Inhibition of Renal Gluconeogenesis in Rats by Ochratoxin

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In kidney-cortex slices from rats fed on 2.0 mg of ochratoxin A/kg per day for 2 days, gluconeogenesis from pyruvate is decreased by 26%, and renal phosphoenolpyruvate carboxykinase activity is lowered by about 55%. Gluconeogenesis from 10 mM-lactate or 20 mM-malate or -glutamine is also significantly decreased. Hepatic phosphoenolpyruvate carboxykinase is unchanged or increased, and hexokinase activity in kidney and liver remains unaffected. We conclude that ochratoxin A *in vivo* is an inhibitor of renal phosphoenolpyruvate carboxykinase activity, which is responsible, at least in part, for the block in renal gluconeogenesis.

Ochratoxin A is a methylisocoumarin derivative produced as a secondary metabolite by certain strains of *Aspergillus ochraceus*, a fungus that is found commonly as a contaminant of grain and peanuts. The LD₅₀ in rats is 20 mg/kg, but is approximately 0.5–1 mg/kg in ducklings (Steyn, 1971), and is therefore nearly as potent as aflatoxin. Pathologically the kidney appears to be affected to the greatest extent, with interstitial fibrosis and proximal-tubular degeneration being the most commonly found lesions (Krogh *et al.*, 1976). This might be expected, since ochratoxin A is bound tightly to circulating albumin (Chu, 1971), and is accumulated in the renal proximal tubules (Elling, 1977; van Walbeek *et al.*, 1971; Chang & Chu, 1977), before being excreted in urine.

The biochemical damage that results from ingestion of the toxin is not well understood. Suzuki *et al.* (1975) have shown that hepatic glycogen stores are depleted within a few hours, and serum glucose concentrations are increased severalfold. We have found that the small pool of kidney glycogen is also decreased (H. Meisner, unpublished work). The lack of other results and the known localization of the toxin in proximal tubules prompted us to examine changes in carbohydrate metabolism in kidney cortex. The most important of these paths is gluconeogenesis (Krebs *et al.*, 1963), which helps to maintain blood glucose concentration. During starvation more than 40% of blood glucose originates from kidney gluconeogenesis (Owen *et al.*, 1969; Kida *et al.*, 1978), and in alloxan-diabetes, this value increases to 55% (Kida *et al.*, 1978). In metabolic acidosis the cortex uses glutamine as substrate to

produce ammonia, which titrates urine acidity (Goodman *et al.*, 1966). In the present study, we examine the effects of ochratoxin A ingestion on renal gluconeogenesis and PEPCK activity, which is the most important regulatory enzyme in this pathway (Utter & Keech, 1963).

Experimental

Crystallization of ochratoxin A

Spores of *A. ochraceus* N.R.R.L. 3174, obtained from C. W. Hesseltine, USDA, Peoria, IL, U.S.A., were maintained on Czapek agar slants (Fisher Scientific Co., Fair Lawn, NJ, U.S.A.). Culture of the fungus, as well as isolation and crystallization of ochratoxin A, were carried out as previously described (Meisner & Chan, 1974; Meisner, 1976). Purity was assessed by t.l.c. and judged to be better than 95%.

Animals

Sprague-Dawley male rats, weighing 100–200 g, obtained from Zivic-Miller Laboratories, Allison Park, PA, U.S.A., were used in all experiments. Ochratoxin A was dissolved in 0.5 M-NaHCO₃, and the concentration determined by measuring the absorbance at 333 nm by using an absorption coefficient of 6100 litre·mol⁻¹·cm⁻¹. Experimental rats were given the toxin via a stomach tube while under light ether anaesthesia, and control rats received an equivalent volume of NaHCO₃. A standard diet of rat chow and water was provided *ad libitum*. In all experiments involving starvation, food was withdrawn 48 h before the animals were killed. After killing, the kidney and liver were immediately removed, and either placed in ice-cold 0.9% NaCl,

Abbreviation used: PEPCK, phosphoenolpyruvate carboxykinase.

or quick-frozen and maintained at -60°C until assayed.

Measurement of renal gluconeogenesis

Cortex was removed from kidneys by free-hand dissection and was sliced uniformly into 0.5 mm thicknesses with a McIlwain tissue chopper. After washing at $0-4^{\circ}\text{C}$ for a few minutes in Krebs & Henseleit (1932) low-bicarbonate buffer to remove endogenous glucose, 4–6 mg (dry wt.) of tissue was distributed among flasks containing 4.0 ml of Krebs–Henseleit bicarbonate-buffered saline, pH 7.4. The flasks were gassed at 0°C with O_2/CO_2 (19:1) for 45 s and stoppered. Gluconeogenesis was initiated by addition of the appropriate substrate, and the flasks were shaken at 100 oscillations/min, at 37°C . Flasks lacking substrate served as controls for any endogenous glucose present. After incubation for 1 h, the slices were removed and dried. The medium was deproteinized with HClO_4 (final concn. 2%) and glucose determined on the neutralized supernatant by using glucose oxidase. Glucose formation is expressed as $\mu\text{mol/h}$ per g dry wt., at 37°C , after subtraction of glucose production in flasks lacking substrate.

Enzyme assays

Homogenate (20%, w/v) of frozen liver and kidney cortex were made in 0.1 M-triethanolamine/HCl buffer (pH 7.2)/0.25 M-sucrose/1 mM-EDTA/1 mM-glutathione (Henning *et al.*, 1966). Homogenates were centrifuged for 1 min in an Eppendorf micro-centrifuge at 16000 rev./min, and supernatants assayed for enzyme activity. PEPCK measurements were based on the method of Lane *et al.* (1969), modified (M. F. Utter, personal communication) to contain in 1 ml: 14 mM- MnCl_2 , 100 mM-imidazole, pH 6.6, 0.14 mM- β -mercaptoethanol, 0.025% Triton X-100, 1.25 mM-phosphoenolpyruvate, 1 mM-NADH,

3 units of malate dehydrogenase, 5 mM-IDP (Na^+) and 50 mM- $\text{NaH}^{14}\text{CO}_3$ ($0.1 \mu\text{Ci}$). The reaction was initiated with 20 μl of homogenate, and stopped at 10 min with 0.25 ml of 20% (w/v) trichloroacetic acid. After centrifuging, the protein-free supernatant was gassed with CO_2 to remove excess $\text{NaH}^{14}\text{CO}_3$, and radioactivity was counted by liquid scintillation. Under these conditions the reaction was linear for 15 min at 30°C . Hexokinase was assayed spectrophotometrically by coupling to glucose 6-phosphate dehydrogenase and measuring the reduction of NADP^+ at 340 nm, as described by Rose & Warms (1967), except that the concentration of ATP was increased to 10 mM, in which case the reaction was linear for 20 min at 30°C .

Blood pH, $p\text{CO}_2$ and HCO_3^-

Ochratoxin A was administered to rats as described previously. Blood was collected by cardiac puncture from control and experimental animals fed on 2 mg of ochratoxin A/kg per day for 48 h. Blood pH and $p\text{CO}_2$ were determined within 15 min with a Radiometer blood gas and pH system. The bicarbonate concentration was calculated from the Henderson–Hasselbach equation by using a pK of 6.1, and a solubility constant for CO_2 of 0.03.

Results

When ochratoxin A is given orally at 2.0 mg/kg per day for 48 h (Table 1), kidney-cortex PEPCK activity in fed rats is decreased by 46%, and gluconeogenesis from pyruvate is decreased by 26%, from 101 to 75 $\mu\text{mol/h}$ per g dry wt. Starvation, although it increases renal gluconeogenesis and PEPCK activity, does not alter significantly the effectiveness of ochratoxin A. In these rats, PEPCK activity is decreased by 64%, and gluconeogenesis by 26%. Measurement of hepatic PEPCK activity revealed

Table 1. *Effect of ochratoxin A on gluconeogenesis, PEPCK and hexokinase in kidney cortex and liver of fed and starved rats*
Rats were given ochratoxin A orally (2.0 mg/kg per day) for 48 h. Starved groups had food withdrawn at the beginning of the experiment. Gluconeogenesis was measured with 20 mM-pyruvate as substrate, as described in the Experimental section. Each value represents mean $\pm$ S.E.M. for six rats. Student's *t* test was used to evaluate statistical significance; n.s., not significant. Enzyme activity is expressed as $\mu\text{mol/min}$ per g wet wt. of tissue.

Nutritional status	Change in body wt. (g)	Ochratoxin A	Renal gluconeogenesis ($\mu\text{mol/h}$ per g dry wt.)	Enzyme activity ($\mu\text{mol/min}$ per g dry wt.)			
				Renal		Hepatic	
				PEPCK	Hexokinase	PEPCK	Hexokinase
Fed	$+11 \pm 2$	–	101 ± 7	4.56 ± 0.43	2.36 ± 0.18	0.87 ± 0.10	1.35 ± 0.05
	-1 ± 3	+	75 ± 8	2.45 ± 0.14	2.26 ± 0.13	1.58 ± 0.02	1.30 ± 0.18
			$P < 0.01$	$P < 0.001$	n.s.	$P < 0.001$	n.s.
Starved	-31 ± 3	–	176 ± 6	9.80 ± 0.41	1.69 ± 0.11	3.24 ± 0.28	0.95 ± 0.11
	-31 ± 2	+	130 ± 14	3.47 ± 0.42	1.36 ± 0.07	3.38 ± 0.18	0.73 ± 0.10
			$P < 0.001$	$P < 0.001$	$P < 0.01$	n.s.	n.s.

Table 2. *Effect of substrate on gluconeogenesis in kidney-cortex slices from rats fed on ochratoxin A*

Rats were fed on ochratoxin A (1.91 mg/kg) for 7 days. At day 5, all groups were starved for 48 h before being killed. All substrate concentrations were 20 mM, except for lactate which was 10 mM. Values represent means $\pm$ S.E.M. for six rats. Student's *t* test was used to evaluate statistical significance; n.s., not significant.

Ochratoxin A	Gluconeogenesis (μ mol/h per g dry wt.)					
	Malate	Glutamine	Lactate	Pyruvate	Glycerol	Fructose
—	141 $\pm$ 11	50 $\pm$ 4	114 $\pm$ 12	115 $\pm$ 4	20 $\pm$ 3	249 $\pm$ 31
+	101 $\pm$ 13	34 $\pm$ 6	70 $\pm$ 6	45 $\pm$ 7	35 $\pm$ 15	251 $\pm$ 31
	<i>P</i> < 0.01	<i>P</i> < 0.01	<i>P</i> < 0.001	<i>P</i> < 0.001	n.s.	n.s.
Inhibition (%)	28	32	39	61	—	—

that ochratoxin A causes an 82% increase in fed rats, from 0.87 to 1.58 μ mol/min per g, but no change in starved animals. It is likely that hepatic PEPCK activity is a reflection of the nutritional state of the rat, the activity being lowest in fed, and highest in starved, animals. Fed animals treated with ochratoxin A represent a semi-starved state, having lost 1 g body weight, and therefore show intermediate PEPCK activity. Little or no change occurs in hepatic or renal hexokinase activity in any condition.

The effects of different substrates on renal gluconeogenesis in ochratoxin A-fed rats are shown in Table 2. After the toxin had been given for 7 days at 1.9 mg/kg per day, gluconeogenesis in these rats from malate, glutamine, lactate and pyruvate is inhibited by 28, 32, 39 and 61% respectively, and that from glycerol or fructose is unaffected. Although not shown, renal PEPCK activity is decreased by 60–80% under these conditions.

Renal gluconeogenesis is known to be affected by acid–base balance (Goodman *et al.*, 1966), and therefore the effect of ochratoxin A on blood pH and $p\text{CO}_2$ was studied. Oral administration of 2 mg of toxin/kg per day for 48 h changed blood pH from 7.394 to 7.376, which was not significant. Blood $p\text{CO}_2$ was unchanged, and the HCO_3^- concentration was lowered slightly, from 22.4 to 18.8 mM. Since metabolic acidosis increases gluconeogenesis, the slight decrease in pH and inhibition of gluconeogenesis occur in directions opposite to those that would be expected if the toxin's effect was mediated through acid–base balance.

Discussion

This study has demonstrated that *in vivo* ochratoxin A is a selective inhibitor of renal PEPCK activity and gluconeogenesis, with no effect, at least at the concentrations we have used, on liver PEPCK or renal hexokinase activity. Other inhibitors of gluconeogenesis in perfused liver have been discovered, namely quinolinate, which blocks PEPCK (Veneziale *et al.*, 1967), and amino-oxyacetate, an inhibitor

of glutamine–oxaloacetate aminotransferase (Williamson *et al.*, 1970), but neither is known to be an inhibitor of PEPCK activity in kidney *in vivo*. The importance of PEPCK in kidney cortex was established by Henning *et al.* (1966), who showed that higher renal rates of gluconeogenesis during starvation or cortisol treatment are related to increases in PEPCK and pyruvate carboxylase activities. The decrease in renal gluconeogenesis therefore is likely to be due to inhibition of PEPCK activity, which is decreased to a greater extent than gluconeogenesis. The fact that glucose synthesis from glutamine and malate was also inhibited by ochratoxin A strengthens the interpretation that the block occurs at PEPCK. The possibility that other enzymes are also decreased, for example pyruvate carboxylase, cannot, of course, be ruled out.

The decrease in renal gluconeogenesis caused by oral administration of ochratoxin A can be related to the localization of the toxin in the proximal convoluted tubules. It is well established that most of the PEPCK activity resides in this area of the renal cortex (Guder & Schmidt, 1974; Schmidt & Guder, 1976), and that the toxin is also found here (Elling, 1977). Hexokinase, on the other hand, is a glycolytic enzyme found in low amounts in the proximal tubules, and in considerably higher quantities in the thick ascending limb of Henle, and distal convoluted tubule (Schmidt *et al.*, 1975). Hexokinase activity is not affected, presumably because this enzyme is localized in an area of the kidney where ochratoxin A is not found.

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