Metabolic Activation of Carcinogenic Aromatic Amines by Dog Bladder and Kidney Prostaglandin H Synthase

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ABSTRACT

Microsomal enzyme preparations from dog liver, kidney, and bladder were used to assess the prostaglandin H synthase-catalyzed activation of carcinogenic aromatic amines to bind covalently to proteins and nucleic acids. Benzidine, a urinary bladder carcinogen, bound to protein of bladder transitional epithelial and renal inner and outer medullary microsomes and was dependent upon addition of arachidonic acid, but not upon reduced nicotinamide adenine dinucleotide phosphate. Bladder transitional epithelial microsomes also activated o-dianisidine, 4-aminobiphenyl, and 2-naphthylamine to bind to protein and transfer RNA and benzidine and o-dianisidine to bind DNA. Cosubstrate and inhibitor specificities were consistent with activation by prostaglandin H synthase. Binding of benzidine to protein was not observed with either hepatic or renal cortical microsomes upon addition of arachidonic acid or reduced nicotinamide adenine dinucleotide phosphate.

Prostaglandin H synthase and mixed-function oxidase-catalyzed bindings of 2-naphthylamine to protein and to transfer RNA were compared using liver and bladder microsomes. Only mixed-function oxidase-catalyzed binding was observed in liver, and only prostaglandin H synthase-catalyzed binding was observed in bladder. The rate of binding catalyzed by bladder microsomes was considerably greater than that catalyzed by hepatic microsomes. In addition, the bladder content of prostaglandin H synthase activity was approximately 10 times that of kidney inner medullary, a tissue reported to have a relatively high content of this enzyme in other species. These results are consistent with involvement of bladder transitional epithelial prostaglandin H synthase in the genesis of primary aromatic amine-induced bladder cancer.

INTRODUCTION

Several primary aromatic amines have been identified as being responsible for an elevated incidence of bladder cancer in industrial workers (3, 5, 28). The dog is susceptible to aromatic amine-induced bladder cancer and is therefore an appropriate animal model to study mechanisms by which certain aromatic amines induce transitional cell carcinoma (9). Metabolic activation of carcinogenic aromatic amines by N-oxidation has been demonstrated in several species and has been shown to involve both hepatic cytochrome P-450 and flavin-containing monooxygenases (8). It has been hypothesized that these N-hydroxy metabolites are transported to the bladder as N-glucuronide conjugates where they can be hydrolyzed by the acid pH of urine to form reactive electrophiles that bind to bladder transitional epithelial DNA (13, 15, 28). In contrast, hepatic metabolism of the carcinogenic aromatic amine benzidine to an N-hydroxy intermediate has been demonstrated only following N-acetylation. The latter is then activated by N-O-acyltransferase to an electrophilic reagent which has been implicated in liver tumor formation in the rat (20, 22). The rat, which rapidly N-acetylates aromatic amines, is not susceptible to benzidine-induced bladder cancer (5, 9, 28). However, the dog, which is unable to N-acetylate benzidine and other amines, is susceptible to benzidine-induced bladder cancer but not to liver cancer (17, 26). Thus, N-acetylation has been proposed as a detoxification pathway for bladder carcinogenesis. Humans that are slow acetylators appear to have an increased risk of developing urinary bladder cancer (18).

Metabolism of aromatic amines by PHS3 has been proposed as an additional pathway for carcinogen activation (34, 36), resulting in electrophilic intermediates which bind to DNA (14). The aromatic amines benzidine, 2-naphthylamine, and 2-aminofluorene are metabolized to mutagenic products by PHS (30). Mono- and diacetylated benzidine are poor substrates for PHS activation (23), which is consistent with N-acetylation as a detoxification pathway for aromatic amine bladder carcinogenesis (18). Target tissue metabolism is a salient feature of the proposed activation of aromatic amines by PHS. However, neither the presence of PHS nor PHS activation of aromatic amines has been demonstrated with bladder transitional epithelial tissue from a susceptible species. This study evaluates the presence of PHS and its activation of several aromatic amines by beagle liver, kidney, and bladder transitional epithelial tissue. In addition, the relative rates of 2-naphthylamine binding to macromolecules catalyzed by mixed-function oxidase and PHS enzymes were assessed with liver and bladder transitional epithelial tissue.

MATERIALS AND METHODS

Materials. [U-14C]Benzidine (25.7 Ci/mmol) and [5,6,8,9,11,12,14,15-3H]arachidonic acid (78.2 Ci/mmol) were purchased from New England Nuclear, Boston, MA. α-[U-rin1-14C]Dianisidine (8.97 mCi/mmol) was purchased from Pathfinder Laboratories, Inc., St. Louis, MO, and provided to us by Dr. R. K. Lynn, Smith, Kline and French Laboratories, Philadelphia, PA. 2-[5,6,7,8-3H]Naphthylamine (47 mCi/mmol) and 4-[2,2'-3H]amino-4'-diocetylaminobiphenyl (55 mCi/mmol) were obtained from Dr. R. Roth, Midwest Research Institute (Kansas City, MO). 4-Aminobiphenyl and o-dianisidine were purchased from Aldrich Chemical Co., Milwaukee, WI. DPEA was a gift from Eli Lilly and Co., Indianapolis, IN. 2-Naphthylamine, glutathione, benzidine, tRNA, indomethacin, methemoglobin (type 1, bovine), NADPH, nicotinic acid, and bovine serum albumin (Fraction V

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powder) were purchased from Sigma Chemical Co., St. Louis, MO. 5,8,11,14-Eicosatetraenoic acid (arachidonic acid) and 11,14,17-eicosatrienoic acid were purchased from Nu Chek Prep, Inc., Elysian, MN. Stock fatty acid solutions were stored at -80°C and aliquots were diluted in ethanol immediately prior to use. Prostaglandins were a generous gift of The Upjohn Co., Kalamazoo, MI. Scintillation fluid (Ready-Solv HP/β) was purchased from Beckman Instruments, Fullerton, CA. All other chemicals were purchased in the highest possible grade from standard sources. Silica Gel G thin-layer chromatography plates were obtained from Merck AG, Darmstadt, Germany. Beagles were purchased from Eldridge Laboratory Animals, Barnhart, MO.

Preparation of Microsomes. Beagles were anesthetized with sodium thiopental (20 mg/kg i.v.). The kidneys, livers, and bladders were quickly removed and placed in ice-cold 0.85% NaCl solution (35). After the capsule was removed, kidneys were bisected in the sagittal plane, and the cortex, outer medulla, and inner medulla were separated by careful dissection. Bladders were opened and stretched flat on a corkboard, and the transitional epithelial tissues were carefully removed with scalpel and forceps (2). Transitional epithelial tissues were routinely examined microscopically. All tissues were minced, washed free of hemoglobin, and homogenized with two 15-sec bursts at 30-sec intervals using a Polytron homogenizer in 3 to 5 volumes of 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 10 mm dithiothreitol. The homogenates were centrifuged at 10,000 × g for 15 min, and the subsequent supernatant fractions were centrifuged at 105,000 × g for 60 min. All pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) with a Potter-Elvehjem Teflon:glass homogenizer to give a final concentration of about 10 mg of microsomal protein/ml. Aliquots of these suspensions were stored frozen at -80°C. Protein content was determined by the method of Lowry et al. (19), using bovine serum albumin as the standard.

Incubation Conditions, Extraction, and Analysis of Aqueous-soluble Products of Aromatic Amine Metabolism. The reaction mixture contained 0.2 to 1.0 mg microsomes, 0.1 M potassium phosphate buffer (pH 7.4), 0.0012 mw methemoglobin, 0.1 mw radiolabeled aromatic amine (diluted from 10 mw solutions in dimethyl sulfoxide:ethanol, 4:1), and various test substances in a final volume of 0.1 ml. Reactions were initiated with either 0.3 mw arachidonic acid or 1.5 mw NADPH containing 15 mw MgCl₂ and incubated at 37°C for 2 min (34). The reactions were stopped by the addition of 0.4 ml of an aqueous solution of the corresponding unlabeled aromatic amine (1 mw) followed by 1 ml ethyl acetate:ethyl ether (1:1, v/v). Following seven 1-ml extractions with organic solvent, cold 0.6 M TCA was added to the aqueous phase, and the samples were centrifuged at 2500 × g for 10 min. The resulting precipitates were washed repeatedly with 1 ml of 0.6 M TCA until the radioactivity of the supernatant was similar to background. Pellets were washed with ethanol and solubilized in 1.0 ml water. Aliquots were taken for determination of radioactivity. Previous experiments demonstrated that DNA and tRNA were present in excess. Results are expressed as pmol aromatic amine bound per mg microsomal protein per min.

Determination of [³H]Arachidonic Acid Metabolism by Dog Tissue Microsomes. For comparison of the rate of benzidine metabolism with arachidonic acid metabolism by dog bladder PHS, reaction mixtures were identical with those used to assess aqueous-soluble products of benzidine metabolism except that radiolabeled arachidonic acid was used instead of benzidine. For comparison of arachidonic acid metabolism by different dog tissues, reaction mixtures contained 0.2 to 1.0 mg microsomal protein, 0.1 mw potassium phosphate buffer (pH 7.8), 0.0012 mw methemoglobin, 1 mw glutathione, and 0.02 mm [³H]arachidonic acid in a final volume of 0.1 ml incubated at 37°C for 3 min (2). Inhibitors were preincubated with microsomes for 2 min before initiation with arachidonic acid. Reactions were stopped by addition of 0.4 ml 1.0 M sodium citrate buffer, pH 3.0. Samples were extracted twice with equal volumes of ethyl acetate, dried under N₂, dissolved in acetone, and spotted on Merck silica gel thin-layer chromatographic plates. Plates were developed with a solvent system containing chloroform, methanol, and acetic acid (90:5.5, v/v/v) and scanned on a Packard 7230 radiochromatogram scanner. Fatty acid, hydroxy fatty acid (ricinoleic acid), and prostaglandin standards were visualized with iodine, areas corresponding to these standards were scraped into scintillation vials, and the radioactivity was determined (2). Incubation with indomethacin gave the same blank values as did incubation without microsomes. Results represent the differences in arachidonic acid and prostaglandin E₂ recovered from the thin-layer chromatographic plates after incubations with and without indomethacin.

RESULTS

The conversion of benzidine to aqueous-soluble products by dog microsomes is shown in Chart 1. Following addition of arachidonic acid, a significant increase in aqueous-soluble benzidine derivatives was observed with microsomes prepared from bladder transitional epithelium, inner medulla, and outer medulla. Arachidonic acid-initiated benzidine metabolism was not observed with hepatic or renal cortical microsomes. Furthermore, aqueous-soluble benzidine metabolites were not detected following addition of NADPH to liver, kidney, or bladder microsomes.

Bladder microsomal activation of benzidine was further characterized by the experiments reported in Table 1. Benzidine binding to TCA-precipitable material was completely inhibited by 0.1 mm indomethacin. Glutathione blocked binding and increased TCA-soluble product(s). 11,14,17-Eicosatetraenoic acid was ineffective as a cosubstrate for benzidine activation.

A more detailed assessment of the effect of increasing concentrations of glutathione on benzidine binding is presented in Chart 2. At all concentrations of glutathione tested, there was a direct relationship between the glutathione-mediated decrease in benzidine binding and the increase in TCA-soluble material such that no change in total benzidine metabolism was observed. Using liquid chromatography with electrochemical detection as described by Rice and Kissinger (29), analysis of this TCA-soluble material demonstrated the presence of a benzidine:glutathione conjugate with an elution profile identical to that observed following horseradish peroxidase-catalyzed oxidation of benzidine (29). Metabolism of [³H]arachidonic acid was also measured with bladder microsomes using the same incubation conditions with which benzidine metabolism was examined (Chart 3). Micro-
Metabolic Activation of Aromatic Amines

Chart 1. Aqueous-soluble metabolites of benzidine catalyzed by beagle liver, kidney, and bladder microsomes. Results represent the mean of the sum 3 to 5 TCA-soluble and -insoluble values. Bars, S.D.

Table 1

Characteristics of \(^{14}C\)-benzidine metabolism by dog bladder transitional epithelial microsomes

Complete reaction mixture contained 0.21 mg bladder transitional epithelial microsomal protein, 0.1 \(^{14}C\)-benzidine, 0.0012 \(^{14}C\)-methemoglobin, and additions as indicated in a final volume of 0.1 ml phosphate buffer, pH 7.8.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (mM)</th>
<th>TCA soluble</th>
<th>TCA insoluble</th>
<th>Aqueous-soluble metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>0.3</td>
<td>51 ± 4(b)</td>
<td>198 ± 18</td>
<td>249 ± 22</td>
</tr>
<tr>
<td>+ glutathione</td>
<td>1.0</td>
<td>190 ± 18</td>
<td>75 ± 12</td>
<td>265 ± 30</td>
</tr>
<tr>
<td>+ indomethacin</td>
<td>0.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NADPH</td>
<td>1.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11,14,17-eicosatrienoic acid</td>
<td>0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a Represents combined TCA-soluble and -insoluble fractions.
*b Mean ± S.D. of 3 to 5 determinations.
*c ND, no metabolism detected.

Benzidine, o-dianisidine, 4-aminobiphenyl, and 2-naphthylamine were all activated by dog bladder transitional epithelial PHS to bind to protein and tRNA (Table 3). The binding of benzidine and o-dianisidine to tRNA was significantly greater than that of arachidonic acid and for synthesizing prostaglandin E\(_2\) as compared to renal inner medullary tissue. In each tissue, the synthesis of prostaglandin E\(_2\) was completely prevented by indomethacin.

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metabolism of arachidonic acid and synthesis of prostaglandin E₂ by beagle liver, kidney, and bladder microsomes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Liver</th>
<th>Kidney</th>
<th>Cortex</th>
<th>Outer medulla</th>
<th>Inner medulla</th>
<th>Bladder transitional epithelial tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid metabo-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6 ± 1</td>
<td>46 ± 5</td>
<td>483 ± 31</td>
</tr>
<tr>
<td>lized</td>
<td></td>
<td></td>
<td></td>
<td>2 ± 0.6</td>
<td>30 ± 3</td>
<td>440 ± 36</td>
</tr>
</tbody>
</table>

a ND, no metabolism detected.

b Mean ± S.D. of 3 determinations.

<table>
<thead>
<tr>
<th>Amine bound/mg protein/min</th>
<th>Protein (nmol)</th>
<th>tRNA (pmol)</th>
<th>DNA (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzidine</td>
<td>0.18 ± 0.02</td>
<td>3.3 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>0.33 ± 0.02</td>
<td>3.6 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>4-Aminobiphenyl</td>
<td>0.11 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>2-Naphthylamine</td>
<td>0.14 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

a In all cases, 1.0 mM glutathione inhibited 60 to 80% and 0.1 mM indomethacin completely inhibited binding.

b Mean ± S.D. of 3 to 5 determinations.

The rates of arachidonic acid metabolism and prostaglandin E₂ synthesis were greater in dog bladder epithelium than in renal medulla. The similarity in the rates of arachidonic acid metabolism and prostaglandin E₂ synthesis suggests that PHS is the major route of bladder and medullary microsomal arachidonic acid metabolism. Furthermore, no arachidonic acid metabolism was observed with indomethacin (Chart 3), an inhibitor of PHS but not lipoxygenase. Lipid peroxides generated from either the fatty acid cyclooxygenase component of PHS or other lipoxygenases could serve as cosubstrates for prostaglandin hydroperoxidase catalysis of aromatic amines. However, no lipoxygenase activity has been demonstrated with bladder epithelial tissue.

The larger relative amount of PHS activity in bladder compared to inner medulla in the dog was not observed in corresponding rabbit tissues (21). Bladder transitional epithelial PHS-catalyzed benzidine binding is about 4 times greater than is binding in the inner medulla in the dog. In contrast, when PHS-catalyzed binding of the 5-nitrofurans heterocyclic aromatic amine carcinogen 2-amino-4-(5-nitro-2-furyl)thiazole was assessed in the rabbit, the renal inner medulla was approximately 3 to 4 times more active than was the bladder transitional epithelium (21). Renal inner medullary and seminal vesicle microsomes have been consistently shown to contain high concentrations of PHS in the several species examined (4). Within the kidneys, the distribution of PHS has shown the inner medulla > outer medulla > cortex in several species (4, 16, 33). This was also observed in the beagle.

The relative amounts of PHS and mixed-function oxidase enzyme activities were different in dog liver and bladder. Hepatic mixed-function oxidase catalyzed the macromolecular binding of 2-naphthylamine, which was initiated by NADPH and inhibited by DPEA. Bladder mixed-function oxidase-catalyzed binding of 2-naphthylamine was not detected. In contrast, PHS-catalyzed binding of this carcinogenic aromatic amine was demonstrated with bladder transitional epithelium but not with liver. This is consistent with the lack of hepatic PHS metabolism of arachidonic acid. The rate of bladder PHS-catalyzed 2-naphthylamine binding to macromolecules was considerably greater than hepatic mixed-function oxidase-catalyzed binding.

Bladder mixed-function oxidase has been proposed as an inhibitor of bladder PHS.
alternative to hepatic mixed-function oxidase for activation of aromatic amine carcinogens (25, 27). Brill observed low levels of NADPH-dependent 2-naphthylamine metabolism in dog liver and bladder microsomes using an assay involving induction of ferrihemoglobin (1). In addition, Kadlubar et al. (27) detected bovine bladder mixed-function oxidase metabolism of 2-naphthylamine using high-pressure liquid chromatography to detect an N-hydroxy product. However, 2-naphthylamine metabolism was not observed with dog bladder or liver (27). The present study reports mixed-function oxidase-catalyzed binding of 2-naphthylamine to protein and tRNA by liver but not by urinary bladder microsomes of the dog (Table 4).

This is the first demonstration of PHS-catalyzed binding of o-dianisidine to macromolecules. Josephy et al. (11) have reported an o-dianisidine cation free radical produced from oxidation by the horseradish peroxidase:H2O2 system. Preliminary experiments indicate that a free radical is generated during PHS metabolism of o-dianisidine. A benzidine free radical cation has been shown to result from peroxidatic oxidation of benzidine by PHS and horseradish peroxidase (32). This may be the reactive aromatic amine intermediate which binds nucleic acids. However, similar studies have not been performed with 4-aminoazopyrine or 2-naphthylamine.

In conclusion, the demonstration of PHS activity with dog urinary bladder transitional epithelial microsomes and epithelial PHS-catalyzed activation of aromatic amines is consistent with the hypothesis that PHS plays a role in aromatic amine-induced bladder carcinogenesis. Support for this hypothesis is provided by in vivo studies in which aspirin, an inhibitor of arachidonic acid, initiated cooxidation by PHS (21, 37), (a) prevented early formation of the carcinogen N-hydroxy-2-aminofluorene and its covalent binding to nucleic acids. However, it has been shown to result from peroxidatic oxidation of benzidine by PHS and horseradish peroxidase (32). This may be the reactive aromatic amine intermediate which binds nucleic acids. However, similar studies have not been performed with 4-aminoazopyrine or 2-naphthylamine.

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