin A transport than did PAH, suggesting that octanoate and ochratoxin A were possibly accessing one or more common pathways in addition to the PAH transporter. In a study using the intact perfused rat kidney, Trimble (1979) reported evidence for mediated peritubular transport of octanoate that is insensitive to the presence of PAH. Although concluding that this fatty acid transport pathway could be the second pathway for peritubular ochratoxin A transport is tempting, that conclusion is complicated by the observation that octanoate clearance in rat kidney is not inhibited by probenecid (Trimble, 1979). Thus, the ability of octanoate to inhibit ochratoxin A uptake in rabbit proximal tubules may indicate that a fatty acid transport pathway may be involved in the transport of ochratoxin A, but more work will be required to support this conclusion.

The inhibition of ochratoxin A transport by piroxicam is of some interest. Piroxicam has been shown to protect rat kidney from ochratoxin A-induced nephrotoxicity (Baudrimont et al., 1995). The basis of this protective effect was suggested to involve competition between piroxicam and ochratoxin A for binding to plasma proteins, thereby reducing a “mobile reserve” of ochratoxin A that can result in prolonged exposure to target tissues to the toxicant. In addition, piroxicam may prevent activation of ochratoxin A through oxidation by the prostaglandin pathway. The present results suggest that piroxicam could also protect the kidney by reducing the transport of ochratoxin A into proximal tubule cells.

In conclusion, peritubular ochratoxin A transport is a high-affinity, low-capacity process that involves the peritubular organic anion (PAH) transporter. However, a second, PAH-insensitive (probenecid-sensitive) transport pathway may play a role in the accumulation of this mycotoxin in isolated rabbit renal proximal tubules. The ability of octanoic acid to inhibit ochratoxin A transport to a greater extent than PAH suggests that a separate fatty acid transport pathway could be involved in the accumulation of ochratoxin A by suspensions of rabbit renal proximal tubules.