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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · OCTOBER 2006

Impact Factor: 3.53 · DOI: 10.1021/tx060138g · Source: PubMed

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Genotoxicity of the Hydroquinone Metabolite of Ochratoxin A: Structure-Activity Relationships for Covalent DNA Adduction

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Received June 22, 2006

Ochratoxin A (OTA) is a mycotoxin that shows potent nephrotoxicity and renal carcinogenicity in rodents. One hypothesis for OTA-induced tumor formation is based on its genotoxic properties that are promoted by oxidative metabolism. Like other chlorinated phenols, OTA undergoes an oxidative dechlorination process to generate a quinone (OTQ)/hydroquinone (OTHQ) redox couple that may play a role in OTA-mediated genotoxicity. To determine whether the OTQ/OTHQ redox couple of OTA contributes to genotoxicity, the DNA adduction properties, as evidenced by the ^{32}P-postlabeling technique, of the hydroquinone analogue (OTHQ) have been compared to OTA in the absence and presence of metabolic activation (pig kidney microsomes) and within human bronchial epithelial (WI26) and human kidney (HK2) cells. Our experiments show that OTHQ generates DNA adduct spots in the absence of metabolic activation. These adducts are ascribed to covalent DNA adduction by OTQ generated through autoxidation of the hydroquinone precursor, OTHQ. Although OTA does not interact with DNA in the absence of metabolism, the OTQ-mediated DNA adduct spots noted with OTHQ are also observed with OTA following treatment with pig kidney microsomes and NADPH, suggesting that OTA undergoes oxidative activation to OTQ by cytochrome P450 or enzymes with peroxidase activity. Comparison of DNA adduction by OTHQ and OTA in human cell lines shows that OTQ-mediated adduct spots form in a dose- and time-dependent manner. The adduct spots form at a faster rate with OTHQ, which is consistent with more facile generation of OTQ from its hydroquinone precursor. These results establish structure−activity relationships for OTA-mediated DNA adduction and provide new evidence for the potential role of the OTQ/OTHQ redox couple in OTA-induced genotoxicity.

Introduction

Ochratoxin A (OTA,[1] Figure 1) is a mycotoxin produced as a secondary metabolite by some *Aspergillus* and *Penicillium* fungal species (*1*). The toxin consists of a *para*-chlorophenolic moiety containing a dihydroisocoumarin group that is amide linked to L-phenylalanine. OTA is nephrotoxic and to date is one of the most potent renal carcinogens studied by the National Cancer Institute/National Toxicological Program (NCI/NTP) (*2*). OTA is associated with the fatal human kidney disease Balkan endemic nephropathy (BEN) (*3*, *4*). BEN is a chronic nephropathy endemic to restricted areas of the Balkans where high levels of OTA are found in food. Although controversial, OTA is suspected of being the etiological agent of BEN and its associated urinary tract cancers. The IARC has classified OTA as a possible human carcinogen (group 2B) on the basis of sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans (*5*).

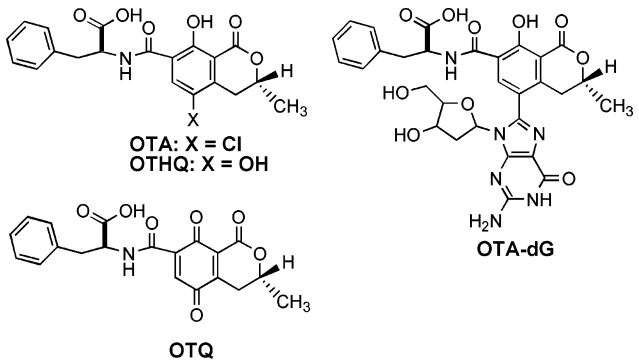

Figure 1. Structures of OTA, OTHQ, OTQ, and OTA-dG.

Although the mode of carcinogenic action of OTA is unknown, one hypothesis is based on its genotoxic properties, which follow from its oxidative metabolism by certain cytochrome P450 enzymes (*6*−*8*), or enzymes with peroxidase activities (*9*−*12*). The genotoxicity of OTA may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action. Support for an indirect mechanism involving OTA-mediated oxidative stress includes studies revealing OTA-dependent lipid peroxidation and free radical formation in mammalian cells (*13*−*15*), decreased levels of vitamin E in the plasma of rats (*16*), and depletion of glutathione in the liver of mice following OTA treatment (*17*). OTA causes oxidative DNA damage *in vitro* (*17*−*19*) and in rodents (*20*,

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[1] Abbreviations: OTA, ochratoxin A (*N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]carbonyl}-3-phenyl-L-alanine); OTHQ, ochratoxin A-hydroquinone; OTQ, ochratoxin A-quinone; OTA-dG, carbon (C)-bonded ochratoxin A-deoxyguanosine; PCP, pentachlorophenol; TCP, trichlorophenol; TCHQ, tetrachlorohydroquinone; NP1, nuclease P1.

10.1021/tx060138g CCC: $33.50
Published on Web 08/15/2006

21). Since the induction of oxidative DNA damage is considered to be a premutagenic genotoxic event (*22*), it has been speculated that oxidative DNA damage in combination with proliferative response might lead to cell-specific malignant transformation within the kidney following OTA treatment (*21*).

Evidence for a direct mode of OTA-mediated genotoxicity involving covalent DNA adduction has been derived from the sensitive ^{32}P-postlabeling assay (*23*−*25*). It has been demonstrated that OTA facilitates guanine-specific DNA adducts *in vitro* (*26*, *27*), and oral dosing of rats or pigs with OTA generates several lesions (*28*), one of which comigrates with a postlabeled 3′,5′-bisphospho-carbon (*C*)-bonded C8-dG OTA adduct standard (OTA-dG); the OTA-dG adduct precursor (Figure 1) of OTA-dG-3′-MP used for postlabeling has been fully characterized by LC/MS and 2D NMR spectroscopy (*29*). Levels of OTA-dG from rat kidney following chronic exposure to OTA are estimated at ∼1.5 adducts per 10^8 DNA bases (*30*). Because DNA adduction has been highly correlated with carcinogenesis (*31*, *32*), these results suggest that the induction of oxidative DNA lesions coupled with direct DNA adducts contribute to OTA-mediated carcinogenicity (*30*, *33*).

Despite positive ^{32}P-postlabeling evidence for DNA adduction by OTA, other researchers question the ability of OTA to act as a direct genotoxin carcinogen (*34*−*36*). One argument against OTA-mediated DNA adduction has been that the OTA metabolites identified are considered to be less toxic than OTA, and it is unlikely that their mechanisms of formation involve electrophilic intermediates (*36*). However, we have shown that the oxidation of OTA generates a quinone (OTQ)/hydroquinone (OTHQ) redox couple (Figure 1) through oxidative dechlorination of OTA (*37*−*39*). Other chlorinated phenols, such as pentachlorophenol (PCP) and 2,4,6-trichlorophenol (TCP), are known to undergo oxidative activation to generate benzoquinone electrophiles (*40*−*42*) that contribute to CP-mediated DNA adduction *in vivo* (*43*, *44*). The reduced tetrachlorohydroquinone (TCHQ) metabolite of PCP is an established genotoxin (*45*, *46*) that stimulates oxidative DNA damage (*47*, *48*) and covalent DNA adduction (*43*, *44*). This background information coupled with the recent finding that administration of OTA to rats by gavage generates the ochratoxin hydroquinone metabolite (OTHQ) in urine (*49*) suggested the potential importance of OTHQ in OTA-mediated genotoxicity. Thus, the present study was performed to determine whether OTHQ contributes to OTA-mediated DNA adduction in vitro and in human bronchial epithelial (WI26) and kidney (HK2) cells using the ^{32}P-postlabeling method for adduct detection. The results of our studies show that the OTQ/OTHQ redox couple of OTA does indeed contribute to OTA-mediated DNA adduction in human cells as expected.

Experimental Procedures

Caution: *The work described involves the handing of hazardous agents and was, therefore, conducted in accordance with NIH guidelines for the Laboratory use of Chemical Carcinogens.*

Materials. OTA (benzene free, CAS# 303-47-9) was purchased from Sigma (Saint Quentin Fallavier, France). OTHQ was available in the laboratory at the University of Guelph, Canada and was chemically synthesized from 4-methoxyphenol as a mixture of diastereomers, as outlined previously (*37*). The following enzymes were purchased: proteinase K (used as received), RNase A, RNase T1 (boiled 10 min at 100 °C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water) were from Sigma (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France), nuclease P1 (NP1), and T4 polynucleotide kinase were from Roche diagnostics (Meylan, France). [γ ^{32}P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France); Dubelco's Eagle's minimum essential medium (D-EMEM) and Roswell Park Memorial Institute medium (RPMI) were prepared with Gibco products (Cergy Pontoise, France); phosphate saline buffer, trypsine, fetal calf serum, streptomycin, and penicillin were from Life-Technologies (Cergy-Pontoise, France); rotiphenol (phenol saturated with TRIS-HCl at pH 8) was from Rothsichel (Lauterbourg, France); salmon testis DNA was from Sigma and was purified before use; cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA); Whatman N° 1 paper (ref 6130932) was from VWR (France) and PEI/cellulose TLC plates used for ^{32}P-postlabeling analyses were prepared in the laboratory at Toulouse, France. All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, hydrochloric acid, acetic acid, and sodium dihydrogen phosphate) were of normal grade.

Microsomal Preparation and Incubation. Pig kidney was homogenized in a buffer solution (0.15 M KCl and 50 mM NaH_2PO_4 at pH 7.4) with fluorosulfonyltoluene (10 μL/mL) and aprotinine (1 μL/mL). The buffer volume was three times the organ weight. After an initial centrifugation at 9000*g* for 20 min, the supernatant was taken and ultracentrifuged at 105 000 *g* for 1 h. The pellets were homogenized in 1 to 2 mL of pH 7.4 buffer containing 1 mM EDTA, 1 mM dithiothreitol (DTT), and 20% glycerol. The mixture was centrifuged again at 105 000*g* for 1 h to obtain a final concentration of about 10 mg of protein, which was stored at −80 °C prior to analysis. All steps of the isolation were carried out at 4 °C. The BCA method (dosage of total protein level with cupric reagent and bicinchoninic acid) was employed to measure protein levels in the isolated microsomes. A sample of each microsomal preparation (10 μL) was diluted with a 0.9% NaCl solution (90 μL). To 50 μL of this dilution, 1 mL of the cupric reagent was added, and the mixture was measured spectrophotometrically at 562 nm and compared to a standard curve determined with 0.2−1.2 mg/mL of solutions of bovine serum albumin.

Microsomal reactions with OTA were carried out in 200 μL of TRIS-HCl (50 mM) at pH 7.4 containing 1 mM EDTA, 70 μg of pig kidney microsomes, and 70 μg of salmon testis DNA that was purified before use. This mixture was incubated for 3 min at 37 °C, and then 10 μL of NADPH (stock solution 10 mg/mL) was added, and the mixture was incubated for 45 min at 37 °C.

Cell Culture. Human bronchial epithelial cells (WI26; ATCC CCL-95.1) and human kidney cells (HK2; CRL-2190) were provided by ATCC (American Type Culture Collection, Manassas, Virginia). WI cells were cultured in RPMI and HK2 in D-EMEM medium containing 44 mM $NaHCO_3$, 5% fetal bovine serum (FBS), 2% vitamins, 2% of nonessential amino acids, 1% streptomycin, and 1% penicillin, for 48 h at 37 °C under 5% CO_2. After trypsin digestion, the cells were re-suspended in this medium to obtain 1 × 10^6 cells per mL and treated as follows. The cells were incubated for 2, 7, or 24 h in the presence of 0.5, 1, or 2.5 μM OTA or OTHQ. All experiments were performed in triplicate, and the results are expressed as average values.

Isolation of DNA. Cell pellets were homogenized in 0.8 mL of a solution containing NaCl (0.1 M), EDTA (20 mM), and Tris-HCl at pH 8 (50 mM) (SET) in an ice bath. To the homogenate, 100 μL of a 20% solution of sodium dodecylsulfate was added, and following incubation for 10 min at 65 °C, 800 μL of potassium acetate (6 M, pH 5) was added. The reaction mixture was kept at 0 °C for 30 min. After centrifugation for 25 min at 0 °C (10 000*g*), the supernatant, which contained nucleic acids, was collected, and nucleic acids were precipitated overnight at −20 °C by adding 2 volumes of cold ethanol. The DNA pellets were collected and washed once with 1 mL of 90% ethanol and dissolved in 500 μL of SET (15 min at 37 °C). The total extract was mixed with 10 μL of a mixture of RNase A (20 mg/mL) and RNase T1 (10 000 U/mL) and incubated for 1 h at 37 °C; this treatment was repeated twice. Samples were then treated with 25 μL of proteinase K solution (20 mg/mL SET) for 1 h at 37 °C. After digestion, 500 μL of rotiphenol was added, and the mixture was moderately shaken for

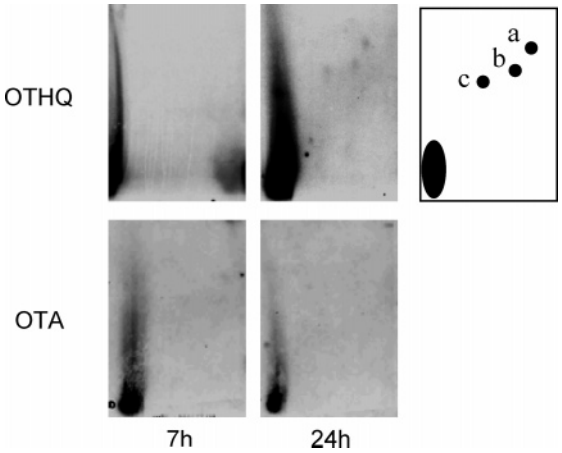

Figure 2. ^{32}P-postlabeling analysis of salmon sperm DNA following incubation for 7 and 24 h with OTHQ (1 μM) or OTA (1 μM) at 37 °C in 20 mM TRIS-HCl at pH 7.4 and 0.1 M KCl. The scheme of adduct numbering is also shown.

20 min at room temperature and centrifuged for 15 min at 15 °C (10 000 *g*). The aqueous phase was collected after two extractions. After a final extraction with one volume of chloroform/isoamyl alcohol (24:1), the aqueous phase was collected, and 50 μL of sodium acetate (3 M, pH 6) was added. The DNA was precipitated by the addition of two volumes of cold ethanol overnight at −20 °C, and the precipitate was collected by centrifugation at 10 000*g* for 30 min. The DNA pellet was washed four times with 90% ethanol. DNA was dissolved in deionized water and tested for purity by UV−vis spectroscopy.

^{32}P-Postlabeling Analysis of DNA Adducts. The equivalent of 4 μg of DNA were dried in vacuum, dissolved in 10 μL of the mix containing 1 μL of micrococcal nuclease (2 mg/mL corresponding to 500 U), spleen phosphodiesterase (15 mU/μg DNA), 1 μL of sodium succinate (200 mM), and 1 μL of calcium chloride (100 mM, pH 6), and digested at 37 °C for 4 h. The digested DNA was then treated with 5 μL of the mix containing 1.5 μL of NP1 (4 mg/mL), 1.6 μL of $ZnCl_2$ (1 mM), and 1.6 μL of sodium acetate (0.5 M, pH 5) at 37 °C for 45 min. The reaction was stopped by the addition of 3 μL of Tris base (500 mM). The DNA adducts were labeled as follows: to the NP1 digest, 5 μL of the reaction mixture containing 2 μL of bicine buffer (bicine (800 μM), dithiothreitol (400 mM), $MgCl_2$ (400 mM), and spermidine (400 mM) adjusted to pH 9.8 with NaOH), 10 U of polynucleotide kinase T4, and 100μCi of [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) was added and the mixture incubated at 37 °C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed by chromatography on PEI/cellulose TLC plates (D1) in 3 M NaH_2PO_4 buffer at pH 5.7 overnight. The origin (4 cm) areas containing labeled adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which was run (D2) for 3 h in 4.8 M lithium formate and 7.7 M urea at pH 3.5. A further (D3) migration was performed after turning the plate 90° anticlockwise in 1 M NaH_2PO_4 and 4.5 M urea at pH 6.4 for 3 h. Finally, the chromatogram was washed in the same direction in 1.7 M NaH_2PO_4 at pH 6 for 2 h (D4). Adduct profiles were analyzed qualitatively and semiquantitatively by autoradiography of the plates, carried out at −80 °C for 48 h in the presence of an intensifying screen, using a radio-analytical system of image analysis (AMBIS, Lablogic).

Results

DNA Adduction without Metabolic Activation. Initial experiments were carried out to determine whether OTHQ can react covalently with DNA in the absence of metabolic activation. Like other hydroquinones, OTHQ undergoes autoxidation ($t_{1/2}$ = 11 h at pH 7.4, 37 °C) to generate the superoxide and the quinone electrophile OTQ (*37*, *39*). Thus, OTHQ is more reactive than OTA, which is not oxidized by O_2 alone (*50*), and therefore, it was anticipated that OTHQ may be capable of covalently interacting with DNA through an autoxidative process.

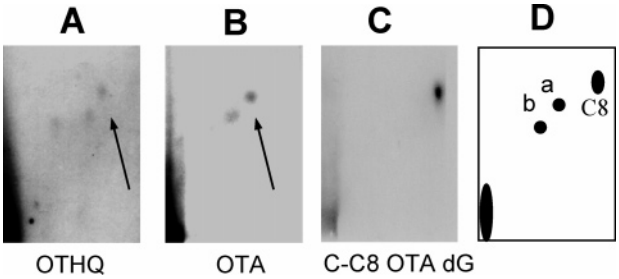

Figure 3. ^{32}P-postlabeling analysis of salmon sperm DNA following incubation for 24 h at 37 °C in 20 mM TRIS-HCl at pH 7.4 and 0.1 M KCl with (A) OTHQ (1 μM) in the absence of added cofactors and (B) OTA (1 μM) in the presence of pig kidney microsomes and NADPH. (C) Autoradiogram of postlabeled 3′,5′-bisphospho-OTA-dG adduct standard. (D) Numbering of DNA adducts observed in B and C; C8 corresponds to the adduct standard.

Figure 2 shows 2D thin layer chromatography (TLC) maps obtained following the treatment of salmon sperm DNA with OTHQ or OTA (1 μM) at 37 °C in 20 mM TRIS-HCl at pH 7.4 containing 0.1 M KCl. For OTHQ, no discernible adduct spots were detected following 7 h of incubation. However, after 24 h, three faint adduct spots (a, b, and c) were observed. These OTHQ-mediated DNA adducts do not comigrate with the postlabeled 3′,5′-bisphoso-OTA-dG adduct standard (Figure 1); the 3′,5′-bisphospho-OTA-dG adduct standard migrates faster, suggesting that the OTHQ-mediated DNA adducts are less polar. For OTA, no DNA adducts were detected following 7 or 24 h of incubation time (Figure 2), which is consistent with the fact that unlike OTHQ, OTA does not undergo an oxidative process to generate an electrophilic species in the absence of added cofactors (*50*).

DNA Adduction with Metabolic Activation. To draw a comparison between DNA adduction mediated by OTHQ with OTA in vitro, pig kidney microsomes in the presence of NADPH was employed to activate OTA. Figure 3 shows the adduct pattern for OTHQ by autoxidation versus the adduct pattern generated by OTA following treatment with the pig kidney microsomes and NADPH. With microsomal activation, OTA yields two clear adduct spots (a and b, indicated by the arrow) that possess the same migration properties as two of the adduct spots (a and b) detected from the autoxidation of OTHQ. This result shows a direct correlation between DNA adduction mediated by the autoxidation of OTHQ with DNA adduction by OTA following microsomal activation. This result also correlates with our earlier findings that OTHQ reacts with GSH to form a conjugate that is also produced by OTA and GSH following activation by rat liver microsomes and NADPH (*39*). It is also important to point out that the adduct spots detected in Figure 3 do not comigrate with the postlabeled 3′,5′-bisphospho-OTA-dG adduct standard (Figure 1).

DNA Adduction in WI26 Cells. Further insight into the role of the OTQ/OTHQ redox couple in OTA-mediated DNA adduction was derived from a study of the dose- and time-dependence of adduct spot formation in human cell lines. Figure 4 shows the adduct pattern obtained following OTHQ (0.5 μM) or OTA (0.5 μM) treatment of human bronchial epithelial (WI26) cells that have been employed previously to study OTA metabolism and DNA adduction (*6*, *11*). For OTHQ, two adduct spots noted in Figure 2 from the autoxidation of OTHQ were visible following 2 h of incubation time (spots indicated by the

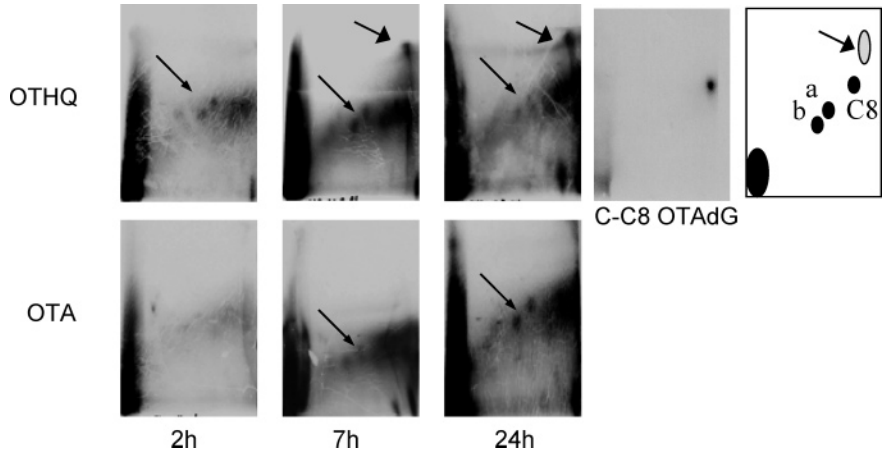

Figure 4. TLC maps of ^{32}P-labeled DNA adducts obtained following treatment of human bronchial epithelial cells (WI26) with OTHQ (0.5 μM) or OTA (0.5 μM) for 2, 7, and 24 h. The autoradiogram of the postlabeled 3′,5′-bisphospho-OTA-dG adduct standard (C-C8 OTAdG) and the scheme of DNA adducts are also shown.

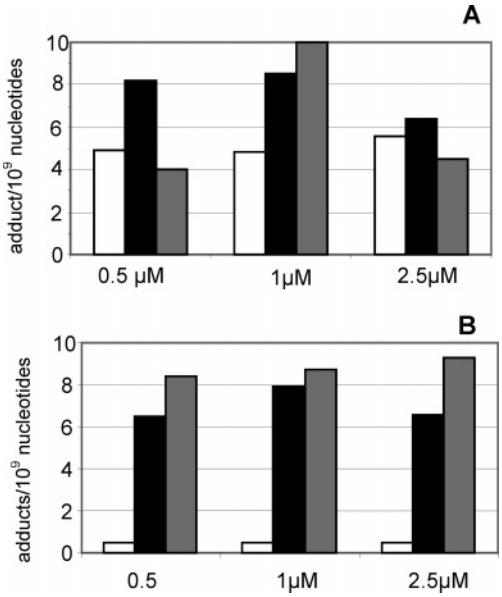

Figure 5. Dose- and time-dependence of two common adduct spots (a and b) in WI26 cells for (A) OTHQ and (B) OTA at 0.5, 1.0, and 2.5 μM following 2 h (white), 7 h (black), and 24 h (gray) of treatment. All experiments were performed in triplicate, and the results (adducts per 10^9 nucleotides) are expressed as average values.

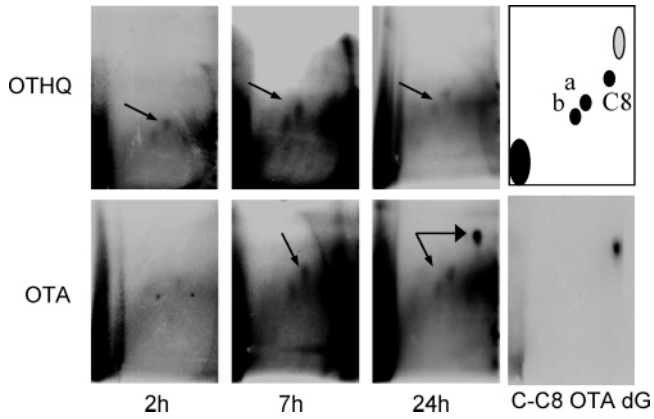

Figure 6. TLC maps of ^{32}P-labeled DNA adducts obtained following the treatment of human kidney cells (HK2) with OTHQ (1 μM) or OTA (1 μM) for 2, 7, and 24 h. The autoradiogram of the postlabeled 3′,5′-bisphospho-OTA-dG adduct standard and the scheme of DNA adducts are also shown.

arrow in Figure 4). After 7 h, a new polar adduct that migrates close to the solvent front is also visible, and this spot is indicated by the larger arrow head. This adduct also does not comigrate with the postlabeled 3′,5′-bisphospho-OTA-dG adduct standard (Figure 1); it migrates too fast and, thus, is more polar than the C8 adduct standard. After 24 h, the two adduct spots have decreased in intensity, while the more polar adduct spot remains. In the case of OTA, no discernible adduct spots were detected following 2 h of incubation. However, after 7 h, the two adduct spots noted in the TLC maps for OTHQ were visible, and after 24 h, they were observed to grow in intensity.

Figure 5 shows the dose- and time-dependence of adduct spot formation for OTHQ (Figure 5A) and OTA (Figure 5B) in WI26 cells. Relative adduct levels are expressed as adducts/10^9 nucleotides and represent the combined levels of the two common adduct spots (a and b) for OTHQ and OTA, which are indicated by the small arrow head in Figure 4. Levels of the polar adduct (indicated by the larger arrow head) for OTHQ are not given. For OTHQ at 0.5 μM, adduct levels peak at 7 h and then decline after 24 h, which may be due to repair. At 1 μM OTHQ, a general increase in adduct levels is observed over the 24 h of incubation time, whereas with the highest dose tested (2.5 μM), a decrease in adduct levels is observed, which may be due to the onset of OTHQ-mediated cytotoxicity. For OTA, adduct levels remain fairly constant for the three doses (0.5−2.5 μM) and exhibit an increase over the 24 h of incubation time with undetectable adduct levels at 2 h for the three doses tested.

DNA Adduction in HK2 Cells. Figure 6 shows the adduct pattern obtained following OTHQ (1.0 μM) or OTA (1.0 μM) treatment of human kidney (HK2) cells, the target organ of OTA-mediated carcinogenicity. Generally, the adduct pattern was similar to the pattern observed in WI26 cells; the two adduct spots (a and b) indicated by the small arrow head form at a faster rate with OTHQ than with OTA. However, in HK2 cells, OTA shows a very clear and new adduct spot (indicated by the larger arrow head) following 24 h of incubation. This adduct was not detected for OTHQ in both cell lines, nor was it formed for OTA in WI26 cells under the time frame of the experiments. This adduct comigrates with postlabeled 3′,5′-bisphospho-OTA-dG (*28*) and was tentatively ascribed to the formation of the C8 OTA-dG adduct on the basis of its chromatographic properties. From the labeling efficiency of the OTA-3′-GMP adduct standard (*28*), the amount of adduct ascribed to OTA-dG present in Figure 6 was ∼18 adducts/10^9 nucleotides, which

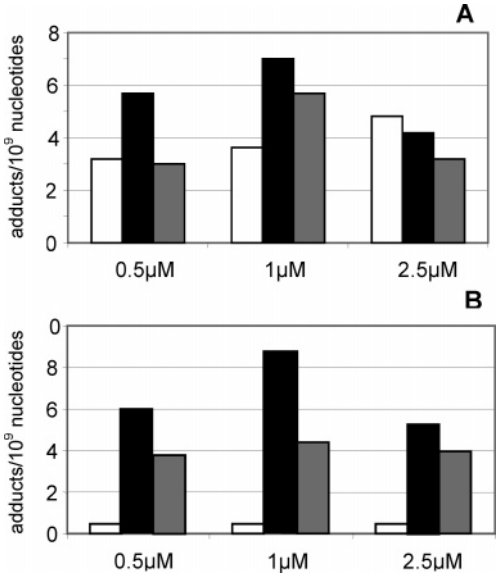

Figure 7. Dose- and time-dependence of DNA adducts a and b in HK2 cells for (A) OTHQ and (B) OTA at 0.5, 1.0, and 2.5 μM following 2 h (white), 7 h (black), and 24 h (gray) of treatment. All experiments were performed in triplicate, and the results (adducts per 10^9 nucleotides) are expressed as average values.

is roughly 2-fold higher than the levels of adducts a and b formed by both OTHQ and OTA.

Figure 7 shows dose- and time-dependence of adducts a and b for OTHQ (Figure 7A) and OTA (Figure 7B) in HK2 cells, whereas Figure 8 shows a comparison between OTHQ and OTA (both at 0.5 μM) in the two cell lines. As shown in Figure 8, adduct levels of the two common spots (a and b) are slightly higher in WI26 cells than that in HK2 cells. In HK2 cells, OTA follows the pattern observed for OTHQ with the adduct levels peaking at 7 h and declining following 24 h of incubation, in contrast to the adduct pattern noted for OTA in WI26 cells.

Discussion

The aim of this study was to determine the role of the OTQ/OTHQ redox couple in OTA-mediated DNA adduct formation. Previous efforts have shown that the oxidation of OTA generates the quinone derivative OTQ (*37*−*39*), analgous to the oxidation of other chlorinated phenols (*40*−*42*). An oxidative mechanism is known to play a role in OTA-mediated genotoxicity (*6*−*12*), suggesting that the OTQ/OTHQ redox couple would contribute to OTA-mediated DNA adduction, as established for the TCHQ metabolite of PCP (*43*, *44*). Biological studies have predicted a role for OTQ in OTA-mediated DNA adduction (*51*, *52*), but direct evidence to support this hypothesis has been lacking.

To assay for OTA-mediated DNA adduct formation, the sensitive ^{32}P-postlabeling technique was employed. This technique does not provide structural information, and it has been argued that adduct spots attributed to OTA treatment may be due to oxidative stress or the modulation of endogenous DNA base modification (*36*). However, in the present work, we have utilized the reference hydroquinone metabolite OTHQ for comparison to DNA adduction mediated by the parent OTA. The hydroquinone OTHQ is known to undergo autoxidation (*37*) to furnish the quinone electrophile OTQ (*39*). The ^{32}P-postlabeling results presented in Figure 2 show adduct spots for OTHQ treatment of DNA in the absence of metabolism. This represents the first case in which an OTA metabolite has been shown to react directly with DNA in the absence of added cofactors. Because the ^{32}P-postlabeling technique with NP1 enrichment is sensitive to bulky adduct detection and no additives have been added to the OTHQ/DNA mixture, it is difficult to argue that the observed adduct spots are derived from endogenous sources. Thus, the adduct spots detected in Figure 2 were ascribed to covalent DNA adduction by OTQ generated through the autoxidation of its hydroquinone precursor. The observation of these reference adduct spots for OTHQ autoxidation made it possible to determine whether OTA forms the same adduct spots following microsomal activation or within human cells.

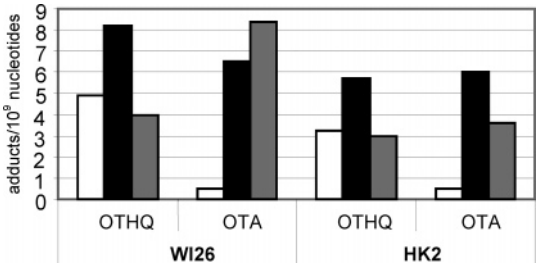

Figure 8. Time-dependence of DNA adduct formation in WI26 and HK2 cells for OTHQ and OTA at 0.5 μM following 2 (white), 7 (black), and 24 h (gray). All experiments were performed in triplicate, and the results (adducts per 10^9 nucleotides) are expressed as average values.

The postlabeling results presented in Figure 3 show a direct correlation between DNA adduction mediated by OTHQ autoxidation with OTA following treatment with pig kidney microsomes and NADPH. Pig kidney microsomes are a good model for the bioactivation of OTA in human kidneys, the toxin's target organ. That OTA forms the same adduct spots as the autoxidation of OTHQ argues strongly that the microsomal system converts OTA into the OTQ electrophile. This is consistent with our earlier findings (*37*−*39*), the well-known fact that chlorinated phenols undergo P450-catalyzed oxidations to form benzoquinones (*40*, *41*, *53*), and our predictions from biological studies on the modulation of OTA-mediated DNA adduction by thiol nucleophiles (*51*, *52*). The adduct spots detected in Figure 3 do not comigrate with the postlabeled 3′,5′-bisphospho-OTA-dG adduct standard (Figure 1) that migrates at a faster rate, suggesting that the OTQ electrophile is not responsible for C8-dG adduct formation that is produced by the photoactivation of OTA (*29*). This finding is consistent with the known tendency of quinone electrophiles to react with nucleophilic nitrogen atoms on the DNA bases (*54*−*56*); C8-dG adduct formation has not been reported for a quinone electrophile.

A correlation between OTHQ- and OTA-mediated DNA adduction was also found with both human bronchial epithelial (WI26) and kidney (HK2) cells. In each cell line, the two adduct spots (a and b) detected from the in vitro activation of OTA (Figure 3) and from OTHQ autoxidation (Figures 2 and 3) were formed in a time- and dose-dependent manner (Figures 4−8). For OTHQ, the adduct spots ascribed to OTQ-mediated DNA adduction formed faster in the cell lines than that from autoxidation (2 h vs 24 h), suggesting that metabolic activation facilitates the conversion of OTHQ into OTQ. The two adduct spots from OTHQ treatment also formed faster than the spots from OTA treatment (2 h vs 7 h), which is consistent with OTQ formation being a more facile process from OTHQ. Although clear kinetic differences in adduct formation was observed, the total peak adduct levels of OTQ-mediated adducts displayed by both OTHQ and OTA were roughly the same (8−10 adducts/10^9 nucleotides), and increasing the toxin dose typically inhibited DNA adduction, which was attributed to the onset of cytotoxicity. These results suggest that the efficiency of DNA adduction

by the OTQ electrophile is poor. This species is a monoanion that would be electrostatically repelled by DNA, and like most quinone electrophiles, it reacts readily with GSH (*39*). We have also monitored the aqueous decomposition of OTHQ, and the OTQ electrophile could not be detected directly, suggesting its limited lifetime in aqueous media (*37*). Taking these factors into consideration, it is not surprising that OTQ forms low levels of covalent DNA adducts.

In human kidney cells, an adduct spot for OTA treatment that comigrates with the postlabeled 3′,5′-bisphospho-OTA-dG standard was also noted following 24 h of incubation (Figure 6). This adduct forms at a slower rate than adducts attributed to OTQ, and it is not generated by the OTHQ metabolite. The regioselectivity for C8 attachment at dG by OTA suggests the intermediacy of a radical species that may involve reductive dehalogenation of OTA to form a carbon-centered radical, as discussed previously (*30*).

In summary, the present experiments provide new ^{32}P-postlabeling evidence for DNA adduction by the hydroquinone metabolite (OTHQ) of the chlorophenolic mycotoxin ochratoxin A (OTA). OTHQ reacts directly with DNA to form adduct spots that are ascribed to covalent attachment by the quinone electrophile (OTQ) generated by OTHQ autoxidation. OTHQ is the first OTA derivative to react covalently with DNA in the absence of metabolism. The adduct spots generated by OTHQ treatment are also produced by the parent OTA following activation with pig kidney microsomes or in human cell lines. In human cells, clear kinetic differences in adduct formation were visible, which highlight the more facile OTQ formation from the OTHQ precursor; OTQ-mediated adducts form slower than OTA. The present experiments support the hypothesis that the OTQ electrophile participates in OTA-mediated DNA adduction and that the observed adducts may contribute to carcinogenesis. Further work is in progress to chemically identify these adducts and confirm their presence in human tissue by mass spectroscopy.

Acknowledgment. This research was supported by the EU project 'Mechanisms of ochratoxin A induced carcinogenicity as a basis for an improved risk assessment' Ochratoxin A – Risk assessment : contract N° QLK1-CT-2001-01614 and "Région Midi-Pyrénées" program on Food safety 2000-2005 n° 0301160 (A.P.L).

Note Added after ASAP Publication. The caption for Figure 8 was incorrect in the version published ASAP August 15, 2006; the corrected version was published ASAP September 18, 2006.

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