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Aryl hydrocarbon hydroxylase activity in rat mammary fibroblasts grown in primary culture

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A requirement for the carcinogenic actions of polycyclic aromatic hydrocarbons (PAH) is their oxidative metabolism to a reactive intermediate(s) that covalently binds to cellular DNA [1–3]. The microsomal mixed function oxidase aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH, flavoprotein-linked monooxygenase, EC1.14.14.1) catalyzes the initial conversion of the parent PAH to epoxide intermediates, which is believed to be the rate-limiting step in PAH-induced carcinogenesis [4, 5]. Previous studies have demonstrated that one of the most susceptible target organs for PAH is the mammary gland of the Sprague–Dawley rat [6–8]. A single, intragastric intubation of 7,12-dimethylbenz[*a*]anthracene (DMBA) to 50 to 60-day-old, virgin Sprague–Dawley rats results in an 80–100 per cent incidence of adenocarcinomas in the mammary glands. Our laboratory has developed techniques for the isolation of epithelial cells and fibroblasts from the rat mammary fat pad [9, 10]. In addition, we have characterized PAH-inducible AHH activity in freshly isolated rat epithelial cells and in those grown in a hormone-supplemented medium [11, 12]. In the mammary fat pad of the intact rat, fibroblasts are in close association with and, in fact, form the support system for, the epithelial cells of the mammary ducts and alveoli [13]. The present study determines that contribution which the fibroblasts may give to the overall metabolism of PAH by the various cell types that constitute the rat mammary gland.

Materials and Methods

Female, virgin Sprague–Dawley rats (50- to 60-days-old; Charles River Breeding Laboratories, Wilmington, MA) were maintained on a diet of Wayne Lab-Blox (Allied Mills, Inc., Chicago, IL) and water *ad lib.* under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (24.0 ± 1.5°).

Isolation of rat mammary fibroblasts. The rats were killed by CO_2 asphyxiation, and the abdominal and inguinal mammary fat pads were removed using aseptic procedures. The fat pads were cleared of all lymph nodes, minced with a razor blade, and added to a dissociation medium containing 0.45% collagenase (CLS, Type II, Worthington Biochemical Corp., Freehold, NJ) in medium 199-Earle's salts (M199-E) with 5% fetal bovine serum (FBS). After incubation at 37° for 60 min, the mixture was filtered through 1–2 layers of 157 nylon mesh (Martin Supply Co., Baltimore, MD) which removed all the undissociated tissue. Lipocytes were removed by centrifugation at 1000 rpm for 15 min, and the resultant cell pellet was suspended and incubated in M199-E containing 10,000 units DNase for 10 min at 37°. The suspension was centrifuged at 600 rpm for 6 min, and the rat mammary fibroblasts were isolated in the top half of the gradient. Previous studies [8, 11] have shown that the lower half of the gradient contained large clumps and chains of epithelial cells.

In vitro *culture conditions.* Isolated mammary fibroblasts were examined for viability by trypan blue exclusion, counted, and added to a culture medium comprised of M199-E, 50 μg/ml gentamicin, and 20% charcoal adsorbed FBS. The fibroblasts were seeded in 100 mm Falcon petri dishes at a concentration of 1×10^6 cells/dish and were incubated at 37° in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed after the initial 24 hr to remove the unattached cells and, thereafter, every 48 hr. The day of plating was designated day 0, and all experiments were done on day 7 when the cells had grown to confluency.

In vitro *induction of AHH activity.* Inducers of mammary fibroblast AHH activity were added to the culture medium in either dimethylsulfofide (DMSO) or water at a final concentration of 0.1%. Control cells received an equal volume of solvent (0.1%) which was not shown to alter AHH activity. Furthermore, no morphological changes were observed after the addition of inducer and/or solvent. Following the induction period (16 hr or as indicated), the medium was decanted, and cells were washed twice with phosphate buffered saline (PBS) and removed with gentle scraping into 10 ml PBS. Each dish was rinsed with 10 ml PBS, and the fractions were combined and centrifuged at 500 *g* for 15 min at 37°. The cell pellet was resuspended and homogenized in a glass–glass Potter–Elvehjem homogenizer.

AHH determination. The fluorometric procedure described by Nebert and Gelboin [14] was used to determine AHH activity in mammary fibroblasts. AHH activity measurements were done in triplicate for homogenates of fibroblasts harvested from four 100 mm petri dishes. One unit of hydroxylase activity was defined as the amount of enzyme that catalyzed the formation of hydroxylated products and resulted in the fluorescence equivalent of 1 pmole of 3-hydroxybenzo[*a*]pyrene in 60 min at 37°. The incubation mixture contained 80 μmoles Tris–Cl buffer (pH 7.5), 3 μmoles $MgCl_2$, 0.36 μmole NADPH, 0.66 μmole glucose-6-phosphate, 0.15 units glucose-6-phosphate dehydrogenase, 80 μmoles benzo[*a*]pyrene and 0.6 to 1.7 mg cell homogenate protein in a final volume of 1 ml. Incubations were carried out in a Dubnoff metabolic incubator at 37° for 60 min. The reaction was stopped and the phenolic metabolites were extracted and measured as described previously [11]. All samples were read against 3-hydroxy-BP

Table 1. Dose–response of DMBA-induced AHH activity in mammary fibroblasts*

DMBA concn (μM)	AHH activity	
	units/mg protein	Induced/Control
	31 ± 6	
0.1	56 ± 3†	1.8
1.0	125 ± 4†,‡	4.0
10.0	193 ± 18†,§	6.2
100.0	48 ± 5†	1.6

* DMBA, at the indicated concentrations, was added to the fibroblast culture medium for 16 hr after which AHH activity was measured as described in Materials and Methods. Values are means ± S.E.; four to nine determinations for each group.

† $P < 0.05$ (vs control activity).

‡ $P < 0.05$ (vs 0.1 μM DMBA-induced AHH activity).

§ $P < 0.05$ (vs 1.0 μM DMBA-induced AHH activity).

Table 2. Effects of charcoal adsorption on serum-induced AHH activity in rat mammary fibroblasts*

Treatment	Serum (%)	Protein (mg/plate)	AHH activity (units/mg protein)
Unadsorbed	5	1.3 ± 0.1	43 ± 4
	10	1.7 ± 0.2	30 ± 4†
	20	2.9 ± 0.1†,‡	17 ± 3†,‡
Charcoal adsorbed	5	0.7 ± 0.1	79 ± 6§
	10	1.0 ± 0.1	59 ± 6†,§
	20	1.6 ± 0.1†,‡,§	38 ± 5†,‡,§

* Fibroblasts were isolated from the rat mammary fat pads as described previously. The cells were added to the culture medium which contained the indicated serum concentrations. After day 7, AHH activity was measured as described in Materials and Methods. Values are means ± S.E.; four to seven determinations for each FBS concentration.

† $P < 0.05$ (vs 5% unadsorbed or adsorbed FBS respectively).

‡ $P < 0.05$ (vs 10% unadsorbed or adsorbed FBS respectively).

§ $P < 0.05$ (vs 5, 10 or 20% unadsorbed FBS respectively).

which was provided by the NCI Chemical Repository. Protein concentrations were determined spectrophotometrically, using bovine serum albumin as a standard [15].

Results

Kinetics of AHH induction. Basal AHH activity in rat mammary fibroblasts after 7 days in primary culture was low [approximately 30 pmoles·(mg protein)$^{-1}$·hr^{-1}] (Table 1). The addition of 0.1, 1.0, 10.0 or 100 μM DMBA increased AHH activity 1.8-, 4.0-, 6.2- and 1.6-fold respectively.

Figure 1 summarizes the time course of DMBA-induced AHH activity in mammary fibroblasts during logarithmic growth. The addition of 10 μM DMBA to the culture medium increased hydroxylase activity 5.5-fold after 8 hr. The magnitude of AHH induction continued to rise until peak induction (6.5-fold over basal activity) occurred 16 hr after DMBA treatment. After 16 hr, AHH activity fell substantially; in fact, hydroxylase activity 36 hr after DMBA treatment was similar to that in untreated cells. For comparison, maximal DMBA-induced AHH activity in confluent cultures of rat mammary fibroblasts was approximately 60–70 per cent that in the same cells during logarithmic growth.

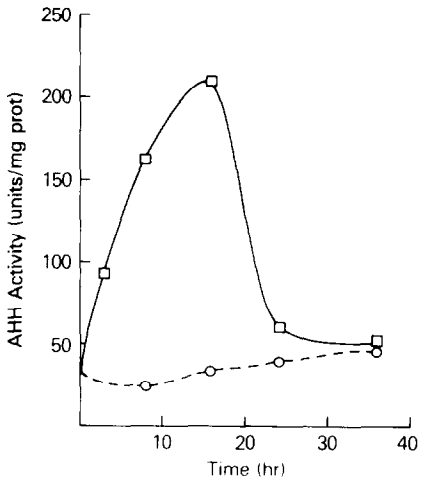

Fig. 1. Time course of DMBA-induced AHH activity in rat mammary fibroblasts. Fibroblasts were isolated from the mammary fat pads of Sprague–Dawley rats and grown for 6 days in primary culture. The plates were then divided into two groups: control (○---○) and DMBA-treated (□—□). DMBA (10 μM) was added to the culture medium in DMSO. Control cells received DMSO only (0.1%). At the times indicated, the cells from four confluent 100 mm petri dishes were pooled, and AHH activity was measured for each group. Each point is the mean of three separate experiments whose standard error did not exceed 10 per cent.

FBS effects on hydroxylase activity. In a previous report [12], we demonstrated AHH induction by FBS in rat mammary epithelial cells grown in primary culture. Furthermore, those findings indicated that FBS treated with 1% activated charcoal lost its ability to induce AHH activity in those cells. The present findings demonstrate that, while the amount of protein/100 mm petri dish rose with increasing concentrations of FBS, fibroblast AHH activity fell (Table 2). For example, rat mammary fibroblasts grown in the presence of 5% unadsorbed FBS yielded 1.3 mg protein/dish and AHH activity was 43 pmoles·(mg protein)$^{-1}$·hr^{-1}. However, if fibroblasts were grown in the presence of 20% unadsorbed FBS, the protein/plate rose to 2.9 mg, but AHH activity fell to 17 pmoles·(mg protein)$^{-1}$·hr^{-1}. Similar findings were observed in cells grown with charcoal adsorbed FBS. However, at the three serum concentrations tested (5, 10 and 20%), AHH activity in fibroblasts grown with charcoal adsorbed FBS was approximately 2-fold greater than in cells grown in unadsorbed serum (Table 2).

Other in vitro *inducers.* Numerous compounds are *in vitro* inducers of AHH activity in rat mammary fibroblasts (Table 3). The addition of 10 μM benz[*a*]anthracene (BA) or β-naphthoflavone (β-NF) increased AHH activity in homogenates of mammary fibroblasts 8.9- and 4.6-fold respectively. Benzo[*a*]pyrene (BP) addition, 0.1, 1.0 or 10.0 μM, increased fibroblastic AHH activity 3.4-, 5.2- and 10.9-fold, whereas only 1.0 μM 3-methylcholanthrene (3-MC) induced enzyme (2.1-fold) activity in these cells. Both sodium phenobarbital and norepinephrine were weak inducers of AHH activity in the mammary fibroblasts. Sodium phenobarbital, added at 100 μM, increased AHH activity 1.7-fold, whereas 10.0 and 100.0 μM norepinephrine induced AHH activity 1.6- and 1.8-fold respectively.

Discussion

In previous studies [11, 16], we investigated AHH activity in epithelial cells that were either isolated from

Table 3. Other *in vitro* inducers of AHH activity in mammary fibroblasts*

Inducer	Concn (μM)	AHH activity	
		Units/mg protein	Induced/control
		28 ± 4	
Benz[*a*]anthracene	10.0	250 ± 31†	8.9
β-Naphthoflavone	10.0	129 ± 8†	4.6
Benzo[*a*]pyrene	0.1	96 ± 7†	3.4
	1.0	146 ± 16†	5.2
	10.0	305 ± 30†	10.9
3-Methylcholanthrene	0.1	30 ± 4	1.1
	1.0	59 ± 4†	2.1
	10.0	34 ± 5	1.2
Sodium phenobarbital	100.0	49 ± 6†	1.7
Norepinephrine	10.0	45 ± 6†	1.6
	100.0	52 ± 4†	1.8

* Mammary fibroblasts were grown in primary culture for 6 days at which time the appropriate inducer was added to the medium for 16 hr. AHH activity was measured as described in Materials and Methods. Values are means ± S.E.; three to ten determinations for each compound at the indicated concentrations.

† $P < 0.05$ (vs control AHH activity).

mammary fat pads of virgin, Sprague–Dawley rats or grown in primary culture in a hormone-supplemented medium. The present study was designed to determine whether the stromal elements of the rat mammary fat pads also contain the inducible AHH enzyme and to compare its characteristics with those of the epithelial cells. Indeed, isolated rat mammary fibroblasts grown *in vitro* for 7 days contained a basal level of AHH activity which was approximately 65 per cent that measured in the epithelial cells grown in primary culture. Rat mammary fibroblast AHH activity was inducible by a variety of polycyclic aromatic hydrocarbons (PAH) and, in particular, induction by DMBA was dose-dependent, with peak activity having occurred 16 hr after the addition of 10 μM DMBA to the growth medium. The time course for AHH induction by DMBA is similar for both fibroblasts and epithelial cells [16]. However, maximum induction of AHH activity in the mammary epithelial cells occurred with 1.0 μM DMBA, which is 10-fold less than the 10.0 μM needed to induce peak fibroblast activity. This observation suggests that, although the cellular processes for DMBA induction of AHH activity might be similar for both mammary epithelial cells and fibroblasts, the epithelial cells are somewhat more sensitive to enzyme induction by DMBA than are the fibroblasts. A number of factors, such as inducer uptake and metabolism, and/or the *in vitro* culture conditions, could contribute to this apparent difference. In any case, after peak AHH induction by DMBA in the fibroblasts, AHH activity, by 36 hr, had returned to control levels. The failure of DMBA or any other PAH to maintain an induced level of AHH activity has been noted in other cells [17] and can be partially explained by a toxic response to the inducer or its metabolite(s) and/or by the failure of an inducer metabolite to maintain an elevated AHH activity. Other studies [14, 18] have shown that the readdition of inducer during the time of AHH decay can maintain an elevated level of enzyme activity, suggesting inducer depletion may partially mediate AHH decline.

Studies have demonstrated that untreated, heat-inactivated or dialyzed FBS can induce AHH activity in a variety of cell types *in vitro* [11, 19, 20]. We have reported [11] that treatment of FBS with 1% activated charcoal removes the constituent(s) that increases AHH activity in control and DMBA-treated rat mammary epithelial cells. In contrast, AHH activity in mammary fibroblasts grown in the presence of untreated FBS is significantly less than that in the same cells grown with charcoal adsorbed FBS. This finding suggests that the serum factor(s) that increases AHH activity in mammary epithelial cells and stimulates protein synthesis in mammary fibroblasts has little or no effect on fibroblast AHH activity. The results suggest, therefore, that regulation of AHH activity in rat mammary fibroblasts and epithelial cells is dependent, in part, on the characteristics of each cell type. Indeed, the relative inducibility of AHH activity in the fibroblasts revealed BP as the most potent inducer, followed by BA, DMBA, β-NF and 3-MC. The order of potencies for these same inducers in mammary epithelial cells was BA > DMBA > BP > β-NF > 3-MC. The present study further establishes an additional framework for future investigations focused on carcinogen metabolism by rat mammary tissue. Additional studies are needed to investigate whether any qualitative differences exist with BP or other PAH metabolism in mammary fibroblasts and epithelial cells. Further study should also consider the existence of intercellular regulation that would modulate the metabolic activity in either cell type.

In summary, the present study demonstrates PAH metabolism by rat mammary fibroblasts grown in primary culture. Basal AHH activity was low, yet inducible by a variety of PAH. The characteristics of AHH activity in mammary fibroblasts were significantly different from those described for mammary epithelial cells, suggesting that the regulation of PAH metabolism is dependent on each cell type.

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Chemical Carcinogenesis Program
NCI-Frederick Cancer Research Center
Frederick, MD 21701, *U.S.A.*

JOHN W. GREINER*

* Send reprint requests to: Dr. John W. Greiner, Laboratory of Biology, NIH, NCI, Bldg. 37, Room 2A19, Bethesda, MD 20205, U.S.A.

REFERENCES

1. H. V. Gelboin, *Cancer Res.* **29**, 1272 (1969).
2. P. L. Grover and P. Sims, *Biochem. J.* **110**, 159 (1968).
3. R. C. Moshel, W. M. Baird and A. Dipple, *Biochem. biophys. Res. Commun.* **76**, 1092 (1977).
4. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, *Nature, Lond.* **252**, 326 (1974).
5. D. R. Thakker, H. Yagi, A. Y. H. Lu, W. Levin, A. H. Conney and D. M. Jerina, *Proc. natn. Acad. Sci. U.S.A.* **73**, 3381 (1976).
6. C. Huggins, G. Briziarelli and H. Sutton, *J. exp. Med.* **110**, 25 (1959).
7. T. L. Dao, *Science* **145**, 810 (1969).
8. S. Z. Haslem and H. S. Bern, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4020 (1977).
9. L. B. Malan, D. H. Janss, A. B. DeAngelo, S. P. Kelley and M. A. Dailey, *In Vitro* **12**, 329 (1976).
10. D. H. Janss, L. B. Malan, E. I. Hadaway, A. B. DeAngelo and S. P. Kelley, *J. Toxic. envir. Hlth* **3**, 359 (1977).
11. J. W. Greiner, L. B. Malan-Shibley and D. H. Janss, *Chem. Biol. Interact.* **27**, 323 (1979).
12. J. W. Greiner, L. B. Malan and D. H. Janss, *Proc. Am. Ass. Cancer Res.* **19**, 164 (1977).
13. G. Semmer, *J. invest. Derm.* **63**, 27 (1974).
14. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243**, 6242 (1968).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. J. W. Greiner, A. H. Bryan, L. B. Malan-Shibley and D. H. Janss, *J. natn. Cancer Inst.* **64**, 1127 (1980).
17. H. V. Gelboin and F. J. Wiebel, *Ann. N.Y. Acad. Sci.* **170**, 529 (1971).
18. M. A. Bittner and R. W. Ruddon, *Molec. Pharmac.* **12**, 966 (1976).
19. J. E. Gielen and D. W. Nebert, *J. biol. Chem.* **247**, 7591 (1972).
20. J. P. Whitlock and H. V. Gelboin, *J. biol. Chem.* **249**, 2616 (1974).

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Dopamine receptor alterations with aging in mouse and rat corpus striatum

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Several recent experimental studies have demonstrated age-related changes in various neurotransmitter systems of mammalian brain [1–7]. Although the synaptic mechanisms of both cholinergic and dopaminergic systems are apparently altered in old animals, dopaminergic synapses seem particularly vulnerable in aging. Samorajski [1] reported decreased striatal dopamine synthesis in old rats. Jonec and Finch [8] measured reduced dopamine uptake in hypothalamic and striatal synaptosomes from aged mice. Govoni *et al.* [9] and Puri and Volicer [10] have shown that dopamine-stimulated adenyl cyclase activity is significantly lower in the striata of 20- to 24-month-old rats than it is in 2- to 3-month-old animals. Similar decreases were found in several brain regions, including striatum, of aged rabbits [11]. Furthermore, Bertler [12] and Carlsson and Winblad [13] reported a significant decline in the dopamine content of human striatum at senesence. To describe such changes in greater detail, we examined the age-related alterations of dopaminergic neurotransmission directly at the receptor level by examining the binding properties of radiolabeled haloperidol, a potent dopamine receptor antagonist and neuroleptic agent. Binding was assayed in homogenates of corpora striata dissected from mouse and rat brains from various age groups encompassing young adult to senescent animals [14].

Specific binding of [^{3}H]haloperidol (New England Nuclear Corp., Boston, MA, sp. act 19.74 Ci/mmole) to post-synaptic dopaminergic receptors was determined by the method of Burt *et al.* [15] in homogenates of isolated caudate-putamen (striatum) of rat (Charles River, CD) and mouse (C57BL/6J) brains. The tissues were coded such that the experimenter had no prior knowledge of the age group. Homogenates were prepared by a 1:20 (w/w) dilution of 0.32 M sucrose buffered with 15 mM Tris–HCl, pH 7.4. Total binding of [^{3}H]haloperidol and non-specific binding in the presence of 1 μM excess unlabeled haloperidol were determined in 15 mM Tris–HCl buffer, pH 7.4, containing 5 mM NaEDTA and 1 mM ascorbate. Following a 30-min incubation of tissue (0.2 to 0.4 mg protein) and ligand at 22°, filtration through Whatman GF/B glass fiber filters and rapid washing with 12 ml of buffer separated free and bound ligand. Radioactivity on the filters was determined in a Packard Tri-Carb scintillation counter at an efficiency of 38 per cent. Receptor number and affinity were determined by Scatchard analysis of specific binding of five concentrations (0.4 to 9.8 nM) of [^{3}H]haloperidol. Protein concentrations were measured by the method of Lowry *et al.* [16] using bovine serum albumin (BSA) as a standard.

In the first group of mouse brains, specific binding of [^{3}H]haloperidol was assessed at 2.8 nM, a saturating ligand concentration, in striata of animals 4, 10, 24 and 32 months of age. Four or five animals were independently studied in each age group. Preliminary experiments demonstrated that the K_d for [^{3}H]haloperidol was 1.35 nM in normal mouse brain striatal homogenates. The animals were decapitated, and the brains were rapidly removed, placed on ice, and dissected as outlined by Glowinski and Iverson [17]. As indicated in Table 1, the striatal tissues were kept frozen at −20° or −80° for 2–6 weeks prior to the binding studies. Table 1 shows that specific [^{3}H]haloperidol binding increased 8-fold in the mature (24-month) mice compared