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Metabolism of Ochratoxin A by Primary Cultures of Rat Hepatocytes

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Association of ochratoxin A with cultured rat hepatocytes occurs at 4°C, and the saturation level in the medium is 0.3 mM ochratoxin A, with maximal binding after 60 min. At 37°C the level of cell-associated ochratoxin A increased up to 6 h and remained at 2 nmol of toxin per mg of cell protein for 30 h. With increasing concentrations of ochratoxin A, increasing amounts of the toxin accumulated in the cells; saturation occurred at a concentration of 0.3 mM. Ochratoxin A was metabolized by hepatocytes at 37°C. (4*R*)-4-Hydroxyochratoxin A appeared in the medium at a maximal level (about 30 nmol/mg of cell protein) at an ochratoxin A concentration of 0.25 mM after 48 h of incubation. Small amounts of (4*S*)-4-hydroxyochratoxin A were detected only after incubation for 22 h or longer.

Ochratoxin A, which is a dihydroisocoumarin derivative linked over a 7-carboxy group to L-phenylalanine by an amide bond, is a secondary metabolite produced by various species of the fungal genera *Aspergillus* and *Penicillium* (Fig. 1). Among other toxic effects, this compound has been shown to cause nephropathy in swine (11). It is also presumed to be involved in a fatal chronic kidney disease which affects people in certain districts of Bulgaria, Romania, and Yugoslavia (12).

Equal amounts of (4*R*)- and (4*S*)-4-hydroxyochratoxin A have been isolated recently from incubations with a pig liver microsomal fraction containing ochratoxin A and NADPH. These metabolites were also formed by rat liver microsomes in a ratio of about 8:1 (20).

Previous studies have shown that when ochratoxin A is given to rats intraperitoneally or orally, (4*R*)-4-hydroxyochratoxin A but not (4*S*)-4-hydroxyochratoxin A is found in the urine or feces (unpublished data). Since rat liver microsomes are able to form both epimers from ochratoxin A, this study was undertaken to examine the metabolism of ochratoxin A in a homogenous population of liver cells. In this paper we describe the uptake of ochratoxin A by cultured rat hepatocytes and the formation and release of (4*R*)- and (4*S*)-4-hydroxyochratoxin A.

MATERIALS AND METHODS

Chemicals. Ochratoxin A was purchased from Sigma Chemical Co. Calculations of concentrations were based on a molecular extinction coefficient of 5,550 M^{-1} cm^{-1} at 333 nm (15).

(4*R*)- and (4*S*)-4-hydroxyochratoxin A were isolated as described elsewhere (20). A molecular extinction coefficient of 6,400 M^{-1} cm^{-1} at 334 nm for (4*R*)-4-hydroxyochratoxin A (8) was used to calculate the concentrations of these compounds (Størmer et al., in press).

Animals. Male Wistar rats (approximate body weight, 300 g) were fed an ordinary laboratory chow. The animals were given water and food ad libitum until perfusion was started at about 9 a.m.

Perfusion and incubation media. Rat livers were preperfused with a modified calcium-free Hanks solution (6) containing (per liter) 9.0 g of NaCl, 0.5 g of KCl, and 2.4 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and the pH was adjusted to 7.4 to 7.5 by adding 1 M NaOH. After about 10 min of preperfusion with this buffer, the livers were perfused with another buffer containing (per liter) 4.0 g of NaCl, 0.5 g of KCl, 0.7 g of $CaCl_2 \cdot 2H_2O$, 24.0 g of HEPES, and 0.5 g of collagenase, and the pH was adjusted to 7.4 to 7.5 with 1 M NaOH. A third buffer was used for preincubation and washing of cells. This buffer (buffer E) contained (per liter) 8.5 g of NaCl, 0.4 g of KCl, 0.06 g of $Na_2HPO_4 \cdot 2H_2O$, 0.047 g of $KH_2PO_4 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 4.76 g of HEPES, and 0.22 g of $CaCl_2$, and the pH was adjusted to 7.5 by adding 1 M NaOH. An arginine-free Dulbecco modified Eagle medium (DME medium) (5) supplemented with 25 mM glucose, 0.4 mM ornithine, and 10 mM HEPES was obtained from Flow Laboratories, Irvine, Scotland, and was used as the standard incubation medium for the cultured liver cells. Gentamicin and fetal calf serum were purchased from Flow Laboratories.

Isolation and cell culture of hepatocytes. The isolation of rat hepatocytes was based upon the method described by Berry and Friend (1) and modified by Seglen (17), except that sterile conditions were maintained throughout. Briefly, the liver was removed from an animal and perfused in vitro, first with the calcium-free Hanks solution and then with the buffer containing calcium and collagenase (see above). The liver

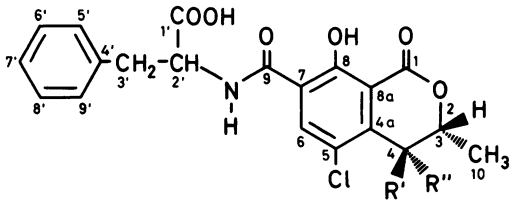

FIG. 1. Ochratoxin A (R′ = R″ = H) and its two metabolites, (4*R*)-4-hydroxyochratoxin A (R″ = OH; R′ = H) and (4*S*)-4-hydroxyochratoxin A (R″ = H; R′ = OH).

cells started to separate after approximately 10 min of perfusion with the collagenase solution, and a cell suspension was obtained by shaking the liver gently in buffer E. The cells were incubated for 20 to 30 min at 37°C in this buffer and then washed three to five times by centrifugation, as described previously (4).

The yield of hepatocytes was 2×10^8 to 4×10^8 cells per liver, with 93 to 97% trypan blue (0.04%) dye exclusion. Cells were placed into 60-mm sterile plastic dishes (Costar, Cambridge, Mass.) at a concentration of 2.5×10^6 cells per dish in 3 ml of arginine-free DME medium containing 20% fetal calf serum and 50 μg of gentamicin per ml, and the dishes were maintained at 37°C in a 95% air–5% CO_2 atmosphere. After 4 to 5 h under these conditions, the hepatocytes became attached to the dishes and started to spread out, as previously described (3). At this time the medium was replaced by serum-free DME medium, and the cells were incubated with ochratoxin A for different periods of time at 37°C. After incubation the medium from the cells was collected and centrifuged at 300 × *g* for 5 min to get rid of a small number of hepatocytes; 1 ml of 2 ml of cell-free medium was extracted with methanol-chloroform (2:1) after 0.1 ml of 1 M HCl was added. The hepatocytes were washed six times with 20 mM phosphate (pH 7.4)–0.9% NaCl. The cells were then centrifuged at 300 × *g* for 5 min. The cell pellet was suspended in 0.5 ml of water, and samples for protein measurements were removed before the cells were extracted the same way as the medium. The amounts of cell-associated and metabolized ochratoxin A differed up to fivefold when different cultures of hepatocytes were used. The protein in each culture dish was measured by the method of Lowry et al. (14), using bovine serum albumin as a standard. The uptake, metabolism, and release of ochratoxin A and its metabolites are reported as amount per milligram of liver cell protein.

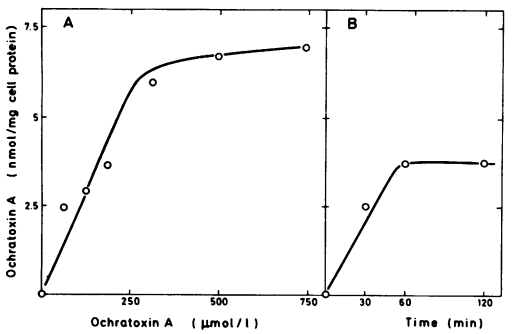

FIG. 2. (A) Effect of increasing ochratoxin A concentration on the amount of cell-associated ochratoxin A. Cultured rat hepatocytes were incubated for 120 min at 4°C. (B) Effect of incubation time on the amount of cell-associated ochratoxin A. Cultured rat hepatocytes were incubated with 120 μM ochratoxin A at 4°C.

Thin-layer chromatography, visualization and quantitative determination of ochratoxin A and (4*R*)- and (4*S*)-4-hydroxyochratoxin A. The solutions described above were spotted onto silica gel plates precoated with 0.25 mm of silica gel containing gypsum and a UV light (254 nm) indicator (Macherey-Nagel Co., Düren, Germany). The solvent system used was chloroform-diethyl-ether-acetic acid (17:2:1) (20), and ochratoxin A and its two hydroxylated metabolites were visualized by UV exposure. This thin-layer chromatography system separated the three compounds completely. The plates were placed at the filter surface of a transilluminator with an intensity of 6,000 μW/cm² at a wavelength of 365 nm.

For quantitative determinations of ochratoxin A and (4*R*)- and (4*S*)-4-hydroxyochratoxin A, the plates were scanned with a Vitatron model LTD 100 densitometer equipped with a mercury lamp (exitation at 366 nm and emission at 460 nm). The recorded areas of the spots were compared with standards of ochratoxin A and (4*R*)-4-hydroxyochratoxin A. Equal amounts of (4*R*)- and (4*S*)-4-hydroxyochratoxin A showed equal fluorescence intensity when they were subjected to fluorescent scanning after thin-layer chromatography (20).

RESULTS

Incubation of ochratoxin A with cultured rat liver cells at 4°C. At 4°C no substrate was metabolized, and internalization promoted by endocytosis was negligible (18). When increasing concentrations of ochratoxin A were incubated with hepatocytes at 4°C, a linear increase in cell-associated ochratoxin A was observed up to a concentration of about 300 μM (Fig. 2A). Above this concentration the amount of cell-associated substrate leveled off. When 120 μM ochratoxin A was incubated with cultured rat hepatocytes at 4°C (Fig. 2B), there was a linear increase in the amount of cell-associated ochratoxin A up to 60 min. After this time there was no further increase in the amount of substrate associated with the cells.

Incubation of ochratoxin A with cultured hepatocytes at 37°C. With increasing incubation time there was a decrease in the amount of ochratoxin A recovered from the medium (Fig. 3A). The amount of ochratoxin A associated with the cells reached a maximal level at about 6 h and remained at this level for up to 24 h (Fig. 3B). After this time the ochratoxin A level in the cells decreased.

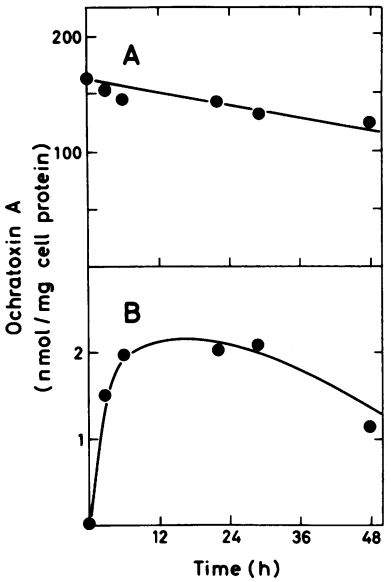

FIG. 3. Effect of incubation time on the amount of ochratoxin A recovered in the medium (A) and associated with the cells (B). Cultured rat hepatocytes were incubated with 250 μM ochratoxin A at 37°C.

There was a linear increase in the amount of cell-associated ochratoxin A up to a concentration of 300 μM with increasing amounts of the toxin in the medium and with an incubation time of 22 h (Fig. 4). The amount of ochratoxin A associated with the cells also increased up to a concentration of 750 μM, but the increase was significantly less than when the concentration was below 300 μM. The maximal amount of ochratoxin A associated with the cells was about 10 nmol/mg of protein.

Formation of (4*R*)-4-hydroxyochratoxin A by cultured hepatocytes. When the cells were incubated with ochratoxin A at 4°C, (4*R*)- and (4*S*)-4-hydroxyochratoxin A were not detected either in the cells or in the incubation medium.

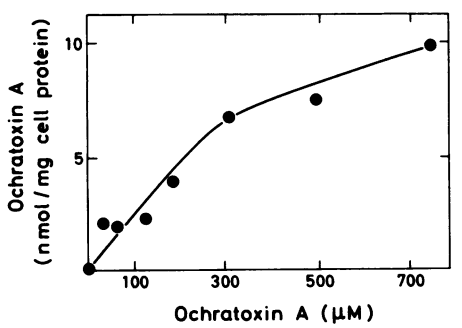

FIG. 4. Effect of increasing concentration of ochratoxin A on the amount of cell-associated ochratoxin A. Cultured rat hepatocytes were incubated for 22 h at 37°C.

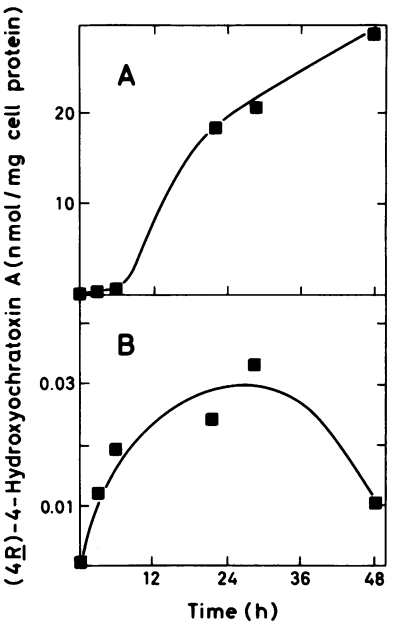

FIG. 5. Effect of incubation time on the amount of (4*R*)-4-hydroxyochratoxin A recovered in the medium (A) and associated with the cells (B). Cultured rat hepatocytes were incubated with 250 μM ochratoxin A at 37°C.

(4*R*)-4-Hydroxyochratoxin A was detected both in the incubation medium and in the washed cell pellet when the cells were incubated at 37°C. There was a lag period of about 6 to 8 h before significant amounts of (4*R*)-4-hydroxyochratoxin A appeared in the medium. After this time the hydroxylated product recovered in the medium increased for 48 h (Fig. 5A). After about 24 h the amount of (4*R*)-4-hydroxyochratoxin A in the medium was about 700 times greater than the amount associated with the hepatocytes for the same concentration of ochratoxin A (250 μM) (Fig. 5). The amount of (4*R*)-4-hydroxyochratoxin A recovered, which was associated with the cells, increased up to about 28 h (Fig. 5B). At 48 h the amount associated with the hepatocytes showed a marked decrease. The difference between the amount of hydroxylated product in the medium (Fig. 5A) and the amount in the cells (Fig. 5B) increased to approximately 3,000-fold at 48 h. At this time the amount of (4*R*)-4-hydroxyochratoxin A associated with the hepatocytes had decreased, whereas the amount in the medium was still increasing.

The amount of (4*R*)-4-hydroxyochratoxin A recovered from the medium showed a saturation

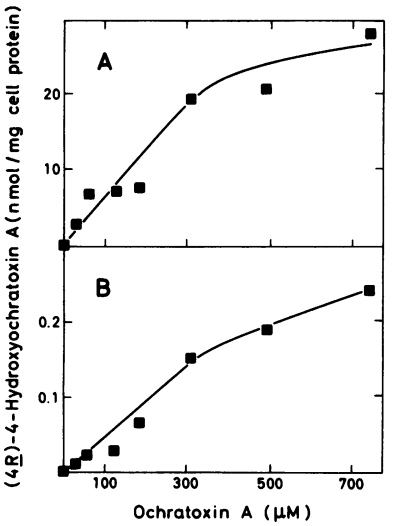

FIG. 6. Effect of increasing concentration of ochratoxin A on the amount of (4*R*)-4-hydroxyochratoxin A recovered in the medium (A) and associated with the cells (B). Cultured rat hepatocytes were incubated for 22 h at 37°C.

level at a concentration of ochratoxin A in the medium of about 300 μM (Fig. 6A). The amount of (4*R*)-4-hydroxyochratoxin A recovered from the cells increased linearly up to an ochratoxin A concentration of about 300 μM in the medium. Above this level there was a smaller increase in the formation of the hydroxylated product with increasing amounts of substrate (Fig. 6B). When hepatocytes were incubated in the presence of 496 nmol of ochratoxin A per dish for 48 h at 37°C, 4.4% of the substrate was converted to (4*R*)-4-hydroxyochratoxin A (Fig. 5A).

Formation of (4*S*)-4-hydroxyochratoxin A. With ochratoxin A concentrations above 100 μM and incubation times of 22 h or longer at 37°C, (4*S*)-4-hydroxyochratoxin A could be detected in the medium (data not shown). The ratio between the formation of the (4*S*) and (4*R*) isomers was about 1/100. No (4*S*)-4-hydroxyochratoxin A was detected in the hepatocytes.

DISCUSSION

Isolated hepatocytes have the capacity to metabolize a wide variety of substances (4, 5a), including toxins (10). Our results show that cultured rat hepatocytes are able to take up and hydroxylate up to 4.5% of the ochratoxin A added. This shows that these cells are able to hydroxylate ochratoxin A with a high yield. When ochratoxin A was given to rats in vivo, 0.5 to 1.5% of the ochratoxin A administrated was metabolized to (4*R*)-4-hydroxyochratoxin A (unpublished data). Even at a concentration of 750 μM, ochratoxin A did not promote cell detachment or cell death for up to 48 h.

At 4°C ochratoxin A associated with the cells at a level of about 7.5 nmol/mg of cell protein (Fig. 2A). Our data do not allow us to conclude that ochratoxin A is bound only to the surface of the hepatocytes at 4°C or that the substrate penetrates the plasma membrane and is localized inside the cells. It is possible that the whole ochratoxin A molecule could penetrate the plasma membrane and bind to a cytosolic acceptor molecule, as reported for androgens (7), fatty acids (16), bilirubin, azodye carcinogens, and steroids (9, 13). However, ochratoxin A is composed of one hydrophobic part (phenylalanine), which could probably be inserted into the lipid bilayer of the plasma membrane, and a hydrophilic part (the isocoumarin moiety), which would be dissolved in the extracellular water phase. This latter explanation would require endocytosis to get the toxin internalized. Maximal accumulation of ochratoxin A in the cells occurred after 3 to 6 h of incubation. Before this only small amounts of (4*R*)-4-hydroxyochratoxin A were formed. The lag in the formation of the hydroxylated product cannot be explained as a lag in binding of the toxin to the plasma membrane since there was maximal binding after 60 min of incubation (Fig. 2B). One explanation of the apparent lag may be that ochratoxin A induces an enzyme system(s) that participates in the hydroxylation of ochratoxin A. Another possibility is induction of transport systems responsible for uptake or release of ochratoxin A and its hydroxylated metabolites. After 3 to 6 h of incubation of ochratoxin A with hepatocytes at 37°C, 10 to 20 times more (4*R*)-4-hydroxyochratoxin A was recovered in the medium than in the cells (Fig. 5A and B). After 24 to 48 h of incubation the amount of (4*R*)-4-hydroxyochratoxin A in the medium was 700 to 3,000 times more than the amount associated with the hepatocytes. This suggests that once the toxin has been hydroxylated, the hepatocytes release the product quickly.

In vitro experiments showed that (4*R*)- and (4*S*)-4-hydroxyochratoxin A were formed in a ratio of 8:1, whereas in the hepatocytes the ratio was about 100:1. The reason for finding only trace amounts of (4*S*)-4-hydroxyochratoxin A produced by the hepatocytes could be that this metabolite is more susceptible to conversion to other compounds than (4*R*)-4-hydroxyochratoxin A. Previous results (20) have indicated that the microsomal hydroxylation system for ochratoxin A is cytochrome P-450 and that different species of this protein are involved in the formation of the two epimeric forms of 4-hydroxyochratoxin A. These two systems could also be

affected differently by compounds produced by the cells or present in the DME medium. It has been reported that ochratoxin A inhibits protein and RNA synthesis in cultured hepatoma cells and that this effect could be reversed by the addition of phenylalanine (2). The DME medium used in our experiments contained 364 μM L-phenylalanine. This may be sufficient to reverse the possible effect of ochratoxin A upon protein synthesis and may explain why the cells were viable and able to hydroxylate ochratoxin A in increasing amounts for up to 48 h.

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