Reabsorption of the Nephrotoxin Ochratoxin A Along the Rat Nephron In Vivo

MATTHIAS ZINGERLE, STEFAN SILBERNAGL and MICHAEL GEKLE
Physiologisches Institut, Universität Würzburg, D-97070 Würzburg, Germany
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ABSTRACT
Ochratoxin A (OTA) is a widespread nephrotoxin excreted to a substantial degree via the kidney. We investigated whether [3H]OTA is reabsorbed from the tubular lumen along the nephron and thus recycled within the kidney. Superficial early proximal and early distal tubules of male Wistar rats were micropunctured in situ. Microinfusion of OTA into superficial nephrons showed that it was reabsorbed in proximal as well as distal parts of the nephron. Reabsorption during early distal microinfusion was not saturable in the range of 0 to 5 x 10^{-4} mol/liter and accounted for 20% of OTA infused. Reabsorption during early proximal microinfusion was partially saturable and reached values up to 70% of OTA infused. The apparent $K_m$ for OTA reabsorption was 2.36 x 10^{-6} mol/liter and maximum transport rate 970 fmol/min/nephron. OTA reabsorption was pH dependent and decreased from 70 to 40% during proximal infusion and from 20 to 10% during distal infusion when pH increased from 6.0 to 7.4. The dipeptides carnosine and glycylsarcosine reduced OTA reabsorption significantly. L-Phenylalanine showed no significant inhibitory action. From our results we conclude: 1) there is substantial reabsorption of OTA along the nephron; 2) one-third of reabsorption takes place in the distal tubule and/or the collecting duct, two-thirds in the proximal tubule; 3) “distal” reabsorption can be explained at least in part by nonionic diffusion, because it was not saturable but pH dependent; 4) “proximal” reabsorption was in part mediated by the H^+-dipeptide cotransporter; 5) reabsorption of filtered and secreted OTA delays its excretion and may lead to accumulation of the toxin in renal tissue and 6) inhibition of OTA reabsorption (e.g., by urine alkalinization) should help to accelerate OTA excretion and thus reduce its toxicity.

The kidney plays an important role in OTA toxicity. On the one hand it is the main target of this mycotoxin and on the other hand it plays an important role in OTA excretion (Kuiper-Goodman and Scott, 1989; Delacruz and Bach, 1990). OTA impairs renal hemodynamics (Gekle and Silbernagl, 1993a), urine concentrating mechanisms (Gekle et al., 1993; Krogh et al., 1974), secretion of organic anions (Gekle and Silbernagl, 1994) and increases the incidence of renal adenoma and carcinoma (National Toxicology Program, 1989; Kuiper-Goodman and Scott, 1989). Due to the ubiquitous occurrence of OTA in improperly stored food and animal food there is a high risk of exposure for man and animals. Once ingested OTA is reabsorbed very effectively from the gastrointestinal tract and reaches the circulation (Kumagai and Aibara, 1982). In blood, OTA is bound to more than 99% to serum proteins (mainly albumin), contributing to its long half-life in the body (Chu, 1971; Chu, 1974). Renal elimination of OTA contributes to at least 50% of total clearance (Kuiper-Goodman and Scott, 1989). Because effective filtration is hindered by the binding to albumin, the main route of OTA into the tubular lumen is secretion in the proximal tubule (Gekle and Silbernagl, 1994; Stein et al., 1984; Sokol et al., 1988). The concentration of free OTA in final urine exceeds the concentration of free OTA in plasma (Gekle and Silbernagl, 1994). The fate of OTA which has reached the tubular lumen is not clear so far. There is only little information available from cell culture studies (Gekle et al., 1993b) with respect to possible reabsorption of OTA. For this reason we investigated possible reabsorption of OTA along the nephron by in vivo microinjection and determined the underlying mechanisms of transport. Our results show that the mycotoxin is reabsorbed in proximal and distal sections of the nephron by the H^+-dipeptide cotransporter (Silbernagl et al., 1987) and probably in part by nonionic diffusion. Reabsorption of OTA is of toxicological importance because it contributes to the long half-life of the mycotoxin in the body and could furthermore lead to recycling and accumulation in certain parts of the kidney (Bauer et al., 1995).

Materials and Methods
Preparation of animals for microinfusion experiments in vivo. Male Wistar rats (Charles River, Sulzfeld, Germany) weighing
210 to 290 g were fed on an Altromin standard diet and had free access to water. The rats were anesthetized with 120 mg/kg body weight Inactin (Byk-Gulden, Constance, Germany). After inserting a polyethylene tube into the trachea and two catheters into the jugular vein, the animals were infused i.v. with Ringer solution at a rate of 50 ml/min. The kidney was prepared for micropuncture as previously described (Gekle and Silbernagl, 1994).

Microinfusion experiments. After identification of the nephron section by i.v. injection of lissamine green SF (Chroma-Gesellschaft, König, Germany) at a bolus dose of 0.02 ml of a 100 g/liter solution titrated with NaOH to pH 7.4, the tubule was micropunctured using glass capillaries. They had ground tips (outer tip diameter 10–12 mm) and were mounted on a microperfusion pump (Sonnener and Deetjen, 1964). Puncture sites were 1) the earliest superficial loop of the proximal tubule (“early proximal”), which represents a distance from the glomerulus of ~0.3-1.2 mm and 2) the first superficial loop of the distal tubule (“early distal”). The microinfused solution was a Ringer solution (see below) containing [14C]inulin and [3H]-OTA. Microinfusion at a rate of 20 nl/min lasted for 10 min. Starting shortly before microinfusion, the ipsilateral urine was collected from a urethral catheter in 15-min fractions for 60 min, and radioactivity of each fraction was counted in a liquid scintillation spectrometer (Packard Instruments, Frankfurt, Germany). Urinary [3H]-OTA recovery (fractional excretion) was calculated from ([3H]urine · [3H]perfusion)/([14C]urine · [3H]perfusion). FR is 1 – fractional excretion. As a control, the urine of the contralateral kidney was collected from a bladder catheter. Radioactivity did not exceed background level.

Materials. The Ringer solution consisted of (in mmol/l): 156.4 Na+, 5.4 K+, 1.7 Ca2+, 162.8 Cl–, and 2.4 HCO3–, pH 7.4. In the experimental series in which the pH dependence of OTA reabsorption was tested the Ringer solution was buffered with 10 mmol/liter N-tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid (pKa = 7.5) and 10 mmol/liter 2-(N-morpholino)ethanesulfonic acid (pKa = 6.4). This solution was titrated to pH 7.4 or 6.0. 14C-inulin (0.4 GBq/g) was obtained from Du Pont de Nemours (Dreieich, Germany), 3H-OTA (1.37·1014 Bq/mol) from Moravek Biochemicals (Brea, CA), N-tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid and 2-(N-morpholino)ethanesulfonic acid from Serva (Heidelberg, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). The purity of 1H-OTA was checked by high performance liquid chromatography as described previously (Gekle and Silbernagl, 1994).

Statistics. Two to three tubules were studied per rat and the data are presented as mean values ± S.E.M. n gives the number of tubules studied. Significance was tested by unpaired t test. Differences were considered significant if P < .05. Curve fitting was performed by the least square method (Sigma Plot; Jandel, Corte Madera, CA).

Results

Up to 70% of OTA (10−6 mol/l) infused into the rat nephron is reabsorbed (fig. 1), depending on the site of infusion and on the pH of the microinfusate. In the “distal” parts of the nephron (distal tubule and collecting duct) FR was ~20%. Thus, up to 50% of OTA was reabsorbed in the proximal tubule and possibly in the short loops of Henle (“proximal” part of the nephron) (fig. 1A). “Distal” reabsorption was not saturated when infusing OTA concentrations up to 5·10−4 mol/liter, because FR did not decrease significantly compared to FR when 10−6 mol/liter OTA was infused (fig. 1C). The constancy of “distal” FR made it possible to determine “proximal” FR as the difference of total FR minus 20% (= “distal” FR).

In contrast to “distal” FR, “proximal” FR reabsorption was a saturable process as shown in figures 1B and 2. FR decreased with increasing concentrations of OTA in the infusion solution. Figure 2A shows the dependence of “proximal” reabsorption (determined after subtraction of “distal” reabsorption as described above) on OTA concentration. Analysis of the data either by nonlinear curve fitting or after linearization (fig. 2b, Hill plot) yields an apparent Km of 236·10−6±59·10−6 mol/liter. The maximum transport rate was estimated as 970 ± 75 fmol/min (fig. 2A).

To determine a possible pH dependence of OTA transport, FR at two different pH values of the microinfusate was determined (10−6 mol/liter OTA; solutions buffered as described in “Materials and Methods”). As shown in figure 3 there was a clear dependence on pH for “proximal” and “distal” reabsorption. Total FR was ~70% at pH 6.0 but only ~35% at pH 7.4. “Distal” FR was ~20% at pH 6.0 and ~10% at pH 7.4. Thus, “proximal” FR decreased from 50 to 25% when pH increased from 6.0 to 7.4.

Glycylsarcosine and carnosine (10−2 mol/liter), both substrates for the H+-dipeptide cotransporter in the nephron (Silbernagl et al., 1987), inhibited FR of OTA significantly as shown in figure 4. By contrast, “distal” FR was not affected by the addition of dipeptides: FR during infusion with 10−6
mol/liter $^3$H-OTA was 21 ± 1% ($n = 6$) in the absence and 27 ± 4% ($n = 3$) in the presence of 20 $\times$ 10$^{-2}$ mol/liter glycylsarcosine. L-Phenylalanine (2 $\times$ 10$^{-2}$ mol/liter), a substrate of the high capacity carrier for neutral amino acids (Silbernagl, 1992), led to a small reduction of FR that did not reach significance. Under control conditions (10 $\times$ 10$^{-6}$ mol/liter $^3$H-OTA) FR was 61.7 ± 5.4% ($n = 6$) and in the presence of 20 $\times$ 10$^{-2}$ mol/liter L-phenylalanine 53.5 ± 1.4% ($n = 4$).

**Discussion**

The results of this study show clearly that OTA, once it has reached the tubular lumen either by secretion or filtration, undergoes significant reabsorption along the nephron. About two-thirds of total reabsorption (which amounts to 60–70% of the tubular load) occurs before OTA reached the distal tubule, i.e., in the proximal convoluted tubule and/or in the short loops of Henle. The remaining reabsorption takes place in the distal tubule and/or the collecting duct.

Thus, the long half-life of OTA in the body (Delacruz and Bach, 1990) is probably not only the result of its binding to plasma proteins but also due to tubular reabsorption. The low specific activity of $^3$H-OTA made it impossible to reduce
its concentration in the perfusion solution below \(10^{-6}\) mol/liter to investigate reabsorption in the nanomolar range. Yet, as \(K_m\) was 236 \(\times 10^{-6}\) mol/liter reabsorption in the low micromolar and nanomolar range can be assumed as being proportional to the concentration of OTA. Thus, the FR determined during infusion with \(10^{-6}\) mol/liter is the same as for lower concentrations that occur during natural exposure (Kuiper-Goodman and Scott, 1989). Furthermore, manipulation of OTA reabsorption (as discussed below) should be a tool to increase its rate of elimination and consequently reduce its toxicity. Probably, the beneficial effect of bicarbonate gavage in murine ochratoxicosis (Yong et al., 1987) can be explained in this way. Luminal uptake of OTA by renal epithelial cells may also increase the cellular content of the toxin and thereby enhance its cytotoxic actions.

Reabsorption in the “distal” parts of the nephron was not saturable in the concentration range investigated. Thus, transport was either due to simple diffusion or mediated by a low-affinity transporter. A nonsaturable transport for OTA has been also described for the gastrointestinal tract (Kumagai and Aibara, 1982) and it was concluded that absorption is due to diffusion. Furthermore, “distal” reabsorption decreased with increasing pH in the infusion solution. Because OTA is a weak organic acid with a \(pK_a\) value of 7.1 (Kuiper-Goodman and Scott, 1989) an increase in pH from 6.0 to 7.4 reduces the fraction of unchanged (nondissociated) OTA from 93 to 33%. In the nondissociated form, OTA is well dissolvable in nonpolar solvents (Kuiper-Goodman and Scott, 1989) and probably also well membrane permeable. Thus, the pH-dependence of “distal” OTA reabsorption can be explained by nonionic diffusion. In this case OTA would be trapped within tubular cells, where pH is in the range of 7.1, and the intracellular concentration should be 2-fold the extracellular one. Accumulation of OTA in tubular cells of renal medulla and papilla (Bauer et al., 1995) can be explained, at least in part, by intracellular ionic trapping. Our data do not indicate the involvement of a \(H^+\)-driven dipeptide cotransport in “distal” transport. Yet, a small but significant reabsorption of glycylsarcosine during early distal microinfusion has been shown (Silbernagl et al., 1987). Thus, there is the possibility that a low affinity \(H^+\)-dipeptide cotransporter that was not saturated under our experimental conditions contributed to OTA reabsorption. In collecting duct-derived Madin Darby canine kidney cell monolayers a pH-dependent and dipeptide-inhibitable reabsorptive transport of OTA has been shown (Schwerdt et al., 1996). These data point to the possibility of dipeptide carrier-mediated reabsorption of OTA in renal collecting duct.

In contrast to “distal” reabsorption, “proximal” reabsorption was saturable pointing to a carrier-mediated transport. Furthermore, OTA transport was also pH dependent with decreasing FR as pH increased. This transport pattern can be well explained by the involvement of the proximal tubular \(H^+\)-dipeptide cotransporter (Silbernagl et al., 1987). The apparent affinity of “proximal” reabsorption for OTA (236 \(\times 10^{-6}\) mol/liter) is almost the same as as for the high affinity dipeptide carrier (240 \(\times 10^{-6}\) mol/liter) (Silbernagl et al., 1987). Furthermore, FR of OTA was significantly reduced in the presence of the dipeptides glycylsarcosine or carnosine that are both substrates of the \(H^+\)-dipeptide cotransporter in the proximal convoluted tubule (Silbernagl et al., 1987). Thus, our results show that OTA is reabsorbed, at least in part, via the \(H^+\)-dipeptide cotransporter of the proximal tubule. These results are in good agreement with the data obtained for proximal tubule-derived OK cells (Gekle et al., 1993b). Uptake across the apical membrane of OK cells was also pH-dependent and inhibitable by dipeptides. The reason for the incomplete inhibition of OTA reabsorption by the dipeptides might be that due to the constant flow of native tubular fluid the concentration of the dipeptides at the transporter site was diluted to suboptimal concentrations. In this case the carrier would not have been saturated completely. Furthermore, the low-affinity \(H^+\)-dipeptide cotransporter, with a \(K_m\) value of \(2 \times 10^{-3}\) mol/liter in brush-border vesicles (Silbernagl et al., 1987) may also be involved. Obviously, this transporter was not saturated. As already discussed above, nonionic diffusion could also be involved to some extent in OTA reabsorption because the pH of tubular fluid drops from 7.4 to \(\sim 6.7\) along the proximal tubule.

According to previous studies (Friis et al., 1988; Sokol et al., 1988; Gekle and Silbernagl, 1994) the renal organic anion transport system is responsible for the blood-to-lumen translocation of OTA in the proximal tubule. Thus, this transport system also should lead to an enrichment of OTA in proximal tubular cells. Reabsorption of the toxin by the \(H^+\)-dipeptide cotransporter and/or by nonionic diffusion reduces transepithelial net-secretion and thereby its elimination. Furthermore, reabsorption enhances the accumulation of OTA within proximal tubular cells but is probably the predominant mechanism for accumulation in postproximal tubular cells, that have no organic anion transport system. Of course, proximal secretion in the prerequisite for postproximal reabsorption and accumulation.

L-phenylalanine did reduce OTA reabsorption in tendency but not significantly. Thus, although OTA is a substrate of phenylalanine hydroxylase (Creppy et al., 1990) and interacts with phenylalanine t-RNA synthetase (Konrad and Roschenthal, 1977) it seems not to be a substrate of the amino acid carrier(s) responsible for L-phenylalanine reabsorption. We cannot exclude completely that the effective concentration of L-phenylalanine at the carrier site was also diluted and a possible inhibitory effect masked. Due to the lower affinity of L-phenylalanine reabsorption (\(K_m\) \(\sim 6 \times 10^{-3}\) mol/liter) (Silbernagl, 1992) as compared to the high affinity dipeptide carrier a reduction of the effective concentration would lead more readily to a lack of effect as compared to dipeptides.

Our data indicate that the \(H^+\)-dipeptide cotransporter is the predominant carrier system for OTA reabsorption in nephron sections before the distal tubule. Although, the proximal tubule seems to be the most probable candidate for the site of reabsorption, the thick ascending limb of the short loops of Henle can not be excluded as an additional site of reabsorption because there is evidence for dipeptide reabsorption in this part of the nephron (Silbernagl et al., 1987).

In conclusion, our data show that there is substantial reabsorption of OTA along the nephron. One-third of the reabsorption takes place in the distal tubule and/or the collecting duct, two-thirds in the proximal tubule and/or the loop of Henle. “Proximal” reabsorption was, at least in part, mediated by the \(H^+\)-dipeptide cotransporter. “Distal” reabsorption possibly involves nonionic diffusion, because it was not saturable but pH dependent. Reabsorption of filtered and secreted OTA delays its excretion and may lead to accumu-
lation of the toxin in renal tissue, thus enhancing its toxicity. Inhibition of OTA reabsorption (e.g., by urine alkalinization) should help to accelerate OTA excretion and thus reduce its toxicity.

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References


Send reprint requests to: Dr. Michael Gekle, Physiologisches Institut, Röntgenring 9, D-97070 Würzburg, Germany.