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 DNA 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, whereas 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-acetyltransferase to an electrophilic reactant 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 PHS 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 [U-15N]arachidonic acid (78.2 Ci/mmol) were purchased from New England Nuclear, Boston, MA. a-[U-ring-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-[U-14C]2-naphthylamine (47 mCi/mmol) and 4-[(2,2'-3H)aminobiphenyl (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 El Lilly and Co., Indianapolis, IN. 2-Naphthylamine, glutathione, benzidine, tRNA, indomethacin, methemoglobin (type 1, bovine), NADPH, nicotinyl acid, and bovine serum albumin (Fraction V DPEA was a gift from Eli Lilly and Co., Indianapolis, IN. 2-Naphthylamine, glutathione, benzidine, tRNA, indomethacin, methemoglobin (type 1, bovine), NADPH, nicotinyl acid, and bovine serum albumin (Fraction V

1 This work was supported by the Veterans Administration, the Environmental Protection Agency, and the National Center for Toxicological Research.

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Received October 31, 1983; accepted January 27, 1984.

MAY 1984
powder) were purchased from Sigma Chemical Co., St. Louis, MO. 5,8,11,14-Eicosatetraenoic acid (arachidonic acid) and 11,14,17-icosatrienoic acid were purchased from Nu Chek Prep, Inc., Elyria, 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 105,000 x g for 30 min, and the supermatant fractions were centrifuged at 105,000 x g for 60 min. 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 mM potassium phosphate buffer (pH 7.8), 0.0012 mM methemoglobin, 0.1 mM radiolabeled aromatic amine (diluted from 10 μM 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 mM arachidonic acid or 1.5 mM NADPH containing 15 μM MnCl₂ 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 mM) 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 x 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 dissolved in 0.05 ml of 1.0 N NaOH at 60°C for 30 min, diluted to 0.5 ml with distilled water, aliquoted into scintillation vials, and analyzed for radioactivity. Radioactivity in the soluble fraction was also determined.

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. Gluthathione blocked binding and increased TCA-soluble product(s). 11,14,17-Eicosatrienoic 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 [14C]benzidine metabolism by dog bladder transitional epithelial microsomes

<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*</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>

* Represents combined TCA-soluble and -insoluble fractions.

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

β ND, no metabolism detected.

The relationship between arachidonic acid metabolism and prostaglandin E2 synthesis was assessed using dog liver, kidney, and bladder microsomes (Table 2). Neither arachidonic acid metabolism nor prostaglandin E2 synthesis was observed with liver or renal cortical microsomes. In contrast, both activities were observed with renal medullary and bladder transitional epithelial tissue, and the rate of prostaglandin E2 synthesis was similar to the rate of arachidonic acid metabolism. Bladder tissue exhibited about a 10-fold greater potential for metabolizing arachidonic acid and for synthesizing prostaglandin E2 as compared to renal inner medullary tissue. In each tissue, the synthesis of prostaglandin E2 was completely prevented by indomethacin.

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...
Table 2
Metabolism of arachidonic acid and synthesis of prostaglandin E2 by beagle liver, kidney, and bladder microsomes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Arachidonic acid metabolism</th>
<th>Prostaglandin E2 synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cortex</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>6 ± 1^b</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>46 ± 5</td>
<td>30 ± 3</td>
</tr>
<tr>
<td>Bladder transitional epithelial tissue</td>
<td>483 ± 31</td>
<td>440 ± 36</td>
</tr>
</tbody>
</table>

^a ND, no metabolism detected.
^b Mean ± S.D. of 3 determinations.

Table 3
Metabolic activation of aromatic amines by PHS in dog bladder microsomes

<table>
<thead>
<tr>
<th>Substrate</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^d</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.

observed with 4-aminobiphenyl or 2-naphthylamine. PHS-catalyzed benzidine and o-dianisidine binding to DNA was detected but was less than that observed with tRNA. 4-Aminobiphenyl and 2-naphthylamine have been shown previously to be activated to bind to DNA by PHS prepared from solubilized ram seminal vesicle microsomes (14).

The bindings of 2-naphthylamine to protein and to tRNA by NADPH- and arachidonic acid-dependent enzymes in dog liver and bladder were compared (Table 4). In the liver, NADPH- but not arachidonic acid-dependent binding was observed; the NADPH-dependent binding was inhibited 90 to 100% by addition of DPEA. In the bladder, arachidonic acid- but not NADPH-dependent binding was observed; the former was completely inhibited by addition of indomethacin.

**DISCUSSION**

This study provides the first demonstration that bladder transitional epithelium from a species (dog) susceptible to aromatic amine-induced bladder cancer contains PHS and is capable of activating a variety of aromatic amine precarcinogens by a PHS-dependent pathway. Dog renal inner and outer medullary microsomes were approximately one-fourth and one-eighth, respectively, as active as the bladder in activating benzidine. Aqueous-soluble products of benzidine metabolism were observed following addition of arachidonic acid but not NADPH. Hepatic and renal cortical metabolism of benzidine was observed following any condition tested. This is consistent with the lack of detectable PHS activity in liver and renal cortex and the need for prior N-acetylation of benzidine before NADPH-dependent mixed-function oxidation activation. Unsaturated fatty acid cosubstrate (arachidonic acid) and inhibitor (indomethacin) specificities were consistent with bladder PHS catalysis of all the aromatic amines tested (7, 31). The amount of bladder PHS-catalyzed binding to protein was similar for all the aromatic amines tested. However, PHS-catalyzed binding of benzidine and o-dianisidine to nucleic acid was much greater than that observed with 4-aminobiphenyl and 2-naphthylamine.

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 co-substrates 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-nitrofuran 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 oxidative enzyme activities were different in dog liver and bladder. Hepatic mixed-function oxidation catalyzed the macromolecular binding of 2-naphthylamine, which was initiated by NADPH and inhibited by DPEA. Bladder mixed-function oxidation-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 oxidation-catalyzed binding.

Bladder mixed-function oxidation has been proposed as an
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 ferric-hemoglobin (1). In addition, Poupko 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-aminobiphenyl 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 binding, initiated cooxidation by PHS (21, 37). (a) Prevented early morphological bladder lesions (6), and (b) reduced the incidence of bladder tumors when coadministered with an heterocyclic aromatic amine urinary bladder carcinogen (24).

ACKNOWLEDGMENTS

The authors wish to thank Sandy Meliere for secretarial assistance.

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