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PROSTAGLANDIN ENDOPEROXIDE SYNTHETASE-MEDIATED METABOLISM OF CARCINOGENIC AROMATIC AMINES AND THEIR BINDING TO DNA AND PROTEIN

F.F. Kadlubar, C.B. Frederick, C.C. Weis, and T.V. Zenser†

National Center for Toxicological Research (HFT-110),
Food and Drug Administration, Jefferson, AR 72079
and †Veterans Administration Medical Center,
St. Louis, MO 63125

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The arachidonic acid-dependent metabolism of the carcinogens, 2-naphthylamine, 4-aminobiphenyl, 2-aminofluorene, benzidine, and N-methyl-4-aminoazobenzene was mediated by a prostaglandin endoperoxide synthetase preparation. Phenacetin, a suspected carcinogen, was not a substrate but its deacetylated metabolite, p-phenetidine, was rapidly oxidized. For each arylamine, extensive metabolism (14-81%) was observed, resulting in high levels of products bound covalently to protein. A low level of binding to added DNA was also detected for each substrate, except p-phenetidine and N-methyl-4-aminoazobenzene. Chromatography of the ethyl acetate-extractable metabolites indicated that the major products were N-oxidized and/or C-oxidized derivatives.

Aromatic amines, which were among the first chemicals to be recognized as human carcinogens, are known to require metabolic N-oxidation as a first step in their conversion to ultimate carcinogenic derivatives (1). Although both hepatic cytochrome P-450 and flavin-containing monooxygenases have been reported to catalyze this reaction (reviewed in ref. 2), their relative importance in extrahepatic tissues is unclear. Prostaglandin endoperoxide synthetase (PES), which catalyzes the initial steps leading to the biosynthesis of prostaglandins, is present in many extrahepatic tissues and is known to regulate a wide variety of cellular processes (3). PES converts arachidonic acid into prostaglandin H_2 and concomitantly generates an activated form of oxygen. Accordingly, recent studies have shown that PES can mediate the arachidonic acid-dependent co-oxidation of several classes of compounds,

ABBREVIATIONS: hplc, high pressure liquid chromatography; PES, prostaglandin endoperoxide synthetase.

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including polycyclic aromatic hydrocarbons (4,5), nitrofurans (6), azo dyes (7), benzidine (6), diethylstilbestrol (8,9), and acetaminophen (10,11). It has been further reported that their oxidation products are chemically reactive and bind covalently to protein (6-11) and/or nucleic acids (4-8). In this communication, we report the arachidonic acid-dependent, PES-mediated metabolism and macromolecular binding of several carcinogenic aromatic amines.

MATERIALS AND METHODS

Ring-labeled [^{3}H]2-naphthylamine (47 mCi/mmol), [^{3}H]2-aminofluorene (45 mCi/mmol), [^{3}H]benzidine (118 mCi/mmol), [^{3}H]4-aminobiphenyl (33 mCi/mmol), [^{3}H]N-methyl-4-aminoazobenzene (90 mCi/mmol), and [^{14}C]phenacetin (5 mCi/mmol) were obtained from Dr. R. Roth, Midwest Research Institute (Kansas City, MO). [^{14}C]p-Phenetidine (5 mCi/mmol) was prepared by deacetylation of [^{14}C]phenacetin with carboxylesterase (12) and was purified by ethyl acetate extraction and high pressure liquid chromatography (hplc) (as described below). 2-Nitrosonaphthalene (13), N^4-(2-naphthyl)-2-amino-1,4-naphthoquinoneimine (14), 4-nitrosobiphenyl (13), and 2-nitrosofluorene (15) were synthesized by published methods and their identities were confirmed by mass spectrometry. DNA (type I) and indomethacin were purchased from Sigma Chemical Co., 4-aminoazobenzene from Aldrich Chemical Co., and arachidonic acid from Nu-Chek Prep, Inc. (Elysian, MN). Solubilized ram seminal vesicle microsomes were used as a source of PES (16).

Unless otherwise indicated, the assay medium (2 ml) for measuring aromatic amine metabolism and covalent binding to DNA and protein contained: 100 mM potassium phosphate buffer (pH 7.8), DNA (1 mg/ml), PES (0.2 mg protein/ml), and 0.05 mM aromatic amine (diluted from 10 mM solutions in dimethyl sulfoxide/ethanol, 4/1). When indicated, 0.1 mM indomethacin was used as an inhibitor by preincubation with PES in the assay medium at 37°C for 3 min. Arachidonic acid (0.1 mM) was added to start the reactions which were carried out at 37°C for 5 min. The medium was then extracted with an equal volume of H_2O-saturated ethyl acetate to recover metabolites and unchanged amine, which were separated by hplc as previously described (17) except that a Beckman Ultrasphere-ODS column was used with 20 mM diethylamine acetate, pH 6.5, in the aqueous solvent. The components were quantitated with a Flo-One HP flow scintillation counter. To estimate covalent binding to DNA and protein, the aqueous phase was further extracted twice with 2 ml H_2O-saturated n-butanol and once with 2 ml H_2O-saturated chloroform. After centrifugation, the aqueous upper phase containing the DNA was removed; and the DNA was purified by phenol extractions, solvent precipitations (18,19) and CsCl centrifugation (20). Bound radioactivity was determined as previously described (19). The lower organic phase, which contained precipitated protein, was washed twice with 2 ml of H_2O. Acetone (10 ml) was then added to the organic phase and the protein was collected by centrifugation. Further protein purification with acetone and ethanol was as previously described (21) and bound radioactivity was determined after solubilization in 0.1 N NaOH.

RESULTS AND DISCUSSION

Initial experiments with incubation mixtures containing both PES and arachidonic acid indicated that the carcinogen, 2-naphthylamine, was rapidly metabolized and became covalently bound to protein and DNA (Table I). Within

Table I. PES-Mediated Metabolism and Macromolecular Binding of Aromatic Amines [a]

Aromatic Amine	Arachidonic Acid-Dependent: Metabolism (%)	Protein Binding (nmol bound/mg)	DNA Binding (pmol bound/mg)
2-Naphthylamine	14 ± 4	16 ± 4	22 ± 7
4-Aminobiphenyl	17 ± 1	8 ± 1	7 ± 2
2-Aminofluorene	46 ± 4	20 ± 6	28 ± 7
Benzidine	81 ± 1	84 ± 16	9800 ± 1500
Phenacetin	<1 [b]	<0.5 [b]	<5 [b]
p-Phenetidine	26 ± 5	28 ± 5	<5 [b]
N-Methyl-4-aminoazobenzene	33 ± 7	18 ± 2	<1 [b]

[a] As described in Materials and Methods, incubations (n = 5) were carried out at 37°C for 5 min using radiolabeled substrates. Metabolism was determined as per cent of substrate recovered by ethyl acetate extraction and hplc analysis. Protein and DNA binding was estimated after reisolation of macromolecules and determination of bound radioactivity.

[b] Judged to be the limit of detection.

4-5 minutes the reaction had gone to completion, and the extent of metabolism and macromolecular binding was proportional to PES concentration (0.1-0.4 mg protein/ml). Addition of indomethacin (0.1 mM), a specific PES inhibitor (3), blocked metabolism and binding by 90%. Complete inhibition was also observed with 1 mM glutathione or 1 mM ascorbic acid. Control incubations without arachidonic acid resulted in levels of binding that were <5% of the complete system. When DNA was added to the medium after the 5-min incubation, DNA binding was reduced by 97%.

Similar results were obtained with other carcinogenic aromatic amines (Table I). Metabolism and binding of the arylamines, 4-aminobiphenyl and 2-aminofluorene, to protein and DNA were comparable to that observed with 2-naphthylamine. Benzidine, as previously reported (22), was considerably more susceptible to co-oxidation and resulted in much higher levels of binding to protein and especially to DNA. Phenacetin, an arylamide, was not a substrate, but its deacetylated derivative, p-phenetidine, was oxidized to a protein-binding metabolite(s); covalent binding to DNA was not detected.

Similarly, N-methyl-4-aminoazobenzene was metabolized to products that bound to protein but not DNA. It should be noted that extensive purification procedures were necessary to remove from DNA the non-covalently bound products as well as protein containing covalently bound aromatic amines. Others have reported peroxidase-mediated binding of N-methyl-4-aminoazobenzene to DNA (7,23), but the isolation procedures may not have been sufficiently rigorous to remove all residual radioactivity. For each of the aromatic amines: metabolism and binding were arachidonic acid-dependent; DNA binding was not detectable if DNA was added after the incubation; and inhibition by indomethacin, as assayed by protein binding, was 90-99%.

Ethyl acetate-extracts of the incubation mixtures, analyzed by reversed-phase hplc, revealed metabolite profiles that were consistent with N- and C-oxidized products. For 2-naphthylamine, two major metabolites were observed which were chromatographically identical to synthetic 2-nitrosonaphthalene and N^4-(2-naphthyl)-2-amino-1,4-naphthoquinoneimine (an addition product of 1,2-naphthoquinoneimine and 2-naphthylamine). For 4-aminobiphenyl and 2-aminofluorene, the major metabolites co-chromatographed with 4-nitrosobiphenyl and 2-nitrosofluorene, respectively; two minor non-polar late-eluting components were also detected for each. Benzidine yielded only one major N-oxidized metabolite, whose electron impact mass spectrum (m/z 364) was consistent with an addition product of benzidine and its diimine derivative. p-Phenetidine metabolism appeared to result in products that were not extracted into ethyl acetate since hplc profiles showed only the arachidonic acid-dependent loss of p-phenetidine. N-Methyl-4-aminoazobenzene gave the most complex metabolite pattern with six different components, the major of which was chromatographically identical to the N-demethylation product, 4-aminoazobenzene.

In this study, PES was shown to mediate the co-oxidation of six different aromatic amines to N- and/or C-oxidation products. Concomitantly, the covalent binding of arylamine residues to DNA or protein was observed. Since PES is distributed in many extrahepatic tissues (24-27) including those sensitive to the carcinogenic action (1,28) of these amines and their acetylated

derivatives (urinary tract, colon), a role for PES in metabolic activation and macromolecular binding *in vivo* appears possible. In this regard, the PES inhibitor, aspirin, has been shown to block the metabolic activation and pathogenesis of lesions in the urinary bladder of rats administered carcinogenic nitrofurans (29). Further studies are in progress to assess the involvement of PES in aromatic amine-induced carcinogenesis and to identify the reactive amine metabolites and their DNA- and protein-bound adducts.

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