Biochemical Pharmacology, Vol. 24, pp. 2111–2116. Pergamon Press, 1975. Printed in Great Britain

DIFFERENTIAL INDUCTION OF MURINE Ah LOCUS-ASSOCIATED MONOOXYGENASE ACTIVITIES IN RABBIT LIVER AND KIDNEY

Steven A. Atlas, Snorri S. Thorgeirsson, Alan R. Boobis, Kenji Kumaki, and Daniel W. Nebert

Section on Developmental Pharmacology, Neonatal and Pediatric Medicine Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

(Received 24 July 1975; accepted 9 September 1975)

The induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity by polycyclic aromatic compounds is associated with the formation of a spectrally distinct form of cytochrome P-450, cytochrome P_1-450 (or P-448), in liver and other tissues of the rat (1, 2) and mouse (3). The appearance of cytochrome P_1-450, initially defined as an increase in the 455 nm/430 nm ratio in the ethyl isocyanide binding spectrum of dithionite-reduced hepatic microsomes (1), is associated with a shift from about 450 to about 448 nm in the Soret maximum of the reduced CO-difference spectrum (2), a change in the n-octylamine difference spectrum (4, 5), and an increase in the g=8.0 signal height seen by electron paramagnetic resonance spectroscopy (6, 7). The capacity of mice to respond to polycyclic aromatic compounds such as MC or BNF[1] is under genetic control (3, 8). In the mouse spectral evidence of the induction of cytochrome P_1-450 is genetically associated with the induction of a number of microsomal monooxygenase activities in addition to aryl hydrocarbon hydroxylase: p-nitroanisole O-demethylase (9), 7-ethoxycoumarin O-deethylase (9), 3-methyl-4-methylaminoazobenzene N-demethylase (9), zoxazolamine hydroxylase (10), 2-acetylaminofluorene N-hydroxylase (11), biphenyl 2-hydroxylase (12), biphenyl 4-hydroxylase (12), acetanilide 4-hydroxylase (12), and naphthalene 1,2-dihydrodiol formation (12). There is also evidence that phenacetin O-deethylase activity (13) and covalent binding of acetaminophen to hepatic macromolecules[2] are similarly associated with the so-called "aromatic hydrocarbon responsiveness," or Ah, locus in the mouse.

In every inbred mouse strain examined (i.e. both "responsive" and "nonresponsive"), treatment with PB is associated with increases principally in a form of cytochrome P-450 other than P_1-450 (3, 14) and with 2- to 4-fold induction of all these monooxygenase activities except for biphenyl 2-hydroxylase (3, 5, 8-12). Administration of TCDD to nonresponsive

[1]The abbreviations used are: MC, 3-methylcholanthrene; BNF, β-naphthoflavone; PB, sodium phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

[2]S. S. Thorgeirsson, unpublished data.

Table 1. SPECTRAL AND ELECTRON PARAMAGNETIC RESONANCE (EPR) STUDIES ON RABBIT LIVER MICROSOMES FOLLOWING TREATMENT WITH VARIOUS INDUCERS

Sexually immature male albino rabbits (New Zealand strain) were treated intraperitoneally with MC or BNF (80 mg/kg) in corn oil 48 hours prior to sacrifice, with TCDD (50 μg/kg) in p-dioxane (0.4 ml/kg) either 3 or 6 days prior to sacrifice, or with PB (60 mg/kg) in 0.85% NaCl daily for 4 days prior to sacrifice. The first day's dose of PB was divided and given 12 hours apart. Control animals received the vehicle p-dioxane or corn oil alone. The animals were starved for 48 hours prior to sacrifice. Microsomes were prepared from liver as described previously (3). Cytochrome P-450 determinations (16), ethyl isocyanide binding spectra (1), n-octylamine binding spectra (4, 5), and EPR spectroscopy (6, 7) were performed by procedures described in the references cited. The values represent the means from three experiments.

Determination	Previous treatment in vivo					
	Control	PB	MC	BNF	TCDD (3 days)	TCDD (6 days)
Cytochrome P-450 Content (nmol/mg protein)	0.81	2.66	2.05	1.93	2.34	2.79
Soret maximum (nm)	450.0	449.7	448.2	448.0	448.2	448.2
Ethyl isocyanide binding spectrum $[\Delta(A_{455}-A_{490})/\Delta(A_{430}-A_{490})]$[a]	0.65	2.80	1.99	1.83	1.76	1.63
n-Octylamine binding spectrum $[\Delta(A_{410}-A_{500})/\Delta(A_{392}-A_{500})]$[b]	1.11	0.95	0.67	0.80	0.69	0.68
EPR spectrum g=8.0 signal height[c]	24	50	178	106		
g=2.27 signal height[c]	29	48	33	27		

[a] Performed at pH 7.5. A spectral maximum of about 452 or 453 instead of 455 nm was seen after MC, BNF, or TCDD treatment.

[b] Values represent a single experiment.

[c] Values are expressed as chart units/gain/mg microsomal protein x 10^{-4}.

as well as responsive strains of mice, however, results in full expression of cytochrome P_1-450 formation and induction of each associated activity examined (8, 11, 14, 15), suggesting that nonresponsive mice have the regulatory and structural genes necessary to respond to a polycyclic aromatic inducer more potent than MC or BNF. Besides cytochrome $P_1$450 induction in the rat and mouse, MC treatment is associated with P_1-450 induction in rabbit (4, 6, 7) and hamster[3] liver and to a lesser degree in guinea pig liver.[3]

An earlier report from this laboratory (6) showed that MC-induced formation of P_1-450 in the rabbit liver is associated with aryl hydrocarbon hydroxylase induction in the kidney but not in the liver. These preliminary data suggested that P_1-450-associated monooxygenase activities differ between rabbit liver and rabbit kidney as well as between rabbit and mouse liver. We have therefore investigated in rabbit liver and kidney the effect of various inducers on the microsomal monooxygenase activities which are associated with the murine

[3] K. Kumaki and A. R. Boobis, unpublished data.

Ah locus.

As seen in Table 1, treatment of rabbits with MC, BNF, or TCDD resulted in the formation of hepatic cytochrome P_1-450 by all the spectral criteria established for the mouse (3, 5-7). The only exception was in the ethyl isocyanide difference spectrum. PB treatment increased the 455 nm/430 nm ratio to the same or greater extent than did MC, BNF, or TCDD, an effect observed in neither the rat (1) nor the mouse.[4] In the rabbit the increased 455 nm/430 nm ratio with PB was not, however, accompanied by the 2- to 3-nm blue spectral shift of the 455-nm peak observed with MC, BNF, or TCDD.

Eight hepatic monooxygenase activities following treatment of rabbits with the various inducers are shown in Table 2. Except for biphenyl 2-hydroxylase and naphthalene 1,2-dihydrodiol formation, all enzyme activities were significantly induced by PB treatment; induction ranged from about 30% for 7-ethoxycoumarin O-deethylase to more than 4-fold for aryl hydrocarbon hydroxylase. Whereas all these hepatic activities are induced 2- to more than 6-fold in responsive strains of mice by MC or BNF (8) and in both responsive and non-responsive strains by TCDD (8, 14, 15), only 2-acetylaminofluorene N-hydroxylase and acetanilide 4-hydroxylase were induced to the same degree in these rabbits by MC, BNF, or TCDD.

Table 2. EFFECT OF VARIOUS INDUCERS ON MONOOXYGENASE ACTIVITIES IN RABBIT LIVER MICROSOMES

The same animals described in Table 1 were used for these enzyme assays. Aryl hydrocarbon hydroxylase (17), p-nitroanisole O-demethylase (9), 7-ethoxycoumarin O-deethylase (9), and 2-acetylaminofluorene N-hydroxylase (11) activities were assayed according to procedures described in the references cited. Biphenyl 4-hydroxylase and biphenyl 2-hydroxylase assays were performed by a modification of the method of Creaven et al. (18). The conversion of [^{14}C]naphthalene to [^{14}C]naphthalene-trans-1,2-dihydrodiol was measured by a modification of the method of Oesch and Daly (19), using radiometric rather than ultraviolet spectroscopic analysis. All enzyme activities are expressed as pmol of product formed per min per mg of microsomal protein and represent the mean value of determinations on 2 to 4 animals in three separate experiments.

Monooxygenase activity	Previous treatment in vivo					
	Control	PB	MC	BNF	TCDD (3 days)	TCDD (6 days)
2-Acetylaminofluorene N-hydroxylase	41	97	330	480	300	530
Acetanilide 4-hydroxylase	200	520	780	480	770	690
Aryl hydrocarbon hydroxylase	97	450	100	75	110	120
7-Ethoxycoumarin O-deethylase	2270	2930	920	810	1130	840
p-Nitroanisole O-demethylase	5430	9220	6420	5330	7830	5780
Naphthalene 1,2-dihydrodiol formation	500	600	600	360	630	570
Biphenyl 4-hydroxylase	1110	2990	1720	1660	2230	1950
Biphenyl 2-hydroxylase	<100	<100	<100	<100	<100	<100

[4] G. F. Kahl, K. Kumaki, and D. W. Nebert, manuscript in preparation.

Biphenyl 4-hydroxylase was slightly (about 50%) but significantly ($.02<\underline{P}<.05$) induced. Biphenyl 2-hydroxylase was not detectable[5] in any of the samples, confirming a previous study (18). There occurred a marked decrease in 7-ethoxycoumarin O-deethylase activity following MC, BNF, or TCDD treatment. This finding suggests that, during induction of rabbit liver cytochrome P_1-450, other forms of cytochrome P-450 either diminish in concentration[6] or are structurally altered so that the substrate specificity is changed.

Corresponding monooxygenase activities are shown in Table 3 for rabbit kidney microsomes. Once again, no detectable biphenyl 2-hydroxylase activity was found. All other activities are induced by MC or TCDD except for 2-acetylaminofluorene N-hydroxylase and *p*-nitroanisole O-demethylase. Measured activities of these latter two are, however, just at the level of sensitivity of the enzyme assay employed; hence, significant induction may in fact be obscured. Nevertheless, the patterns of response to MC or TCDD apparently differ in the liver and kidney from the same animal. This conclusion is in agreement with a recent study of biphenyl 4-hydroxylase activity in rabbit liver and kidney following treatment with TCDD (20). The patterns of response to polycyclic hydrocarbon inducers in the mouse also differ between hepatic and various nonhepatic tissues (3, 8, 21). We realize that some of these differences could be due to different pH optima for the enzymes between rabbit and rat or mouse. We are presently carrying out experiments to prove or disprove this possibility.

Our results indicate that cytochrome P_1-450 differs in its substrate specificity among rabbit liver, rabbit kidney, and mouse liver. Recent studies on partially purified and reconstituted P_1-450 from rat and rabbit liver (22) suggest that these cytochromes differ catalytically as well; these investigators were unable, however, to find any MC-inducible activity in the rabbit system. We have reported here that two activities, 2-acetylaminofluorene N-hydroxylase and acetanilide 4-hydroxylase, are markedly induced by MC in rabbit liver. Furthermore, ring hydroxylation of 2-acetylaminofluorene in the 3-position is also induced about 5-fold in the liver of MC-treated rabbits.[2] Although these activities include both N-hydroxylation and ring hydroxylation, it is of interest that all three activities are

[5]We have recently found that, concomitant with the presence of detectable biphenyl 2-hydroxylase activity, aryl hydrocarbon hydroxylase may be inducible in weanling New Zealand albino rabbits. The apparent loss of biphenyl 2-hydroxylase activity with increasing age has been reported previously (18). We hope to determine whether the absence of aryl hydrocarbon hydroxylase inducibility in the older rabbits studied here is due to maturation or to genetic heterogeneity. Nevertheless, the eight enzymatic activities whose regulation is closely linked in the mouse are differentially inducible in these older rabbits.

[6]Electrophoretic analysis of hepatic microsomal proteins from mice which had received pulses of [^{14}C]leucine or [^{3}H]leucine also supports the possibility that increased synthesis, or incorporation into the membrane, of one form of cytochrome may occur concomitantly with decreased synthesis or incorporation of other forms of P-450 (14).

Table 3. EFFECT OF VARIOUS INDUCERS ON MONOOXYGENASE ACTIVITIES IN RABBIT KIDNEY MICROSOMES

The same animals described in Tables 1 and 2 were used. Except for higher pH (15-17), the same enzyme assay conditions used for hepatic microsomes were used for kidney microsomes. Kidney microsomes were prepared as described previously (6, 7). All activities are expressed as pmol of product formed per min per mg of microsomal protein and represent the mean values of determinations on two animals.

Monooxygenase activity	Previous treatment *in vivo*			
	Control	MC	TCDD (3 days)	TCDD (6 days)
2-Acetylaminofluorene N-hydroxylase	26	24		
Acetanilide 4-hydroxylase	11	26	25	22
Aryl hydrocarbon hydroxylase	4.7	15	26	13
7-Ethoxycoumarin O-deethylase	<10	29	79	66
p-Nitroanisole O-demethylase	1020	960	1030	1010
Naphthalene 1,2-dihydrodiol formation	31	47	49	47
Biphenyl 4-hydroxylase	39	170	220	180
Biphenyl 2-hydroxylase	<100	<100	<100	<100

associated with two substrates possessing N-acetyl moieties, suggesting a possible structural specificity of the MC-induced form(s) of hepatic cytochrome P-450 in the rabbit.

Although the hepatic monooxygenase systems in rabbit and mouse might not be analogous, it is possible that the particular cytochrome(s) associated with MC-inducible 2-acetylaminofluorene N-hydroxylase and acetanilide 4-hydroxylase activities are different from the cytochrome(s) associated with MC-inducible aryl hydrocarbon hydroxylase activity in the mouse as well. This idea does not detract from the concept of the murine *Ah* locus controlling the polycyclic aromatic hydrocarbon responsiveness of a wide variety of enzymatic activities. This concept suggests, rather, that a single genetic regulatory system may control the expression of a multiplicity of induction-specific proteins.

In summary, eight inducible monooxygenase activities, previously shown to be associated with the genetic locus (or loci) (8) controlling the formation of mouse cytochrome P_1-450, have been studied in liver and kidney microsomes from rabbit. Despite spectral evidence for cytochrome P_1-450 induction in rabbit liver, of these eight activities only the N- and ring-hydroxylation reactions involving two N-acetylarylamines, 2-acetylaminofluorene and acetanilide, are induced more than 2-fold. Four of the monooxygenase activities not inducible in rabbit liver are, however, inducible in rabbit kidney. These data support the hypothesis that there are multiple forms of inducible cytochrome P_1-450 that differ from organ to organ as well as from species to species. Consequently, it is unwarranted to assume that enzymatic activities whose regulation is linked in the mouse are regulated similarly in other species, including man.

ACKNOWLEDGMENT: We thank Dr. Hideo Kon for his help with the EPR determinations and Ms. Nancy M. Jensen for valuable technical assistance.

REFERENCES

1. Sladek, N. E. and Mannering, G. J. (1966) Biochem. Biophys. Res. Commun. 24, 668-674.

2. Alvares, A. P., Schilling, G., Levin, W., and Kuntzman, R. (1967) Biochem. Biophys. Res. Commun. 29, 521-526.

3. Gielen, J. E., Goujon, F. M., and Nebert, D. W. (1972) J. Biol. Chem. 228, 753-756.

4. Jefcoate, C. R. E., Gaylor, J. L., and Calabrese, R. L. (1969) Biochemistry 8, 3455-3463.

5. Nebert, D. W., Gielen, J. E., and Goujon, F. M. (1972) Mol. Pharmacol. 8, 651-666.

6. Nebert, D. W. and Kon, H. (1973) J. Biol. Chem. 248, 169-178.

7. Nebert, D. W., Robinson, J. R., and Kon, H. (1973) J. Biol. Chem. 248, 7637-7647.

8. Nebert, D. W., Robinson, J. R., Niwa, A., Kumaki, K., and Poland, A. P. (1975) J. Cell. Physiol. 85, 393-414.

9. Nebert, D. W., Considine, N., and Owens, I. S. (1973) Arch. Biochem. Biophys. 157, 148-159.

10. Robinson, J. R. and Nebert, D. W. (1974) Mol. Pharmacol. 10, 484-493.

11. Thorgeirsson, S. S., Felton, J. S., and Nebert, D. W. (1975) Mol. Pharmacol. 11, 159-165.

12. Atlas, S. A., Daly, J. W., and Nebert, D. W. (1975) Fed. Proc. 34, 755.

13. Poppers, P. J., Levin, W., and Conney, A. H. (1974) Pharmacologist 16, 262.

14. Haugen, D. A., Coon, M. J., and Nebert, D. W. J. Biol. Chem., in press.

15. Poland, A. P., Glover, E., Robinson, J. R., and Nebert, D. W. (1974) J. Biol. Chem. 249, 5599-5606.

16. Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370-2379.

17. Nebert, D. W. and Gielen, J. E. (1972) Fed. Proc. 31, 1315-1325.

18. Creaven, P. J., Parke, D. V., and Williams, R. T. (1965) Biochem. J. 96, 879-885.

19. Oesch, F. and Daly, J. (1972) Biochem. Biophys. Res. Commun. 46, 1713-1720.

20. Hook, G. E. R., Haseman, J. K., and Lucier, G. W. (1975) Chem.-Biol. Interactions 10, 199-214.

21. Niwa, A., Kumaki, K., and Nebert, D. W. (1975) Arch. Biochem. Biophys. 166, 559-564.

22. Kawalek, J. C. and Lu, A. Y. H. (1975) Mol. Pharmacol. 11, 201-210.