In Vivo Metabolism of Topically Applied Benzo[a]pyrene-4,5-oxide in Neonatal Rat Skin

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The metabolism of benzo[a]pyrene (BP)-4,5-oxide in the skin and liver of neonatal rats was studied after topical application of the arene oxide in vivo. The metabolism of BP-4,5-oxide was time-dependent and showed a 2-h maximum for BP-4,5-dihydrodiol formation in both skin and liver. Product formation was also dose-dependent. Inhibitors of epoxide hydrolase such as clotrimazole, 1,1,1,-trichloropropene oxide, and cyclohexene oxide largely abolished the formation of BP-4,5-dihydrodiol. The rapid biotransformation of arene oxides such as BP-4,5-oxide in the skin emphasizes the potential importance of epoxide hydrolase in the activation and inactivation of polycyclic aromatic hydrocarbons. Furthermore, the topically applied arene oxide also penetrated the skin and was rapidly metabolized in the liver as well.

The skin is an important interface between the body and the environment and can become a portal of entry for environmental pollutants, some of which can evoke toxicity including carcinogenicity in cutaneous tissue [1]. Benzo[a]pyrene is a ubiquitous environmental pollutant and is a known skin carcinogen [2]. It is generally accepted that polycyclic aromatic hydrocarbons (PAHs) such as BP, must be metabolically activated to dihydrodiol-epoxides which covalently bind nucleophilic sites on cellular macromolecules such as DNA to initiate the process of carcinogenicity [2–7]. Three successive enzymatic steps are involved in the generation of dihydrodiol-epoxides of BP. First, turnover by the cytochrome P-450-dependent monooxygenase followed by epoxide hydrolase and again by monooxygenase [2,3,7–10]. It follows that monooxygenases and epoxide hydrolase play important roles in the metabolism of PAHs. Also of importance is the availability of some as yet unidentified critical concentrations of reactive metabolites of PAHs in the target organ for the initiation of tumor induction. The balance between the ability of an organ to generate, detoxify, and eliminate reactive epoxide intermediates may be a critical factor in determining the susceptibility or resistance of that organ to PAH-induced carcinogenesis. Therefore, detailed studies on the mechanism of these enzymatic pathways are warranted in the skin.

We have reported previously the presence of aryl hydrocarbon hydroxylase, several other monooxygenases, epoxide hydrolase, and glutathione-S-transferase activities in cutaneous tissue [11–13]. BP-4,5-oxide, a stable arene oxide, is a model substrate for the study of epoxide hydrolase and glutathione-S-transferase activities [14–16]. Furthermore, it is a weak mutagen [17] and is relatively stable under physiologic conditions [18]. In this study we report that BP-4,5-oxide topically applied to neonatal rats is metabolized to BP-4,5-dihydrodiol and other water-soluble metabolites in the skin and liver.

MATERIALS AND METHODS

Chemicals

[G-^{3}H]BP-4,5-oxide (sp act 282.5 mCi/mmol; radiochemical purity greater than 99%), chromatographically pure labeled BP-4,5-oxide, and BP-4,5-dihydrodiol were obtained from the Midwest Research Institute (Kansas City, Missouri) through the Chemcial Repository of the National Cancer Institute. Cyclohexene oxide and 1,1,1-trichloropropene oxide (TCPO) were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Clotrimazole, β-glucuronidase, D-saccharic acid 1,4-lactone, and aryl sulfatase were purchased from Sigma Chemical Co. (St. Louis, Missouri). Whatman Linear-K silica gel TLC plates were obtained from Fisher Scientific Co. (Cleveland, Ohio). All solvents were of high-pressure liquid chromatography grade and were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Michigan).

Animals

Sperm-positive Sprague-Dawley rats of known insemination date were obtained from the Holtzman Rat Farm (Madison, Wisconsin). Neonatal rats born in situ were allowed to suckle until the 4th day after birth, withdrawn from their mothers, and used in the experiments. The animals' weight at the time of the experiment ranged from 9.5–10.5 g. The advantages of using neonatal rodents for studies on cutaneous drug metabolism have been described previously [11].

Treatment of Animals

The animals were treated topically with [^{3}H]BP-4,5-oxide (180 nmol/10 g body wt in 100 μl of acetone) and sacrificed at 0, 1, 2, 6, and 24 h after dosing for the time-dependent experiment. For the dose-dependent study, the rats were treated topically with 36, 90, 180, 360, 540, and 720 nmol [^{3}H]BP-4,5-oxide per 10 g body wt 2 h prior to sacrifice. In the studies where the effects of various inhibitors were studied, the animals were treated topically with single applications of cyclohexene oxide (2 mg/10 g), TCPO (2 mg/10 g), or clotrimazole (2 mg/10 g) 24 h prior to sacrifice. All animals were then treated with a single topical application of [^{3}H]BP-4,5-oxide (180 nmol/10 g) 2 h prior to sacrifice. The animals were killed by decapitation and the skin and liver removed. All subsequent operations were carried out at 0–4°C. The skin and liver were then minced with scissors in 0.1 M phosphate buffer pH 7.4. The minced tissue was homogenized with 6 separate bursts of a Polytron Tissue Homogenizer (Brinkman Instruments, Westbury, New York) equipped with an ST-10 generator.

Isolation and Analysis of BP-4,5-oxide Metabolites

BP-4,5-dihydrodiol and water-soluble metabolites were analyzed in extracts prepared from the skin and liver homogenates. Tissue homogenates were extracted 3 times with 2 volumes of ethyl acetate:acetone (2:1, v/v). The organic and aqueous phases were separated by centrifugation and the organic residue dried under a stream of nitrogen and then dissolved in 2 ml of methanol for thin-layer chromatographic analysis. The method for TLC separation of BP-4,5-oxide and BP-4,5-dihydrodiol has been described earlier [19,20]. Aliquots (0.1 ml) were applied to TLC plates and developed in tanks containing benzene:ethanol (95:5, v/v). BP-4,5-oxide and BP-4,5-dihydrodiol on the plates were identified on the basis of reference standards. The R_f of BP-4,5-oxide under the conditions of chromatography was 0.9, while the R_f of BP-4,5-dihydrodiol was 0.2–0.3. After development, the plates were air dried, and BP-4,5-dihydrodiol and BP-4,5-oxide were quantified by counting samples scraped from TLC plates on a Packard

TriCarb 460CD liquid scintillation spectrometer. Aliquots of the aqueous phase were also counted to determine the amount of water-soluble metabolites present.

Aqueous samples were analyzed further for the presence of glucuronide and sulfate conjugates of the dihydrodiol as described by Di-Giovanni et al [21]. For the determination of glucuronide conjugates, 1 ml of the aqueous sample was combined with 1 ml of 0.1 M potassium phosphate buffer pH 6.8 and incubated at 37°C for 2 h with 2000 Fishman units β-glucuronidase per ml. The addition of 20 mM D-saccharic acid 1,4-lactone completely inhibited the release of glucuronide conjugates under these conditions. In addition, ascorbic acid (1 mg/ml) was routinely added to incubations to prevent any spontaneous oxidation. For the determination of sulfate conjugates, 1 ml of the aqueous sample was combined with 1 ml of 0.2 M sodium acetate buffer pH 5.0 and incubated at 37°C for 4 h with aryl sulfatase (30 units/ml) plus 20 mM D-saccharic acid 1,4-lactone. After incubation, the reactions were terminated by adding 2 volumes of ice-cold ethyl acetate:acetone (2:1, v/v). Samples were then centrifuged, and the organic phase was counted for radioactivity, a blank for each sample containing all reactants except enzyme was employed and the values obtained were subtracted from the appropriate sample.

RESULTS

Time and Dose Dependent Metabolism of BP-4,5-oxide by the Neonatal Rat

A single topical application of BP-4,5-oxide to neonatal rats resulted in the formation of BP-4,5-dihydrodiol as well as water-soluble metabolites. The data in Table I indicate that the metabolism of BP-4,5-oxide to BP-4,5-dihydrodiol by the rat skin is time-dependent. The formation of BP-4,5-dihydrodiol in liver as well as in skin was maximum at 2 h after topical application of BP-4,5-oxide to rats. However, substantial amounts of BP-4,5-dihydrodiol were recovered even 24 h following a single topical application of BP-4,5-oxide. The formation of BP-4,5-dihydrodiol in liver was always higher (2–6 times) than that occurring in the skin. Within minutes (0 h) after topical application of BP-4,5-oxide the amount of dihydrodiol recovered from the liver was 1.3 times that of the skin; at 1, 2, 6, and 24 h after dosing this was 1.8-, 6.1-, 6.4-, and 3.0-fold higher, respectively. A similar trend for time dependency was observed in the formation of water-soluble metabolites of topically applied BP-4,5-oxide in the liver and skin (Table I).

Increasing concentrations of BP-4,5-oxide applied topically to the skin of neonatal rats resulted in a linear increase in the recovery of BP-4,5-dihydrodiol (Fig 1). The largest increment in diol formation occurred between doses of 180 and 360 nmol/animal. There was approximately 50% more dihydrodiol formed at the 360-nmol dose than at the 180-nmol dose (Fig 1).

Metabolism of BP-4,5-oxide to Glucuronide and Sulfate Conjugates by Neonatal Rat Skin

The water-soluble metabolites obtained after ethyl acetate extraction were enzymatically digested with β-glucuronidase

TABLE I. *Time-dependent metabolism of topically applied BP-4,5-oxide in neonatal rat skin and liver*

Time[a] (h)	BP-4,5-dihydrodiol formation (nmol/animal)		Water-soluble metabolite formation (nmol/animal)	
	Skin[b]	Liver	Skin	Liver
0	1.6 ± 0.2[c]	3.0 ± 0.2	3.7 ± 0.4	16.0 ± 0.8
1	4.1 ± 0.1	7.4 ± 0.4	14.4 ± 0.6	23.9 ± 1.1
2	7.7 ± 0.3	47.2 ± 1.4	15.1 ± 0.6	26.4 ± 1.4
6	5.2 ± 0.2	33.2 ± 1.1	9.6 ± 0.3	25.2 ± 1.2
24	4.8 ± 0.2	14.4 ± 0.8	8.2 ± 0.3	13.6 ± 0.7

[a] For each time point determination 4 animals were treated topically with [³H]BP-4,5-oxide (180 nmol/animal) and sacrificed at the specified time intervals.

[b] Skin and liver homogenates were prepared and the organic solvent-extractable material was applied to TLC plates as described in *Materials and Methods*. The aqueous phase remaining after organic extraction contained the water-soluble metabolites.
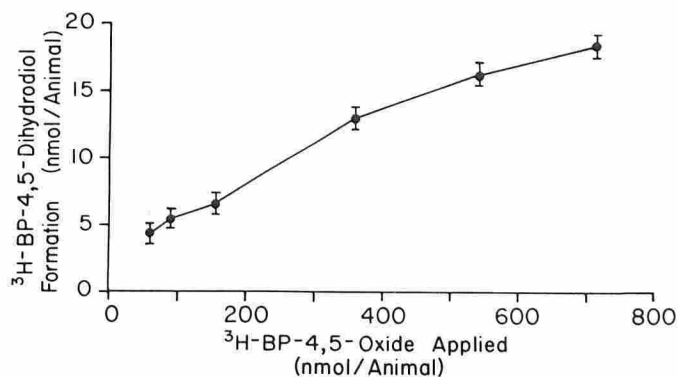
[c] Mean ± SEM of 4 experiments.

FIG 1. Dose-dependent metabolism of BP-4,5-oxide in rat skin. Neonatal rats (9.5–10.5 g body wt) were topically treated with various concentrations of [³H]BP-4,5-oxide and sacrificed 2 h after dosing. Data represent mean ± SEM of 4 separate experiments.

TABLE II. *Water-soluble metabolism of BP-4,5-oxide by neonatal rat skin*

Conjugate[a]	[³H]BP-4,5-dihydrodiol-conjugate[b] (pmol/ml)	Percent of total aqueous phase
BP-4,5-dihydrodiol-β-glucuronide	20.2 ± 0.8[c]	0.12
BP-4,5-dihydrodiol-sulfate	24.4 ± 1.1	0.18

[a] Neonatal rats were treated topically with [³H]BP-4,5-oxide (180 nmol/animal) 2 h prior to sacrifice. Skin homogenates were prepared and extracted 3 times with ethyl acetate. The aqueous phase was then treated with either β-glucuronidase (2000 units/ml) and 20 mM D-saccharic acid 1,4-lactone or aryl sulfatase (30 units/ml) and 20 mM D-saccharic acid 1,4-lactone. These reaction mixtures were incubated at 37°C for 2–4 h.

[b] Organic extracts were counted to determine the amount of [³H]BP-4,5-dihydrodiol bound to conjugate.

[c] Mean ± SEM of 4 experiments.

and aryl sulfatase. The formation of BP-4,5-dihydrodiol-β-glucuronide and sulfate conjugates in neonatal rat skin are depicted in Table II. Dihydrodiol conjugation with glucuronide and sulfate represents only a fraction of the total aqueous metabolism of the hydrocarbon. Less than 1% of the aqueous metabolites were conjugated with glucuronide and sulfate (Table II).

Inhibition of BP-4,5-dihydrodiol Formation by Epoxide Hydrolase Inhibitors in the Skin

To further verify that the formation of BP-4,5-dihydrodiol from BP-4,5-oxide in neonatal rat skin occurred enzymatically, several known inhibitors of epoxide hydrolase were evaluated. Animals treated topically with cyclohexene oxide, TCPO, or clotrimazole showed significant reduction in BP-4,5-dihydrodiol formation (Fig 2). Cyclohexene oxide, TCPO, and clotrimazole reduced the level of dihydrodiol formation by 51, 56, and 72% respectively.

DISCUSSION

The data presented here indicate that topical application of BP-4,5-oxide to skin resulted in the metabolic conversion to BP-4,5-dihydrodiol and to water-soluble metabolites and conjugates. BP-4,5-dihydrodiol formation was maximum at 2 h, after which there was a gradual decrease in the amount of BP-4,5-dihydrodiol formed. The decrease in the formation of the dihydrodiol after 2 h may relate to its binding to macromolecules such as DNA, RNA, and protein. The decrease in water-soluble metabolites after 2 h may be due to the detoxification and excretion of conjugated metabolites. Our data also suggest a linear increase in BP-4,5-dihydrodiol formation as a function
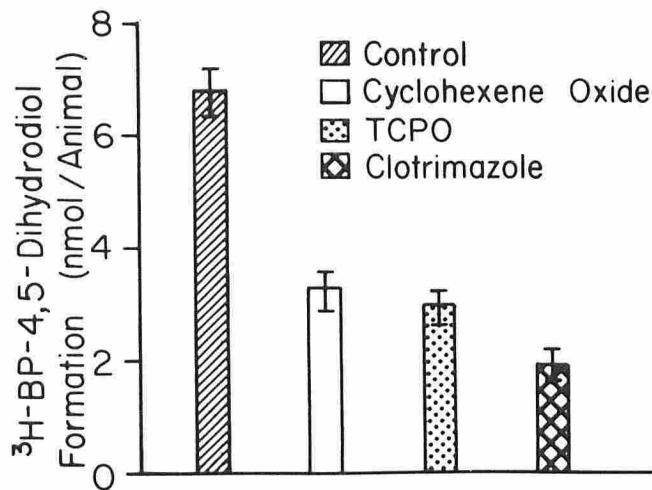
FIG 2. Inhibition of the metabolism of BP-4,5-oxide to BP-4,5-dihydrodiol in rat skin. Neonatal rats (9.5–10.5 g body wt) were topically treated with acetone (control), cyclohexene oxide (2 mg/animal), TCPO (2 mg/animal), or clotrimazole (2 mg/animal) 24 h prior to [^{3}H]BP-4,5-oxide application. All animals were then topically treated with [^{3}H]BP-4,5-oxide (180 nmol/animal) 2 h before sacrifice. Data represent mean ± SEM of 4 separate experiments.

of increasing BP-4,5-oxide concentration after topical application to the skin.

The formation of water-soluble conjugates in neonatal rat skin was also observed in this study. We found very limited amounts of both glucuronic and sulfuric acid conjugates 2 h after topical application of BP-4,5-oxide. This may be due to the limited amount of substrate present in the skin. The presence of glucuronide and sulfate conjugates of BP-4,5-dihydrodiol in vitro [22,23] and in isolated perfused organ studies [24,25] has been shown previously. The formation of these conjugated metabolites of arene oxides in skin has not previously been demonstrated.

Our data also indicate that the metabolism of BP-4,5-oxide to BP-4,5-dihydrodiol in skin is enzyme-mediated since several inhibitors of epoxide hydrolase [26] largely abolished the formation of BP-4,5-dihydrodiol in the skin. The imidazole antifungal agent clotrimazole was the most effective inhibitor in this respect. Clotrimazole has previously been shown to be a potent inhibitor of the cytochrome P-450-dependent microsomal enzyme aryl hydrocarbon hydroxylase [27]. Our unpublished observations have suggested that clotrimazole is also a potent inhibitor of microsomal epoxide hydrolase in vitro.

In summary, our data indicate that the topically applied model arene oxide, BP-4,5-oxide, is metabolized to BP-4,5-dihydrodiol and to sulfate and glucuronide conjugates in rat skin. Furthermore, topically applied BP-4,5-oxide was shown to be rapidly metabolized in the liver, suggesting that the skin exposure to some carcinogenic compounds may result in percutaneous absorption of the parent compound and/or its metabolites which in turn could produce effects in other body tissues.

REFERENCES

1. Maibach H: Cutaneous pharmacology and toxicology. Annu Rev Pharmacol Toxicol 16:401–411, 1976
2. Slaga TJ, Bracken WM, Dresner S, Levin W, Yagi H, Jerina DM, Conney AH: Marked differences in the skin tumor-initiating activities of the optical enantiomers of the diasteromeric benzo[a]pyrene 7,8-diol-9,10-epoxides. Cancer Res 39:67–71, 1979
3. Heidelberger C: Chemical carcinogenesis. Annu Rev Biochem 44:79–121, 1975
4. Conney AH: Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. Cancer Res 42:4875–4917, 1982
5. Huberman E, Sachs L, Yang SK, Gelboin HV: Identification of mutagenic metabolites of benzo[a]pyrene in mammalian cells. Proc Natl Acad Sci USA 73:607–611, 1976
6. Wood AW, Levin W, Lu AYH, Yagi H, Hernandez O, Jerina DM, Conney AH: Metabolism of benzo[a]pyrene and benzo[a]pyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes. J Biol Chem 251:4882–4890, 1976
7. Thakker DR, Yagi H, Lu AYH, Levin W, Conney AH, Jerina DM: Metabolism of benzo[a]pyrene: Conversion of (±)-trans-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene to highly mutagenic 7,8-diol-9,10-epoxides. Proc Natl Acad Sci USA 73:3381–3385, 1976
8. Jerina DM, Daly JW: Arene oxides: a new aspect of drug metabolism. Science 185:573–582, 1974
9. Sims P, Grover PL, Swaisland A, Pal K, Hewer A: Metabolic activation of benzo[a]pyrene proceeds by a diol-epoxide. Nature 252:326–328, 1974
10. Miller EC, Miller JA: Biochemical mechanisms of chemical carcinogenesis, Molecular Biology of Cancer. Edited by H Busch. New York, Academic Press, 1974, pp 377–402
11. Bickers DR, Dutta-Choudhury T, Mukhtar H: Epidermis: a site of drug metabolism in neonatal rat skin. Studies on cytochrome P-450 content and mixed-function oxidase and epoxide hydrolase activity. Mol Pharmacol 21:239–247, 1982
12. Mukhtar H, Bickers DR: Drug metabolism in skin. Comparative activity of the mixed-function oxidases, epoxide hydrolase, and glutathione S-transferase in liver and skin of neonatal rats. Drug Metab Dispos 9:311–314, 1981
13. Mukhtar H, Bickers DR: Aminopyrine N-demethylase activity in neonatal rat skin. Biochem Pharmacol 30:3257–3260, 1981
14. Grover PL, Hewer A, Sims P: Formation of K-region epoxides as microsomal metabolites of pyrene and benzo[a]pyrene. Biochem Pharmacol 21:2713–2726, 1972
15. Bend JR, Smith BR, Ball LM, Mukhtar H: Alkene and arene oxide metabolism in hepatic and extrahepatic tissues—pharmacological and toxicological aspects, Conjugation Reactions in Drug Biotransformation. Edited by A Aitio. Amsterdam, Elsevier North-Holland, 1978, pp 3–16
16. Nemoto N, Gelboin HV, Habig WH, Ketley JN, Jakoby WB: K-region benzo[a]pyrene-4,5-oxide is conjugated by homogenous glutathione-S-transferases. Nature 255:512–521, 1975
17. Wislocki PG, Wood AW, Chang RL, Levin W, Yagi H, Hernandez O, Dansette PM, Jerina DM, Conney AH: Mutagenicity and cytotoxicity of benzo[a]pyrene arene oxides, phenols, quinones and dihydrodiols in bacterial and mammalian cells. Cancer Res 36:3350–3357, 1976
18. Conney AH, Wood AW, Levin W, Lu AYH, Chang RL, Wislocki PG, Goode RL, Holder GM, Dansette PM, Yagi H, Jerina DM: Metabolism and biological activity of benzo[a]pyrene and its metabolic products, Biological reactive intermediates: Formation, toxicity and inactivation. Edited by DJ Jollow, JJ Kocsis, R Snyder, H Vainio. New York, Plenum Press, 1977, pp 335–357
19. Lee IP, Mukhtar H, Suzuki K, Bend JR: Metabolism of benzo[a]pyrene 4,5-oxide by isolated perfused rat testis. Biochem Pharmacol 32:159–161, 1983
20. Jerina DM, Dansette PM, Lu AYH, Levin W: Hepatic microsomal epoxide hydrase: a sensitive radiometric assay for hydration of arene oxides of carcinogenic aromatic hydrocarbons. Mol Pharmacol 13:342–351, 1977
21. DiGiovanni J, Miller DR, Singer JM, Viaje A, Slaga TJ: Benzo[a]pyrene metabolism in primary cultures of mouse epidermal cells and untransformed and transformed epidermal cell lines. Cancer Res 42:2579–2586, 1982
22. Nemoto N, Gelboin HV: Enzymatic conjugation of benzo[a]pyrene oxides, phenols and dihydrodiols with UDP-glucuronic acid. Biochem Pharmacol 25:1221–1226, 1976
23. Nemoto N, Takayame S, Gelboin HV: Enzymatic conversion of benzo[a]pyrene phenols, dihydrodiols and quinones to sulfate conjugates. Biochem Pharmacol 26:1825–1829, 1977
24. Lee IP, Nagayama J: Metabolism of benzo[a]pyrene by the isolated perfused rat testis. Cancer Res 40:3297–3306, 1980
25. Mukhtar H, Lee IP, Fourman GL, Bend JR: Epoxide metabolizing enzyme activities in rat testes: postnatal development and relative activities in interstitial and spermatogenic cell compartments. Chem Biol Interact 22:153–165, 1978
26. Oesch F: Mammalian epoxide hydrases: inducible enzymes catalyzing the inactivation of carcinogenic and olefinic compounds. Xenobiotica 3:305–340, 1973
27. Kahl R, Frederici DE, Kahl GF, Ritter W, Krebs R: Clotrimazole as an inhibitor of benzo[a]pyrene metabolite-DNA adduct formation *in vitro* and of microsomal monooxygenase activity. Drug Metab Dispos 8:191–196, 1980