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PROSTAGLANDIN H SYNTHASE-MEDIATED REACTION OF CARCINOGENIC ARYLAMINES WITH tRNA AND HOMOPOLYRIBONUCLEOTIDES

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SUMMARY: Prostaglandin H synthase mediates the reaction of an extensive series of carcinogenic arylamines with tRNA. Structure-activity relationships suggest that benzidine is especially reactive due to extended conjugation between the 4,4'-diamino groups. In trapping experiments with homopolyribonucleotides, benzidine reacts with polyguanylic acid but 4-aminobiphenyl reacts with polycytidylic acid. The nitrenium ion of 4-aminobiphenyl (formed by N,O-acyltransferase activation of N-hydroxy-4-acetylaminobiphenyl) reacts primarily with polyguanylic acid and to a lesser extent with polyadenylic acid. The results suggest that arylamine activation by prostaglandin H synthase does not involve nitrenium ion formation.

Arylamines are an important class of carcinogens that require oxidative metabolism to become carcinogenic (1). A key activation step in a number of tissues is N-hydroxylation catalyzed by cytochrome P-450 - or flavin-containing monooxygenases (1,2). The N-hydroxylated derivatives or their esters can decompose to nitrenium ions that covalently bind to DNA and induce mutations, a critical step in cell transformation (1-3). It has recently been reported that arylamines can also be co-oxidized during prostaglandin biosynthesis by the peroxidase activity of PGH synthase; substrates oxidized include arylamines and aryldiamines (4-7), alkylarylamines (6-8), alkylamines (8), aminoazobenzenes (6,9), and aminophenols and acetamidophenols (10-12). Furthermore, benzidine and several arylmonamines are metabolized to protein- and DNA-binding derivatives during prostaglandin biosynthesis in ram seminal

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Abbreviations: PGH synthase, prostaglandin H synthase; GMP, guanosine-5'-monophosphate.

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vesicle microsomes (6). These observations, together with the distribution of PGH synthase (13) suggest that this enzyme may contribute to arylamine carcinogenesis in extrahepatic tissues. Since the major identifiable products of arylmonoamine metabolism by PGH synthase *in vitro* are the nitroso derivatives (6), which can be formed by oxidation of hydroxylamines, the reactive intermediates which bind to DNA might be nitrenium ions. We have investigated the mechanism of arylamine activation by this enzyme by comparing the base specificity of the reactive intermediate to that of the nitrenium ion generated by *N,O*-acyltransferase-catalyzed activation of arylhydroxamic acids. Our results show that the derivative which binds to nucleic acid is not a nitrenium ion.

MATERIALS AND METHODS

Arachidonic acid (Nu-Chek Prep, Inc., Elysian, MN), GMP (P.L. Biochemicals, Inc.), tRNA (Calbiochem-Behring Corp.), indomethacin, and polycytidylic acid (Sigma Chemical Co.) were obtained as indicated; other homopolyribonucleotides were from Miles Laboratories. Radiochemicals were obtained as described below and purities were determined by analytical thin-layer radiochromatography. [^{14}C(U)]Benzidine (New England Nuclear, >96% purity, 1.04 mCi/mmole) in chloroform was titrated with acetic anhydride to obtain the mono- and diacetyl derivatives; these were purified by silica gel column chromatography, using ethyl acetate/$CHCl_3$ (1/3) to elute *N*-acetylbenzidine (>99%) and 100% ethyl acetate to elute *N,N'*-diacetylbenzidine (>99%). [Ring-^{3}H]-2-aminofluorene (>99%, 33.3 mCi/mmole) was prepared by reducing [ring-^{3}H]-2-nitrofluorene with hydrazine (14); the nitro starting material was prepared from [ring-^{3}H]fluorene (Amersham) by nitration (15). [Ring-^{3}H]-2-aminonaphthalene (>98%, 60 mCi/mmole) was prepared by reducing [ring-^{3}H]-2-nitronaphthalene (Midwest Research Institute, Kansas City, MO) with hydrazine (14). [Ring-^{3}H]Acetaminophen (>98%, 10 mCi/mmole) was purchased from New England Nuclear. [Ring-^{3}H]-*N*-hydroxyphenacetin (>98%, 109 mCi/mmole) was purchased from Midwest Research Institute. [Ring-^{3}H]-1-aminopyrene (>97%, 27.9 mCi/mmole) was prepared by reducing [ring-^{3}H]-1-nitropyrene with zinc (16); the nitro starting material was prepared from [ring-^{3}H]pyrene (Amersham) by nitration (17). [Ring-^{3}H]-6-aminobenz[a]pyrene (>96%, 5.9 mCi/mmole) was prepared by reducing [ring-^{3}H]-6-nitrobenz[a]pyrene with zinc (16); the starting material was prepared by nitration (18) of [ring-^{3}H]benz[a]pyrene (Amersham). 2-Amino-4-(5-nitro-2-furyl)[2-^{14}C]thiazole (>98%, 24 mCi/mmole) and *N*-[4-(5-nitro-2-furyl)-2-[2-^{14}C]thiazolyl]formamide (>98%, 55 mCi/mmole) were prepared as described (19). [Ring-^{3}H]-4-aminobiphenyl (>99%, 41 mCi/mmole) was prepared by reducing [ring-^{3}H]-4-nitrobiphenyl (Amersham) with hydrazine (14). 4,4'-[^{14}C]Methylene-bis(2-chloroaniline) (>99%, 7.3 mCi/mmole) was prepared by Amersham and kindly donated by the Michigan Toxic Substance Control Commission.

The standard assays for PGH synthase-mediated binding (1.0 ml) contained 0.086M phosphate buffer (pH 7.8), nucleic acid (1 mg tRNA or 2.5 umole homopolyribonucleotide phosphate), ram seminal vesicle microsomes (0.17-0.34 mg microsomal protein; prepared as in ref. 20), and 50 uM radioactive substrate. After preincubation (37°, 3 minutes), the reaction was started by adding 0.1 mM arachidonic acid. The reaction was stopped 5 minutes later by extracting with phenol, and the nucleic acid binding was determined as described previously (21). In addition, the *N*,*O*-acyltransferase-catalyzed binding of [ring-^{3}H]*N*-hydroxy-4-acetylaminobiphenyl (>98%, 14.2 mCi/mmole (22)) to nucleic acid was assayed as described previously (21), using rat liver cytosol as the enzyme preparation. For both enzymes, control incubations were conducted by withholding the nucleic acid until the end of the incubation. To search for a specific adduct, the PGH synthase assay was modified as follows: nucleic acid was replaced by GMP (5mg/ml), the labeled substrate was 4-aminobiphenyl (100 uM), and synthetic, unlabeled 8-(*N*-4-aminobiphenyl)guanosine-5'-monophsophate (23) was added as a carrier (100ug/ml). Recovery and analysis for this adduct was conducted as described previously (24).

RESULTS AND DISCUSSION

Incubation of benzidine with ram seminal vesicle microsomes and arachidonic acid resulted in reaction of approximately 15% of the benzidine with tRNA (Table 1). This reaction appeared to be catalyzed by PGH synthase since it was diminished >98% by heat inactivation of the enzyme, omission of arachidonic acid, or addition of the specific inhibitor indomethacin (0.1 mM, data not shown). The low values in control incubations, in which tRNA was added at the end of the incubation rather than at the beginning, showed that the reactive product was short-lived and that the tRNA isolation procedure effectively removed unbound benzidine and its metabolites as well as any labeled proteins that might have been present.

A number of other *N*-substituted aryl compounds were also tested - all of which are known or suspected carcinogens (Table 1). Although the net binding levels were no more than 2% of that of benzidine, results with the complete system were at least 5-10 times greater than the control for all compounds except the two nitrofurans and 4,4'-methylene-bis(2-chloroaniline). Macromolecular binding of six of these compounds has been reported previously (6,10,11,25).

Of particular interest in Table 1 are the low levels of reaction for several compounds structurally related to benzidine, i.e. *N*-acetylbenzidine, *N*,*N*'-diacetylbenzidine, 4-aminobiphenyl, and 4,4'-methylene-bis(2-chloroaniline). This implies that the unusually high reactivity of benzidine in this system could be due to the potential for extended conjugation between 4,4'-amino substituents. In contrast to these results, *N*-acetylbenzidine is

Table 1: PGH Synthase-catalyzed Reaction of Various Substrates with tRNA.

Substrate	Adduct Formation (pmoles/mg tRNA) complete system	control	net
benzidine	7821	216	7605
2-aminofluorene	144	6	138
2-aminonaphthalene	110	5	105
acetaminophen	118	18	100
N,*N*-diacetylbenzidine	64	10	54
N-acetylbenzidine	64	13	51
N-hydroxyphenacetin	50	4	46
1-aminopyrene	50	5	45
6-aminobenzo[a]pyrene	55	11	44
2-amino-4-(5-nitro-2-furyl)-thiazole	66	22	44
4-aminobiphenyl	31	3	28
N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide	76	53	23
4,4'-methylene-bis-(2-chloroaniline)	8	9	0

Standard incubations contained labeled substrates, arachidonic acid, ram seminal vesical microsomes, and tRNA as described in MATERIALS AND METHODS. For control incubations, tRNA was added at the end of the incubation, rather than at the beginning. Nucleic acid adduct formation was assayed as described previously (21). The results are expressed as the mean of duplicate determinations, which typically differed from each other by 5-15% in the complete system. Results from different experiments were normalized, using benzidine as a reference.

much more mutagenic than benzidine in liver microsome-mediated mutagenicity assays (26) and also constitutes the major DNA adduct in rodent liver *in vivo* (27). This difference may be a reflection of the fact that the liver is relatively low in PGH synthase activity (5,13) and high in *N*-hydroxylase activity (2).

The mechanism of activation of arylmonoamines during PGH synthase-mediated metabolism was investigated by examining adduct formation between 4-aminobiphenyl and specific trapping agents. For comparison, the *N*,*O*-acyltransferase-catalyzed activation of *N*-hydroxy-4-acetylaminobiphenyl was also included. The latter reaction proceeds through loss of an acetyl group to yield the nitrenium ion of 4-aminobiphenyl and preferentially yields an 8-(*N*-4-aminobiphenyl)guanine adduct (23,24). Although this adduct could be generated by substituting GMP for tRNA in the acyltransferase assay (24), it could not be detected following incubation of 4-aminobiphenyl with GMP in the

Table 2: PGH Synthase- and *N*,*O*-Acyltransferase-catalyzed Reactions of Benzidine and 4-Aminobiphenyl With Homopolyribonucleotides.

	Relative Nucleotide Substitution With		
	4-Aminobiphenyl		Benzidine
Polynucleotide	PGH Synthase	*N*,*O*-Acyltransferase	PGH Synthase
tRNA	100	100	100
polyadenylic acid	<0.5	36	0.6
polyguanylic acid	6	83	59
polycytidylic acid	53	<0.5	0.7
polyuridylic acid	<0.5	<0.5	<0.2

PGH synthase-catalyzed adduct formation was assayed as described in Table 1, except that tRNA was replaced by homopolyribonucleotides as shown below. Comparative data for the nitrenium ion of 4-aminobiphenyl were obtained from the rat liver acyltransferase-catalyzed activation of *N*-hydroxy-4-acetylaminobiphenyl. The recovery of individual polynucleotides was determined following each incubation; the results were expressed as pmoles arylamine bound/umole polynucleotide P, and then corrected for the controls (i.e. late addition of polynucleotide) before being normalized to the tRNA values. The relative amounts of the two enzymes used were such that they gave the same extent of binding of 4-aminobiphenyl to tRNA (18.6 and 18.2 pmoles/umole P for PGH synthase and acyltransferase respectively); the benzidine-substituted tRNA contained 3,360 pmoles/umole P.

presence of PGH synthase and arachidonic acid. However, the synthetic, unlabeled adduct which was included in the incubation was reisolated and gave the expected HPLC profile; this procedure would have detected 7.5 pmoles of labeled adduct/ml incubation mixture. In a different approach, the base specificity of the reactive intermediate was determined by substituting homopolymers for tRNA in the binding assays (Table 2). Binding occurred primarily with polycytidylic acid in the PGH synthase-catalyzed reaction, but the nitrenium ion in the acyltransferase incubations reacted preferentially with polyguanylic acid and also with polyadenylic acid.

These results strongly suggest that the nucleic acid-binding derivative formed from 4-aminobiphenyl during metabolism by PGH synthase is not an arylnitrenium ion. Since arylnitrenium ions are the reactive end-products of *N*-oxidative pathways, this further suggests that *N*-hydroxylation is not quantitatively significant in PGH synthase-mediated activation of 4-aminobiphenyl. Although arylnitroso compounds can react with protein (28), and have recently been reported as the major product of the PGH synthase-catalyzed oxidation of arylmonoamines (6), they do not react with nucleic acid (29,30). It is possible that hydroxylamines are the major initial metabolites of arylmonoamines, but that under the strongly oxidative assay conditions, they are rapidly converted to nitroso derivatives rather than undergoing heterolytic decomposition to yield nitrenium ions. If this occurs with 4-aminobiphenyl, it is unlikely that *in vitro* binding of any of the arylmonoamines in Table 1 results from nitrenium ion formation. However, the strongly oxidizing conditions of these incubations may not accurately reflect the conditions *in vivo* and the formation of arylhydroxylamines in intact cells cannot be ruled out. Indeed, it has been reported that following administration of 4-aminobiphenyl to dogs, a C-8 substituted guanine derivative is the major DNA adduct in the urinary bladder (31), which is a primary target organ for 4-aminobiphenyl carcinogenesis in this species (32). The urinary bladder epithelium contains PGH synthase activity (33), but nitrenium ions may also arise from urinary hydroxylamine *N*-glucuronides (34).

Table 2 also shows that benzidine, in contrast to 4-aminobiphenyl, reacted primarily with polyguanylic acid. However, it seems unlikely that this reaction would involve sequential formation of a hydroxylamine and a nitrenium ion due to the oxidizing conditions noted above. Two-electron oxidation of benzidine could yield a diimine that would be expected to undergo an addition reaction; indeed, the major recoverable benzidine metabolite is consistent with an addition product between benzidine and its diimine derivative (6). This diimine can also react with sulfhydryl compounds (35) and thus presumably combines with protein. It is unclear, however, whether the diimine reacts with nucleic acid, since the analogous quinone imine derivatives of aminophenols react readily with protein, but not with nucleic acid (36-38). An alternative mechanism would involve a free radical analogous to that detected recently for 3,5,3'5'-tetramethylbenzidine (4); it has been reported that benzidine quenches the free radical signal normally present during PGH synthesis (39).

Other studies have shown that PGH synthase-dependent epoxidation of polycyclic hydrocarbon derivatives can be differentiated from that catalyzed by cytochrome P-450 on the basis of substrate specificity (40,41), protein identity (42,43), and mechanism (44). This suggests that cooxidation during prostglandin biosynthesis is a pathway that is distinct from and complementary to mixed-function oxidase-dependent pathways of xenobiotic metabolism. The present results extend this concept to aromatic amines by demonstrating that the nucleic acid-binding derivatives generated by PGH synthase are distinct from the nitrenium ions resulting from mixed-function oxidase-dependent metabolism.

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