Metabolism and Nucleic Acid Binding of N-Hydroxy-4-acetylamino-biphenyl and N-Acetoxy-4-acetylamino-biphenyl by Cultured Human Uroepithelial Cells

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ABSTRACT

Metabolic activation of N-hydroxy-4-acetylamino-biphenyl (N-OH-AABP) and N-acetoxy-4-acetylamino-biphenyl (N-OAc-AABP), the proximate carcinogenic metabolites of the human bladder carcinogen 4-aminobiphenyl (ABP), was examined in human uroepithelial cells (HUC). Bioconversion was studied by incubating HUC cultures with [3H]-N-OAc-AABP or [3H]-N-OH-AABP. Three organo-soluble metabolites, N-OH-AABP, 4-acetylamino-biphenyl (AABP), and ABP were identified in ethyl acetate extracts from cultures exposed to N-OAc-AABP. Similarly, AABP and ABP were characterized as the major metabolites from cultures treated with N-OH-AABP. Incubation of N-OAc-AABP with HUC microsomes in vitro yielded primarily the O-deacetylation product N-OH-AABP. The HUC microsomes also catalyzed the N-deacetylation of N-OAc-[14C]-AABP, N-OH-[14C]-AABP, and [3H]-AABP. The O- and N-deacetylase activities for N-OAc-AABP were 55.9 and 38.2 nmol/min, respectively. These O- and N-deacetylase activities were both blocked by paraoxon. Incubation of [3H]-N-OH-AABP or [3H]-N-OH-AABP with HUC microsomes and tRNA or DNA showed that 23.0 and 8.0 nmol of N-OAc-AABP and 74.5 and 25.2 pmol of N-OH-AABP were bound per mg protein/mg RNA or DNA, respectively. In comparison, the acetyl-CoA-dependent HUC cytosol-mediated bindings of [3H]-N-OH-ABP to RNA and DNA were 801 and 447 pmol/mg nucleic acid/mg protein. The HUC microsome-mediated bindings of N-OAc-AABP and N-OH-AABP to nucleic acids were inhibited by paraoxon, whereas the cytosol-mediated binding of N-OH-ABP was insensitive to paraoxon inhibition. Chromatography of the DNA hydrolysate obtained from the in vitro incubation of [3H]-N-OAc-AABP or [14C]-N-OH-AABP with HUC microsomes showed N-deoxyguanosine-8-yl]-4-aminobiphenyl as the major adduct, based on comparison with authentic synthetic standard. These results show that human uroepithelia contain microsomal acetyl transferases that are capable of converting the proximate metabolites N-OAc-AABP and N-OH-AABP of the human bladder carcinogen ABP, to reactive electrophiles that bind to DNA. The occurrence of these acetyl transferases in the target organ of the human bladder carcinogen ABP suggests that metabolic activation of some proximate metabolites of ABP could occur directly in HUC and could play a pivotal role in susceptibility to aryl-amine/acetaldehyde induced human bladder cancers.

INTRODUCTION

ABP and several other related arylamines have been shown to be causally involved in the induction of human urinary bladder cancers (1–3). ABP induces a wide spectrum of tumors in a number of experimental animals (4–6). Arylamines, including ABP, were used in chemical industries and occur in cigarette smoke and in other sources (7–9). Hemoglobin-ABP adducts have been detected in the blood of smokers as well as non-smokers, although at much greater levels among smokers, who show an increased risk for bladder cancer (10, 11). Thus, ABP and related aryl and heterocyclic amines represent an important class of environmental contaminants.

The various metabolic activation pathways that generate reactive electrophiles from arylamines or amides have been extensively studied in experimental animals (reviewed in Ref. 12). The activation of arylamines and acetamides in general involves N-oxidation by hepatic enzymes followed by conjugation with acetate, sulfate, or glucurate (12–16). Additional activation pathways by peroxidative metabolism, deacetylation, intramolecular N→O-trans-acetylation and intermolecular trans-acetylation have been documented (17–20). In bladder carcinogenesis, the N-glucuronides formed by hepatic metabolism are postulated to be transported to the urinary bladder where they are hydrolyzed to the N-hydroxy derivatives (16, 21, 22). Under the acidic conditions of urine, the latter are thought to generate the aryl nitrenium ions that interact with critical cellular nucleophiles to initiate neoplasia (16). Alternatively, the procarcinogens or their proximate metabolites could be activated by the enzyme systems that are present in the urothelium. Specifically, with arylamines, in addition to N-glucuronide conjugates of N-hydroxyarylamines, O-glucuronide conjugates of N-hydroxyarylamides are excreted as metabolites in urine (23). These glucuronide conjugates could be hydrolyzed under alkaline conditions or by β-glucuronidases to hydroxamic analogues, which then could enter the uroepithelia. Which of these metabolites represents the proximate carcinogenic species in humans is not completely understood.

A number of studies have shown that urinary bladder tissues metabolically activate various carcinogens, including ABP (24–27). This is further supported by studies on DNA-adduct formation (28, 29), uroepithelial cell-mediated mutagenesis (30, 31), and DNA-repair synthesis (32, 33). Most of these studies used experimental animal systems, and species differences preclude the extrapolation of these data to humans. We have developed a HUC culture system that permits us to grow and propagate a large number of cells to conduct metabolism and other biochemical and molecular biological studies (34, 35). HUC cultured using this system have been characterized and shown to represent normal human uroepithelial cells, the target cell for ABP carcinogenesis (36). In this system, we showed recently that N-OH-ABP, N-OH-AABP, and N-OAc-AABP cause tumorigenic transformation and neoplastic progression of an immortalized cell line of HUC (37). Using this cell culture system, we determined whether ABP or its proximate carcinogenic N-hydroxy metabolites, N-OH-ABP, N-OH-AABP, and N-OAc-AABP, are activated to reactive electrophile species by HUC. In the present study, we demonstrate that normal human uroepithelia possess acetyl transferases that catalyze the conversion of N-OH-ABP, N-OAc-AABP, and N-OH-AABP to reactive electrophiles that bind to DNA. These results suggest that N-OAc-AABP resulting from O-acetylation of N-OH-ABP, N-deacetylation of N-OAc-AABP, or trans-acetylation of N-OH-AABP might play a determinate role in the initiation of
bladder neoplasia in humans and that susceptibility to ABP-induced bladder carcinogenesis might in part be determined by these acetyl transferases.

MATERIALS AND METHODS

Chemicals. NBP, ABP, paraaxon, 2′-deoxyguanosine, calf thymus DNA, and diithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO. Yeast tRNA was purchased from Calbiochem Corp., La Jolla, CA. Acetyl chloride, dimethyl sulfoxide, acetic anhydride, 10% palladium on activated carbon, trifluoroacetic anhydride, 2,6-dichlorobenzoyl chloride, deuto chloroform, deuto dimethyl sulfoxide, and tetramethyl silane were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. The sources of biochemicals for tissue culture were the same as those cited earlier (34-36). ABP and NBP were recrystallized from ethanol, and the crystalline preparations were determined to be greater than 99% pure based on HPLC and TLC. N-OH-ABP was synthesized from NBP by ammonium sulfide reduction using published methods (38) and was recrystallized from warm benzene. N-OAC-AABP was synthesized by acetylation of N-OH-ABP using acetyl chloride in the presence of triethylamine. N-OH-AABP was prepared by hydrolysis of N-OAC-AABP using a method described for the synthesis of N-hydroxy-2-acetylaminofluorene (39). The structural authenticities of these chemicals were confirmed by UV, infrared, proton nuclear magnetic resonance, and mass spectral analyses. The chromatographic purities of these chemicals were >99%.

Synthesis of Labeled Substrates. [3H]4-Nitrobiphenyl (571 mCi/mmol) uniformly labeled on the aromatic rings was purchased from Midwest Research Institute, Kansas City, MO. [3H]N-OH-ABP, [3H]N-OH-AABP, and [3H]N-OAC-AABP were prepared using procedures similar to those described above for the synthesis of unlabeled compounds with the exception that the precursors were diluted appropriately with unlabeled compounds to yield the required specific activities. [3H]AABP was prepared by acetylation of [3H]ABP with acetic anhydride in the presence of triethylamine. [3H]AABP was obtained by catalytic hydogenation of [3H]NBP using 10% palladium on activated carbon. N-OH-[4C]AABP and N-O-[14C]Ac-AABP were synthesized by acetylation of N-OH-ABP and N-OH-AABP, respectively, using [1-14C]acetyl chloride (50 mCi/mmol; purchased from ICN Radiochemicals, Irvine, CA). N-OAC-[14C]AAABP was prepared by acetylation of N-OH-[14C]AABP using acetyl chloride in the presence of triethylamine. [3H-C]H2AABP was prepared by acetylation of ABP using [3H]acetic anhydride (50 mCi/mmol; purchased from New England Nuclear Research Products, Boston, MA) in the presence of triethylamine. The radiochemical purities of these compounds were assessed by HPLC interfaced to a Flow One-Beta radioactivity detector (Radiomatic Instruments and Chemicals Co., Inc., Tampa, FL) and were determined to be >95% pure.

Cell Culture Methods. The techniques used to culture HUC have been described in detail elsewhere (34, 36). Fresh specimens of normal ureter, available as a byproduct of kidney transplantation surgery were used to initiate the primary cultures. Following culture for 7–10 days, the explants were removed and discarded. The cells were dispersed using 0.1% EDTA and then replated at a density of about 1.5–2 × 10^6 cells/100-mm dish. The cells were allowed to attach and grow for 2–3 days, following which the media were replaced with serum-free F12* medium (35). The test chemicals dissolved in dimethyl sulfoxide as 10× stock solution were added to each culture. The concentrations of chemicals used for these studies were based on cytotoxicity experiments that demonstrated dose-dependent responses in HUC (35). In these earlier reports, the cytotoxic responses were quantified based on viability measurements made 5–7 days after exposure of HUC to the carcinogens. This was done in order to account for any latent effect that these chemicals might exert on HUC, as in long term mutagenicity or transformation experiments. In the present metabolic studies, the viability measurements were made immediately following exposure of HUC to these chemicals, which generally showed less cytotoxicity than when viable cell counts were measured after 5–7 days of growth. In these experiments, we found that a single exposure of HUC to 10 μM N-OH-AABP or N-OAc-AABP for as long as 24 h did not significantly affect cell viability. Measurement of the relative stability of these chemicals under the culture conditions revealed that ABP, AABP, and N-OH-AABP were relatively stable with a half-life greater than 24 h. In contrast, N-OAC-AABP was less stable and had a half-life of about 5 h. For this reason, metabolism studies with N-OAC-AABP on HUC cultures were done for a duration of 4 h, unless otherwise specified. With all chemicals, the medium was removed at the end of incubation period, and the cells were rinsed with 0.25 M sucrose. The layer of cells was dispersed with 0.1% EDTA and viable cell counts were done as described (36). The cells were pelleted by centrifugation at 1000 × g for 10 min; the media and the sucrose washings were pooled, then extracted immediately 5 times with an equal volume of ethyl acetate. The organic phase was separated, evaporated to dryness, and analyzed for metabolites by chromatographic and spectroscopic methods.

Analysis of the Metabolites. HPLC was performed in a Hewlett Packard 1090M liquid chromatograph equipped with a diode array detector and interfaced with a Flow One-Beta radioactivity detector. The instrument was fitted with a 25-cm Hamilton PRP-1 Poly(styrene-divinylbenzene) polymer column. The chromatogram was run isocratically under a constant flow rate of 1.0 ml/min using a mixture (50:50, v/v) of acetonitrile and water. The absorbance of the eluates was continuously monitored at 265 nm. The UV absorption spectra of the eluates were continuously monitored by the diode array detector, and the spectra of components of interest were processed at the end of the chromatographic analyses.

TLC was performed on 0.1-mm-thick E. Merck Silica gel 60/Kiesel gur F-254 plates (obtained from Brinkmann Instruments, Inc., Westbury, NY). The TLC solvent systems used and the Rf values determined for N-OAC-AABP, N-OH-AABP, AABP, and ABP, respectively, were: carbon tetrachloride:ethanol (94:6), 0.6, 0.2, 0.3, and 0.4; and chloroform:triethylamine (85:15), 0.9, 0.7, 0.4, and 0.8. The TLC plates were cut into 0.3-cm strips and radioactivity monitored using Tracer Analytic Mark III liquid scintillation counter, after adding 5 ml of organic counting solvent (Amersham Corp., Arlington Heights, IL).

Mass spectral analyses were performed by direct injection of the sample into the ion source at an initial inlet temperature of 150°C, in a Kratos-MS-50 mass spectrometer.

O-Deacetylase Activities of Microsomes. Qualitative assessment of O-deacetylase activities was carried out using N-OAC-AABP, wherein the formation of N-OH-AABP was examined by HPLC. For quantitative studies, the amount of [14C]acetate released into the aqueous phase following incubation of N-O-[14C]Ac-AABP, (label at the acetyl group attached to the O-function, 2.0 μCi/μmol) with microsomes was measured. Incubations were conducted at 37°C and the final concentrations of the components were: substrate, 0.5 mM; sodium phosphate buffer, 50 mM, pH 7.4; diithiothreitol, 0.5 mM; and microsomal protein, 0.1–0.5 mg/ml. Following incubation of substrate with the microsomes, 1 ml of 0.1 M sodium acetate, pH 7.0, was added as a carrier and the incubate was extracted 3 times with ethyl acetate. The organic phase was back-washed once with a small volume of water and the washing was pooled with the original aqueous phase and brought to a known volume with water. An aliquot was taken for measurement of radioactivity by scintillation methods. Based on the specific activity of the substrate and the amount of labeled acetate released into the aqueous phase, the O-deacetylase activities were calculated. Appropriate controls were incubated and processed under identical conditions to correct for non-enzymatic hydrolysis of the substrate.

O-Deacetylase (carboxylesterase) activities were quantitated spectrophotometrically by measuring the yellow phenol liberated from 4-nitrophenyl acetate using a published method (40). Typically the assay mixture contained in a 2-ml volume 1 μmol of 4-nitrophenyl acetate and 0.1 μmol of Tris, pH 8.0. Following addition of the buffer to the substrate, the spontaneous hydrolysis, if any, was measured for 1 min at 37°C, using a Shimatzu spectrophotometer. The enzyme reaction was started by addition of 40 μg of microsomal protein in 40 μl of buffer, and 40 μl of the release of 4-nitrophenol was monitored spectrophotometrically continuously for 2 min using a recorder. The increase in absorbance was linear up to 1.5 units of absorbance. Based on the linear portion of the graph and the estimate of the molar extinction coefficient of 16,400
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N-Deacetylation (Amidase) Activities of HUC Microsomes. N-Deacetylase activities were monitored by measuring the amounts of radioactive activity released into the aqueous phase following incubation of N-OH-[14C]AABP (label at the acetyl function, 11.5 Ci/µmol), N-OAc-[14C]-AABP (label at the acetyl group attached to the N-function, 2.19 Ci/µmol), or N-[14CH3]-AABP (label at the methyl of the acetyl function, 14.4 Ci/µmol) with microsomes. The assay conditions for deacetylation were similar to those described above for O-deacetylation of N-O-[14C]Ac-AABP. For these experiments, about 100 × 10^6 cells from four different HUC cultures were pooled, and the microsomes were prepared and used in these assays. Based on the specific activities of the substrates, the amounts of product formed were calculated and expressed as nmol/mg protein/min.

Nucleic Acid Binding Assays. Binding of [3H]N-OH-AABP and [3H]-N-OAc-AABP to tRNA was measured using a modification of an earlier published method (15). The volume of the reaction mixture was 1.3 ml and contained 0.05 M phosphate buffer, pH 7.0; tRNA, 1.5 mg/ml; dithiothreitol, 0.5 mM; and [3H]N-OH-AABP (110 Ci/µmol) or [3H]-N-OAc-AABP (7.5 Ci/µmol), 0.5 mM. Incubations were carried out at 37°C for 30 min, and the reaction was terminated by the addition of an equal volume of buffer-saturated phenol. After centrifugation at 8000 × g for 10 min, the aqueous layer was removed and the RNA was precipitated by adding 9 volumes of 95% ethanol containing 2% sodium acetate. The RNA was collected by filtering through a glass fiber filter. The RNA was dried in a vacuum. The residue was chromatographed on a Sephadex G-25 column and eluted with buffer containing 0.1 MTris, pH 7.5. The RNA was precipitated by adding 2 volumes of 95% ethanol containing 2% sodium acetate. The tRNA was collected by filtering through a glass fiber filter. An aliquot of the RNA solution was diluted appropriately to measure the absorbance at 260 nm. Another aliquot was taken for measurement of radioactivity by liquid scintillation counting. Based on the extent of radioactivity incorporated into RNA and the specific activities of the substrates, the amounts of label bound per unit weight of nucleic acids were estimated. Twenty absorbance units at 260 nm were estimated to be equivalent to 1 mg of nucleic acid.

Measurement of binding of [3H]N-Oac-AABP or [3H]N-OH-AABP to DNA in vitro by HUC microsomes was performed using a modification of the procedure described by Beland et al. (41). The incubation conditions were similar to those described for N-deacetylation reaction except that calf thymus DNA was added exogenously to give a final concentration of the procedure described by Beland et al. (41). The incubation conditions were similar to those described for A'-deacetylation reaction (42). Samples (1 ml) contained 0.05 M phosphate buffer, pH 7.4, 2.0 mg RNA or DNA, 0.1 mM [3H]-AABP, and 0.3 mg HUC microsomal protein. After preincubation for 5 min at 37°C, arachidonic acid (0.1 mM) was added to initiate the reactions, which were terminated by addition of buffer-saturated phenol after an additional 5-min incubation at 37°C. For inhibition of PHS, indomethacin (0.25 mM) was added prior to the 3-min preincubation. Analysis of the DNA Adducts. DNA containing adduct(s) was enzymatically hydrolyzed to nucleosides (43). Nucleoside adducts were extracted into n-butanol as described (43) and analyzed by HPLC using a C-18 reverse-phase column. Conditions of HPLC analyses are given in the legend to Fig. 6. Synthetic N-(4'-OH)-yABP was prepared by interaction of N-acetoxy-4-trifluoroacetyl-aminobiphenyl with deoxyguanosine, using a modification of an earlier reported procedure (44). N-Hydroxy-4-trifluoroacetyl-aminobiphenyl (200 mg) (44) was acetylated in ether at 0°C with acetic anhydride in the presence of triethylamine. After the reaction, the ether solution was added to 2'-deoxyguanosine (100 mg) in dimethyl sulfoxide (8 ml). The ether was removed in a vacuum and 450 µl of 1 N citrate buffer, pH 5.5, and 1.5 ml of water were added. The reaction was stirred at 45°C for 90 min, then diluted to 50 ml with water and extracted with n-butanol. The butanol layer was back-washed with water to remove dimethyl sulfoxide, then dried in a vacuum. The residue was chromatographed on a Sephadex LH-20 column in 50% aqueous ethanol. Fractions were monitored by TLC (cellulose, 30% acetic acid). Fractions containing the adduct (Rf = 0.4, detected by blue fluorescence in UV) were pooled and dried. Final purification was accomplished on a silica gel column in ethyl acetate-celtern (3:1) to give 5 mg N-(dG2'-yABP). The acetyl adduct N-(4'-OH)-yABP was synthesized by reaction of N-(2',6'-dichlorobenzoyloxy)-4-acetylaminobiphenyl with 2'-deoxyguanosine as described (45). The fast atom bombardment mass spectra and the proton nuclear magnetic resonance spectra of these synthetic standards were consistent with their chemical structures, and they were similar to those described earlier for these adducts (23, 45-47), confirming the structural authenticitys of these synthetic adducts.

RESULTS

Metabolism of [3H]N-OAc-AABP and [3H]N-OH-AABP by HUC. Quantitation of the distribution of radioactivity in the organic and aqueous phases of the HUC culture media exposed to [3H]N-OAc-AABP or [3H]N-OH-AABP revealed that the majority, i.e., about 96.5 and 85% of radioactivity, respectively, was distributed in the ethyl acetate extract. Fig. 1A shows the HPLC profile of the ethyl acetate extract following 4-h exposure of HUC to [3H]N-OAc-AABP, indicating the complete disappearance of the substrate. Two major UV absorbing peaks containing radioactivity were observed at elution times of about 8 and 11 min, in addition to 2 minor metabolites at 3 and 27 min. The early eluting peak was present in small amounts and contained other contaminants that precluded its structural identification. The metabolites corresponding with the retention times of 8 and 11 min were identified as N-OH-AABP and AABP based on their chromatographic and spectral characteristics. Fig. 1B shows the retention times of the synthetic standards N-OH-AABP and AABP along with their absorption spectra obtained from the diode array detector. Both components with retention times of 8 and 11 min showed absorption spectra similar to those observed with synthetic compounds (see Fig. 1, A and B, inserts) thus confirming their structural identity. The metabolites corresponding with the retention times of 8 and 11 min were identified as N-OH-AABP and AABP based on their chromatographic and spectral characteristics. Fig. 1C shows the profile of the extract obtained from cultures after 24-h exposures to [3H]N-OAc-AABP. In these samples, the N-OH-AABP was completely converted to AABP (retention time, 11 min) and furthermore, the amount of the component eluting at 27 min was increased. The retention time and the absorption spectra of the late eluting 27-min component were the same as those observed with AABP (see Fig. 1, B and C). These same metabolites, AABP and ABP, were also observed in cultures exposed to N-OH-AABP (Fig. 1D), thus showing that N-OH-AABP might be an intermediate metabolite in the bioconversion of N-OAc-AABP to AABP. These metabolites were further characterized by silica gel TLC (chloro-
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These metabolites were further characterized by mass spectral analyses.

For isolation of the metabolites for mass spectra, a larger scale preparation of the extract from N-OAc-AABP-treated cultures was injected into HPLC, and the 8- and 11-min fractions were collected, dried, and then injected directly into Kratos MS/25 mass spectrometer using a probe. Fig. 3 shows the mass spectra of these different metabolites. The low resolution mass spectrum of the metabolite 1 (Fig. 3A), which eluted at retention time of 8 min, showed a molecular ion at a m/e of 227, corresponding with N-OH-AABP (C_{14}H_{13}NO_2). Additional fragments at 211 (from loss of oxygen), 185 (from loss of *COCH_2), and 169 (loss of *O and *COCH_2) were observed, consistent with the structure. Similarly, the metabolite with the retention time of 11 min in HPLC showed the molecular ion at a m/e of 211 with the base peak at 169, resulting from the loss of acetyl group (Fig. 3B) and is in compliance with the fragmentation patterns based on its chemical structure. The mass spectrum of the late eluting component (Fig. 3C) showed primarily one major ion with a mass of 169, corresponding with ABP. Based on these spectroscopic and mass spectral characteristics, the metabolites of N-OAc-AABP were identified as N-OH-AABP, AABP, and ABP.

**HUC Microsome-mediated Metabolism of N-OAc-AABP, N-OH-AABP, and AABP.** The detection of N-OH-AABP as a metabolite of N-OAc-AABP suggested that HUC contain deacetylases or esterases that are capable of O-deacetylation. Since microsomes have been known to contain deacetylase(s) and since they have been shown to be capable of activating N-OAc-AABP and N-OH-AABP to reactive electrophiles, we investigated the HUC microsome-mediated activation of N-OAc-AABP. In the case of N-OAc-AABP, deacetylation could conceivably occur either by removal of the acetyl group attached to the O-function (O-deacetylation or esterase activity) or N-function (N-deacetylation or amidase activity). On O-deacetylation of N-OAc-AABP, N-OH-AABP is formed, whereas on N-deacetylation, a highly reactive product, N-OAc-ABP, is formed. In order to test the metabolic capabilities of HUC to catalyze the above reactions, we first examined the metabolism of [3H]-N-OAc-AABP using HUC microsomes. Fig. 4 shows the HPLC profile of the extract of the reaction mixture obtained after

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**Fig. 1.** HPLC profile of the organo soluble metabolites. The media were extracted after exposure for 4 h (A) or 24 h (C) to [3H]N-OAc-AABP or 24-h exposure to [3H]N-OH-AABP (D). B, chromatographic profile of a mixture of synthetic standards of N-OAc-AABP, N-OH-AABP, AABP, and ABP. The UV spectra obtained with the diode array detector are presented in the inserts (A). Inserts at top, left to right: spectra of metabolites with retention times of 8, 11, and 27 min, respectively; B, spectra of N-OH-AABP and AABP (top) and N-OAc-AABP and ABP (bottom); C and D, spectra of metabolites with retention times of 11 and 27 min. The HPLC conditions are described in "Materials and Methods."

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form: triethylamine; 85:15 v:v) wherein the ethyl acetate extracts of the media from [3H]N-OAc-AABP- or [3H]N-OH-AABP-treated cultures showed radioactivity at a R_f of 0.4 and 0.8, corresponding with AABP and ABP, respectively (Fig. 2). Similar results were also observed (data not shown) using carbon tetrachloride:ethanol (94:6) as the developing solvent in TLC, where radioactivity was detected at R_f values of 0.3 and 0.4, corresponding with the region of mobility of AABP and ABP.

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**Fig. 2.** TLC profiles of ethyl acetate extracts. The media after exposure to [3H]N-OAc-AABP or [3H]N-OH-AABP were extracted and analyzed on silica gel TLC plates, using chloroform and triethylamine (85:15) as the developing solvent. The relative mobilities of standards run under similar conditions are marked in the chromatogram.

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the HUC microsome-mediated formation of N-OH-AABP from N-OAc-AABP (Fig. 4B), suggesting the involvement of microsomal deacetylases in this process. Quantitative assessments of O-deacetylase activities were carried out using radioactively labeled N-O-[14C]Ac-AABP (labeled at the acetyl group attached to the O-function). The amount of [14C]acetate released into the aqueous phase following incubation with microsomes was measured after extraction of the unreacted N-OAc-AABP from the reaction mixture with ethyl acetate. The results of these experiments, presented in Table 1, show that HUC exhibit O-deacetylase activities. The occurrence of O-deacetylase(s) in the HUC microsomes was further confirmed by measurement of esterase activities using p-nitrophenyl acetate as a substrate. The esterase activity of HUC microsomes was determined to be about 0.1 µmol/mg protein/min, in comparison to 4 µmol/mg protein/min observed with rat hepatic microsomes.

Similar to O-deacetylation, N-deacetylation was quantified by measurement of labeled acetate released from N-OAc-[14C]-

Fig. 3. Low resolution mass spectra of the metabolites. Metabolites 1 and 2 were isolated by HPLC chromatography (retention time, 8 min and 11 min, A and B, respectively) of the organic extract of the media after 24-h exposure to N-OAc-AABP. Metabolite 3 (C) corresponds with the late eluting component (retention time, 27 min).

incubation of N-OAc-AABP with HUC microsomes. The retention time and the absorption spectrum of the product correspond with N-OH-AABP (Fig. 4A), thus showing that HUC catalyze the conversion of N-OAc-AABP to N-OH-AABP. Paraoxon, an inhibitor of microsomal carboxy esterase(s), inhibited
AABP, N-OH-[14C]AABP, or [3H]-CH3]AABP (labeled at the methyl of acetyl attached to N-function). The results of these experiments presented in Table 1 show that HUC also catalyze the N-deacetylation of these substrates but quantitatively the activities were significantly different. The rates of N-deacetylation of N-OH-AABP and AABP were 40- to 100-fold lower when compared against N-OAc-AABP. In contrast, the N-deacetylase activity of HUC was in a similar range as that observed for O-deacetylation, when N-O-[14C]Ac-AABP was used as the substrate. Similar to O-deacetylation, the N-deacetylase activities of HUC were also blocked by paraoxon, suggesting that similar microsomal deacetylase(s) might be involved in both these deacetylation processes. N-Deacetylation of AABP to ABP was further documented by incubation of HUC-microsomes with [3H]AABP and analyzing the product generated. Fig. 5 shows the HPLC profile of the organic extract of the microsomal incubates of [3H]AABP in the absence (top panel) or presence (bottom panel) of paraoxon. As shown, the deacetylation product, ABP, was observed following incubation with HUC-microsomes, and its formation was blocked by paraoxon.

HUC Microsome-mediated Binding of [3H]N-OAc-AABP, [3H]N-OH-AABP, and [3H]ABP. Since N-deacetylation of N-OAc-AABP results in the formation of N-OAc-ABP, which can generate the reactive electrophilic aryl nitrenium ion that could bind to nucleophiles, we examined the deacetylase-mediated binding of [3H]N-OAc-AABP to nucleic acids. Table 2 shows the binding of [3H]N-OAc-AABP to RNA and DNA following incubation with HUC microsomes. Binding to RNA was about 3 times greater compared to DNA. The microsome catalyzed binding to nucleic acids was inhibited by the deacetylase inhibitor, paraoxon. Similar binding studies with [3H]N-OH-AABP revealed that, although HUC microsomes were effective, the relative amounts of label bound to nucleic acids were markedly (about 300 times) lower compared with [3H]N-OAc-AABP binding. Since bladder tissues have been reported to contain PHS, which is capable of activating arylamines and is inhibited by indomethacin (48), we tested HUC microsomes for arachidonic acid-dependent and indomethacin-sensitive binding of [3H]ABP to nucleic acids. In contrast to the binding of N-OAc-AABP and N-OH-AABP, HUC microsomes were less effective in activating [3H]ABP. Furthermore, the binding was not dependent on arachidonic acid and was not inhibited by indomethacin (Table 2).

HUC Cytosol-mediated Binding of [3H]N-OH-ABP to Nucleic Acids. The binding studies with N-OAc-AABP and N-OH-AABP suggested that N-OAc-ABP might be involved as a reactive intermediate. Since N-OAc-ABP could also be generated by acetyl CoA-dependent O-acetylation of N-OH-ABP, we tested HUC cytosol for acetyl transferase-mediated binding of [3H]N-OH-ABP to nucleic acids. The results presented in Table 2 show that HUC cytosols were very effective in catalyzing the binding of N-OH-ABP to RNA and DNA, and the reaction was insensitive to paraoxon inhibition. The activities were relatively lower than the microsome-mediated binding of N-OAc-AABP, but were much greater than those observed with N-OH-AABP.

Characterization of the DNA-Adducts. The DNA adducts obtained after incubation of [3H]N-OAc-AABP with 2 mg of calf thymus DNA and 100 μg of HUC microsomes, or [3H]N-OH-AABP with 6 mg DNA and 3 mg of microsomes, were enzymatically hydrolyzed to nucleosides as described in “Materials and Methods,” and subjected to HPLC analyses. Fig. 6, A and B, shows the HPLC profiles of the DNA-hydrolysates obtained with labeled N-OAc-AABP and N-OH-AABP, respectively. As shown, the majority of the radioactivity in the DNA-hydrolysates was associated with a peak whose chromatographic mobility (15 min) corresponds with the synthetic standard N-(dG8-yl)ABP adduct (Fig. 6C). This was further confirmed by thin-layer chromatographic analysis of the hydrolysate of the DNA-adducts on cellulose (30% acetic acid) and polyamide-6 (acetonitrile:water:ethanol, 2:5:3), wherein the majority of the radioactivity was detected in the region with a Rf of 0.4 (cellulose) and 0.2 (polyamide-6), the same region where the synthetic N-(dG8-yl)ABP standard adduct also migrates (data not shown). Based on these data, the DNA adduct generated from microsome-mediated activation of N-OAc-

![HPLC profile of the ethyl acetate extract of the microsomal incubates of [3H]AABP in the absence (top panel) or presence (bottom panel) of paraoxon.](image)

**Fig. 5.** HPLC profile of the ethyl acetate extract of the microsomal incubates of [3H]AABP in the absence (top panel) or presence (bottom panel) of paraoxon. The HPLC conditions were the same as those described in “Materials and Methods” except the flow rate was 1.5 ml/min.

**Table 2 HUC enzyme-mediated binding of [3H]N-OAc-AABP, [3H]N-OH-AABP, and [3H]ABP to nucleic acids**

<table>
<thead>
<tr>
<th>Substrate (enzyme)</th>
<th>Inhibitor</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]N-OAc-AABP (microsomes)</td>
<td>Paraoxon (100 μM)</td>
<td>pmol/mg protein/mg nucleic acids</td>
</tr>
<tr>
<td>–</td>
<td>23.0</td>
<td>8.0</td>
</tr>
<tr>
<td>+</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td>[3H]N-OH-AABP (microsomes)</td>
<td>–</td>
<td>74.5</td>
</tr>
<tr>
<td>+</td>
<td>10.9</td>
<td>6.8</td>
</tr>
<tr>
<td>[3H]N-OH-ABP (cytosol)</td>
<td>–</td>
<td>801</td>
</tr>
<tr>
<td>+</td>
<td>737</td>
<td>455</td>
</tr>
<tr>
<td>[3H]ABP (microsomes)</td>
<td>Arachidonic acid (250 μM)</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>–</td>
<td>4.7</td>
<td>35</td>
</tr>
<tr>
<td>+</td>
<td>4.9</td>
<td>27</td>
</tr>
<tr>
<td>+</td>
<td>3.5</td>
<td>28</td>
</tr>
</tbody>
</table>

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A'-OH-AABP and N-OH-AABP has been identified as N-(deoxyguanosine-8-yl)ABP. This adduct has also been identified in hydrolysates of DNA obtained from acetyl CoA-dependent HUC cytosol-mediated binding of [3H]N-OH-ABP (49). Additionally, 2 other minor adducts were detected based on the radioactivity profile of the DNA-hydrolysate derived from samples incubated with [3H]N-OAc-AABP or [3H]N-OH-AABP, respectively. The chromatogram and the spectra of the synthetic N/(dG3-yl)-ABP (retention time, 15 min) and N/(dG3-yl)AABP (retention time, 10.5 min) obtained under the same conditions are shown (C).

DISCUSSION

It has been suggested that the N-hydroxylamines resulting from hepatic metabolism are conjugated to glucuronides, and these N-glucuronide conjugates are excreted in urine and ultimately converted to reactive electrophiles under the acidic conditions of urine (16). In addition, a number of recent studies have shown that metabolic activation of certain procarcinogens or their proximate carcinogenic metabolites could occur in these target organs by the various enzymes associated with these tissues (24–27, 48, 50). In the present study, we have shown that human urinary bladder tissues contain enzymes that catalyze the metabolic activation of N-OH-ABP, N-OAc-AABP, and N-OH-AABP. Furthermore, these studies show that specifically, the microsomal deacetylase(s) are responsible for the activation of N-OH-AABP and N-OAc-AABP, since the enzyme inhibitor paraoxon blocked the HUC-microsome-mediated activation. Measurement of the distribution of deacetylation activity in HUC cytosol and microsomes using N-OAc-AABP or N-OH-AABP revealed that the majority (>90%) is associated with the microsomes. Whether the residual activity observed with the cytosol is due to cytosolic enzymes or to microsomal carryover remains to be investigated. The ratio of O- to N-deacetylase activities in microsomes (Table 1) was about 1.4, which is relatively low compared with the ratios 7 and 9 obtained with rat hepatic and bladder microsomes, reported earlier (51). The relative ratios of O- to N-deacetylase might play a pivotal role in the activation process, since O-deacetylation of N-OAc-AABP generates N-OH-AABP, which is relatively innocuous compared to the N-deacetylation product. In contrast to O-deacetylation, N-deacetylation of N-OAc-AABP results in N-OAc-ABP, which is the putative penultimate carcinogenic intermediate that yields the electrophilic aryl nitrenium ion. Although N-OH-AABP could conceivably generate N-OAc-ABP by trans-acetylation, such enzyme activity in the HUC microsomes is relatively low (Table 2). The results of the binding studies show that HUC microsomes are metabolically more active in converting N-OAc-AABP to reactive species than N-OH-AABP. Similarly, HUC cytosols also contain enzymes that catalyzed the binding of [3H]N-OH-ABP, and on a protein basis, the binding was relatively more effective than activation of N-OH-AABP, but much less than activation of N-OAc-AABP, by microsomes.

The binding of N-OAc-AABP and N-OH-AABP to DNA is covalent in nature, and N-(dG3-yl)ABP has been characterized as the major adduct, with both the chemicals. We did not detect the acetylated N-(dG3-yl)ABP adduct. On HPLC chromatography, the chemically synthesized acetylated adduct elutes ahead of the N-(dG3-yl)ABP adduct (Fig. 6), and no radioactive peak corresponding with the retention time of the acetyl standard was observed in the DNA-hydrolysates generated after interaction of HUC microsomes with [3H]N-OAc-AABP or [3H]N-OH-AABP (Fig. 6). The identification of N-(dG3-yl)ABP adduct is consistent with the postulated deacetylation and trans-acetylation mechanisms for the activation of N-OAc-AABP and N-OH-AABP, respectively, and is in agreement with the published reports on DNA-adducts with arylamines in experimental animals (12, 28, 29, 52). The same DNA-adduct has recently been reported in biopsy samples of human urinary bladder (53, 54). Kadlubar and coworkers (12, 28, 29, 52, 55) have also identified DNA adducts that have been generated in various experimental animals after treatment with different arylamine derivatives, including ABP analogues. These studies in experimental animals with ABP have shown 3 adducts identified as N-(dG3-yl)ABP, N-(deoxyadenosine-8-yl)ABP, and 3-(deoxyguanosine-N3-yl)ABP. Similar adducts have also been identified in vitro following interaction of N-OH-ABP with DNA under acidic conditions, which facilitates the generation of the aryl nitrenium ion (28). The aryl nitrenium ions have been implicated as the ultimate carcinogen in dogs and presumably in human bladder cancer and are thought to arise from N-glucuronide conjugates of N-hydroxylamines that are excreted in urine (16). However, our results suggest that such aryl nitrenium ions could also arise from N-OAc-AABP and N-OH-AABP, possibly involving the deacetylation or trans-acetylation reactions to yield N-OAc-ABP, which might then break down to form the aryl nitrenium ion. N-OAc-ABP could also arise from...
acetyl CoA-dependent activation of N-hydroxylamines by cytosolic acetyl transferases. The data on HUC cytosol-mediated, acetyl CoA-dependent activation of N-OH-ABP suggest that these acetyl transferases are distributed in human urinary bladder tissues. Taken together, these results strongly suggest that in human urothelial cells N-OAc-ABP might act as the penultimate carcinogenic intermediate that could yield the electrophile, arylnitrenium ion. Thus, susceptibility to human bladder carcinogenesis might in part be dependent upon the the intracellular concentration of N-OAc-ABP. Since mammalian tissues contain various acyl transferases, the relative distribution of these enzymes might greatly influence the susceptibility to aryline-induced bladder cancer. In this regard, N-acetyltransferase, which shows polymorphic distribution in humans, has been implicated in human bladder carcinogenesis. Specific mutations in the alleles of this gene (56, 57) have been associated with "slow-acetylator" phenotypes, which have been thought to be at greater risk for arylamine-induced bladder cancer (58, 59).

We have shown recently, using near diploid, SV40-immortalized isogenic cell lines of HUC, that ABP and its N-hydroxy derivatives, namely, N-OH-ABP, N-OH-AABP and N-OAc-AABP, cause tumorigenic transformation and neoplastic progression in vitro (37). Furthermore, in the same system we demonstrated that these chemicals also caused mutations in the hypoxanthine guanine phosphoribosyl transferase gene, an endogenous selectable marker gene (60). Additionally, we have shown that the mutagenic potency of ABP and its N-hydroxy metabolites were lower when tested on an isogenic subclone of Simian virus-HUC that was found to be refractive to transformation in vitro by the same chemicals. Thus, there is a strong correlation between mutagenicity and tumorigenic transformation. The biochemical findings on the metabolic activation of N-Oac-AABP and N-OH-ABP and their DNA-adduct formation are in accordance with the biological effects observed with these chemicals in vitro, and suggests that these proximate metabolite(s) might cause neoplastic transformation by inducing mutations at critical cellular regulatory genes, such as protooncogenes or suppressor genes, an aspect that is currently under investigation.

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Metabolism and Nucleic Acid Binding of \( N \)-Hydroxy-4-acetylaminobiphenyl and \( N \)-Acetoxy-4-acetylaminobiphenyl by Cultured Human Uroepithelial Cells

Santhanam Swaminathan and Catherine A. Reznikoff


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