0022-3565/79/2113-0460$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 211, No. 3

Printed in U.S.A.

Cooxidation of Benzidine by Renal Medullary Prostaglandin Cyclooxygenase[1]

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Accepted for publication August 3, 1979

ABSTRACT

Zenser, Terry V., Michael B. Mattammal and Bernard B. Davis: *Cooxidation of benzidine by renal medullary prostaglandin cyclooxygenase.* J. Pharmacol. Exp. Ther. **211:** 460–464, 1979.

Rabbit hepatic and renal microsomes were used to investigate benzidine metabolism. Metabolism of [^{14}C]benzidine was analyzed in both organic soluble and aqueous fractions of reaction mixtures. Metabolism of benzidine was observed in both outer and inner medullary microsomes in the presence of arachidonic acid, but not under control conditions or with 1.5 mM NADPH. Hepatic and cortical microsomes did not metabolize benzidine under control conditions, with arachidonic acid or with NADPH. Evidence for medullary metabolism of benzidine was only observed in the aqueous fraction. Greater than 95% of the radioactivity recovered in organic extracts of reaction mixtures was authentic benzidine. In the presence of 0.03 mM benzidine and 0.068 mM arachidonic acid, the rate of benzidine metabolism by inner medullary microsomes was 335 pmol/mg of protein per min. This resulted in metabolism of greater than 30% of the benzidine present in the reaction mixture. Outer medullary metabolism was 60% of that in inner medulla. Glutathione (1.0 mM) reduced benzidine metabolism by 30% but increased the precentage of aqueous, non-trichloroacetic acid-precipitable metabolism from approximately 25 to 75%. Formation of (benzidine)-glutathione conjugates was demonstrated. While ethoxyquin completely inhibited benzidine metabolism, benzoate was not inhibitory. The fatty acid requirement for medullary benzidine metabolism is the same as that for prostaglandin cyclooxygenase. In addition, inhibitors of arachidonic acid-dependent benzidine metabolism are also inhibitors of prostaglandin cyclooxygenase. Therefore, specific fatty acid substrate requirements and inhibitor data suggest that prostaglandin cyclooxygenase is mediating renal medullary benzidine metabolism by a cooxidation process.

Benzidine elicits nephrotoxic efffects in certain species and in man, dog and rabbit is a urinary bladder carcinogen (Haley, 1975; Case *et al.*, 1954). Bladder carcinogens are thought to acquire their carcinogenic potential by activation to more carcinogenic forms (Miller, 1978). Because some agents such as β-naphthylamine exert their effects only when the bladder is exposed to urine and because direct implantation of known bladder carcinogens into the bladder may not induce bladder carcinoma (McDonald and Lund, 1954), it has been proposed that activation to more proximal or ultimate carcinogens proceeds before the passage of urine into the bladder. This activation has been postulated to occur in the liver (Miller, 1978). However, definitive studies evaluating potential hepatic metabolism of benzidine have not been reported. In addition, no reports have evaluated the potential renal metabolism of benzidine (Clayson and Garner, 1976). Recent data suggest that the kidney possesses the ability to convert compounds to metabolites with nephrotoxic potential (Zenser *et al.*, 1978, 1979). The purpose of the present study was to investigate possible mechanisms by which benzidine might exert its adverse effects on the kidney and extrarenal collecting system by evaluating benzidine metabolism by microsomes of rabbit liver and renal cortex, outer and inner medulla.

Received for publication March 30, 1979
[1] This work was supported by the Veterans Administration.

Materials and Methods

Materials. [^{14}C(*U*)]benzidine (0.13 mCi/mg) was purchased from New England Nuclear Corp., Boston, MA. [1-^{14}C]Arachidonic acid (56 mCi/mmol) and scintillation fluid (ACS) were purchased from Amersham Searle, Arlington Heights, IL. Indomethacin, NADPH, asprin (acetylsalicylic acid), benzidine dihydrochloride (lot no. 18C-0025-1), sodium benzoate, glutathione, cysteine, 11,14,17-eicosatrienoic acid, and 9,12,15-octadecatrienoic acid were purchased from Sigma Chemical Co., St. Louis, MO. 5,8,11,14-Eicosatretaenoic acid and 8,11,14-eicosatrienoic acid were purchased from Nu Check Prep, Inc., Elysian, MN. 5,8,11,14-Eicosatraynoic acid was the generous gift of Dr. W. E. Scott, Hoffmann-La Roche, Inc., Nutley, NJ; ethoxyquin of Dr. G. L. Romoser, Monsanto Chemical Co., St. Louis, MO; and prostaglandin (PG) E_2 of Dr. John Pike, The Upjohn Co., Kalamazoo, MI. Thin-layer chromatographic plates (Silica Gel F-254) were obtained from EM Laboratories, Elmsford, NJ. All other chemicals were purchased in the highest possible grade from standard sources. Male New Zealand rabbits weighing 2 to 3 kg were obtained from Eldridge Laboratory Animals, Barnhart, MO.

Preparation of microsomes. Rabbits were anesthetized with intravenous sodium thiopental (20 mg/kg i.v.). The kidneys were quickly

removed and placed in ice-cold 0.9% NaCl. Renal inner medullary tissue was isolated by careful dissection, minced, washed free of hemoglobin, and homogenized for 15 sec with a Polytron homogenizer in 3 volumes of 0.1 M phosphate buffer (pH 7.8) containing 20% glycerol and 10^{-4} M dithiothreitol. The homogenate was centrifuged at 10,000 × *g* for 15 min and the subsequent supernatant at 100,000 × *g* for 60 min. This pellet was layered with 1.15% KCl and stored at −40°C. Under these storage conditions there are not detectable changes in either cytochrome P-450 spectra or mixed-function oxidase enzymatic activities for up to 3 months (Zenser *et al.*, 1978). Before use, the microsomal pellets were resuspended in 1.15% KCl by hand homogenization and recentrifuged for 30 min at 105,000 × *g*. Pellets were resuspended in 0.1 M phosphate buffer (pH 7.8) with a Potter Elvehjem Teflon glass homogenizer to give a final concentration of about 10 mg of microsomal protein per ml. Aliquots of these suspensions were either used immediately or stored frozen at −40°C. Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Incubation conditions, extraction and analysis of benzidine metabolism. The reaction mixture contained 0.5 to 2 mg/ml of microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.03 mM [^{14}C]-benzidine (0.25 μCi) and various test substances in a final volume of 0.25 ml at 37°C for 15 min. The reaction was linear between 5 to 15 min of incubation with 0.03 mM benzidine and 0.068 mM arachidonic acid. Addition of fatty acids started the reaction. Inhibitiors were preincubated with microsomes for 5 min at room temperature before the addition of fatty acid. The reaction was stopped by addition of 0.75 mM unlabeled benzidine and 0.5 ml of ethyl acetate–diethyl ether (1:1, v/v). The reaction mixture was extracted until the radioactivity in the organic phase reached a constant value (approximately eight extractions per sample). Pooled organic phases from each individual sample were dried with anhydrous Na_2SO_4, evaporated under N_2 gas and applied to thin-layer chromatography plates. Chromatographic solvent systems used were: methyl ethyl ketone–N,N-diisopropylethylamine (85:5) or benzene–ethanol (70:30) with R_f values for benzidine of 0.70 and 0.53, respectively. Authentic benzidine was identified with an ultraviolet light. Each chromatographed sample was divided into 1-cm sections from the origin to the solvent front, the sections were scraped into scintillation vials, and the radioactivity was determined.

Following organic solvent extraction 0.6 M trichloroacetic acid (TCA) was added to the aqueous phase which was then centrifuged at 2500 × *g* for 10 min. The resulting precipitates were washed with 1 ml of 0.6 M TCA and repeated centrifugation was carried out until the radioactivity of the supernatant was similar to background (approximately eight extractions per sample). Pellets were disolved in 0.2 ml of 1 N NaOH at 60°C for 30 min and diluted to 1 ml with distilled water, aliquoted and analyzed for radioactivity. Radioactivity in the aqueous, non-TCA-precipitable fraction was also determined. Metabolism of benzidine was expressed as picomoles per milligram of protein per minute.

Evidence for the formation of glutathione conjugates of [^{14}C]benzidine products was provided by thin-layer chromatographic analysis. Pooled samples were lyophilized, washed with ether and dissolved in dimethylformamide–dimethylsulfoxide (1:1). Samples were applied to Silica Gel F-254 plates and developed with solvent system I [1-butanol–1-propanol–0.1 N NH_3 (2:1:1)] or II [benzene–ethanol (60:40)]. Unlabeled benzidine was run as a standard with R_f 0.82 or 0.70 in solvent systems I and II, respectively. Radioactivity was determined using a Packard radiochromatogram scanner 7230 series.

Synthesis of prostaglandins. Reaction mixtures contained microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.03 mM benzidine and various concentrations of [^{14}C]arachidonic acid in a final volume of 0.1 ml. Microsomes and reaction mixture were preincubated for 5 min at room temperature before a 10-min incubation at 37°C. The reaction was started by addition of arachidonic acid and stopped by addition of HCl. Heated microsomes served as blanks. All reported values have the blank values substracted. Samples were adjusted to pH 3 to 3.5, extracted with ethyl acetate and evaporated to dryness under N_2 gas as previously described (Mattammal *et al.*, 1979). Samples were applied to Silica Gel F-254 plates and developed with a solvent system containing chloroform–methanol–acetic acid (90:5:5). PGE_2, $PGF_{2\alpha}$, PGD_2 and arachidonic acid standards were identified with iodine vapors and the radioactivity was determined in corresponding zones.

Results

Benzidine metabolism by hepatic and renal cortical, outer and inner medullary microsomes is tabulated in table 1. Metabolism of benzidine (0.03 mM) was not detected in either hepatic or cortical microsomes under control conditions (no addition) or with 1.5 mM NADPH or 0.068 mM arachidonic acid. Neither inner nor outer medullary microsomes metabolized benzidine under control conditions or with NADPH. However, in the presence of arachidonic acid, both inner and outer medullary microsomes demonstrated evidence of significant metabolism of benzidine in the TCA-precipitable and aqueous, non-TCA-precipitable fractions of reaction mixtures. Greater than 95% of the radioactivity recovered in organic extracts of reaction mixtures was authentic benzidine as judged by thin-layer chromatographic analysis whether microsomes were from liver or kidney. In the inner medulla, the total rate of benzidine (metabolism (TCA-precipitable plus non-TCA-precipitable) with 0.068 mM arachidonic acid and 0.03 mM benzidine was 335 pmol/mg of protein per min. This represents metabolism of greater than 30% of the benzidine present in the reaction mixture during the 15-min incubation. In the outer medulla, the total rate of benzidine metabolism was 60% of that observed in the inner medulla.

Microsomal metabolism of benzidine by inner medulla required specific fatty acids (table 2). 5,8,11,14-Eicosatetraenoic (arachidonic acid) and 8,11,14-eicosatrienoic acids (the precursor of monoenic prostaglandins) both initiated dose-dependent metabolism of benzidine while the unsaturated fatty acids, 11,14,17-eicosatrienoic and 9,12,15-octadecatrienoic did not initiate benzidine metabolism. PGE_2, a major product of arachidonic acid-mediated prostaglandin synthesis in the inner medulla, had no effect upon benzidine metabolism.

TABLE 1

Metabolism of [^{14}C]benzidine by renal and hepatic microsomes

All reaction mixtures contained 0.5 to 2 mg of microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.03 mM [^{14}C]benzidine, and where indicated 0.068 mM arachidonic acid or 1.5 mM NADPH. Values represent the mean ± S.E.; *n* = 9. N.D., not detected.

Tissue	Aqueous, TCA-Precipitable	Aqueous, Non-TCA-Precipitable
	pmol/mg protein/min	
Liver		
Control	N.D.	N.D.
NADPH	N.D.	N.D.
Arachidonic acid	N.D.	N.D.
Kidney		
Inner medulla		
Control	N.D.	N.D.
NADPH	N.D.	N.D.
Arachidonic acid	259 ± 10	76 ± 9
Outer medulla		
Control	N.D.	N.D.
NADPH	N.D.	N.D.
Arachidonic acid	146 ± 8	55 ± 7
Cortex		
Control	N.D.	N.D.
NADPH	N.D.	N.D.
Arachidonic acid	N.D.	N.D.

Inhibitors of prostaglandin cyclooxygenase inhibited arachidonic acid-dependent metabolism of benzidine (table 3). Aspirin (1.0 mM), indomethacin (0.1 mM) and meclofenamic acid (0.1 mM) all inhibited benzidine metabolism. 5,8,11,14-Eicosatetraynoic acid (0.1 mM), an acetylene analog of arachidonic acid, completely inhibited benzidine metabolism. The antioxidant ethoxyquin (0.1 mM) completely inhibited benzidine metabolism. Benzoate, a scavenger of hydroxyl radicals (Lai and Piette, 1978), had no effect. Glutathione reduced total benzidine metabolism by 30% but increased the amount of product incorporated into the aqueous, non-TCA-precipitable material. Heated microsomes (3 min at 100°C) did not metabolize benzidine either in the presence or absence of arachidonic acid.

The dose-dependent effect of indomethacin on PGE_2 synthesis and benzidine metabolism is illustrated in figure 1. Indomethacin inhibited the synthesis of PGE_2 and metabolism of benzidine in a similar manner with ID_{50} values of 0.71 and 0.78 μM, respectively. Using the assay conditions in figure 1 (0.025 mM arachidonic acid and 0.03 mM benzidine), the ratio of PGE_2 synthetized/benzidine metabolized was approximately 4:1.

The effects of glutathione and cysteine, another sulfhydryl agent, on arachidonic acid-dependent metabolism of benzidine were further examined (table 4). Cysteine (1.0 mM), like glutathione (1.0 mM), increased the amount of [^{14}C]benzidine incorporated into aqueous, non-TCA-precipitable material. Concentrations of glutathione as low as 0.05 mM had a similar effect. Addition of glutathione (1.0 mM) at the end of the incubation for 15 min did not affect the metabolism of benzidine relative to the control. In separate experiments, the metabolism of [^{14}C]arachidonic acid (0.068 mM) with 0.03 mM benzidine present was examined using inner medullary microsomes. Glutathione increased the amount of PGE_2 synthetized but did not alter the total amount of arachidonic acid metabolized. The percentages of [^{14}C]PGE_2, [^{14}C]$PGF_{2\alpha}$, [^{14}C]PGD_2 and [^{14}C]-arachidonic acid in the control were 4.5 ± 0.3, 2.8 ± 0.4, 3.0 ± 0.3 and 83 ± 2.8%, respectively, and with 1.0 mM glutathione 10.6 ± 0.8, 0.4 ± 0.1, 0.4 ± 0.1 and 82 ± 4.1%, respectively.

Evidence for the formation of ([^{14}C]benzidine)-glutathione conjugates is illustrated in figure 2. Radioscans of control and glutathione (1.0 mM)-treated samples are shown. In the presence of glutathione, a peak was observed which was distinct from benzidine and not observed in the control.

The radioactivity incorporated into the TCA-precipitable material was also investigated. Microsomes were incubated with 0.068 mM arachidonic acid and 0.030 mM benzidine using the standard incubation conditions. Following incubation, the TCA-precipitable material was isolated as described under "Materials and Methods". This material was solubilized with NaOH and then extracted at pH 3.0, 7.5 and 12.5 with the following solvents 1) ethyl ether or 2) chloroform–ethyl ether

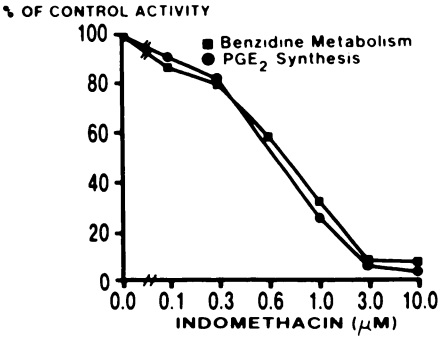

Fig. 1. Effect of indomethacin on PGE_2 synthesis and benzidine metabolism. Reaction mixtures contained 0.14 mg of microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.025 mM arachidonic acid and 0.03 mM benzidine in a final volume of 0.1 ml. Microsomes and reaction mixture were preincubated for 2 min at room temperature before a 10-min incubation at 37°C. For determination of PGE_2 synthesis, [^{14}C]-arachidonic acid was used. For determination of benzidine metabolism, [^{14}C]benzidine was used. Details of methods and analysis of PGE_2 synthesis and benzidine metabolism are provided under "Materials and Methods." The rates of PGE_2 synthesis and total benzidine metabolism (both TCA-precipitable and non-TCA-precipitable aqueous fractions) were 0.64 and 0.15 nmol/mg/min, respectively. These rates each correspond to 100% of their respective control activity. Values represent the mean of four determinations.

TABLE 2

Fatty acid substrate requirements for the cooxygenation of [^{14}C]-benzidine by inner medullary microsomes

Reaction mixtures contained 0.5 mg of microsomal protein, 0.1 M phosphate buffer (pH 7.8), 0.03 mM [^{14}C]benzidine and the indicated concentration of fatty acid. Values represent the mean ± S.E.; n = 6. N.D., not detected.

Additions	Conc.	Aqueous, TCA-Precipitable	Aqueous, Non-TCA-Precipitable
	mM	*pmol/mg protein/min*	
No addition		N.D.	N.D.
5,8,11,14-Eicosatetraenoic acid (arachidonic acid)	0.034	130 ± 6	45 ± 10
	0.068	259 ± 5	76 ± 8
5,8,11,14-Eicosatetraynoic acid	0.068	N.D.	N.D.
Prostaglandin E_2	0.068	N.D.	N.D.
8,11,14-Eicosatrienoic acid	0.068	180 ± 8	30 ± 5
11,14,17-Eicosatrienoic acid	0.068	N.D.	N.D.
9,12,15-Octadecatrienoic acid	0.068	N.D.	N.D.

TABLE 3

Effect of various compounds on arachidonic acid-dependent [^{14}C]-benzidine oxidation by inner medullary microsomes

All reaction mixtures contained 0.068 mM arachidonic acid, 0.03 mM [^{14}C]-benzidine, 0.1 M phosphate buffer (pH 7.8), 0.5 mg of protein and the indicated concentration of test agents. Values represent the mean ± S.E.; n = 6. N.D., not detected.

Additions	Conc.	Aqueous, TCA-Precipitable	Aqueous, Non-TCA-Precipitable
	mM	*pmol/mg protein/min*	
No addition		272 ± 11	91 ± 8
Indomethacin	0.03	N.D.	N.D.
Aspirin	1.0	N.D.	N.D.
5,8,11,14-Eicosatetraynoic acid	0.1	N.D.	N.D.
Ethoxyquin	0.1	N.D.	N.D.
Meclofenamic acid	0.1	N.D.	N.D.
Benzoate	1.0	259 ± 10	76 ± 8
Glutathione	1.0	56 ± 4	180 ± 10

TABLE 4

Effect of glutathione and cysteine on arachidonicacid-mediated covalent binding of [^{14}C]benzidine to protein

Reaction conditions and method of analysis were the same as table 3. Values represent the mean ± S.E.; n = 6.

Additions	Conc.	Aqueous, TCA-Precipitable	Aqueous, Non-TCA-Precipitable
	mM	*pmol/mg protein/min*	
Control		280 ± 21	97 ± 9
Glutathione	1.0	62 ± 5	192 ± 11
	0.05	105 ± 4	135 ± 9
Glutathione (added at the end of incubation for 15 min)	1.0	305 ± 23	105 ± 4
Cysteine	1.0	58 ± 4	186 ± 7

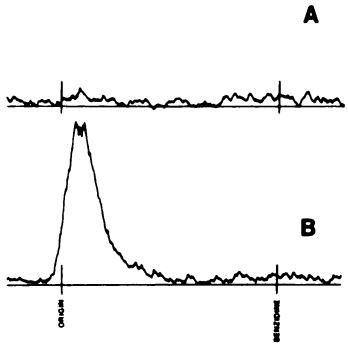

Fig. 2. Effect of glutathione on [^{14}C]-benzidine metabolism. Aqueous, non-TCA-precipitable samples from tables 3 and 4 were pooled, lyophilized, applied to thin-layer chromatographic plates and developed with solvent system I as described under "Materials and Methods." Illustrated is a radioscan of these plates where A represents control and B represents 1.0 mM glutathione-treated samples.

(1:3, v/v). Following extraction, greater than 90% of the radioactivity remained in the aqueous phase.

Discussion

These data indicate that the renal medulla possesses the capacity to metabolize the urinary bladder carcinogen benzidine. A similar capacity was not measurable in either renal cortex or liver. Medullary metabolism of benzidine is not due to either lipoxygenase enzymes or lipid peroxidation. The specific fatty acid requirements and the inhibition by structurally diverse agents such as indomethacin and aspirin are not compatible with lipid peroxidation. Benzoate, which is an effective inhibitor of lipid peroxidation by hepatic microsomes (Lai and Piette, 1978), had no effect upon benzidine metabolism. Fatty acids such as 9,12,15-octadecatrienoic acid and 11,14,17-eicosatrienoic acid which are substrates for lipoxygenases did not support benzidine metaolism (Hamberg and Samuelsson, 1967). Furthermore, lipoxygenases are not inhibited by the nonsteroidal anti-inflammatory agents as was the case for benzidine metabolism in these experiments. Thin-layer chromatographic analysis of [1-^{14}C]arachidonic acid metabolism by inner medullary microsomes did not result in the identification of the characteristic products of the lipoxygenease reaction.

Oxidation of benzidine appeared independent of mixed-function monooxygenases. The liver, which contains mixed-function oxidases, did not metabolize benzidine. Our previous results indicate that the methods used to prepare these microsomes results in hepatic mixed function oxidase activities comparable to those observed by others. These microsomal preparations also exhibit characteristic cytochrome P-450 optical (Zenser *et al.*, 1978) and electron paramagnetic resonance spectra (Armbrecht *et al.*, 1979). Finally, the NADPH-dependent rate of 1,3-diphenylisobenzofuran metabolism was routinely examined for each batch of liver microsomes (Zenser *et al.*, 1979). Therefore, the lack of hepatic metabolism of benzidine is not related to the lack of mixed-function oxidase activity. Inner medulla, which metabolized benzidine, does not contain spectrally detectable cytochrome P-450 (Zenser *et al.*, 1978). NADPH, a cofactor in mixed-function oxidase reactions, had no effect upon benzidine metabolism in any of the tissues tested.

The data are all compatible with benzidine being metabolized by a cooxidative process involving prostaglandin cyclooxygenase. Fatty acids that initiated benzidine metabolism have in common the location of a triene system which terminates at the sixth carbon from the alkyl terminus. Prostaglandin cyclooxygenase has this same substrate requirement (Andersen, 1971). The lack of effect of PGE_2 is consistent with a previous study examining cooxidation of organic compounds by vesicular gland prostaglandin cyclooxygenase (Marnett *et al.*, 1975). The latter study showed that arachidonic acid and the prostaglandin intermediate PGG_2 could initiate cooxidation, but that PGH_2 or PGE_2 could not. In the present study, a variety of structurally diverse inhibitors of prostaglandin cyclooxygenase inhibited arachidonic acid-dependent benzidine metabolism. The close similarity of the indomethacin ID_{50} values for PGE_2 synthesis (0.71 μM) and benzidine metabolism (0.78 μM) suggests that both processes are mediated by prostaglandin cyclooxygenase. Previous studies with rabbit medullary homogenates have reported an indomethacin ID_{50} value of 0.88 μM (Gafni *et al.*, 1978). Therefore, oxidation of benzidine appears to occur by simultaneous oxidation of fatty acids *via* prostaglandin cyclooxygenase with the formation of reactive intermediates of benzidine metabolism.

Glutathione and cysteine altered the pattern of cooxidative metabolism of benzidine. Both compounds increased the amount of aqueous, non-TCA-precipitable benzidine product(s) formed at the expense of the TCA-precipitable product(s). Since this effect of glutathione was not observed following its addition at the end of the incubation, glutathione does not appear to be involved in an exchange reaction with sulfhydryl groups on proteins but rather glutathione appears to be combining with a reactive product(s) formed during the cooxidative process. Evidence for (benzidine)-glutathione conjugates was also illustrated. The capacity of glutathione to inactivate toxic compounds formed during drug metabolism is well documented (Mitchell *et al.*, 1973). These effects of glutathione and cysteine appear independent of their effects on prostaglandin synthesis. Both of these sulfhydryl compounds stabilize the enzyme prostaglandin E isomerase. Glutathione is also an essential cofactor in the conversion of PGH to PGE by this isomerase enzyme (Ogino *et al.*, 1977). Glutathione is not required for the synthesis of PGG or PGH (Miyamoto *et al.*, 1976). Our results demonstrating that 1.0 mM glutathione increased the amount of PGE_2 synthetized while not altering the amount of arachidonic acid metabolized are consistent with these previously published results. The syntheses of $PGF_{2\alpha}$ and PGD_2 were both significantly reduced by the increase in prostaglandin E isomerase activity caused by glutathione.

This demonstration of the involvement of prostaglandins in the metabolism of a carcinogen such as benzidine is consistent with other reports. Marnett *et al.* (1978) have demonstrated the *in vitro* activation of a precarcinogenic metabolite of benzo(a)pyrene to a mutagen by prostaglandin cyclooxygenase. In addition, tumor-promoting phorbol-12,13-diesters and polycyclic hydrocarbon carcinogens stimulate cultured dog kidney cells to produce prostaglandins (Levine and Hassid, 1977; Hassid and Levine, 1977). A number of malignant tumors also produce high levels of prostaglandins (Humes and Stausser, 1974); Tashjian *et al.*, 1972). The antioxidants ethoxyquin and butylated hydroxyanisole which inhibit prostaglandin cyclooxygenase (Vanderhoek and Lands, 1973) inhibit the carcinogenic effect of dietary 7,12-dimethylbenz(a)anthracene on mouse forestomach (Wattenberg, 1972). Antioxidant inhibition of the cutaneous carcinogenic effect of 7,12-dimethylbenz(a)anthracene did not correlate with inhibition of the mixed-function

oxidase enzyme aryl hydrocarbon hydroxylase (Slaga and Bracken, 1977). However, alternative enzyme systems were not investigated.

There are marked species differences with respect to the adverse tissue effects of benzidine (Weisburger *et al.*, 1967). However, carcinoma of the bladder is inducible in the rabbit by administration of benzidine. Benzidine is thought to be transformed to a more effective carcinogen by endogenous metabolism. These data are consistent with the generation of the carcinogenic form of benzidine by inner medullary metabolism. It has been proposed that host differences in benzidine metabolism are a major factor in determining the response of a particular species to benzidine.

In summary, these results are consistent with a previous study demonstrating the relatively high cooxidative potential of the inner medulla (Zenser *et al.*, 1979). Concepts relating to the metabolism of benzidine remain speculative at the present time, but they do provide the opportunity to construct testable hypotheses.

Acknowledgments

Appreciation is expresed to Mr. Harald Buhle and Mr. Mark Palmier for technical assistance and to Miss Sharon Smith and Mrs. Sandy Melliere for secretarial assistance.

References

ANDERSEN, N.: Program notes on structures and nomenclature. Ann. N.Y. Acad. Sci. **180:** 14-23, 1971.

ARMBRECHT, H. J., BIRNBAUM, L. S., ZENSER, T. V., MATTAMMAL, M. B. AND DAVIS, B. B.: Renal cytochrome P450's—Electrophoretic and electron paramagnetic resonance studies. Arch. Biochem. Biophys., **197:** 277-284, 1979.

CASE, R. A. M., HOSKER, M. W., MCDONALD, D. B. AND PEARSON, J. T.: Tumors of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Br. J. Ind. Med. **11:** 75-104, 1954.

CLAYSON, D. B. AND GARNER, R. C.: Carcinogenic aromatic amines and related compounds. *In* Chemical Carcinogens, ed. by C. E. Searle, chap. 8, American Chemical Society, Washington, DC, 1976.

GAFNI, Y., SCHWARTZMAN, M. AND RAZ, A.: Prostaglandin biosynthesis in rabbit kidney medulla: Inhibition *in vitro* vs. *in vivo* by aspirin, indomethacin and meclofenamic acid. Prostaglandins **15:** 759-772, 1978.

HALEY, T. J.: Benzidine revisited: A review of the literature and problems associated with the use of benzidine and its congeners. Clin. Toxicol. **8:** 13-42, 1975.

HAMBERG, M. AND SAMUELSSON, B.: On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxidase. J. Biol. Chem. **242:** 5329-5335, 1967.

HASSID, A. AND LEVINE, L.: Induction of fatty acid cyclooxygenase activity in canine kidney cells (MDCK) by benzo(a)pyrene. J. Biol. Chem. **252:** 6591-6593, 1977.

HUMES, J. L. AND STAUSSER, H. R.: Prostaglandins and cyclic nucleotides in Maloney sarcoma tumors. Prostaglandins **5:** 183-196, 1974.

LAI, C. S. AND PIETTE, L. H.: Spin-trapping studies of hydroxyl radical production involved in lipid peroxidation. Arch. Biochem. Biophys. **190:** 27-38, 1978.

LEVINE, L. AND HASSID, A.: Effects of phorbol-12,13-diesters on prostaglandin production and phospholipase activity in canine kidney (MDCK) cells. Biochem. Biophys. Res. Commun. **79:** 477-484, 1977.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. AND RANDALL, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:** 265-275, 1951.

MARNETT, L. J., REED, G. A. AND DENNISON, D. J.: Prostaglandin synthetase dependent activation of 7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene to mutagenic derivatives. Biochem. Biophys. Res. Commun. **82:** 210-216, 1978.

MARNETT, L. J., WLODAWER, P. AND SAMUELSSON, B.: Co-oxygenation of organic substrates by the prostaglandin synthetase of sheep vesicular gland. J. Biol. Chem. **250:** 8510-8517, 1975.

MATTAMMAL, M. B., ZENSER, T. V., BROWN, W. W., HERMAN, C. A. AND DAVIS, B. B.: Mechanism of inhibition of renal prostaglandin production by acetaminophen. J. Pharmacol. Exp. Ther. **210:** 405-409, 1979.

MCDONALD, D. F. AND LUND, R. R.: The role of the urine in vesical neoplasms. I. Experimental confirmation of the urogenous theory of pathogenesis. J. Urol. **71:** 560-570, 1954.

MILLER, E. C.: Some current perspectives on chemical carcinogenesis in humans and experimental animals. Cancer Res. **38:** 1479-1496, 1978.

MITCHELL, J. R., JOLLOW, D. J., POTTER, W. Z., GILLETTE, J. R. AND BRODIE, B. B.: Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J. Pharmacol. Exp. Ther. **187:** 211-217, 1973.

MIYAMOTO, T., OGINO, N., YAMAMOTO, S. AND HAYAISHI, O.: Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. J. Biol. Chem. **251:** 2629-2636, 1976.

OGINO, N., MIYAMOTO, T., YAMAMOTO, S. AND HAYAISHI, O.: Prostaglandin endoperoxide E isomerase from bovine vesicular gland microsomes, a glutathione-requiring enzyme. J. Biol. Chem. **252:** 890-895, 1977.

SLAGA, T. J. AND BRACKEN, W. M.: The effects of antioxidants on skin tumor initiation and aryl hydrocarbon hydroxylase. Cancer Res. **37:** 1631-1635, 1977.

TASHJIAN, A. H., JR., VOELKEL, E. F., LEVINE, L. AND GOLDHABER, P.: Evidence that the bone resorption-stimulating factor produced by mouse fibrosarcoma cells in prostaglandin E_2. A new model for the hypercalcemia of cancer. J. Exp. Med. **136:** 1329-1343, 1972.

VANDERHOEK, J. Y. AND LANDS, W. E. M.: The inhibition of the fatty acid oxygenase of sheep vesicular gland by antioxidants. Biochim. Biophys. Acta **296:** 382-385, 1973.

WATTENBERG, L. W.: Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxyquin. J. Natl. Cancer Inst. **48:** 1425-1431, 1972.

WEISBURGER, J. H., HADIDIAN, Z., FREDERICKSON, T. N. AND WEISBURGER, E. K.: Host properties determine target, bladder or liver in chemical carcinogenesis. *In* Bladder Cancer, ed. by K. F. Lampe, chap. 3, Aesculapius Publishing, Birmingham, AL, 1967

ZENSER, T. V., MATTAMMAL, M. B. AND DAVIS, B. B.: Differential distribution of mixed function oxidase activities in rabbit kidney. J. Pharmacol. Exp. Ther. **207:** 719-725, 1978.

ZENSER, T. V., MATTAMMAL, M. B. AND DAVIS, B. B.: Demonstration of separate pathways for drug metabolism in the rabbit kidney. J. Pharmacol. Exp. Ther. **208:** 418-421, 1979.

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