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CHANGES OF RENAL mRNA SPECIES ABUNDANCE BY OCHRATOXIN A*

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Abstract—Ochratoxin A is a nephrotoxin produced by certain species of Aspergillus and Penicillium. We have found previously that renal but not hepatic P-enolpyruvate carboxykinase, and the mRNA for this enzyme, are rapidly decreased in rats and swine fed 0.1 to 1 mg/kg body weight for a few days. In the present study, we isolated kidney mRNA from rats fed ochratoxin A for 2–5 days. By screening a rat kidney cDNA library with [^{32}P]RNA, we have identified several renal mRNAs whose concentration is changed within 2 days by the toxin. The transcription rate of each mRNA was measured in nuclei isolated from kidneys of rats fed ochratoxin A. The incorporation of [^{32}P]UMP into P-enolpyruvate carboxykinase mRNA and the synthesis of other RNAs were not affected. Therefore, the toxin changes mRNA abundance at the post-transcriptional level.

Ochratoxin A is a secondary metabolite produced by certain species of Aspergillus and Penicillium. Ingestion of this toxin by rats [1], swine [2], and fowl [3] results in a nephropathic syndrome, with most histological damage localized to the proximal convoluted tubules [4, 5]. We have found that renal gluconeogenesis and cytosolic P-enolpyruvate carboxykinase (PEPCK‡) activity are reduced by as little as 0.1 mg/kg/day in rats [6], and by 0.008 mg/kg/day in swine.§ Although other enzymes that we have measured, for example hexokinase, pyruvate carboxylase, and phosphate-dependent glutaminase, are not affected, the toxin must have pleiotrophic effects, since protein synthesis [6] and the mRNA pool [7] are reduced by 30–40%. To study the mechanism of action of ochratoxin A at the molecular level, we have identified cDNA clones corresponding to species of mRNA that are changed in abundance by the toxin. We found that the transcription rate of these mRNAs was not changed.

METHODS

Isolation of poly (A)$^+$ mRNA. Male Sprague–Dawley rats, weighing 200–300 g, were fed *ad lib.* Animals were given ochratoxin A orally at 2 to 2.5 mg/kg, dissolved in 0.5 M bicarbonate, daily for 1–5 days. The hot phenol method [8] was used to extract RNA from rat kidneys, and the poly (A)$^+$ fraction was separated from total RNA by oligo (dT) affinity chromatography.

cDNA screening. Individual cDNAs from a rat kidney cDNA library were grown in 2 ml volumes at 37° overnight, and isolated by alkaline lysis [9]. Each sample of approximately 1 μg DNA was blotted in duplicate to nitrocellulose paper on a Schleicher and Schuell manifold and hybridized to [^{32}P]RNA taken from control and ochratoxin A treated rats. RNA was labeled *in vitro* by partial hydrolysis at pH 9.5 for 10 min, 90°, followed by labeling with [^{32}P]-γ-ATP and polynucleotide kinase [10] to a sp. act. of 5–10 × 10^6 cpm/μg. The size distribution of labeled RNA was about 200–400 nucleotides. Unincorporated ATP as well as small oligonucleotides were removed by centrifuging through a G50 Sephadex column. Prehybridization and hybridization were at 68° in 0.5 M NaCl, 10 mM Hepes (pH 7.5), 10 mM EDTA, 0.2% SDS, 1X Denhardts, and salmon sperm DNA, as described [11, 12].

Quantitative blotting. [^{32}P]mRNA (2–3 μg) was hybridized to 1 μg of cDNA bound to nitrocellulose filters in the above medium. Filters were washed in 5X SSC at 45°, in 2X SSC at 22°, treated with RNAse A (10 μg/ml) and T1 (5 units/ml) at room temperature for 20 min, and finally boiled in 10 mM EDTA to remove specifically bound [^{32}P]RNA. Efficiency of hybridization, measured by including a specific [^{3}H]cRNA for PEPCK [13], was 0.4 to 0.5. The [^{32}P]RNA bound to specific cDNA filters increased proportionally up to 7 μg poly (A)$^+$ RNA (data not shown), indicating that cDNA was in excess.

Alternately, RNA blots were exposed to X-ray film (Fuji), and the autoradiographs scanned with a soft laser densitometer (Biomed Instruments). The integrated areas of control and ochratoxin A samples were compared, using identical gain settings.

Transcription. Nuclei were isolated as described [11], and frozen at −80° until used. RNA synthesis was measured by a nuclear run-off assay [11, 14], in

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‡ Abbreviations: PEPCK, P-enolpyruvate carboxykinase; EDTA, ethylenediaminotetracetate; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; and 1X SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.
§ H. Meisner and P. Krogh, *Proc. IUPAC Symp. on Mycotoxins, V*, Vienna (1982).

which nascent RNA chains are elongated by bound RNA polymerase, but little or no initiation occurs. The transcription medium contained 2 mM Mn^{2+} and 0.5 mM Mg^{2+}, 12.5% glycerol, 0.03 mM EDTA, 50 mM Hepes (pH 7.5), 0.1 M KCl, 4 mM dithiothreitol, 0.5 mM CTP, 0.5 mM GTP, 1.0 mM ATP, 0.04 mg creatine phosphokinase/ml, 8.8 mM creatine phosphate, and 2–3 × 10^7 nuclei in a total volume of 200 μl. Transcription was started by adding 150 μCi [^{32}P]-α-UTP. Hybridization of [^{32}P]RNA was to 2 μg of filter-bound cDNA. Transcription rates were corrected for efficiency of hybridization by including a [^{3}H]cRNA for PEPCK in each sample [11].

Chemicals. Ochratoxin was crystallized as described previously [6]. The cDNA for PEPCK, a 2.6 Kb insert isolated from a rat kidney cDNA library and cloned into the Pst 1 site of pBR322 [15], was provided by R. W. Hanson, Case Western Reserve University. The rat cDNA library, prepared from whole rat kidneys, was the gift of J. Short and R. W. Hanson, Department of Biochemistry. Restriction enzymes were purchased from Promega Biotek; [^{32}P]-γ-ATP was made from [$^{32}P_i$] and phosphoglycerol kinase (Promega Biotek); [^{32}P]UTP was obtained from the N.E.N. Corp. (sp. act. 400 Ci/mmole); nitrocellulose paper was from Schleicher & Schuell.

RESULTS

Species of renal poly (A)$^+$ mRNA that are changed in concentration by ochratoxin A were identified by first labeling *in vitro* with [^{32}P]-γ-ATP, then hybridizing to individual cDNAs isolated from a rat kidney cDNA library that were bound to nitrocellulose filters. Each cDNA was present in 30 to 50-fold excess, assuming a concentration of 0.5 μg cDNA and an abundance of 0.1% for each species of mRNA. The result of a typical screen is shown in Fig. 1. The same group of cDNAs were hybridized to control [^{32}P]RNA (left panel) and to [^{32}P]RNA from rats fed ochratoxin A (right panel). In lane A2, the reduction of PEPCK mRNA by ochratoxin A can be easily detected. By densitometric scanning the decrease was about 50% (950 vs 409 units), a relative value that is similar to earlier studies [7]. The toxin also increased the concentration of several other species 2- to 5-fold, as measured densitometrically (for example, lane 3A, E, F, G; lane 2F; and lane 1F, G, H). Lane 3G corresponds to cDNA #85 in Tables 1 and 2. Most of the cDNA blots, however, either hybridized weakly to RNA, or showed no change in signal intensity by ochratoxin A.

The size of each mRNA and effect of ochratoxin A on mRNA concentration were determined by

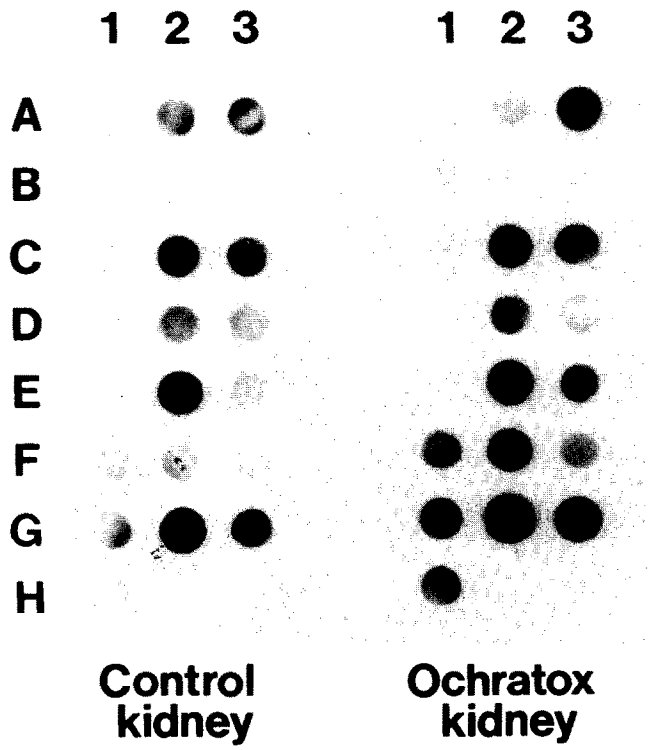

Fig. 1. Identification of renal mRNA species affected by ochratoxin A. Ochratoxin A (2.5 mg/kg) was given orally each day for 2 days to rats, and poly (A)$^+$ mRNA was isolated from whole kidneys and liver as described in Methods. The mRNA was labeled *in vitro* with [^{32}P]-γ-ATP, and steady-state mRNA concentrations were measured by hybridizing [^{32}P]mRNA to cDNA that was bound to nitrocellulose filters.

Table 1. Characteristics of selected renal species of mRNA

cDNA #	Size of cDNA (n.t.)	mRNA size (n.t.)	mRNA concentration (ppm)*	Effect of ochratoxin A
1 (PEPCK)	2535	2680	320	↓
4	1000	2000	500	NC
6	720	760	610	NC
8	690	1180	300	NC
28	770	1180	1010	NC
40	680	820	1110	↓
55	1300	1350	1480	↓
66	400	1520	5700	↓
76	980	1450	5120	↓
85	370	870	1720	↑

The screening of a cDNA prepared from rat kidney mRNA and determination of mRNA concentration are described in Methods. Size of mRNA was measured by northern blotting, and cDNA size by agarose gel electrophoresis using ϕX174 as mol. wt markers. The effect of ochratoxin A on mRNA concentration was determined by northern blots or [^{32}P]RNA hybridization to cDNA. Abbreviations: n.t. = nucleotides, and NC = no change.

$$* \text{ppm} = \frac{\text{cpm(cDNA-pBR322)}}{\text{cpm in total RNA}} \times \frac{100}{\text{hybridization}} \times \frac{\text{RNA size}}{\text{cDNA size}}$$

northern analysis, as depicted in Fig. 2. Renal poly (A)$^+$ RNA from control rats and rats fed ochratoxin A for 2 days were electrophoresed, transferred to nitrocellulose [12], and hybridized to groups of three [^{32}]P-nick-translated cDNAs. Preliminary experiments with single radiolabeled probes identified the size of each mRNA. In this example, the relative amount of mRNA #40 was decreased 50%, #28 was unchanged, while mRNA #2 was decreased 67%, as measured by densitometric scanning.

To remove the possibility that any of the cDNAs selected hybridize to common mRNAs, suspected pairs of these cDNAs were cross hybridized (data not shown). None of the cDNAs listed in Tables 1 and 2 showed sequence homology by this method.

From approximately 1000 cDNA clones that have been screened, the characteristics of selected cDNAs as well as the respective mRNAs are shown in Table 1. The selection was based on abundance of the mRNA, size of the cDNA, and effect of ochratoxin A. Emphasis is placed on mRNA species whose abundance is reduced by the toxin. Most of the cDNAs were greater than 50% of the length of the respective mRNA, and a few were nearly full length. Several of the selected clones (#55, 66 and 76) hybridized to abundant mRNAs, present at 0.2 to 0.6%. This compares to renal PEPCK mRNA, which was only 0.03% in the control fed state.

The effect of ochratoxin A on several selected mRNAs was examined in rats fed 2 mg ochratoxin A/kg body weight daily over a period of 5 days (Fig. 3). The absolute abundance of each mRNA in the total poly (A)$^+$ population was quantitated by hybridizing [^{32}P]RNA to excess filter bound cDNA, and correcting for the mRNA/cDNA size ratio, as well as the efficiency of hybridization. The toxin appeared to have a rapid effect on the abundance of PEPCK, #55, and #76 mRNA. The concentration of PEPCK mRNA was 0.032% (324 ppm) in control fed rats, and decreased by 60% to 0.013% (135 ppm) after 5 days. The mRNAs hybridizing to #55 and #76, which were present initially at 0.15 and 0.51%, were reduced about 30% and 60% respectively. On the other hand, mRNA #6 (0.61%) and #28 (1.0%) were unchanged over this period of time.

The preceding data show that ochratoxin A reduces the concentration of several renal mRNA species. These effects may be caused by changes at the transcriptional or post-transcriptional level. The possibility that transcription is reduced was

Table 2. Effect of ochratoxin A on the transcription rate of individual mRNAs

cDNA #	Effect of ochratoxin A on mRNA	Transcription rate (ppm)	
		Ochratoxin A −	+
1 (PEPCK)	↓	730	870
4	NC*	45	47
8	NC	48	57
28	NC	15	13
40	↓	14	14
76	↓	73	72
85	↑	68	69

Rats were fed 2.5 mg ochratoxin A/kg or vehicle (bicarbonate) alone at 0 and 24 hr. Both groups were starved beginning at 24 hr, and killed at 48 hr. Nuclei were isolated from kidneys, and incorporation of [^{32}P]UMP into specific RNA was measured as described in Methods. The total radioactivity incorporated into RNA was 10–12 × 10^6 cpm. Efficiency of hybridization was 0.49, as measured with [^{3}H]-cRNA from PEPCK. A background incorporation of [^{32}P] onto pBR322 filters, amounting to 10–11 ppm, was subtracted from all samples. Transcription rates were determined in two to four samples for each cDNA, and are expressed as ppm.

$$\text{ppm} \frac{\text{cpm cDNA} - \text{cpm pBR322}}{\text{cpm in total RNA}} \times \frac{100}{\text{efficiency}} \frac{\text{bp mRNA}}{\text{bp cDNA}}$$

* NC = no change.

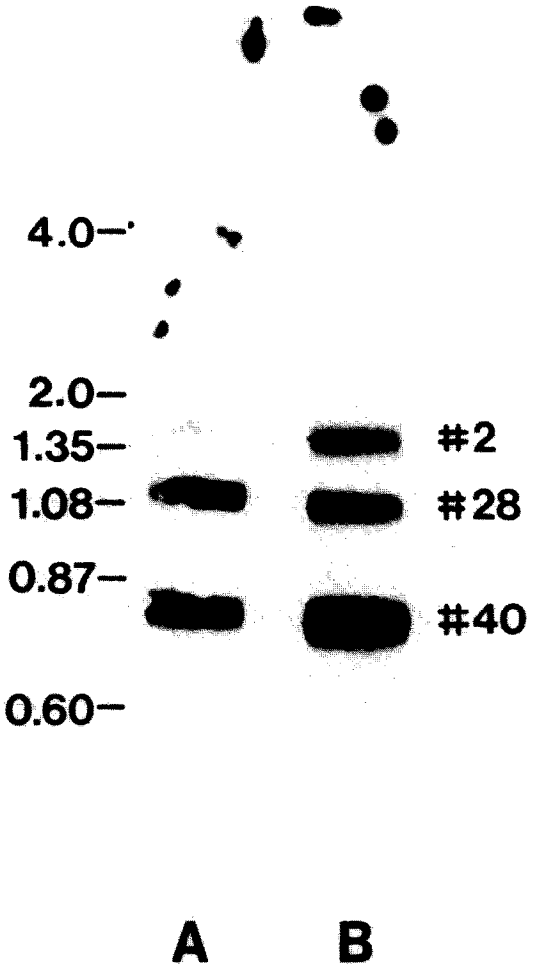

Fig. 2. Northern analysis of mRNA from control and toxin-treated rats. Rats were fed ochratoxin A (2.5 mg/kg) daily for 2 days. Poly $(A)^+$ mRNA was isolated, electrophoresed in a formaldehyde gel containing 1.5% agarose and 2 μg. RNA/lane, and transferred to nitrocellulose. Lanes A and B represent cDNA #2, 28, and 40 that was nick-translated and hybridized to control (label B) or ochratoxin A (lane A) mRNA. Molecular weight markers were poly $(A)^-$ RNA and Hae III-digested ϕX174 DNA.

approached by isolating nuclei from kidneys of control and ochratoxin A-fed rats, and measuring [^{32}P] UMP incorporation into specific RNAs. Table 2 shows that PEPCK RNA synthesis, and the synthesis of other RNAs, were not affected by ochratoxin A. Furthermore, in contrast to the high rate of synthesis of PEPCK RNA, the other mRNA species were transcribed very slowly, on the order of 15–70 ppm.

DISCUSSION

We have screened approximately 1000 cDNA clones from a rat cDNA library with [^{32}P]RNA from control and ochratoxin A-treated rats, and initially selected 40–50 clones for further analysis. The final group of 10–15 cDNAs was chosen based on abundance of the mRNA, effect of the toxin, and a relatively large cDNA size compared to the mRNA size. Several of these cDNAs, including PEPCK, are complementary to renal mRNAs that decrease by 30–60% shortly after feeding ochratoxin A while some are increased 2 to 5-fold in abundance. An increased abundance of an mRNA species may be either a direct effect of the toxin at the transcription or turnover level, or an indirect effect. We have demonstrated previously that the poly$(A)^+$ RNA pool is reduced in toxin-fed rats [7], and any mRNA species not affected would therefore be present in relatively higher concentration. Whether the small reduction in the mRNA pool is sufficient to account for a 2- to 5-fold increase in some mRNAs is, however, questionable.

In nuclear runoff experiments, we have shown that the rate of transcription of the genes coding for ochratoxin A-sensitive RNAs was not affected. This includes the gene for PEPCK, which is transcriptionally regulated in rat kidneys by various hormonal and dietary treatments [16], as well as the genes coding for several unnamed mRNA species. The toxin must therefore affect the concentration of certain mRNAs by a post-transcriptional mechanism. At the post-transcriptional level, the effect of ochratoxin A may be on RNA processing (poly A addition, splicing, or capping), translational efficiency, or degradation rate. Although none of these can be ruled out entirely, we have found that the

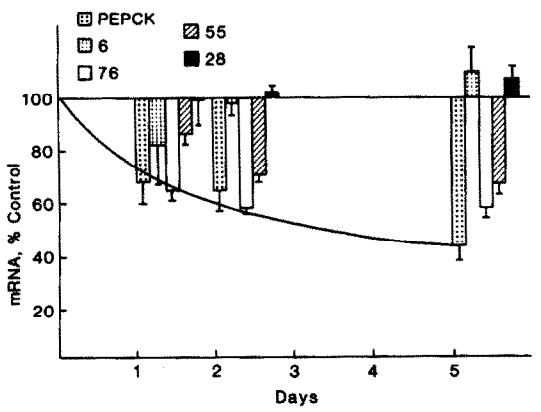

Fig. 3. Effect of time of exposure of rats to ochratoxin A on the concentration of selected renal mRNAs. Poly $(A)^+$ was isolated on days 1, 2, and 5, and labeled *in vitro* with [^{32}P]ATP as described in Methods. Between 2 and 4 μg [^{32}P]RNA was hybridized in duplicate to 1 μg of cDNA that was linearized with ECoRI and bound to nitrocellulose filters. Results are expressed as [cpm in RNA from ochratoxin treated rats/cpm in RNA from control rats × 100] after correcting for ^{32}P that was bound to filters containing 1 μg pBR322. Bars, S.E.M.

translational efficiency of PEPCK mRNA is unchanged in toxin-treated rats (H. Meisner, unpublished observation). In view of the known effects of hormones, etc., on RNA turnover [17], it is most likely that ochratoxin A perturbs this parameter.

The factors that determine whether a particular mRNA is "susceptible" to ochratoxin A are not known. However, it appears that neither the rate of mRNA synthesis nor the rate of degradation of mRNA correlates well. For example, the half-life of PEPCK mRNA has been measured as 30–40 min [18] and the rate of transcription is very high in both kidney and liver. In contrast, #76 had a very low transcription rate (Table 2) and the high abundance (Table 1) indicates a long half-life. The fact that the mRNA for PEPCK and #76 both decreased 50–60% suggests that the rate of turnover is of limited importance in determining ochratoxin A susceptibility.

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