Activation of Some Aromatic Amines to Mutagenic Products by Prostaglandin Endoperoxide Synthetase

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ABSTRACT

Cooxidation of xenobiotics may occur during prostaglandin biosynthesis. The ability of prostaglandin endoperoxide synthetase to cooxidize several aromatic amines and other chemicals to mutagenic products was tested with the standard Salmonella tester strains. The microsomal fraction of ram seminal vesicles, a rich source of prostaglandin endoperoxide synthetase, in the presence of the prostaglandin endoperoxide synthetase substrate arachidonic acid metabolized benzidine, 2-aminofluorene, 2-naphthylamine, and 2,5-diaminoanisole to mutagenic products. 1-Naphthylamine, 2-aminoanthracene, 2-acetylaminofluorene, and 2,4-diaminoanisole were negative or weakly mutagenic. N-Nitrosodimethylamine, N-nitrosomorpholine, the pesticide Aminocarb, and di(2-ethylhexyl)phthalate were not activated to mutagenic products by the ram seminal vesicle microsomal fraction.

INTRODUCTION

Arachidonic acid is metabolized to prostaglandins by PES.3 PES has 2 catalytic activities: a cyclooxygenase activity that converts arachidonic acid to the hydroperoxyendoperoxide, prostaglandin G2; and a hydroperoxidase activity which reduces prostaglandin G2 to the corresponding hydroxyendoperoxide, prostaglandin H2 (11, 15, 17, 22). Prostaglandin H2 is the pivotal substance from which all other prostaglandins arise (7).

In the presence of prostaglandin G2 or other peroxides, the hydroperoxidase can metabolize a number of xenobiotic substances (5, 10). This peroxidase metabolism of xenobiotics by PES is termed cooxidation (12, 14), and PES has been proposed as an additional enzyme system to the cytochrome P-450-NADPH-dependent monooxygenase system for metabolizing chemicals to reactive metabolites, particularly in extrahepatic tissues (10, 19).

Cooxidation of PAH and their derivatives by PES has been extensively studied (5, 10). In addition, the formation of mutagenic metabolites of PAH has been examined using the microsomal fraction from RSV, a source of PES, as the activation system in the Salmonella mutagen test (2, 13). The PES system was more selective than a cytochrome P-450-NADPH-dependent monooxygenase system (Aroclor 1254-induced rat liver 9000 X g supernatant), in that only the dihydrodiols from which the bay-region diol-epoxides can be formed were metabolized to mutagenic products (6, 13).

We have demonstrated N-dealkylation of a number of secondary and tertiary amines by RSV microsomes (20). Also, benzidine, a human and rodent bladder carcinogen, was metabolized by RSV microsomes to reactive metabolites that covalently bound to nucleic acids (26). These results suggested the possible formation of mutagenic metabolites from chemicals other than PAH by PES. In this study, we have examined the ability of PES to metabolize a number of aromatic amines and other compounds to mutagenic products using the RSV microsomal fraction in a modification of the Salmonella test of Ames et al. (1, 6).

MATERIALS AND METHODS

Preparation of RSV Microsomal Fractions. The preparation of RSV microsomes has been described previously (6). For experiments to demonstrate arachidonic acid dependence and inhibition by indomethacin, the RSV microsomes were prepared in 0.15 M KCl (pH 7.4) containing 1% serum albumin and centrifuged at 100,000 X g a second time before storage at −80°C.

Preparation of Rat Liver S-9. The method of preparation has been described previously (6).

Analytical Procedures. PES activity was determined prior to each experiment by the measurement of O2 consumption using a Clark oxygen electrode (Yellow Instrument Co., Yellow Springs, Ohio) after addition of arachidonic acid (400 μM) to the RSV microsomes (1 mg protein per ml) in 0.1 M phosphate buffer, pH 7.8.

Protein concentrations were determined by the method of Lowry et al. (9) using bovine serum albumin Fraction V as a standard.

Reagents and Biochemicals. Glucose 6-phosphate, NADP+, L-histidine-HCl, d-biotin, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, Mo.); arachidonic acid was from NuChek Prep, Inc. (Elysian, Minn.); 2AA was from ICN Pharmaceuticals, Inc. (Plainview, N. Y.); 2AF, 2AAF, 2,4DA (4-methoxy-m-phenylenediamine sulfate trihydrate), 2,5DA (2-methoxy-p-phenylenediamine sulfate monohydrate), and DMN were from Aldrich Chemical Co. (Milwaukee, Wis.); 1NA and DEHP (practical grade) were from Matheson, Coleman, and Bell (Cincinnati, Ohio); 2NA was from J. T. Baker Chemical Co. (Phillipsburg, N. J.); Aminocarb (4-dimethylamino-m-lolyq-methylcaramate) was from Mobay Chemical Co. (Kansas City, Mo.); and NM was from Fluka AG (Switzerland). All chemicals were used as obtained.

Solutions of all test chemicals were prepared immediately prior to each experiment. DMN and NM in distilled water; the remaining test chemicals in dimethyl sulfoxide (Fisher Scientific Co., Plainview, N. J.). Solutions of indomethacin and arachidonic acid were prepared in absolute ethanol (U. S. Industrial Chemicals Co., Tuscola, Ill.).

Salmonella Mutagenesis Assay. Salmonella typhimurium strains G46, TA1535, TA1538, TA98, and TA100 were obtained from Dr. Bruce N. Ames (Berkeley, Calif.) and were maintained as frozen stocks at −80°C as recommended (1). Cultures were grown in Oxoid Nutrient Broth No. 2 at 37°C overnight with limited shaking, followed by fast
shaking to an absorbance at 650 nm of 1.0 (0.8 to 1.2 \times 10^5) colony-forming units/ml and then held on ice until used. The phenotypes of the strains were checked at the time of each experiment by the method of Zeiger et al. (25). The assay was performed as described previously (6). Briefly, mixtures consisting of 0.5 ml RSV microsomal fraction (diluted to 4 mg protein per ml in 60 mM KCl-100 mM phosphate buffer, pH 7.4), 0.1 ml bacterial culture, and 0.05 ml test chemical solution were incubated at 37° for 3 min before the addition of 5 or 10 \mu l arachidonic acid (to 100 \mu M). The complete system was then incubated at 37° for an additional 30 min before addition of top agar and plating. In experiments with indomethacin, the RSV microsomal fraction was preincubated with 5 \mu l indomethacin solution (to 100 \mu M) at 37° for 3 min before the addition of bacteria and test chemical. The control without activation contained buffer in place of RSV microsomal fraction. In incubation mixtures containing rat liver S-9, the 0.5 ml of RSV microsomal fraction was replaced by 0.5 ml of S-9 mix which contained 50 \mu l of S-9 (approximately 2 mg protein) plus cofactors (1), and arachidonic acid was not added.

RESULTS

We have studied the activation of a number of aromatic amines and several other compounds by PES to mutagenic products in a modification of the Salmonella microsome test (1, 6).

The mutagenicities of benzidine, 2AF and 2AAF, and 2,4DA and 2,5DA after activation by RSV microsomes are shown in Charts 1 to 3, respectively. With strain TA98, an approximately 4-fold increase in the number of histidine-independent (his\(^+\)) revertants per plate was obtained at 100 nmol benzidine per plate with no further increase up to 1000 nmol per plate (Chart 1). Both 2AF and 2AAF were activated by RSV microsomes to products mutagenic to strain TA98 (Chart 2). However, 2AAF was only weakly mutagenic; a response above background was obtained only at the highest dose tested (300 nmol/plate). Either benzidine or 2AF was included as a positive control in all subsequent experiments.

Both 2,4DA and 2,5DA were activated to products mutagenic to strain TA1538 by RSV microsomes (Chart 3). A small increase in the number of his\(^+\) revertants per plate was observed with 2,4DA at concentrations up to 100 nmol per plate, but no further increase was observed when it was retested at up to 1000 nmol per plate. The dose response obtained with 2,5DA differed from that obtained with 2,4DA; 2,5DA was inactive at 30 nmol per plate, but the number of his\(^+\) revertants obtained increased linearly between 100 and 1000 nmol per plate, the highest dose tested. In contrast, with the S-9 mix, the dose-response curves for 2,4DA and 2,5DA were similar, with 2,4DA having approximately the same activity as 2,5DA at one-tenth to one-twentieth the concentration of 2,5DA.

The activation of 1NA and 2NA was tested with strains TA1535 and TA98 (Table 1). With strain TA1535, 1NA was inactive with both the RSV microsomes and the S-9 mix at concentrations to 1000 nmol per plate, whereas 2NA was mutagenic over the same concentration range with both systems. Similar results were obtained with strain TA98, except that 1NA was weakly mutagenic with the S-9 mix in this strain.

Also shown in Table 1 are results obtained with 2AA in strain TA98. This compound was negative with RSV microsomes at concentrations which were strongly mutagenic for strain TA98 when tested with the S-9 mix. In the RSV system, no increase in revertants over that found in the control was seen.

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**Chart 1.** Mutagenicity of benzidine in S. typhimurium TA98. Experimental details are given in "Materials and Methods." Points, mean of up to 18 plates counted in up to 6 separate experiments; bars, S.D.; ○, RSV microsomal fraction; △, control (without enzyme); Δ, rat liver S-9.

**Chart 2.** Mutagenicity of 2AF and 2AAF in S. typhimurium TA98. Experimental details are given in "Materials and Methods." Points, mean of 6 plates counted in 2 separate experiments; bars, S.D.; ○, RSV microsomal fraction; △, control (without enzyme); Δ, rat liver S-9.

**Chart 3.** Mutagenicity of 2,4DA and 2,5DA in S. typhimurium TA1538. Experimental details are given in "Materials and Methods." Points, mean of up to 6 plates counted in 2 separate experiments; bars, S.D.; ○, RSV microsomal fraction; △, control (without enzyme); Δ, rat liver S-9.
The remaining compounds tested were the nitrosamines DMN and NM, the phthalate derivative DEHP, and the pesticide Aminocarb. DMN and NM were not mutagenic in strain G46 with the RSV microsomes, although they were mutagenic with the S-9 mix at the same doses (Table 2). Neither DEHP nor Aminocarb was activated by the RSV microsomes or by the S-9 mix to products mutagenic to strains TA98 or TA100 (Table 2). Aminocarb was toxic in the buffer control at the highest dose tested. However, in the presence of RSV microsomal fraction, no toxicity was apparent in the bacterial lawn.

To confirm the involvement of PES in the activation by RSV microsomes, the dependence on arachidonic acid (the PES substrate) and the inhibition by indomethacin (an inhibitor of PES) were examined using 2AF. Under conditions where activation is dependent on the amount of RSV microsomal protein present (Chart 4), the activation of 2AF was stimulated by arachidonic acid (Chart 5A) and inhibited by indomethacin (Chart 5B). In all instances, the effect was dose dependent; however, approximately 50% of the activity was neither inhibited by indomethacin nor dependent on the added arachidonic acid.

**DISCUSSION**

We have studied the activation of a number of aromatic amines and other compounds to mutagenic products by PES using the microsomal fraction from RSV. This tissue was chosen because of its high prostaglandin-biosynthetic activity and the absence of cytochrome P-450-NADPH-dependent mono-oxygenase activity (19).
The majority of the compounds tested were known to be activated to mutagens by the Aroclor 1254-induced rat liver S-9 system commonly used in the Salmonella assay (1). Tester strains known to be sensitive to these agents with rat S-9 were used in this study, and the rat liver S-9 mix was also included as a positive control.

The most mutagenic substance after activation by RSV microsomes was 2AF (Chart 1). Additional evidence suggests that 2AF is metabolized by PES. High-pressure liquid chromatography analyses of metabolites of 2AF in the presence of RSV and arachidonic acid suggest the formation of the hydroxylamine N-hydroxy-2-aminofluorene. 2,2'-Bisazofluorene, the stable condensation product of 2AF and nitrosofluorene, has been isolated (2). N-Hydroxy-2-aminofluorene is thought to be the product of the cytochrome P-450-NADPH-dependent metabolism of 2AF (21). The relative activity of 2AAF to 2AF is lower with the RSV microsomes compared to the S-9 mix. This may be due to low levels of deacetylase present in the RSV microsomes, which would result in reduced formation of N-hydroxy-2-aminofluorene, which is more mutagenic than N-hydroxycyethylaminofluorene (18, 23).

Where activation was dependent on the amount of RSV microsomal protein present, the mutagenicity of 2AF was stimulated by arachidonic acid and partially inhibited by indomethacin (Chart 5). This supports the involvement of PES in the activation by RSV microsomes. In the incubations without exogenous arachidonate, organic hydroperoxides could still be formed by either the action of lipooxygenases on endogenous fatty acids or the autooxidation of fatty acids or other compounds endogenous to the enzyme preparation or the bacteria.

In such cases, RSV microsomes would still be required as a source of peroxidase activity, but neither the formation of the hydroperoxides nor the cooxidation reaction itself would be dependent on exogenous arachidonate. Indomethacin inhibits the cyclooxygenase activity of PES and may similarly be bypassed by endogenous hydroperoxides. There are no known specific inhibitors of the hydroperoxidase (5, 10). It should be noted that the RSV microsomal fraction contains no detectable cytochrome P-450 (results not shown) and does not support NADPH-dependent oxidation of benzo(a)pyrene-7,8-dihydriodiol (19). Also, inhibitors of cytochrome P-450-dependent N-dealkylation had no effect on the metabolism of N-alkyl compounds with RSV microsomal fraction (20).

Benzidine, 2NA, 2,4DA, and 2,5DA were also activated to mutagens by the RSV microsomal fraction; 1NA and 2AA were negative. The lack of activity with 2AA is surprising in view of the activity of both 2AF and 2NA and the high degree of mutagenicity with the S-9 system.

None of the remaining compounds tested (the nitroso compounds DMN and NM, the pesticide Aminocarb, and the phthalate ester DEHP) was activated to mutagenic products by RSV microsomes under the conditions of this study. DMN and NM were activated to mutagens by the S-9 mix, but DEHP and Aminocarb were not (Tables 1 and 2). Unlike the aromatic amines which are activated via an initial N-hydroxylation, DMN, NM, and Aminocarb are activated by α-C-hydroxylation followed by N-dealkylation (3, 4, 8); PES-mediated N-dealkylation of Aminocarb but not DMN has been demonstrated (20).

DEHP, a potent hepatocarcinogen (16), was not activated by the S-9 mix in either this study or a previous study (24), and PES also did not activate this compound to mutagenic products.

In conclusion, we have demonstrated the ability of RSV microsomal fraction, a crude source of PES, to cooxidize a number of potential substrates for N-hydroxylation, with the notable exception of 2AA, to products mutagenic to Salmonella. Neither DMN, NM, and Aminocarb, all of which are potential substrates for N-dealkylation, nor the phthalate ester DEHP was activated to mutagenic products by the RSV microsomal fraction.

REFERENCES

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