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Metabolism and Lack of DNA Reactivity of the Mycotoxin Ochratoxin A in Cultured Rat and Human Primary Hepatocytes

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It is still unclear whether the carcinogenic mycotoxin ochratoxin A (OTA) is bioactivated to DNA-binding metabolites in rodents and humans. Therefore, we have incubated cultured rat and human primary hepatocytes with noncytotoxic concentrations of ^{3}H-OTA ranging from 10^{-7} to 10^{-5} M for 8 h and determined its metabolism and covalent DNA binding. In rat hepatocytes, OTA was metabolized to small amounts of three products, which were further studied by electrospray ionization (ESI)–MS/MS techniques. In addition to 4-hydroxy-OTA, which is a known product of OTA biotransformation, two novel metabolites were detected and tentatively identified as hexose and pentose conjugates of OTA. The in vitro induction with 3-methylcholanthrene (3MC) increased the formation of 4-hydroxy-OTA but did not alter the formation of the conjugated metabolites. No covalent binding of ^{3}H-OTA or its metabolites to DNA was observed in rat hepatocytes with or without 3MC induction with a limit of detection of 2 adducts per 10^9 nucleotides. However, the cellular ratio of reduced glutathione to oxidized glutathione was significantly decreased by treatment with OTA. In cultured human hepatocytes, ^{3}H-OTA was only very poorly metabolized, and no covalent DNA binding was observed. In conclusion, the results of this in vitro study do not support the notion that OTA has the potential to undergo metabolic activation and form covalent DNA adducts in rodents and humans.

KEYWORDS: Ochratoxin A; metabolism; covalent DNA binding; hepatocytes; human

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several species of Aspergillus and Penicillium molds. It contaminates a wide range of food and feed (*1*) and has been implicated in a large number of mycotoxicoses in farm animals (*2*). Besides numerous specific toxic effects (*3*), the mycotoxin has been proven to be carcinogenic in the kidney and liver in mice (*4, 5*) and rats (*6*). In humans, OTA appears to cause a fatal disease called Balkan Endemic Nephropathy (*7*–*9*) which is typically associated with a high incidence of kidney, pelvis, ureter, and urinary bladder tumors (*10, 11*). Therefore, OTA was classified by the International Agency for Research on Cancer as a possible human carcinogen (*12*).

The mechanism of OTA carcinogenesis has been a matter of considerable debate. OTA was first considered a nongenotoxic agent, because the results of several short-term tests for mutagenic activity were negative (*13*–*15*). However, more recent studies investigating various endpoints for genotoxicity gave positive results: OTA induces SOS DNA repair and gene mutations in bacterial systems (*16, 17*), sister chromatid exchange in cultured human lymphocytes (*16*) and in cultured porcine bladder epithelial cells (*18*), unscheduled DNA synthesis in cultured rat as well as in mouse hepatocytes and cultured pig urinary bladder epithelial cells (*19, 20*), DNA single strand breaks in mice spleen in vitro (*21*) and in liver, kidney, and spleen of rat and mice in vivo (*21, 22*), gene mutations in NIH/3T3 cells stably expressing human cytochrome P-450 (CYP450) activities 1A1/2, 3A4, 2C10 (*23*), chromosomal aberrations in cultured human lymphocytes (*24*), micronuclei in cultured ovine seminal vesicle cells (*25*), and clastogenic effects in mice in vivo (*26*). Moreover, in several investigations using the ^{32}P-postlabeling technique, OTA has been shown to induce DNA adducts in kidney, liver, spleen, and urinary bladder of the rat and the mouse in vivo (*27*–*35*) and in various mammalian in vitro systems (*36*–*40*). The adducts in vivo showed a time- and dose dependent response with maximum levels of 103, 42, and 2 adducts per 10^9 DNA nucleotides in the mouse kidney, liver, and spleen, respectively (*29*). However, in studies using ^{3}H-labeled OTA, no covalent OTA derived DNA adducts could be detected in the rat kidney and liver in vivo and in vitro (*35,*

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41). Thus, DNA adducts were detected thus far only when the ^{32}P-postlabeling technique was used.

A number of chemical carcinogens exhibit their genotoxicity after metabolic activation, which is usually mediated by CYP450. The highest activity of CYP450 enzymes is localized in the liver, from both a qualitative and a quantitative point of view. In the present study, we have therefore investigated the metabolism and covalent DNA binding of metabolically stable ^{3}H-labeled OTA in cultured rat hepatocytes. To assess the potential DNA reactivity of OTA in humans, the same studies were carried out in cultured human primary hepatocytes.

MATERIALS AND METHODS

Animals. Adult male Sprague−Dawley rats (R. Janvier, Breeding Center, Le Neufgenest St. Isle, France) weighing 200 to 300 g were housed under controlled conditions with a 12 h light and 12 h dark cycle and free access to water and food.

Chemicals and Reagents. G-^{3}H-OTA (0.6 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany), and G-^{3}H-OTA (15.5 Ci/mmol) and ring-labeled ^{14}C-aflatoxin B_1 (80 mCi/mmol)were purchased from Moravek Biochemicals (Brea, CA). ^{14}C-Verapamil (15 mCi/mmol) and ^{14}C-propafenone (18 mCi/mmol) were synthesized by Knoll AG (Ludwigshafen, Germany). OTA, ochratoxin B (OTB), 3-methylcholanthrene (3MC), calf thymus DNA, β-glucuronidase type LII, sulfatase type VI, ribonuclease type II-A, and Williams E medium were obtained from Sigma Chemie (Deisenhofen, Germany). Collagenase D, glutathione reductase, proteinase K, and carboxypeptidase A were from Roche Mannheim (Mannheim, Germany), collagen (type I) was from Serva Feinbiochemica GmbH (Heidelberg, Germany), and Dulbecco's modified Eagle medium was from Gibco Ltd. (Paisley, UK). Ochratoxin α (OTα) was prepared enzymatically as described by Grosse et al (*38*). The size-fractionated silica suspension for DNA extraction was obtained by following the procedure of Boom et al (*42*). Freshly prepared collagen solution from rat tail was kindly provided by the laboratory of Dr. Hans-Guenter Koebe, Klinikum Groβhadern, München, Germany.

Isolation and Culture of Rat Hepatocytes. Rat hepatocytes were isolated by a two-step collagenase perfusion procedure according to Gebhardt and Jung (*43*) and were seeded on collagen film-coated plastic culture dishes at a density of 10^6 viable cells per 10 cm^2 using Williams E medium supplemented with 10% fetal calf serum (FCS), 2×10^{-6} M L-glutamine, 100,000 U/L penicillin G, 100 mg/L streptomycin sulfate, and 10^{-7} M dexamethasone. The hepatocytes were kept at 37 °C in an incubator under humidified air containing 5% CO_2. At 4 h after seeding, the medium was replaced by supplemented FCS-free Williams E medium in order to remove any unattached nonviable cells. For some studies, CYP450 activity was induced with 3MC: following the medium change 4 h after plating, a final concentration of 1.5×10^{-6} M 3MC was added to the culture medium for 16 h. To control the efficiency of this treatment, the 7-ethoxyresorufin-*O*-deethylase (EROD) activity was measured according to the method of Burke and Mayer (*44*).

Isolation and Culture of Human Hepatocytes. Viable human hepatic tissue biopsies were obtained from three patients (patient 1, female, 55 years; patient 2, male, 51 years; patient 3, female, 26 years) undergoing liver resection of primary or secondary hepatomas, in agreement with the regulations of the local Ethical Committee. Preparation and culture of human hepatocytes were carried out in the laboratory of Dr. Hans-Günter Koebe, Klinikum Groβhadern, München (Germany) as described by Koebe et al (*45*).

Incubation of Hepatocytes with OTA. The radiolabeled OTA was purified by reversed-phase HPLC (see below for metabolite analysis) to >98% chemical and radiochemical purity prior to use. At 16 h after seeding, cultures of hepatocytes from three rats were exposed for 8 h to 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, and 10^{-5} M ^{3}H-OTA dissolved in dimethyl sulfoxide (DMSO); the radioactivity per culture was 13.5 μCi and the final DMSO concentration was 0.5% [v/v]. As well, cultures of human hepatocytes from three patients were exposed for 8 h to 10^{-6} M ^{3}H-OTA with 27 μCi per culture. Following incubation, the supernatant media was collected and the cells were harvested, and both were frozen in liquid nitrogen. Several control incubations were conducted with rat hepatocytes for 8 h, i.e., with culture medium only, with medium containing 0.5% DMSO, and with medium containing 0.5% DMSO and 1.5×10^{-6} M ^{14}C-aflatoxin B_1 (AFB). Moreover, 75.5 μM calf thymus DNA (equivalent to the amount of DNA in 10^6 hepatocytes in the corresponding incubation volume) was incubated for 8 h in sodium EDTA (SE) buffer (25 mM EDTA, 75 mM NaCl, 20 mM sodium phosphate buffer, pH 7.0) with the five ^{3}H-OTA concentrations used in the rat hepatocytes incubations in order to control for possible intercalation into DNA and/or ^{3}H exchange.

Determination of Covalent DNA Binding. Following incubation of the hepatocytes, the culture medium was removed for metabolite analysis (see below), and the DNA was isolated from the cells as follows: monolayers were first washed twice with phosphate buffered saline (PBS). Hepatocytes were then harvested in SE buffer containing 1% (w/v) sodium dodecyl sulfate, and incubated with proteinase K (final concentration 500 μg/mL) for 5 h at 45 °C. After addition of 1/3 volume of cold saturated NaCl solution, the homogenates were extracted with chloroform/isoamyl alcohol (24:1, v/v). The DNA was precipitated by adding an equal volume of 2-propanol; the precipitate was washed three times with ethanol/50 mM tris−HCl buffer pH 7.0 (7:3, v/v) and redissolved in sodium citrate buffer (150 mM NaCl, 15 mM sodium citrate, 20 mM sodium phosphate buffer, pH 7.0). Following incubation with DNAse-free RNAse A (final concentration 100 μg/mL) for 30 min at 37 °C, the DNA was extracted with chloroform/isoamyl alcohol, precipitated, washed, and dissolved as described above. For further purification, 10 volumes of chaotropic buffer (7 M $NaClO_4$, 1% sorbitol w/v, 100 mM tris−HCl, pH 7.0) and 2 volumes of silica suspension prepared according to Boom et al (*42*) were added, and the mixture was incubated at 45 °C for 15 min. The silica-bound DNA was washed once with the chaotropic buffer at 45 °C and three times with ethanol/ 50 mM tris−HCl buffer pH 7.0 (7:3, v/v) at 4 °C, and eluted by applying SE buffer twice for 15 min at 45 °C. This procedure provides a DNA of satisfactory purity but it is sufficiently gentle and does not result in the loss of DNA adducts.

To determine the extent of covalent DNA binding, the DNA concentration was quantified using Hoechst dye no. 33258 in 96-well plates according to the method of Rago et al (*46*). The DNA was then hydrolyzed with trichloroacetic acid using the method of Schmidt and Tannhauser (*47*), and the radioactivity was measured in a Packard Tricarb 2300TR liquid scintillation counter with external standard quench correction using Ultima Gold scintillation cocktail (Packard Instrument Company, Meriden, CT). Each sample was counted such that the 2σ values did not exceed 5%. The limit of detection was determined according to Currie (*48*) using DNA isolated from 10 hepatocyte cultures incubated with DMSO alone (see above) as background value.

Metabolite Analysis. Both the total OTA metabolites in the complete hepatocyte incubations and the extra-hepatocyte OTA metabolites in the supernatant media were recovered by solid-phase extraction and analyzed by reversed-phase HPLC. To determine the total metabolites, hepatocytes were lysed by addition of NaOH to the medium (final concentration 50 mM NaOH for 5 min). Both the alkaline hepatocyte lysates (containing intracellular plus extracellular metabolites) and the supernatants alone (containing only extracellular metabolites) were adjusted to pH 3.0 using acetic acid and subsequently applied to C8 cartridges (Waters, Eschborn, Germany). After the metabolites were washed with water, they were eluted with methanol. The methanol was evaporated under a flow of nitrogen, and the metabolites were dissolved in the initial mobile phase of the HPLC analysis. The recovery of metabolites was monitored by liquid scintillation counting. The HPLC system was from Merck (Darmstadt, Germany) and equipped with a radioactivity detection and quantification system from Raytest (Straubenhardt, Germany). A Symmetry-C8 column (4.6 × 250 mm, 5 μm; Waters, Eschborn, Germany) and a linear solvent gradient changing from 50% solvent B to 100% solvent B in 25 min at a flow rate of 1 mL/min were used. Solvent A was 1.0% aqueous acetic acid (pH 3.0 adjusted with aqueous ammonia) and solvent B was methanol/ acetonitrile (1:1, v/v).

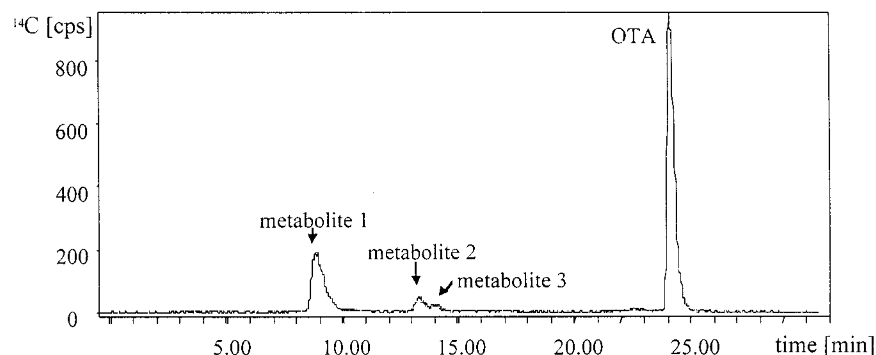


Figure 1. HPLC profile of the extract from the total incubation of rat primary hepatocytes incubated with ^{3}H-OTA (5×10^{-6} M for 8 h).

For the elucidation of the metabolite structures, HPLC fractions containing single metabolites from the extractions of the 5×10^{-6} and 10^{-5} M incubations with ^{3}H-OTA were collected manually, pooled, and subjected to solid-phase extraction on C8 cartridges to collect sufficient material for analysis by mass spectrometry (MS). The injection volumes for fractionation did not exceed 250 μL. About 15 pooled fractions of each metabolite were analyzed. HPLC conditions for HPLC−MS/MS analysis were the same as described above. HPLC−MS/MS was carried out using electrospray ionization in combination with a triple quadrupole mass spectrometer (TSQ 7000, Finnigan MAT, San Jose, CA) operated under the following conditions: capillary temperature 200 °C, (±)-spray voltage 6 kV, octapole voltages 0, 15, and 50 V. Nitrogen was the nebulizing gas at 80 psi. MS/MS experiments were based on collision-induced dissociation occurring in the radio frequency-only collision cell of the triple quadrupole, using a collision energy of 25 eV and xenon as the collision gas at 2.0 mTorr. The mass range monitored was *m/z* 105 to 900.

The incubations with the enzymes β-glucuronidase and sulfatase were carried out according to Weirich et al. (*49*) and to Fowler and Rammler (*50*), respectively, using phenolphthalein glucuronide and *p*-nitrophenol sulfate as controls.

Determination of Cellular Glutathione. Cultures of hepatocytes from 3 rats were incubated with culture medium alone, with culture medium containing 0.5% DMSO, and with culture medium containing 0.5% DMSO and 10^{-7}, 10^{-6}, and 10^{-5} M OTA for 8 h. Following treatment, cells were washed three times with PBS, and 5% 5-sulfosalicylic acid was added to each culture. Hepatocytes were harvested quantitatively with a rubber policeman, then frozen, thawed, and centrifuged at 15,000*g* for 2 min. The determination of the total glutathione concentration was carried out with aliquots of the protein-free supernatants by the enzymatic recycling assay according to Tietze (*51*). The quantification of oxidized glutathione (GSSG) was performed by the enzymatic recycling assay after derivatization of the reduced glutathione (GSH) using vinylpyridine as described by Griffith (*52*).

RESULTS

OTA Cytotoxicity and Metabolism in Rat Hepatocytes. Rat hepatocytes were incubated with 10^{-7}, 5×10^{-7}, 10^{-6}, 5×10^{-6}, or 10^{-5} M ^{3}H-OTA for 8 h. None of these concentrations caused leakage of intracellular lactate dehydrogenase into the culture medium, as was determined using the method of Bergmeyer (*53*). The viability of hepatocytes incubated under these conditions ranged from 89 to 100% of that of the controls.

At the end of the incubation period, the total hepatocyte incubations after lysis of the cells, as well as the supernatant media alone, were analyzed for OTA metabolites. HPLC of all extracts revealed the presence of four radioactive products (**Figure 1**). The major peak coeluted with OTA and therefore represents nonmetabolized parent compound. This was confirmed by HPLC−ESI-MS in the negative and positive ionization mode, which gave rise to the respective $[M - H]^-$ and $[M + H]^+$ ions (**Table 1**). The peaks of metabolites 1, 2, and 3 did not coelute with either of the reference compounds OTB or OTα; nor did treatment with β-glucuronidase, sulfatase, or γ-glutamyltranspeptidase alter retention times. When the metabolites were analyzed by HPLC−ESI-MS in the negative ionization mode, $[M - H]^-$ ions of *m/z* 564, 534, and 418 were observed for metabolites 1, 2, and 3, respectively, with an isotope pattern indicative of one chlorine atom (**Table 1**); analysis in the positive ionization mode gave rise to quasimolecular ions of *m/z* 583, 553, and 420 for metabolites 1, 2, and 3, respectively. As the respective ions for metabolite 3 differ by 16 mass units from that of OTA, it is assumed that metabolite 3 represents monohydroxylated OTA. Collision-induced fragmentation of $[M + H]^+$ *m/z* 420 with high collision energy gave rise to an intense peak at *m/z* 255, representing the hydroxylated isocoumarin moiety released after cleavage of the peptide bond; with OTA, the corresponding ion was found at *m/z* 239. Therefore, it is proposed that metabolite 3 is 4-hydroxy-OTA (**Figure 2**), which has previously been identified as an OTA metabolite (see Discussion).

Table 1. Major Ions (*m/z* values and their relative intensities) in the ESI-Mass Spectra of OTA and Its Metabolites (Only the Ions Containing the ^{35}Cl Isotope Are Given, although Each Listed Ion Contained the Typical Isotope Pattern for One Chlorine Atom)

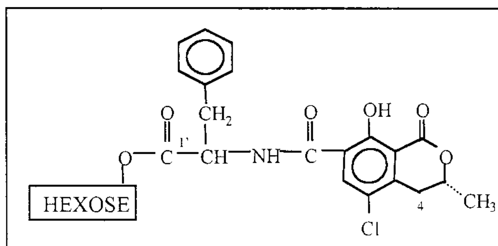
HPLC peak[a]	negative ionization mode	positive ionization mode	MW
1	564 (100%), 402 (41%)	583 (78%), 404 (100%)	565
2	534 (60%), 402 (100%)	553 (96%), 404 (100%)	535
3	418 (100%), 374 (62%)	420 (100%)	419
OTA	402 (100%), 358 (19%)	404 (100%)	403

[a] According to **Figure 1**.

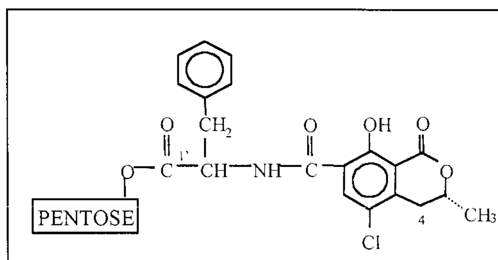
The mass spectra of metabolites 1 and 2 were more difficult to interpret. Assuming that the positive molecule ions reflect the association of NH_4^+, the molecular weight of metabolite 1 would be 565 and thus exceed that of OTA by 162 (**Table 1**). As the mass spectra of metabolite 1 obtained in the negative as well as positive ionization mode display prominent fragment ions for OTA at *m/z* 402 and 404, respectively (**Table 1**), it is likely that the 162 mass units reside in one moiety attached to the unchanged OTA molecule through the carboxyl group. Collision-induced fragmentation of *m/z* 583 with high collision energy gave rise to an intense peak at *m/z* 239, which was also observed with OTA (see above). A possible structure of metabolite 1, consistent with the mass spectrometric data and the chromatographic behavior is that of a hexose conjugate (**Figure 2**). The involvement of the carboxyl group is supported by the fact that, in contrast to OTA and 4-hydroxy-OTA, no loss of *m/z* 44 from the $[M - H]^-$ ion due to decarboxylation was observed (**Table 1**).

Metabolite 2 displayed mass spectra which were quite similar to that of metabolite 1 except that the quasimolecular ions were

Metabolite 1
Acyl hexose conjugate



Metabolite 2
Acyl pentose conjugate



Metabolite 3
4-(*R*)-Hydroxy-OTA

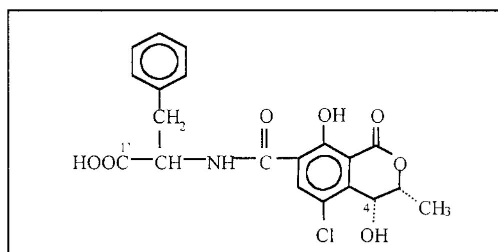


Figure 2. Proposed structures of the OTA metabolites formed in cultured rat primary hepatocytes.

30 mass units lower (**Table 1**). It is therefore proposed that metabolite 2 represents a pentose conjugate of OTA (**Figure 2**).

The formation of $[M + H]^+$ ions from OTA and 4-hydroxy-OTA, but not from the conjugated OTA metabolites 1 and 2 may be explained by the lower proton affinity of the conjugates. A very high ratio of $[M + NH_4]^+$ to $[M + H]^+$ has also been reported for the ESI-mass spectra of the glycosides digitoxin and amygdalin when ammonia-containing solvents were used (*54*).

The tentative identification of metabolites 1 and 2 as glycosides of OTA should be corroborated by enzymatic or chemical hydrolysis. However, the minute amounts available so far precluded this confirmation of the proposed structures.

Although the absolute amounts of metabolites 1−3 in the total hepatocyte incubations increased with increasing concentration of OTA, their relative proportions did not change. When the culture medium alone was analyzed, it was noted that the medium contained about 80% of the total radioactivity but only half the amount of OTA metabolites (with the same pattern) as found in the total incubations. This implies that the OTA metabolites formed in the hepatocytes are not readily released into the medium.

Effect of Treatment with 3MC on OTA Metabolism. When cultured rat hepatocytes were treated in vitro with 3MC, their EROD activity, which reflects principally the activity of CYP450 1A1 in rodents (*44*), increased about 6-fold. Incubation of the induced hepatocytes with ^{3}H-OTA and analysis of the metabolites by HPLC as before showed that no new metabolite was formed but the profile changed in a quantitative manner: metabolite 3 increased about 3-fold in comparison with untreated hepatocytes, whereas the amounts of metabolites 1 and 2 were not altered (**Figure 3**). Thus, induction of hepatocytes with 3MC leads to a marked enhancement of OTA hydroxylation, but does not affect the glycoside conjugation.

Lack of Covalent DNA Binding of OTA in Untreated and Induced Rat Hepatocytes. The DNA was isolated from the rat hepatocytes after incubation with various concentrations of ^{3}H-OTA as described for the metabolism studies, and the covalently bound radioactivity was determined (**Table 2**). Both noninduced and 3MC-induced hepatocytes were used. In each experiment, the covalent binding of OTA was below the limit of detection, which depends on both the quantity of isolated DNA and the amount of applied radioactivity (see Materials and Methods). The genotoxic carcinogen aflatoxin B_1 (AFB) was used as a positive control and gave rise to a high level of DNA adducts under the same conditions (**Table 2**).

Influence of OTA on the GSH/GSSG Ratio in Rat Hepatocytes. When rat hepatocytes were incubated with concentrations of OTA ranging from 10^{-7} to 10^{-5} M for 8 h and the cellular GSH/GSSG ratio was determined, a concentration-dependent decline of this ratio was observed (**Figure 4**). The effect was significant at the highest OTA concentration as compared to that for cells incubated for the same period of time with medium containing 0.5% DMSO (vehicle control). Further, no effect was noted in vehicle control cells compared to that of untreated cells.

OTA Metabolism and Lack of Covalent DNA Binding in Human Hepatocytes. Hepatocytes from three human patients were prepared in the laboratory of Dr. Koebe (Munich, Germany) and characterized with respect to their metabolic capabilities by using the drugs propafenone and verapamil. Both drugs were extensively metabolized (**Table 3**). However, when the human hepatocytes were incubated with 10^{-6} M ^{3}H-OTA

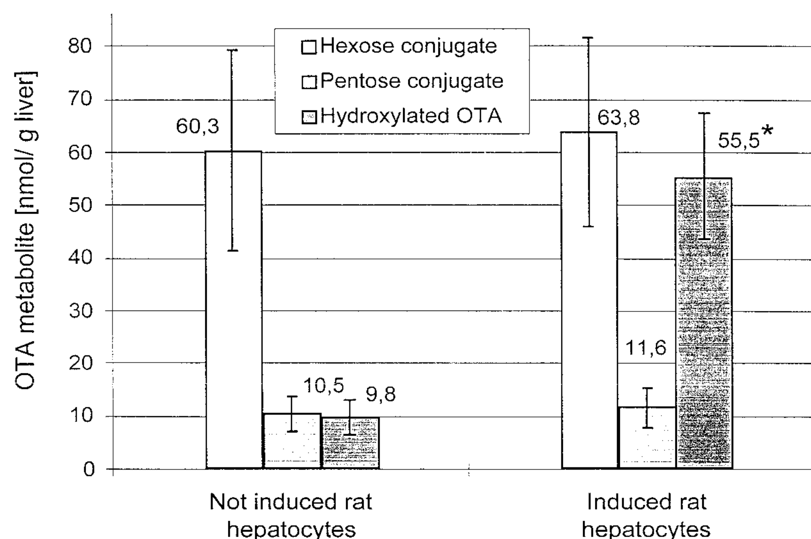


Figure 3. Effect of treatment of rat primary hepatocytes with 3MC on the pattern of OTA metabolites in the medium after incubation with 10^{-5} M ^{3}H-OTA for 8 h. Bars represent mean ± standard deviation of three hepatocyte preparations. * Significant according to Student's *t*-test, two-sided, paired, $p < 0.05$.

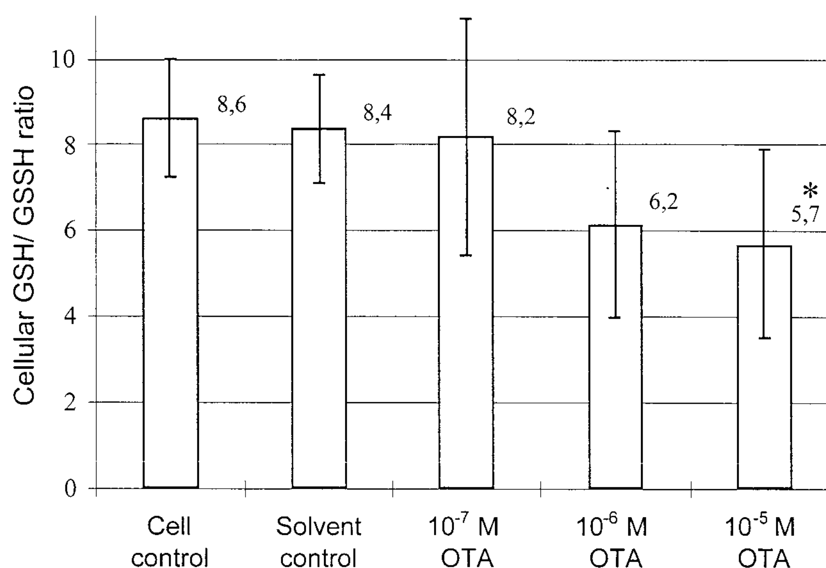


Figure 4. Effect of OTA on the cellular glutathione in rat primary hepatocytes after incubation for 8 h. Cell controls are incubations with medium only, and solvent controls are incubations with medium containing 0.5% DMSO. Bars represent mean ± standard deviation of three hepatocyte preparations. * Significant according to Student's *t*-test, two-sided, paired, $p < 0.05$.

Table 2. Covalent DNA Binding of ^{3}H-OTA and ^{14}C-AFB in Cultured Rat Primary Hepatocytes after Incubation for 8 Hours

compound	concentration	pretreatment	adducts per 10^9 DNA nucleotides (limit of detection)
OTA	10^{-7} M	none	– (2)
	5×10^{-7} M	none	– (2)
	10^{-6} M	none	– (2)
	10^{-6} M	3MC	– (2)
	5×10^{-6} M	none	– (13)
	10^{-5} M	none	– (38)
	10^{-5} M	3MC	– (24)
AFB	1.5×10^{-6} M	none	94,500 (1518)

for 8 h, only 3–6% of the dose was metabolized (**Table 3**). Analysis by HPLC revealed the formation of six OTA metabolites, two of which coeluted with the metabolites 1 and 3 formed by rat hepatocytes (see above). The additional four metabolites had retention times different from those of OTα and OTB and remain unknown at present, as their amounts were too small for structure elucidation by mass spectrometry. The radioactivity bound covalently to DNA after incubation with ^{3}H-OTA was below the limit of detection in two cases and at that limit in the third case (**Table 3**).

DISCUSSION

The purpose of our study was to clarify whether OTA is able to form DNA adducts when metabolized in cultured hepatocytes from rats and humans. Primary hepatocytes are known to express a variety of enzymes involved in the biotransformation of xenobiotics. Indeed, three metabolites of OTA were formed in rat hepatocytes, although in small amounts. One of them was identified by ESI–MS/MS as 4-hydroxy-OTA. This metabolite has also been detected in previous studies on the biotransformation of OTA in liver microsomes from rats, rabbits, pigs, and humans (*55–59*), as well as in cultured rat hepatocytes (*60*). In rat liver microsomes, both the R and S stereoisomers of

Table 3. Metabolism and Covalent DNA Binding of ^{3}H-OTA in Cultured Human Primary Hepatocytes

donor	propafenone metabolism	verapamil metabolism	OTA metabolism	covalent DNA binding of OTA[a]
female, 55 y	100%	73%	3.4%	– (2)
male, 51 y	100%	43%	3.8%	– (4)
female, 26 y	100%	44%	6.1%	2 (2)

[a] Adducts per 10^9 DNA nucleotides. The limit of detection is given in parentheses.

4-hydroxy-OTA were formed; because the R isomer was the predominant metabolite (*59*), it may be assumed that the 4-hydroxy-OTA identified in our study also represents the R form. This assumption is corroborated by the finding that pretreatment of the rat hepatocytes with 3MC, which led to a 6-fold enhancement of EROD activity, caused a pronounced increase in the amount of 4-hydroxy-OTA in our experiments. Zepnik et al. (*59*) reported that hepatic microsomes from 3MC-induced rats gave rise to elevated amounts of 4R- but not 4S-hydroxy-OTA. The failure to detect the S isomer in our study may be due to its minute amounts and/or its coelution with one of the other OTA metabolites.

Metabolites 1 and 2 represent novel biotransformation products of OTA. The ESI-mass spectra imply the presence of an unchanged OTA molecule conjugated with a hexose or pentose moiety, respectively. More studies are required to confirm these tentative structures and to elucidate the nature of the carbohydrate molecules. In general, the conjugation with glucose or other aldoses is common in insects and bacteria but rare in mammals (*61*). Examples are the conjugation of the drug pranoprofene with glucose in mice in vivo and in vitro (*62, 63*) and the conjugation of endogenous substrates such as bilirubin or bile acids with glucose and xylose by different mammalian species, including humans, in vivo (*64, 65*). It is presently unclear why OTA is not conjugated with glucuronic acid in rat hepatocytes. The absence of this and other conjugated metabolites of OTA, e.g., sulfates and glutathione adducts, is clearly demonstrated by the fact that treatment with the corresponding hydrolyzing enzymes had no effect on the observed elution times.

A very small amount of the presumptive hexose conjugate of OTA was also formed in cultured human primary hepatocytes, together with trace amounts of 4-hydroxy-OTA and four as-of-yet unidentified metabolites. The human hepatocytes were highly active in metabolizing the model compounds propafenone, involving CYP450 2D6 and 3A4 (*66, 67*), and verapamil, involving CYP450 3A4 and 1A2 (*68*). The very low capacity of human hepatocytes to biotransform OTA observed in our study is consistent with the reported low activity of the human CYP450 1A2, 3A4, and 2C9-1 toward OTA (*58*), and may, in part, explain why the half-life of OTA is more than 10 times longer in humans than in rats (*69, 70*).

DNA isolated from rat or human hepatocytes incubated with ^{3}H-OTA under various conditions did not contain covalently bound OTA metabolites. Under the conditions used in our study, the limit of detection was as low as 2 adducts per 10^9 DNA nucleotides. These results are in agreement with other studies using radioactively labeled OTA: no covalent binding of radioactivity was observed by Schlatter et al. (*41*) to liver and kidney DNA of rats in vivo, or by Gautier et al. (*35*) to rat kidney DNA in vivo and calf thymus DNA in vitro in the presence of various enzyme systems, including rat and human microsomes. However, evidence for DNA lesions was clearly detected in the rat kidney using the ^{32}P-postlabeling technique in the study of Gautier et al. (*35*) and in other reports mentioned earlier (see Introduction). The ^{32}P-postlabeling method does not distinguish between DNA adducts of the examined chemical and nonspecific adducts, e.g., caused by products of oxidative stress and cytotoxicity. Furthermore, from a quantitative point of view, our study was sufficiently sensitive (limit of detection of 2 adducts per 10^9 bases) to assess the DNA adduct levels obtained in the aforementioned ^{32}P-postlabeling studies. This suggests that those adducts under comparable dosing conditions determined by ^{32}P-postlabeling may not be structurally derived from OTA. Gautier et al. (*35*) proposed that OTA is not activated to a metabolite undergoing covalent DNA binding, but may mediate DNA lesions through its cytotoxicity. Our results support this notion, as they demonstrate the lack of covalent DNA binding of OTA in metabolically active rat and human hepatocytes and a decrease of the GSH/GSSG ratio after incubation with OTA, which may favor a prooxidant state of the cells.

ABBREVIATIONS USED

AFB, aflatoxin B_1; API, atmospheric pressure ionization; CYP450, cytochrome P450; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetate; EROD, 7-ethoxyresorufin-*O*-deethylase; ESI, electrospray ionization; FCS, fetal calf serum; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); HPLC, high-performance liquid chromatography; 3MC, 3-methylcholanthrene; MS, mass spectrometry; OTA, ochratoxin A; OTB, ochratoxin B; OTα, ochratoxin α; PBS, phosphate buffered saline; SE buffer, sodium EDTA buffer.

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