TRANSPLACENTAL CONTROL OF EPOXIDE HYDRATASE AND ITS RELATIONSHIP TO THE CONTROL OF MICROSOMAL MONOOXYGENASE

Franz OESCH

*Department of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland**

Received 13 December 1974
Revised version received 12 February 1975

1. Introduction

Aromatic and olefinic compounds can be transformed by microsomal mono-oxygenase(s) (MO) (EC 1.14.1.1) to epoxides which represent electrophilically reactive species. Accordingly, spontaneous binding of such epoxides to DNA, RNA and protein has been observed. Such alterations of important biomacromolecules can involve disturbancies of the ordered sequences and interrelationships of biochemical events in a cell in such a way as to lead to mutagenicity, carcinogenicity or cell necrosis (for a review see [1]). In the subcellular compartment were these epoxides are formed, the endoplasmatic reticulum, they can be metabolized by epoxide hydratase(s) (EH) (EC 4.2.1.63) to much less reactive dihydrodiols (for a review see [2]; for reactivation of a dihydrodiol by epoxidation at another site, and inactivation of the resulting mixed dihydrodiol–epoxide by epoxide hydratase-catalyzed transformation to a tetrahydrotetrol see [3,4]). Induction of EH would therefore be of great experimental and possibly practical interest, especially if MO responsible for the formation of epoxides, was not concomitantly induced. However, several (unpublished) observations in the rat such as a virtually identical ontogenetic development of EH (determined with [^{3}H]styrene oxide as substrate) and MO (determined with aminopyrine as substrate): 2–3-fold higher EH levels in adult male than in female but no sex difference in young rats corresponding to what is known for MO with respect to a great number of substrates [5]; and induction of EH by representatives of 3 distinctly different groups [6] of MO inducers (phenobarbital, 3-methylcholanthrene, pregnenolone 16α-carbonitrile) indicated that possibly anything that switches on the synthesis of EH may concomitantly do so with respect to (a rate limiting entity of the multicomponent) MO. Since the stage of ontogenetic development required to allow induction would not necessarily be the same for EH and MO if above observations were coinicental rather than really due to a common biosynthetic control of EH and MO, transplacental effects of potential inducing agents on EH and MO were investigated in this study. Several reports have indicated the presence of more than one EH [7] and MO [6,8–11] in liver microsomal fractions. In the present investigation EH is assayed with 7-[^{3}H]styrene oxide and MO with benzo(a)pyrene as substrate. It had been shown in previous studies that increases in specific activity during solubilization and purification of EH were identical with epoxides derived from polycyclic aromatic hydrocarbons and with styrene oxide as substrate [7], and that the former effectively inhibited the hydration of the latter [12] suggesting that the same enzyme(s) is responsible for their hydration.

2. Material and methods

Female Sprague-Dawley rats (250–350 g) were kept together with males (200–300 g) during one

* Present address: Section on Biochemical Pharmacology, Institute of Pharmacology, University of Mainz, Obere Zahlbacherstr. 67 (Hochhaus), D-6500 Mainz, West Germany.

night immediately after the day of littering. The fetal ages referred to in this paper are based on counting the day thereafter as one day after copulation. Stomach tubes were used for transplacental treatment with potential inducers. At the time points indicated the pregnant rats were killed by a blow on the head, fetuses removed and livers of each litter were pooled in 2 vol of ice-cold 0.25 M sucrose containing 10 mM phosphate buffer pH 7.4. Homogenization was done in Potter-Elvehjem homogenizers with a Teflon pestle. Since in preliminary experiments heavy losses of microsomal yield were observed after 8000 *g* centrifugation (cf. also [13]) unbroken cells and nuclei were removed by a centrifugation at 800 *g* for 15 min and the resulting supernatant used for the preparation of a crude microsomal pellet by centrifugation at 100 000 *g* for 1 hr. The pellets were resuspended in one half of the vol of the original homogenate of 10 mM phosphate buffer pH 7.4. MO was assayed with benzo(a)pyrene as substrate. Due to the instability of epoxides derived from aromatic compounds, no assay for quantitating the formation of arene oxides is available. Arene oxides in microsomal preparations, besides undergoing enzymatic hydration and spontaneous binding to tissue components, rearrange non-enzymatically to phenols [14]. Since several MO's may be present in the endoplasmic reticulum [6,8–11] differentially involved in oxidative metabolism at different positions of the benzo(a)pyrene molecule a dual assay was developed essentially by combining a) the method of Nebert and Gelboin [15] and b) a modification (to be described in detail elsewhere) of that of Hayakawa and Udenfriend [16] with an increased sensitivity allowing determinations in fetal liver preparations from non-induced animals. The former method measures the major terminal microsomal metabolite, 3-hydroxybenzo(a)pyrene and (possibly) also some other phenolic metabolites fluorimetrically. Pertinant results are labelled '3-OHBP' in this study. The latter method using [^{3}H]benzo(a)pyrene measures monooxygenase activity with respect to any position at the benzo(a)pyrene molecule although possibly not with even weight especially since the substrate is generally and not uniformly labelled and the percentage retention of tritium during isomerization of intermediate epoxides to terminal phenols (NIH-shift, for a review see [1]) may not be strictly identical at different positions of the molecule although differences in relative retentions would be expected to be small. Pertinent results are labelled 'Overall' in this study. Despite these limitations, the results will provide a valid measure of relative MO activities with respect to (different positions of) the benzo(a)pyrene molecule. For determination of EH with styrene oxide as a substrate, the more sensitive radiometric assay was used [17]. Protein concentrations were estimated as described by Lowry et al. [18] using bovine serum albumin as a standard. The significance of differences between means was established using the student's t-test [19]. $P < 0.05$ was chosen as the level of significance. Where increases in enzyme activity are referred to as 'induction' in this study, presence of higher levels of enzyme protein rather than presence of an activator or removal of an inhibitor was demonstrated by mixing enzyme preparations from controls and treated animals in various proportions which always resulted in activities corresponding to the sum of the two components. However, no specific underlying mechanism was established.

3. Results and discussion

EH activity of the fetal rat liver was significantly (although quite weakly) increased after transplacental treatment with phenobarbital (table 1). Interestingly, MO activity was also increased, as already observed by some workers [20] but not by others who used somewhat lower doses [21]. Similar requirements with respect to time and dosage of treatment, and similar magnitudes of increase enforced the view that possibly MO and EH induction were under a common biosynthetic control. However, pregnenolone 16α-carbonitrile transplacentally induced EH significantly but only slightly while MO was greatly induced (table 1). These marked differences in relative increases may indicate a different control of EH and MO induction. On the other hand they may simply be due to different turnover rates. Use of 3-methylcholanthrene or benzo(a)pyrene as potential transplacental inducers led to a complete dissociation, MO being greatly induced while EH remained unchanged (increased MO activities in fetal rat after transplacental treatment with polycyclic hydrocarbons have

Table 1
Microsomal enzyme activities in fetal rat liver after transplacental treatment with potential inducers

Treatment	Mono-oxygenase				Epoxide hydratase	
	'3-OHBP'[a]		'Overall'[a]			
	pmol OH-benzpyrene/mg protein per min	% of controls	dpm over blank/mg protein per min[b]	% of controls	nmol styrene glycol /mg protein per min	% of controls
None	6.0 ± 1.6	100 ± 27	1′200 ± 150	100 ± 12	0.87 ± 0.07	100 ± 8
Phenobarbital:						
1 day[c]	–	–	1′310 ± 110	109 ± 9	1.05 ± 0.14	121 ± 16
2 days[c]	–	–	1′580 ± 160	132 ± 13	1.34 ± 0.29	154 ± 33
3 days[c]	–	–	1′800 ± 110*	150 ± 9*	1.32 ± 0.07*	152 ± 8*
4 days[d]	8.2 ± 1.1	137 ± 18	2′060 ± 270*	172 ± 22*	1.24 ± 0.11*	143 ± 12*
Pregnenolone 16α-carbonitrile						
4 days[d]	25.2 ± 2.4*	420 ± 60*	3′410 ± 330*	284 ± 28*	1.27 ± 0.11*	146 ± 12*
3-Methylcholanthrene						
1 day[c]	216.0 ± 15*	3′600 ± 250*	7′000 ± 610*	583 ± 51*	0.95 ± 0.11	109 ± 13
Benzo(a)pyrene						
1 day[c]	124.0 ± 14*	2′060 ± 240*	3′890 ± 170*	324 ± 14*	0.84 ± 0.13	97 ± 14

Pregnant rats were treated intragastrally with 80 mg/kg of phenobarbital in water; 60 mg/kg of pregnenolone 16α-carbonitrile in propane-1,2-diol or 20 mg/kg of 3-methylcholanthrene or 60 mg/kg of benzo(a)pyrene in sunflower oil. No significant differences in enzyme activities were observed between untreated and vehicle-treated controls. All rats were killed 20 days after copulation which was always 24 hr after the last dose of potential inducer. Values represent means ± S.E.M. of at least 4 determinations.

[a] See text.
[b] 1 pmol substrate = 330 dpm.
[c] One dose daily.
[d] Two doses daily.
* Increase at the $P < 0.05$ level statistically significant.

been observed [21–23]. Table 2 shows that this complete dissociation holds for any stage of ontogenetic development at which MO and EH activities can be measured without prior induction, that is from day 17 to 20 of fetal age. These increases in MO in contrast to unchanged EH levels were always observed 24 hr after treatment with the polycyclic hydrocarbon, still leaving the question open whether the dissociation referred to induction as such or possibly just to the time course of induction. Fig.1 shows that during the entire time course of rise of MO activity and of its return to control levels EH activity remained unchanged.

The great differences in MO induction with respect to '3-OHBP' as compared to 'Overall' after transplacental treatment with 3-methylcholanthrene or benzo(a)pyrene (and, although to a smaller but still significant extent, also after pregnenolone 16α-carbonitrile) (tables 1 and 2, fig.1) indicate that more than one MO is involved in oxidative metabolism of benzo(a)pyrene and differentially measured with this dual assay, these different increases being explainable by a different control of induction or by a different turnover rate, either assumption being incompatible with one molecular species.

Apart from other possible explanations, this selectivity of transplacental induction of MO under conditions not leading to changes in EH may possibly explain why carcinogenic polycyclic aromatic hydrocarbons which do not induce liver tumors in adult rats [24–26], where they induce both MO and EH, can do so transplacentally [27,28], inducing

Table 2
Prenatal enzyme levels and inducibility by 3-methylcholanthrene at different stages of ontogenetic development

Fetal age (days)	Monooxygenase						Epoxide hydratase		
	'3-OHBP'[a]			'Overall'[a]			nmol styrene glycol/mg protein per min		% of age-matched controls
	pmol OH-benzpyrene/mg protein per min		% of age-matched controls[c]	dpm over blank/mg protein per min[b]		% of age-matched controls			
	Controls[c]	Treated		Controls	Treated		Controls	Treated	
15	trace	–	–	trace	–	–	trace	–	–
17	2.2 ± 1.0	76.4 ± 7.0*	3500*	428 ± 66	3,940 ± 960*	920*	0.38 ± 0.06	0.30 ± 0.04	79
18	2.7 ± 0.7	74.7 ± 9.1*	2800*	458 ± 58	3,700 ± 610*	810*	0.44 ± 0.09	0.50 ± 0.04	114
19	4.1 ± 1.4	94.2 ± 12.9*	2300*	846 ± 132	4,960 ± 420*	590*	0.61 ± 0.06	0.59 ± 0.06	97
20	5.7 ± 1.8	158.0 ± 19.5*	2800*	1,260 ± 195	7,480 ± 620*	590*	0.95 ± 0.05	1.06 ± 0.10	112

Pregnant rats were treated intragastrally with a single dose of 20 mg/kg of 3-methylcholanthrene in sunflower oil 24 hr prior to sacrifice. No significant differences in enzyme activities were observed between untreated and vehicle-treated controls. Values represent means ± S.E.M. of at least 4 determinations.

[a] See text.

[b] 1 pmol substrate = 330 dpm.

[c] Control values and % increases represent rough approximations since the former were near the experimental blank (see large standard errors).

* Increase at the $P < 0.05$ level statistically significant.

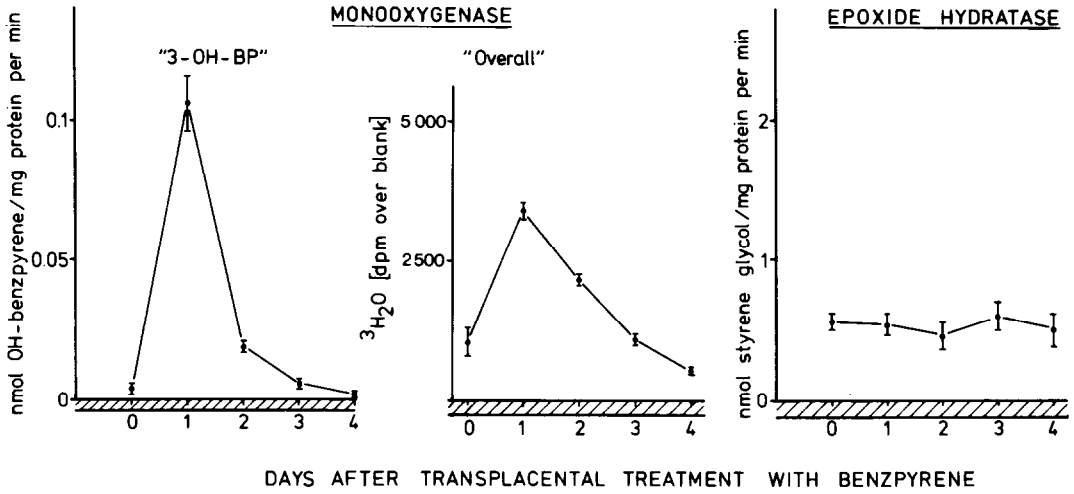

Fig.1. Time course of fetal liver enzyme activities after a single dose of benzo(a)pyrene. Pregnant rats were treated intragastrally with 60 mg/kg of benzo(a)pyrene in sunflower oil and were killed 0 (controls), 1, 2, 3 and 4 days thereafter which always was at 19 days of fetal age. The hatched areas below the zero line represent the experimental blank. 'Overall' monooxygenase activities are expressed in terms of dpm over blank/mg protein per min. 1 pmol benzpyrene (substrate) had 330 dpm. Values represent means ± S.E.M. of at least 4 determinations. Epoxide hydratase activities after treatment were at the $P < 0.05$ level not significantly different from controls.

in the fetal liver MO but not EH. Rates of hydration in control preparations even higher than those of MO after transplacental induction (tables 1 and 2, fig. 1) do not preclude this speculation since concentrations of metabolically produced unstable epoxides may be well below that required to saturate EH. Thus in vivo rates of hydration may be considerably slower than the ones determined in vitro under saturating conditions.

It therefore appears that (at least) two MO's are differentially involved in oxidative metabolism at different sites of the benzo(a)pyrene molecule, and that induction of EH, clearly, is not under common control with either of them. A selective induction of the inactivating EH should therefore not be intrinsically impossible, although it might practically prove difficult due to the great ease of induction of MO [29–31].

Acknowledgements

Advice by Dr Eva Schlede from the University of Berlin, Germany, on controlled mating of rats to allow transplacental treatment at known fetal ages, excellent technical assistance by Mr K. Suda, and financial support by NIH grant CA12928, and by the Swiss National Foundation for Scientific Research, Grant 3.877.72, are gratefully acknowledged. The author thanks Dr P. Bentley from his laboratory for critically reviewing the manuscript.

References

[1] Daly, J. W., Jerina, D. M. and Witkop, B. (1972) Experientia 28, 1129–1149.
[2] Oesch, F. (1973) Xenobiotica 3, 305–340.
[3] Booth, J. and Sims, P. (1974) FEBS Lett. 47, 30–33.
[4] Swaisland, A. J., Hewer, A., Pal, K., Keysell, G. R., Booth, J., Grover, P. L. and Sims, P. (1974) FEBS Lett. 47, 34–38.
[5] Kato, R. (1974) Drug Metabolism Reviews 3, 1–32.
[6] Conney, A. H., Lu, A. Y., Levin, W., Somogyi, A., West, S., Jacobsson, M., Ryan, D. and Kuntzman, R. (1973) Drug Metabolism and Disposition 1, 199–210.
[7] Oesch, F., Jerina, D. M. and Daly, J. W. (1971) Arch. Biochem. Biophys. 144, 253–261.
[8] Sims, P. (1970) Biochem. Pharmacol. 19, 2261–2275.
[9] Mannering, G. J., Sladek, N. E., Parli, C. J. and Shoeman, D. W. (1969) in: Microsomes and Drug Oxidations (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. and Mannering, G. J., eds.) pp. 303–330, Academic Press, New York.
[10] Nebert, D. W., Considine, N. and Owens, I. S. (1973) Arch. Biochem. Biophys. 157, 148–159.

[11] Comai, K. and Gaylor, J. L. (1973) J. Biol. Chem. 248, 4947–4955, and further references therein.
[12] Oesch, F., Kaubisch, N., Jerina, D. M. and Daly, J. W. (1971) Biochemistry N.Y. 10, 4858–4866.
[13] Ackermann, E. and Rane, A. (1971) Chem.–Biol. Interactions 3, 233–234.
[14] Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P. and Udenfriend, S. (1970) Biochemistry N.Y. 9, 147–156.
[15] Nebert, D. W. and Gelboin, H. V. (1968) J. Biol. Chem. 243, 6242–6249.
[16] Hayakawa, T. and Udenfriend, S. (1973) Analyt. Biochem. 51, 501–509.
[17] Oesch, F., Thoenen, H. and Fahrländer, H. J. (1974) Biochem. Pharmacol. 23, 1307–1317.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[19] Snedecor, G. W. and Cochran, W. G. (1967) Statistical Methods, Iowa State University Press, Ames.
[20] Pantuck, E., Conney, A. H. and Kuntzman, R. (1968) Biochem. Pharmacol. 17, 1441–1447.
[21] Welch, R. M., Gommi, B., Alvares, A. P. and Conney, A. H. (1972) Cancer Res. 32, 973–978.
[22] Nebert, D. W. and Gelboin, H. V. (1969) Arch. Biochem. Biophys. 134, 76–89.
[23] Schlede, E. and Merker, H. J. (1972) Naunyn-Schmiedebergs Arch. Pharmacol. 272, 89–100.
[24] Dunning, W. F., Curtis, M. R. and Segaloff, A. (1946) Cancer Res. 6, 256–262.
[25] Miller, E. C. and Miller, J. A. (1967) Proc. Soc. Exp. Biol. Med. 124, 915–919.
[26] Marquardt, H., Sternberg, S. S. and Philips, F. S. (1970) Chem. Biol. Interactions 2, 401–403.
[27] Shay, H., Gruenstein, M. and Weinberger, M. (1952) Cancer Res. 12, 296.
[28] Tomatis, L., Turusov, V. and Guibbert, D. (1972) in Topics in Chemical Carcinogenesis (Nakahara, W., Takayama, S., Sugimura, T. and Odashima, S., eds.) pp. 445–461, University Park Press, Baltimore.
[29] Conney, A. H. (1967) Pharmacol. Rev. 19, 317–366.
[30] Remmer, H. (1971) in: Proc. Second Int. IUPAC Congress, Vol. II, Pesticide Chemistry (Tahori, A. S., ed.) pp. 167–196, Gordon and Beach, New York.
[31] Gielen, J. E. and Nebert, D. W. (1972) J. Biol. Chem. 247, 7591–7602.