CYTOCHROME P-450-LINKED ACTIVATION OF 3-HYDROXYBENZO(α)PYRENE

Jorge Capdevila, Bengt Jernström, Helena Vadi and Sten Orrenius

Department of Forensic Medicine, Karolinska Institutet,
S-104 01 Stockholm 60, Sweden.

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Summary

Incubation of 3-hydroxybenzo(α)pyrene with rat lung microsomes in the presence of NADPH and oxygen results in the formation of a metabolite which binds covalently to DNA. The reaction is inhibited by carbon monoxide and α-naphthoflavone and markedly induced by pretreatment of the rats with 3-methylcholanthrene.

Introduction

The metabolism of carcinogenic polycyclic hydrocarbons results in the formation of reactive epoxides which can bind covalently to tissue nucleophiles including DNA (1-9), or undergo subsequent transformation to less reactive products such as phenols, dihydrodiols, quinones and various conjugates (10-16). It is now generally assumed that the carcinogenic effect is due to the interaction of the reactive epoxide formed from the parent hydrocarbon with cellular macromolecules and that subsequent transformation results in the conversion of this epoxide into less reactive and toxic products. On the other hand, previous work by Borgen et al. (17) and Sims and collaborators (18) has indicated that certain products derived from the parent epoxide, such as the 7,8-dihydrodiol of benzo(α)pyrene (BP) may be reactivated by a second oxidative step to produce a more stable epoxide also capable of binding to DNA.

During recent studies of BP hydroxylation by lung microsomes from 3-methylcholanthrene (3-MC) pretreated rats, we ob-

served that phenolic products formed during the reaction underwent rapid further metabolism (19). This reaction was dependent on NADPH and oxygen and inhibited by carbon monoxide (50%) and α-naphthoflavone (60%) suggesting the involvement of the 3-MC inducible form of cytochrome P-450 and the production of a new oxygenated metabolite, possibly an epoxide, from 3-hydroxybenzo-(α)pyrene (3-OH-BP). The present study was designed to further investigate the formation and reactivity in terms of binding to DNA of this new metabolite.

Materials and Methods

Lung microsomes from either control or induced (20 mg/kg bodyweight of 3-MC administered intraperitoneally daily during 3 days) rats were isolated as follows: Lungs from male rats of the Sprague-Dawley strain (200-250 g) were perfused *in situ* with icecold 0.15 M KCl, removed, and dissected free of larger bronchi, minced and homogenized in 0.15 M phosphate buffer, pH 7.5, containing 10% glycerol, using a glass-teflon homogenizer. The resulting homogenate was diluted to a 20% suspension and spun twice at 12,000 x g for 15 min. The supernatant was chromatographed on a Sepharose 2B column essentially as described by Tangen *et al.* (20). The microsomes were pelleted by centrifugation at 105,000 x g for 90 min and resuspended in 0.25 M sucrose to ∿1 mg protein/ml before use.

Labelled 3-OH-BP was isolated from a 10 ml incubation mixture consisting of 10 mg microsomal protein (microsomes isolated according to Ernster *et al.* (21)) derived from livers of 3-MC treated rats, 100 nmoles (^{3}H)BP (spec. act, 25 Ci/mmol), diluted with 700 nmoles unlabelled BP, 60 μmoles isocitrate, 1.8 units isocitrate dehydrogenase, 10 μmoles $NADP^+$, 50 μmoles $MgCl_2$, 50 nmoles $MnCl_2$ and 0.5 nmoles Tris-HCl buffer, pH 7.5. The incubation was carried out for 1 hour at 37° in red light. After extraction of the microsomal suspension with 4 x 10 ml ethylacetate, the extract was treated with Na_2SO_4, concentrated to dryness in vacuum and the residual material dissolved in 1 ml benzene which in turn was loaded on a column (23 x 1 cm) packed with aluminium oxide (activity grade III, Woelm) equilibrated with benzene. Unmetabolized BP was removed by washing the column with benzene. By changing the elutant to 5% ethanol in benzene, the tightly adsorbed material in the top of the column was moved. 2 ml fractions were collected and examined for phenolic compounds by TLC developed with 5% ethanol in benzene and using authentic 3-OH-BP as a standard (kindly supplied by Dr H.V. Gelboin, NIH). Fractions with the same Rf-value as the standard were pooled, treated with Na_2SO_4 and reduced in volume to 1 ml. A contamitant with a slightly higher Rf-value in the TLC system used was removed by further chromatography on an aluminium oxide column (5 x 1 cm; activity grade I, Woelm) using 5% ethanol in benzene as elutant. 3-OH-BP was recovered by subsequent elution with ethanol. Identification of the product was achieved by fluorescence spectroscopy

(22), mobility in thin layer chromatography using three different solvent systems (23) and elution pattern in high-pressure liquid chromatography using a methanol-water gradient (15, 16). The compound was kept under nitrogen in aceton at -20° and had a radioactivity of 6.85×10^6 cpm/nmol.

Lung microsomes from either control or induced rats were incubated with 3-OH-(^{3}H)BP at 37° for 30 or 60 min. The incubation mixture contained in a final volume of 2 ml the following components: 1 mg microsomal protein, 400 µg calf thymus DNA (type I, Sigma Chemical Co, St. Louis, Mo.), 4.5 nmoles 3-OH-(^{3}H)BP added in 100 µl acetone, 2 µmoles $NADP^+$, 12 µmoles isocitrate, 0.36 units of isocitrate dehydrogenase, 10 µmoles $MgCl_2$, 10 nmoles $MnCl_2$ and 100 µmoles Tris-HCl, pH 7.9. When used 10 nmoles α-naphthoflavone was added in 100 µl acetone. The reaction was stopped by adding sodium dodecyl sulphate (1% final concentration) and 0.5 ml of a 100 mM EDTA solution and the mixture allowed to stand for 30 min at 37° in presence of 50 µg/ml of pancreatic RNase (Type I-A, Sigma, St. Louis, Mo.) pretreated for 10 min at 80°. 2 ml of SSC (0.15 M NaCl + 0.014 M sodium citrate, pH 7.5) were added to the incubation mixture which was subsequently extracted once with phenol (one volume) and twice (1 volume each time) with a mixture of chloroform-isoamylic alcohol (9:1). After dialyzing for 2 x 12 hours against 500 volumes of SSC, DNA was precipitated with cold ethanol containing 2% sodium acetate (2 volumes). The pellet obtained after centrifugation was washed with ether and redissolved in SSC. An amount corresponding to approximately 100 µg DNA (based on UV absorption at 260 nm) was mixed with an appropriate volume of a CsCl solution in SSC and the refractive index of the resulting mixture was adjusted to 1.400 by adding solid CsCl. Density equilibrium was attained by centrifugation at 35,000 rpm in a SW 41 rotor of a Spinco-Beckman ultracentrifuge for 70 hours at 15 - 20°. 31 equal volume fractions were collected from the bottom at each tube. After measuring UV absorption at 260 nm of each fraction, the DNA was precipitated with TCA, 5% final concentration, filtrated with nitrocellulose membrane filters (Sartorius Membranen Filter GmbH, Göttingen, Germany) and washed with 5% TCA. Radioactivity was measured in a Beckman 350 scintillation counter using toluene-PBD as scintillation mixture.

Results and Comments

Table I shows the amount of radioactivity bound to DNA after incubation of lung microsomes with labelled 3-OH-BP under various conditions. From the results it is clear that binding to DNA required enzymatic activity, was time and NADPH dependent and increased more than 10-fold by 3-MC pretreatment of the animals. Furthermore, the reaction was inhibited in the presence of carbon monoxide or α-naphthoflavone.

Figure 1 shows the sedimentation pattern of the DNA isolated from the reaction mixture after incubation for 60 min at 37° of

TABLE I

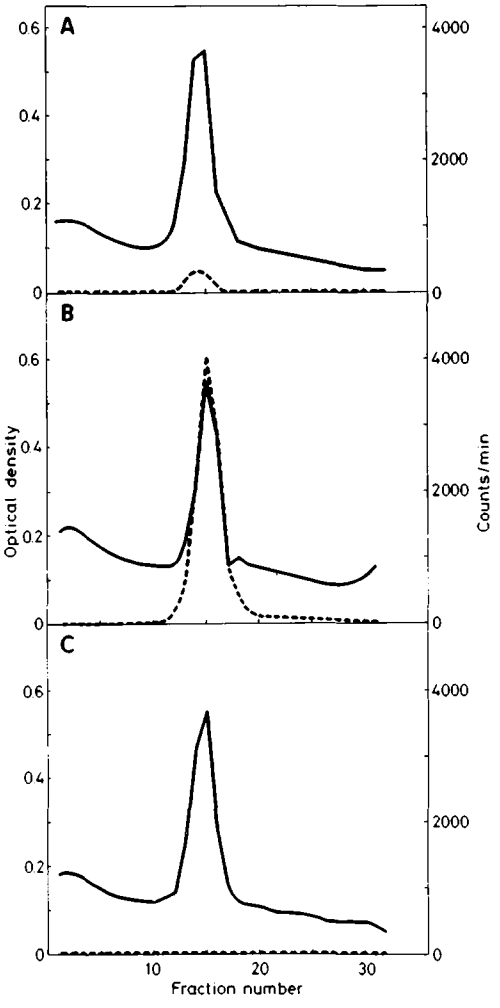
DNA bound radioactivity after incubation of lung microsomes with 3-hydroxy(^{3}H)benzo(α)pyrene in the presence of calf thymus DNA

System	Incubation time, min	counts/min /mg DNA	pmole bound radioactive metabolite/mg DNA
Boiled microsomes	60	821	0.12
Control microsomes	60	10569	1.55
3-MC microsomes	30	96236	14.05
3-MC microsomes	60	148426	21.70
- NADPH	60	1800	0.3
+ CO I[1]	60	82079	12.0
+ CO II[2]	60	45715	6.7
+ α-naphthoflavone[3]	60	70502	10.3

1/ 8% CO, 15% O_2, 77% N_2
2/ 40% CO, 4% O_2, 56% N_2
3/ 5 μM

labelled 3-OH-BP with lung microsomes from control (A) and 3-MC pretreated (B) rats in the presence of a NADPH generating system and with lung microsomes from 3-MC treated rats in the absence of NADPH (C). In the latter case, there was no radioactivity associated with the DNA fraction, whereas a relatively low activity was recovered in the DNA fraction deriving from the incubation with the control microsomes (A). Cosedimentation of the radioactive material and DNA, which was obvious in the case of the induced microsomes incubated in the presence of NADPH (B), strongly suggests the formation of a covalent bond between DNA and the product formed from 3-OH-BP.

Thus, it is obvious that a phenolic product of BP, such as 3-OH-BP can be converted to a reactive metabolite able to bind covalently to DNA and that this reaction appears to be cytochrome P-450 dependent and highly inducible by 3-MC pretreatment of the



Fig, 1. Distribution of UV absorption and radioactivity between various fractions isolated by CsCl gradient centrifugation of DNA incubated with lung microsomes in the presence of 3-hydroxy(^{3}H)-benzo(α)pyrene. A, control microsomes plus NADPH; B, 3-MC induced microsomes plus NADPH; C, 3-MC-induced microsomes minus NADPH. Incubations were for 60 min at 37° as described in Materials and Methods. ——, OD at 260 nm; ---, cpm.

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animals. In preliminary experiments we have also found liver microsomes capable of catalyzing the further metabolism of 3-OH-BP to a product which binds to DNA. This reaction is also markedly stimulated by 3-MC pretreatment of the rats. Although the liver

microsomes are more active in catalyzing the overall disappearance of 3-OH-BP from the incubation medium as compared to lung microsomes, the extent of formation of a product that binds to DNA is similar with microsomes from both tissues. Of course, when incubated with BP, microsomes from both tissues catalyze the formation of products which bind to DNA - the liver being far more active in this respect.

We do not as yet know the chemical nature of this new reactive metabolite formed from 3-OH-BP. However, since its production appears to be catalyzed by cytochrome P-450, we would like to propose that the reaction involves the formation of an epoxide which in turn may be more stable than the primary epoxide due to the presence of the 3-hydroxy group (cf. 17). Our present effort is directed towards the isolation and identification of this metabolite and the investigation of its mutagenic and carcinogenic activity.

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References

1. Boyland, E. (1950) Biochem. Soc. Symp. 5, 40
2. Grover, P.L. and Sims, P. (1968) Biochem. J. 110, 159
3. Gelboin, H.V. (1969) Cancer Res. 29, 1272
4. Grover, P.L., Hewer, A. and Sims, P. (1971) FEBS Lett. 18, 76
5. Kinoshita, N. and Gelboin, H.V. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 824
6. Grover, P.L., Hewer, A. and Sims, P. (1972) Biochem. Pharmacol. 21, 2713
7. Meunier, M. and Chaveau, J. (1973) FEBS Lett. 31, 327
8. Waterfall, J.F. and Sims, P. (1973) Biochem. Pharmacol. 22, 2469
9. Grover, P.L. (1974) Biochem. Pharmacol. 23, 333
10. Boyland, E. and Sims, P. (1965) Biochem. J. 95, 788
11. Boyland, E. and Sims, P. (1965) Biochem. J. 97, 7
12. Jerina, D.M., Daly, J.W., Witkop, B., Zaltman-Nirenberg, P. and Udenfriend, S.J. (1970) Biochemistry, 9, 147
13. Selkirk, J.K., Huberman, E. and Heidelberger, C. (1971) Biochem. Biophys. Res. Commun. 43, 1010
14. Jerina, D.M. and Daly, J.W. (1974) Science 185, 573
15. Selkirk, J.K., Croy, R.G. and Gelboin, H.V. (1974) Science 184, 169

16. Holder, G., Yagi, H., Dansette, P., Jerina, D.M., Levin, W., Lu, A.Y.H. and Conney, A.H. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4356
17. Borgen, A., Darvey, N., Castagnoli, N., Crocker, T.T., Rasmussen, R.E. and Wang, I.Y. (1973) J. Med. Chem. 66, 502
18. Sims, P., Grover, P.L., Swaisland, A., Pal, K. and Hewer, A. (1974) Nature 252, 326
19. Capdevila, J., Jernström, B., Jakobsson, S. and Orrenius, S. (1975) Submitted to Cancer Res.
20. Tangen, O., Jonsson, J. and Orrenius, S. (1973) Anal. Biochem. 54, 597
21. Ernster, L., Siekevitz, P. and Palade, G.E. (1962) J. Cell Biol. 15, 541
22. Kinoshita, N., Shears, B. and Gelboin, H.V. (1973) Cancer Res. 33, 1937
23. Sims, P. (1966) Biochem. Pharmacol. 16, 613