MOLECULAR PHARMACOLOGY, 13, 1181-1186

SHORT COMMUNICATION

Subcellular Events Occurring during Aryl Hydrocarbon Hydroxylase Induction: No Requirement for Metabolism of Polycyclic Hydrocarbon Inducer

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(Received April 22, 1977)
(Accepted June 6, 1977)

SUMMARY

KANO, ITSU, GIELEN, JACQUES E., YAGI, HARUHIKO, JERINA, DONALD M. & NEBERT, DANIEL W. (1977) Subcellular events occurring during aryl hydrocarbon hydroxylase induction: no requirement for metabolism of polycyclic hydrocarbon inducer. *Mol. Pharmacol.*, 13, 1181-1186.

The induction of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.2) activity in the rat Reuber hepatoma H-4-II-E established cell line was studied with 3-methylcholanthrene and its K-region oxide and diol and with benzo[*a*]pyrene and 23 of its oxygenated derivatives, including 12 phenols, three diols, three quinones, three oxides, and two diol-epoxides. Compared with the parent benzo[*a*]pyrene molecule, the naturally occurring benzo[*a*]pyrene 6,12-quinone is approximately as potent, the chemically synthesized 12-hydroxy derivative is at least 50% more potent, and the chemically synthesized 4-hydroxy derivative is about one-half as potent in inducing the hydroxylase activity. The naturally occurring benzo[*a*]pyrene 7,8-oxide is also about one-half as potent as benzo[*a*]pyrene, and all other analogues examined are poorer inducers than the four compounds mentioned. *trans*-7,8-Dihydroxy-7,8-dihydrobenzo[*a*]pyrene is more polar than benzo[*a*]pyrene and enters the cells in culture more readily than benzo[*a*]pyrene. Knowing the amount of polycyclic hydrocarbon metabolized and the percentage of various metabolites of benzo[*a*]pyrene that exist in these cells, we have found no metabolite sufficiently potent to induce the hydroxylase activity more than the parent benzo[*a*]pyrene molecule. It is concluded, therefore, that metabolism of the parent polycyclic hydrocarbon is not a requirement for the induction process to proceed.

INTRODUCTION

The inductions of tyrosine aminotransferase by dexamethasone (1, 2), of glutamine synthetase by hydrocortisone (3), of alkaline phosphatase by hydrocortisone (4), and of aryl hydrocarbon hydroxylase activity[4] by polycyclic aromatic compounds

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[4] With benzo[*a*]pyrene as the substrate *in vitro*, "aryl hydrocarbon hydroxylase activity" is equated with the rate of formation of 3-hydroxybenzo[*a*]pyrene (5) and probably other phenols [such as 9-hydroxybenzo[*a*]pyrene (6)] having similar wavelengths of fluorescent activation and emission.

ISSN 0026-895X

(9–11) are among the best characterized enzyme induction processes in eukaryotic tissue culture experimental model systems. Aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons differs from the other three induction processes in that the induced enzyme in turn (usually) metabolizes the inducer (6–8, 11–16).

The steroid inducers bind with high specificity to a cytosolic receptor protein (17, 18); similar evidence now exists for polycyclic aromatic inducers in mouse liver (19) and in established cell lines in tissue culture (20). The cytosolic androgen-binding receptor in the rat has a much higher affinity for testosterone than for dihydrotestosterone in the immature uterus, the mature testicular seminiferous tubules, and the immature ovary, whereas the same receptor (or a cytosolic protein having many similar biophysical properties) has a much higher affinity for dihydrotestosterone than for testosterone in the ventral prostate, the epididymis, and the preputial gland (21). The amount of cytosolic testosterone 5α-reductase (22), the affinity of the androgen-binding receptor for testosterone compared with dihydrotestosterone, and the capacity of the testosterone-receptor complex relative to the dihydrotestosterone-receptor complex to translocate into the nucleus are all important factors (23) from an experimental as well as a physiological *in vivo* point of view in each tissue. Hence, for example, if the 5α-reductase were low in a dihydrotestosterone-responsive tissue exposed to testosterone, the androgen effect would be low because of the poor metabolic conversion of testosterone to dihydrotestosterone.

The induced aryl hydrocarbon hydroxylase level has sometimes been noted (24, 25) to parallel the basal hydroxylase level in several different cell culture systems. Accordingly, the possibility exists that metabolism of the parent polycyclic hydrocarbon is essential for the induction process. To test this hypothesis, one cannot add chemical inhibitors of monooxygenase activity such as α-naphthoflavone, metyrapone, SKF 525-A, or piperonyl butoxide in an attempt to block aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons, because these inhibitors dissolved in the growth medium can be good inducers (26, 27). Also, one cannot test this hypothesis by inhibiting cytochrome P-450-mediated metabolism *in vivo* with carbon monoxide, because of the much higher affinity of carbon monoxide for hemoglobin and cytochrome a_3 than for P-450. By knowing what percentage of a polycyclic hydrocarbon inducer is metabolized to what product, however, we were able to compare in this report the inducing capacities of metabolites of MC[5] or BP with that of the parent polycyclic hydrocarbon.

The metabolism of numerous polycyclic hydrocarbons – including BP, MC, BA, 7,12-dimethylbenz[*a*]anthracene, and dibenz[*a*,*c*]anthracene – in cell culture has been studied extensively (16, 28–33). When 13 μM polycyclic hydrocarbon (about 3 μg/ml) is added to the growth medium (30, 31), the *maximal* rate of metabolism of any of these polycyclic hydrocarbons by the P-450-mediated monooxygenase system(s) in 12 hr is about 5% of the total added (i.e., about 0.65 μM, or 0.65 nmole/ml). This rate represents between 0.2 and 1.0 fmole of metabolites per cell. Lower concentrations of polycyclic hydrocarbon result in rates less than this maximal rate (31). With BP, if any product represented 50% of the total metabolites (16), that product would exist in these cultures at no more than 0.325 μM. We therefore examined various metabolites of MC and BP (Fig. 1 and Table 1) to see whether any intermediate or product, at its estimated

Whereas the 9-hydroxy product is known to be formed via an arene oxide (7), it is not known at present whether the 3-hydroxy product is formed via a direct hydroxylation or in a two-step process via an arene oxide. Consequently, low amounts of epoxide hydratase can lead to higher amounts of these phenolic benzo[*a*]pyrenes formed, relative to total benzo[*a*]pyrene metabolized (8), compared with conditions when epoxide hydratase concentrations are not rate-limiting.

[5] The abbreviations used are: MC, 3-methylcholanthrene; BP, benzo[*a*]pyrene; BA, benz[*a*]anthracene; BP-4,5-oxide (and others), the BP metabolite having an arene oxide in the 4,5-position; BP-4,5-diol (and others), the BP metabolite having a *trans*-dihydrodiol in the 4,5-position; 1-HO-BP (and others), 1-hydroxybenzo[*a*]pyrene; BP-1,6-Q (and others), benzo[*a*]pyrene 1,6-quinone.

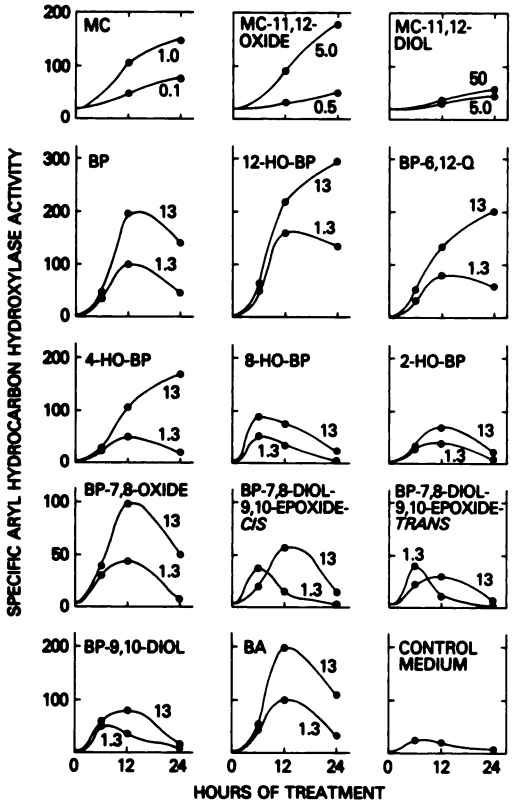

FIG. 1. *Effects of MC and its K-region oxide and diol, of BP and nine of its metabolites, of BA, or of control growth medium alone on aryl hydrocarbon hydroxylase induction in rat Reuber hepatoma H-4 established cell cultures*

No morphological toxicity of these compounds at these concentrations was evident from light microscopy and rate of cell division. In experiments shown in both figures and Table 1, the cells were plated at about 0.5×10^6/60-mm plastic dish (0.5×10^6 in 3.0 ml) and allowed to reach logarithmic growth during the next 2 days, as previously described (26, 27, 34). The test compound was then added at the indicated concentration, and cells from two dishes were assayed separately at each indicated time for the hydroxylase activity, as previously described (26, 27, 34); the *points* represent the means of duplicate determinations. BP-7,8-diol-9,10-epoxide-*cis* refers to (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BP-7,8-diol-9,10-epoxide-*trans* refers to (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. The number next to each line indicates the micromolar concentration of the test compound. One unit of the hydroxylase activity is defined (35) as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of the 3-hydroxybenzo[*a*]pyrene recrystallized standard. Specific activity denotes units per milligram of cell protein. It should be noted that the units per milligram of total cellular protein on the ordinate differ for BP-7,8-oxide and the *cis*- and *trans*-diol-epoxide isomers compared with the ordinate scales for the other 12 compounds. The MC-11,12-oxide and MC-11,12-diol were generous gifts in 1972 from Dr. Peter Sims. The syntheses of these BP derivatives have been described (reviewed in ref. 36).

physiological concentration, would induce aryl hydrocarbon hydroxylase activity as effectively as the parent compound at its optimal concentration. This would require that any product at 0.325 μM in the growth medium be at least 40 times as potent an inducer as BP. Clearly, none of the metabolites of BP or MC tested (Fig. 1 and Table 1) is capable of inducing the hydroxylase activity at its estimated physiological concentration—by a factor of greater than 20. We therefore conclude that metabolism of the parent polycyclic hydrocarbon molecule is not required for the hydroxylase induction process to proceed.

It is of interest that the two metabolites that induce the enzyme at least as well as BP are 12-HO-BP and BP-6,12-Q. However, the 1,6- and 3,6-quinones and 3-HO-BP and 6-HO-BP are not inducers. An oxygen atom at C-12 therefore has little effect on blocking the induction process (although it does not enhance induction), yet oxygen atoms at C-3 and C-6 impede the induction process. Moreover, 4-HO-BP and 5-HO-BP are somewhat active as inducers,[6] whereas metabolites containing 1 or 2 oxygen atoms in the benzylic ring (C-7, -8, -9, and -10) are quite poor inducers. The *cis*- and *trans*-diol-epoxide isomers might be quite poor inducers (Fig. 1) simply on the basis of their extremely short half-lives in aqueous solutions (39). Some of these data could provide useful information on the nature of the active binding site of the cytosolic receptor for polycyclic aromatic inducers (19, 20). For example, modification of BP in position 12 does not affect the inducing properties of the chem-

[6] Although all 12 phenols of BP were examined, it should be noted that only the 3-HO-BP and 9-HO-BP, and small amounts of the 1-HO-BP (12) and 7-HO-BP, appear to be formed metabolically *in vivo* or *in vitro* (38).

TABLE 1

Effects of other BP metabolites not illustrated in Fig. 1 on aryl hydrocarbon hydroxylase induction in H-4 cell cultures

Experimental conditions are described in the legend to Fig. 1. No morphological toxicity at these concentrations of these metabolites was evident from light microscopy and logarithmic growth rate (cell division). Similar experiments with the hydroxylase induction in an epithelial cell line cloned from Buffalo rat liver (37) showed BA-5,6-diol to be about 1000 times less effective an inducer than BA. The possible contamination of fluorescence in the cell cultures from the added BP metabolite, which might have interefered with the hydroxylase assay, was examined by adding 13 μM 3-HO-BP, 12-HO-BP, or BP-6,12-Q to the cell cultures for 30 min [at which time the intracellular concentration of the chemical is maximal (30) but the actual rise in induced activity has not yet begun (31)]. Fluorescence of the cell homogenate was examined with and without extraction into 1.0 N NaOH, and with and without the usual enzyme assay (30-min incubation at 37°, extraction with cold hexane–acetone, followed by extraction of the organic phase with 1.0 N NaOH). No fluorescence beyond that found in cells grown in control medium alone was found.

Test compound	Concentration in growth medium	Treatment time	Specific hydroxylase activity[a]	Test compound	Concentration in growth medium	Treatment time	Specific hydroxylase activity[a]
	μM	hr			μM	hr	
1-HO-BP	13	6	45	11-HO-BP	13	6	70
	1.3	6	13		1.3	6	28
	0.13	6	18		0.13	6	16
3-HO-BP	13	6	22	BP-1,6-Q	1.3	6	14
	1.3	6	22		0.13	6	21
	0.13	6	12		0.013	6	19
5-HO-BP	13	12	62	BP-3,6-Q	1.3	6	11
	1.3	6	23		0.13	6	11
	0.13	6	17	BP-4,5-diol	1.3	6	33
6-HO-BP	13	12	30		0.13	6	32
	1.3	6	24	BP-7,8-diol	1.3	6	20
	0.13	6	22		0.13	6	18
7-HO-BP	13	6	25	BP-4,5-oxide	1.3	6	22
	1.3	6	20		0.13	6	23
	0.13	6	18	BP-9,10-oxide	1.3	6	27
9-HO-BP	13	6	28		0.13	6	26
	1.3	6	24	BP	13	12	195
	0.13	6	20		1.3	12	100
10-HO-BP	1.3	6	18		0.13	6	37
	0.13	6	14		0.013	6	25
				Control medium		6	24

[a] Specific hydroxylase activity is defined in the legend to Fig. 1.

ical at all. Hence attachment of BP via position 12 to an affinity column might be more efficacious in purifying the cytosolic receptor protein (19) than attachment to such a column via C-3 or C-6.

It might be argued that these polar BP metabolites do not enter the cell as readily as BP and that the poor inducing capacity might be explained on this basis. However, numerous studies (40–46) have demonstrated transformation, mutagenicity, toxicity, or stimulation of DNA synthesis when various oxygen-containing metabolites of polycyclic hydrocarbons—including BA, dibenz[*a*,*h*]anthracene, 7-methylbenz[*a*]anthracene, 7-bromomethylbenz[*a*]anthracene, 7-bromomethyl-12-methylbenz[*a*]anthracene, MC, and BP—were added to mammalian cells in culture. These results strongly indicate that such oxygen-containing metabolites do enter cells in culture readily.

Figure 2 illustrates that radiolabeled BP-7,8-diol actually enters the cell cultures more rapidly, and in a greater amount, than BP. The maximal intracel-

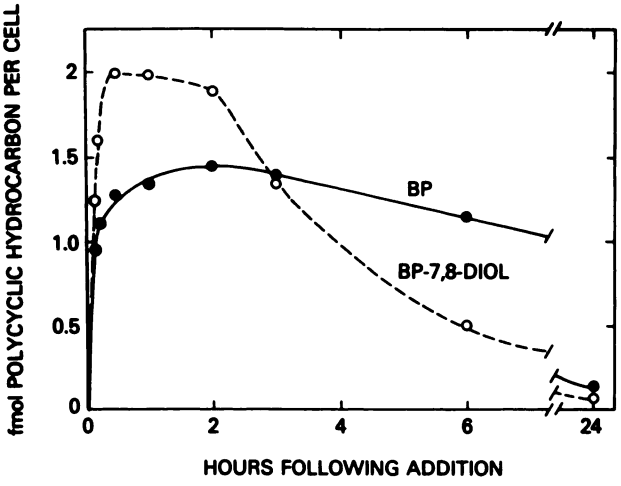

FIG. 2. *Uptake of BP or BP-7,8-diol by H-4 cells in culture*

[*G*-^{3}H]BP (20 Ci/mmole) or [^{3}H]9,10-BP-7,8-diol (29.9 mCi/mmole) (36) was dissolved in a minimal amount of acetone and added at 0.05 μCi (in 2.0 ml of growth medium) per dish to logarithmically growing H-4 cells. Concentrations of both chemicals were adjusted with nonradioactive material to make a 13 μM final concentration. Cells from three dishes at each indicated time were analyzed separately for radioactivity, as previously described (30); the *points* represent the means of triplicate determinations. A second experiment yielded similar results. The harvested cells were centrifuged at 1000 × *g* for 10 min, resuspended in Dulbecco's phosphate-buffered saline (0.85% NaCl), and recentrifuged three times. The washed cell pellet was then homogenized in 0.4 ml of the phosphate-buffered saline with a Potter-Elvehjem glass homogenizer with Teflon pestle; two 0.05-ml aliquots were taken for protein determination (47), and 0.20 ml was added to a scintillation vial. One milliliter of "NCS" (Nuclear-Chicago Solubilizer) was added, and the mixture was kept at 45° overnight, following which 10 ml of the liquid scintillation mixture were added (30). The radioactivity was converted to femtomoles of polycyclic hydrocarbon estimated per cell. Whether this material is all in the cell or how much is adsorbed to the cell surface has not been determined. However, previous experiments of this type with BA showed (30) about 50% of the BA bound to a crude nuclear fraction, about 20% to a crude mitochondrial fraction, about 20% to a crude microsomal fraction, and about 10% in the post-105,000 × *g* supernatant fraction.

lular concentrations attained were 10.2% and 7.8% of the total BP-7,8-diol and BP added, respectively. As might be expected, the polar diol is also excreted (presumably after further metabolism) from the cell more rapidly than BP; about 0.5 fmole of the diol per cell remains after 6 hr, compared with more than 1.0 fmole of BP per cell. The gradual decrease in intracellular BP concentration seen in Fig. 2 after about 2 hr, in spite of a large excess of extracellular polycyclic hydrocarbon, was also observed with BA (30) and is not understood. It is therefore concluded that, because metabolites as polar as BP-7,8-diol enter the cell even more readily than BP, the poor inducing capacity of these oxygen-containing metabolites cannot be explained on the basis of decreased hydrophobicity and that metabolism of the parent polycyclic hydrocarbon molecule is unnecessary during the process of aryl hydrocarbon hydroxylase induction.

ACKNOWLEDGMENTS

We thank Dr. Olavi Pelkonen and Dr. Mortimer B. Lipsett for helpful suggestions in reviewing this manuscript, and Ms. Ingrid E. Jordan for her expert secretarial assistance.

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