Biochemical Pharmacology, Vol. 32, No. 1, pp. 159–161, 1983.
Printed in Great Britain.

0006-2952/83/010159-03 $03.00/0
Pergamon Press Ltd.

SHORT COMMUNICATIONS

Metabolism of benzo[*a*]pyrene 4,5-oxide by isolated perfused rat testis

(*Received* 29 *March* 1982; *accepted* 2 *July* 1982)

Current evidence suggests that both cancer and mutations induced by several polycyclic aromatic hydrocarbons (PAH) are due to metabolically activated diol epoxides which react with nucleophilic sites of DNA [1–7]. Consequently, the balance between metabolic activation and detoxication systems in target organs and their constitutive cells may contribute to mutagenic and carcinogenic and/or necrotic processes in both liver and extrahepatic tissues. With respect to PAH-induced testicular toxicity, it has been shown that the treatment of adult males with benzo[*a*]pyrene (BP) or 7,12-dimethylbenzanthracene (DMBA) produces a significant increase in early embryonic death in mice and premeiotic spermatogenic cell damage in rats [8–10]. In addition, the incidence of dominant lethal mutations induced by BP treatment in various mouse strains was reported to be significantly different [11].

We have reported previously the presence of aryl hydrocarbon hydroxylase (AHH), epoxide hydrolase (EH), and glutathione transferase (GSH-T) activities in rat testicular tissues [12, 13]. Specific AHH activity and cytochrome P-450 content were 2-fold greater in microsomes from interstitial cells than in those from microsomes prepared from the germ cell compartment. In contrast, specific EH and GSH-T activities in the germ cell compartment were about twice those in the interstitial cells. Although specific AHH activity in testicular microsomes was only 5% of that in hepatic microsomes, the ability of interstitial as well as germ cells to enzymatically activate BP may be an important consideration for genotoxicity of germ cells.

Since the metabolism of arene oxides by male germ cells can be affected by various factors not present in cell-free systems, such as the blood–testis barrier (BTB), the isolated perfused testis may better mimic the *in situ* testicular metabolism of PAH. BP 4,5-oxide was selected for the present study because the BTB is highly permeable to it (the transfer rate constant is predicted to be 0.1 min^{-1} based on previously published data), it is a relatively stable k-region arene oxide, and it is a substrate for both EH and GSH-T of the rat testis [13, 14].

Materials and methods

Chemicals. [G-^{3}H]Benzo[*a*]pyrene 4,5-oxide (spec. act: 10 mCi/mmole; radiochemical purity 99%), chromatographically pure unlabeled benzo[*a*]pyrene 4,5-oxide (BP 4,5-oxide), and benzo[*a*]pyrene 4,5-dihydrodiol (BP 4,5-dihydrodiol) were obtained from the Midwest Research Institute, Kansas City, MO, through a National Cancer Institute Contract (1-CD33387), which is supplemented by the National Institute of Environmental Health Sciences. Benzo[*a*]pyrene was obtained from the Eastman Kodak Co., Rochester, NY and was further purified by elution from an alumina G column (1.4 × 20 cm) with acetone. HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Type XI), and NADP were purchased from the Sigma Chemical Co., St. Louis, MO.

Animals. Adult male CD Sprague–Dawley rats (250–300 g body wt) were obtained from the Charles River Breeding Laboratories Inc., Wilmington, MA, and were used throughout the experiments. Animals were housed three per cage and allowed free access to a rodent diet (NIH 31) and drinking water. Animals were maintained under defined conditions of illumination (14 hr light, 10 hr dark) and temperature (25°).

Preparation of testes for perfusion studies. Animals were killed and the testes were removed. The testicular artery was immediately cannulated under a stereomicroscope. Details of the procedures were described earlier [12]. In each experiment, four testes were perfused simultaneously in the dark. The effluent from the testicular vein was collected at 15-min intervals over a 90-min period for analysis of the metabolites. [^{3}H]BP 4,5-oxide was added to the perfusion medium to give final concentrations of 1.3, 3.6 and 5×10^{-6} M.

Isolation and analysis of BP 4,5-oxide metabolites. BP 4,5-dihydrodiol and water-soluble metabolites were recovered from both the effluent and the tissues (at the end of perfusion). The testes were homogenized and were extracted three times with 2 vol. of ethyl acetate–acetone (2:1, v/v). The organic and water phases were separated by centrifugation and the sample residue from both the effluent and the tissue was dissolved in 0.1 ml of ethyl acetate for thin-layer chromatographic analysis. The assay details for BP 4,5-dihydrodiol and water-soluble metabolites are described elsewhere [12, 13, 15–18].

Statistical analysis. Analysis of regression and of significant differences between control and experimental values was performed with Student's *t*-test, using the MINITAB II Statistical Computor Program and PDP 11-70,

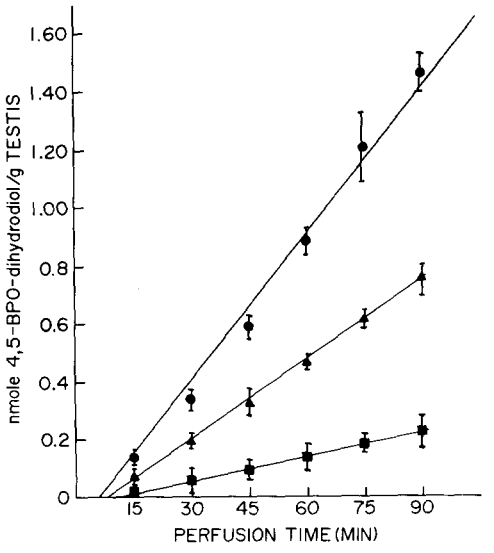

Fig. 1. Rate of BP 4,5-dihydrodiol appearance in the testis perfusate as a function of time and BP 4,5-oxide concentration. Each point is the mean ± S.E. of three to four experiments. Key: 1.3×10^{-6} M (■), 3.6×10^{-6} M (▲), and 5.0×10^{-6} M (●). The equation for each curve is as follows: Y = −0.04 + 0.0029X (■), Y = −0.076 + 0.0094X (▲), and Y = −0.109 + 0.017X (●).

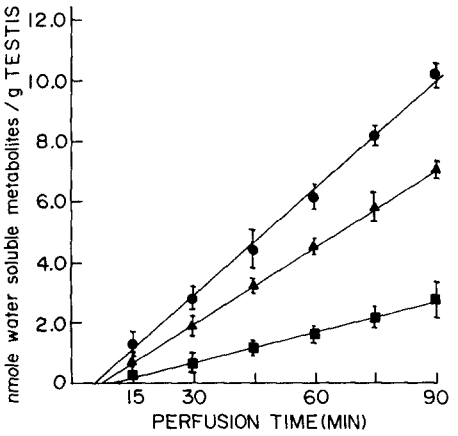

Fig. 2. Rate of appearance of water-soluble metabolites in the testis perfusate as a function of time and BP 4,5-oxide concentration. Each point is the mean ± S.E. of three to four experiments. Key: 1.3×10^{-6} M (■), 3.6×10^{-6} M (▲), and 5.0×10^{-6} M (●). The equation for each curve is as follows: $Y = -0.300 + 0.032X$ (■), $Y = -0.560 + 0.084X$ (▲), and $Y = -0.770 + 0.119X$ (●).

RSX 11M operating systems [19]; the level of significance chosen for the difference between mean was $P < 0.05$.

Results and discussion

Metabolism of BP 4,5-oxide by the isolated perfused testis. The release of BP 4,5-dihydrodiol into the effluent medium at various times during the constant perfusion of rat testis with 1.3, 3.6, or 5.0×10^{-6} M BP 4,5-oxide is shown in Fig. 1. These data indicate that, after an initial lag period, the release of BP 4,5-dihydrodiol was linear for the 90-min perfusion period. From the slopes, the rates of BP 4,5-dihydrodiol release over the 90-min perfusion periods were estimated to be at least 2.9, 9.4, and 16.9 pmoles $\cdot$ min^{-1} $\cdot$ (g testis)$^{-1}$ respectively. The total BP 4,5-dihydrodiol formation in both the effluent and the testicular tissue was 17 nmoles/g testis at 60-min perfusion. Lag times between the onset of perfusion and the release of BP 4,5-dihydrodiol at 1.3, 3.6 and 5.0×10^{-6} M of BP 4,5-oxide concentrations were approximately 13.8, 8.1 and 6.4 min respectively. The lag periods can be attributed to the time required for epoxide hydration and the transfer of the metabolites from the intracellular site of hydrolysis in both germ cells and interstitial cell compartments to the effluent medium.

Formation of water-soluble metabolites by the isolated perfused testis. The release of water-soluble metabolites into the effluent medium at various times during the constant perfusion [13.3 ml perfusate $\cdot$ (g testis)$^{-1}$ $\cdot$ hr^{-1}] of the testis with 1.3, 3.6, and 5.0×10^{-6} M of BP 4,5-oxide is demonstrated in Fig. 2. From the slopes, the rates of water-soluble metabolite release into effluent during the 90-min perfusion periods were estimated to be at least 32.4, 84.3 and 118.7 pmoles $\cdot$ min^{-1} $\cdot$ (g testis)$^{-1}$ respectively. The total water-soluble metabolite formation in both effluent and the tissue was approximately 27.4 nmoles/g testis at 60 min (Table 1). During the early phase of perfusion, the lag period between the onset of perfusion and the release of water-soluble metabolites into the effluent perfusate was determined at 1.3, 3.6, and 5.0×10^{-6} M BP 4,5-oxide; these values were 9.0, 6.7, and 6.5 min respectively. The lower the concentration, the longer the latent period. The amount of water-soluble metabolites in testis at the completion of perfusion was fifteen times greater than that of BP 4,5-dihydrodiol. This reflects the greater specific GSH-T activities (than EH activities) in rat testes and/or the slow release of intracellular thioether metabolites to the vascular system.

Comparison of BP 4,5-oxide metabolites in the effluent and the testicular tissues. Table 1 presents a comparison of BP 4,5-oxide metabolites in the effluent and the testicular tissues at the conclusion of the experiments. In contrast to BP 4,5-oxide metabolites in the effluent, BP 4,5-dihydrodiol and the water-soluble metabolites in the testicular tissues were 0.77 and 18.4 nmoles/g testis by 60 min of perfusion respectively. The ratios of total BP 4,5-dihydrodiol and water-soluble metabolites localized in the testis to those in the effluent were 0.85 and 2.92 respectively. Thus, the distribution of BP 4,5-dihydrodiol between the two compartments was at about unity, which suggests that BP 4,5-dihydrodiol readily moved into the effluent medium from the tissue compartment. In contrast, the water-soluble metabolites did not readily pass from the tissue compartment to the vascular compartment. More than 90% of the total metabolites formed from BP 4,5-oxide were found in the water-soluble fraction. This is consistent with the high GSH-T activity present in this tissue.

We have reported previously that permeability characteristics of various chemicals and drugs across the blood–testis barrier resembled those across the blood–brain barrier, and that permeability rates correlated well with partition coefficients and molecular size [14]. From these data,

Table 1. BP 4,5-oxide metabolites in the rat testis and in the perfusate after 60 min of perfusion*

	Isolated perfused testis			
BP 4,5-oxide metabolites	Effluent	Tissue (nmoles total metabolites formed/g testis)	Total metabolites	Ratio† (tissue/effluent)
BP 4,5-dihydrodiol	0.91 ± 0.05 (3)	0.77 ± 0.12 (3)	1.7	0.85
Water-soluble metabolites	6.3 ± 0.4 (3)	18.4 ± 2.8‡ (3)	24.7	2.92
Ratio of water-soluble metabolites to BP 4,5-dihydrodiol	6.9	24.0	14.7	3.5
Total metabolites§	7.2	19.2	26.4	2.67

* Final concentration of BP 4,5-oxide in the perfusate was 5.0×10^{-6} M; the testis was perfused for 60 min at 32° at the rate of 13.3 ml $\cdot$ hr^{-1} $\cdot$ (g testis)$^{-1}$. Mean values are ± S.E.

† Ratio of BP 4,5-oxide metabolites in testicular tissues to that of the effluent.

‡ Water-soluble metabolites in the testicular tissues following perfusion were significantly greater than in the effluent of the perfused testis ($P < 0.05$).

§ Total metabolites represent the sum of BP 4,5-dihydrodiol and the water-soluble metabolites.

the permeability rate constants for highly lipid soluble BP 4,5-oxide and BP 4,5-dihydrodiol are predicted to be greater than 0.1 min^{-1}, while permeability rate constants for water-soluble polar conjugates from the testicular cells will be very low. Thus, the blood–testis barrier may play an important role in limiting the rate of transfer of water-soluble metabolites formed in the germ cell compartment to the vascular compartment.

The rates of release of BP 4,5-dihydrodiol [0.9 nmole · hr^{-1} · (g testis)$^{-1}$] and water-soluble metabolites [6.3 nmoles · hr^{-1} · (g testis)$^{-1}$] in the effluent medium of isolated testis were only 6 and 9% of those found in the isolated perfused rat liver [BP 4,5-dihydrodiol: 16 nmoles · hr^{-1} · (g liver)$^{-1}$; water-soluble conjugates: 70 nmoles · hr^{-1} · (g liver)$^{-1}$ [20]], at the identical concentration of BP 4,5-oxide (5.0×10^{-6} M), consistent with the much slower rate of total metabolism. It has been demonstrated that the venous return emerging from the testis returns via the unique venous network of the pampiniform plexus surrounding the internal spermatic artery, where countercurrent heat exchange to cool the arterial blood flowing to the testis as well as venous-arterial transfer of various steroids and chemicals against the concentration gradients occurs [21–25]. Therefore, venous BP 4,5-oxide, BP 4,5-dihydrodiol, and other BP metabolites could continue to recycle between the venous pampiniform plexus and the internal spermatic artery and return right back into the testis, thus making germ cells even more vulnerable to mutagenic BP metabolites.

In summary, the metabolism of a relatively stable arene oxide, BP 4,5-oxide, was studied in an isolated perfused rat testis preparation. In this testis preparation, the release of both BP 4,5-dihydrodiol and water-soluble metabolites was linear for up to 90 min and, consequently, the enzymatic systems responsible for the metabolism and the cellular transport of the substrate and its metabolites appear viable for 90 min. The formation of BP 4,5-dihydrodiol in the effluent and in the testicular tissue was 0.91 and 0.77 nmole · hr^{-1} · (g testis)$^{-1}$ respectively, and thus the data suggest that BP 4,5-dihydrodiol readily passes into the effluent medium. In contrast, the formation of soluble metabolites in the effluent and in the testicular tissue compartment was 6.3 and 18.4 nmoles · hr^{-1} · (g testis)$^{-1}$, respectively, suggesting that water-soluble metabolites did not readily pass from the tissue compartment to the vascular system. The slower release of conjugated water-soluble metabolites may have resulted from a lower permeability of these relatively large polar molecules across the blood–testis barrier. Thus, the isolated perfused testis preparation may mimic *in situ* testicular metabolism of PAH more closely than do cell-free systems.

In conclusion, the isolated perfused testis system may serve as a useful model to study pharmacokinetic characteristics of the blood–testis barrier, toxication and detoxication mechanisms for xenobiotics, DNA damage and repair systems in male gonads.

† Author to whom correspondence should be addressed.
‡ Present address: Department of Dermatology, Case Western Reserve University, Cleveland, OH, U.S.A.
‖ Present address: Department of Physiology, Nippon Veterinary and Zootechnical College, Tokyo, Japan.

* *Laboratory of Reproductive and Developmental Toxicology*
National Institute of Environmental Health Sciences
Research Triangle Park NC 27709, U.S.A.

INSU P. LEE*·†
HASAN MUKHTAR‡·§
KATSUSHI SUZUKI*·‖
JOHN R. BEND§

§ *Laboratory of Pharmacology*
National Institute of Environmental Health Sciences
Research Triangle Park NC 27709, U.S.A.

REFERENCES

1. H. V. Gelboin, *Cancer Res.* **29**, 1272 (1969).
2. P. L. Glover and P. Sims, *Biochem. Pharmac.* **22**, 661 (1973).
3. W. Levin, A. W. Wood, H. Yagi, P. M. Dansette, D. M. Jerina and A. H. Conney, *Proc. natn. Acad. Sci. U.S.A.* **73**, 243 (1976).
4. C. Malaveille, H. Bartsch, P. L. Grover and P. Sims, *Biochem. biophys. Res. Commun.* **66**, 693 (1975).
5. H. Marquardt, P. L. Grover and P. Sims, *Cancer Res.* **36**, 2059 (1976).
6. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, *Nature, Lond.* **252**, 326 (1974).
7. P. G. Wislocki, A. W. Wood, R. L. Chang, W. Levin, H. Yagi, O. Hernandez, P. M. Dansette, D. M. Jerina and A. H. Conney, *Cancer Res.* **36**, 3350 (1976).
8. K. A. Ahlquist, *J. Reprod. Fert.* **12**, 377 (1966).
9. S. S. Epstein, E. Arnold, J. Andrea, W. Bass and W. Bishop, *Toxic. appl. Pharmac.* **23**, 288 (1972).
10. E. Ford and C. B. Huggins, *J. exp. Med.* **118**, 27 (1963).
11. W. M. Generoso, K. T. Cain, M. Krishna and S. W. Huff, *Proc. natn. Acad. Sci. U.S.A.* **76**, 435 (1979).
12. I. P. Lee and J. Nagayama, *Cancer Res.* **40**, 3297 (1980).
13. H. Mukhtar, I. P. Lee, G. L. Fourman and J. R. Bend, *Chem. Biol. Interact.* **22**, 153 (1978).
14. K. Okumura, I. P. Lee and R. L. Dixon, *J. Pharmac. exp. Ther.* **194**, 89 (1975).
15. J. R. Bend, Z. Ben-Zvi, J. Van Anda, P. M. Dansette and D. M. Jerina, in *Carcinogenesis* (Eds. R. I. Freudenthal and P. W. Jones), Vol. 1, pp. 63–75. Raven Press, New York (1976).
16. D. M. Jerina and J. R. Bend, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio), p. 207. Plenum Press, New York (1977).
17. D. M. Jerina, P. M. Dansette, A. Y. H. Lu and W. Levin, *Molec. Pharmac.* **13**, 342 (1977).
18. M. O. James, J. R. Fouts and J. R. Bend, *Biochem. Pharmac.* **25**, 187 (1976).
19. T. A. Ryan, Jr., B. L. Joiner and B. F. Ryan, *Minitab Reference Manual.* Duxbury Press, Boston (1981).
20. B. R. Smith and J. R. Bend, *Cancer Res.* **39**, 2051 (1979).
21. B. A. Cross and I. A. Silver, *J. Reprod. Fert.* **3**, 377 (1962).
22. B. P. Setchell and N. T. Hinks, *J. Reprod. Fert.* **20**, 197 (1969).
23. M. J. Free and R. A. Jaffe, *Endocrinology* **97**, 169 (1975).
24. M. J. Free and Nguyen-Duc-Kien, *Proc. Soc. exp. Biol. Med.* **143**, 284 (1973).
25. M. J. Free, R. A. Jaffe, S. K. Jain and W. R. Gomes, *Nature, New Biol.* **244**, 24 (1973).