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Aryl Hydrocarbon Hydroxylase, Epoxide Hydrase, and 7,12-Dimethylbenz[a]anthracene-Produced Skin Tumorigenesis in the Mouse

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SUMMARY

Mouse skin tumorigenesis initiated by 7,12-dimethylbenz[a]anthracene and promoted by repeated applications of phorbol ester is unrelated to genetic differences in the extent of aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons in inbred or hybrid C57BL/6N and DBA/2N mice. Thus, if aryl metabolism of 7,12-dimethylbenz[a] anthracene is a necessary step in the initiation of chemical carcinogenesis, the constitutive level of the hydroxylase activity in the skin of these mice is sufficient. Levels of hepatic epoxide hydrase activity are the same among inbred and hybrid C57BL/6N and DBA/2N mice and, in contrast to the hydroxylase, are not inducible by 3-methylcholanthrene. Epoxide hydrase activity could not be detected in the skin of these mice.

The initial event in the oxidative metabolism of polycyclic hydrocarbons is catalyzed by aryl hydrocarbon hydroxylase (1, 2), a substrate-inducible mono-oxygenase found mainly in mammalian liver microsomes but also present in other tissues (2–5), including skin (6, 7). The initial products formed by aryl hydroxylases such as aryl hydrocarbon hydroxylase from mono-(8), bi-(9), and polycyclic (10) hydrocarbons are reactive epoxides (arene oxides), which can (a) be converted to *trans*-dihydrodiols by the action of microsomal epoxide hydrase(s) (9–13), (b) rearrange spontaneously to phenols (9, 14, 15), (c) conjugate with glutathione (9, 10, 14), or (d) combine covalently with cellular nucleic acids, histones (16), and other proteins (17). In cell culture, K-region epoxides are many times more active than the parent polycyclic hydrocarbons and the corresponding phenols and *cis*- and *trans*-dihydrodiols in producing malignant transformation (18, 19). Moreover, the magnitude of mutagenicity produced by K-region epoxides correlates well with the known carcinogenicity of the parent aromatic hydrocarbons *in vivo* (20). Induction of aryl hydrocarbon hydroxylase activity by aromatic hydrocarbons occurs in cell culture (1) and in many tissues of different animal species (1, 2, 21). Epoxide hydrase is inducible by 3-methylcholanthrene in Sprague-Dawley rats (13). Therefore the constitutive and inducible levels of either aryl hydrocarbon hydroxylase or epoxide hydrase (or both) present in any tissue may be im-

portant for the magnitude of tumorigenesis evoked by epoxide intermediates.

Recently we have reported (22–25) that aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons occurs in some inbred strains of mice but not in other strains. This expressed function segregates as a single autosomal dominant gene, for which we have proposed the *ah* locus (23–25). Hence, in any mouse which is homozygous or heterozygous for the allele *Ah*, the aryl hydrocarbon hydroxylase system is inducible generally as an all-or-none response in all tissues regularly containing the inducible enzyme (23–25). It is therefore possible within the same hybrid litter to study mice possessing the inducible aryl hydrocarbon hydroxylase and mice that do not have it. The question is then raised: Can one find a relationship between 7,12-dimethylbenz[*a*]-anthracene-produced skin tumor formation, epoxide hydrase activity, and genetic differences in the extent of aromatic hydrocarbon-inducible aryl hydrocarbon hydroxylase activity among *AhAh*, *Ahah*, and *ahah* mice? The pH optima of the control hepatic aryl hydrocarbon hydroxylase activity from *ahah* mice and from *AhAh* mice are distinctly different (23–25). Other dissimilarities in aromatic hydrocarbon metabolism between these strains of mice—such as the position or amount of epoxide formation or the amount of covalent interaction with cellular macromolecules—may also exist and influence the rate of tumor formation.

The experiments were carried out with C57BL/6N mice, which have the inducible aryl hydrocarbon hydroxylase in various tissues, DBA/2N mice, in which the enzyme is relatively nonresponsive to aromatic hydrocarbons (22–25), and the appropriate hybrids. The skin of 3-week-old weanlings was treated once with 20 μg of DMBA,[1] and phorbol ester was applied three times a week as described in the legend to Table 1. We arbitrarily chose a relatively low dose of carcinogen, since a larger dose would be likely to cause tumors in all animals. The constitutive and inducible aryl hydrocarbon hydroxylase (23–25) and epoxide hydrase[2] activities generally are maximal in these mice at about 3 weeks of age[3], and at 4 months of age are 80–90% of these maximal values. After no more tumors appeared, which was at about 4 months of age, each mouse was killed 24 hr after an intraperitoneal dose of MC, and the aryl hydrocarbon hydroxylase and epoxide hydrase specific activities were determined in the liver microsomes. By measuring the hepatic microsomal aryl hydrocarbon hydroxylase activity 24 hr after administration of MC, we could determine with certainty (23–25) whether this enzyme system was genetically inducible or noninducible in many other tissues of that mouse, such as kidney, bowel, lung, or skin.

Table 1 shows that DMBA did not produce skin tumors in any of the C57BL/6N (i.e., B6) inbred mice and caused tumors in 19 out of 21 inbred DBA/2N (i.e., D2) mice. The hepatic aryl hydrocarbon hydroxylase system was inducible by MC about 4–5-fold in the inbred B6 mice and was not inducible in the inbred D2 mice. The skin aryl hydrocarbon hydroxylase activity was induced by MC about 8-fold in the B6 mice and less than 2-fold in D2 mice. Thus no tumors were found in B6 mice even though their skin aryl hydrocarbon hydroxylase activity was about 5 times more inducible by polycyclic hydrocarbons than in the D2 mice. These data suggest that low or noninducible enzyme activity may be correlated with skin tumor formation caused by DMBA. However, inflammation produced by the repeated applications of phorbol ester was considerably more marked in the D2 inbred strain than in the B6 strain; thus tumorigenesis related to phorbol ester-produced skin irritation may be a more important factor than the aryl hydrocarbon hydrox-

[1] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; MC, 3-methylcholanthrene.

[2] Total epoxide hydrase activity was measured with [^{3}H]styrene oxide as substrate. At least in liver, the same epoxide hydrase appears to be responsible for hydration of styrene oxide and polycyclic arene oxides such as phenanthrene 9,10-oxide (11, 12). However, such an assay provides no information on the levels of epoxide hydrase possibly present as part of a tightly coupled aryl hydrocarbon hydroxylase–epoxide hydrase system (see the text).

[3] Unpublished observations.

TABLE 1

Lack of correlation between DMBA-produced skin tumorigenesis and inducible aryl hydrocarbon hydroxylase and epoxide hydrase specific activities in inbred and hybrid mice

To one ear of each weanling mouse, 20 μg of DMBA in 20 μl of acetone were applied once. For the next 3 months, 1 μg of phorbol ester (Schuchardt-USA, Katonah, N. Y.) in 20 μl of acetone was applied three times a week to the opposite ear. All tumors appeared within a 2-week period after approximately 2 months of continuous phorbol ester treatment; generally the papillomas were multiple. Four weeks after no further tumors had appeared, the presence or absence of inducible hydroxylase activity was determined for each individual mouse: MC, 80 mg/kg of body weight, was administered intraperitoneally, and liver microsomes were prepared 24 hr later as previously described (23–25). The assay for aryl hydrocarbon hydroxylase was carried out as usual (1, 23–25); 1 unit of activity is defined (1) as that amount of enzyme catalyzing the formation per minute at 37° of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene. The hepatic aryl hydrocarbon hydroxylase specific activity is thus expressed as units per milligram of microsomal protein. The skin aryl hydrocarbon hydroxylase activity was determined by assaying a tissue homogenate from individual weanlings 24 hr after 200 μg of MC in 50 μl of acetone had been applied to the skin at the nape of the neck. The skin hydroxylase specific activity is therefore expressed (7) as units per milligram of skin homogenate protein in weanling mice, the age at which the carcinogen was always applied. Epoxide hydrase activity was assayed as previously described (13) in the same liver microsomal fraction used for the determination of aryl hydrocarbon hydroxylase activity, and is expressed as nanomoles of [^{3}H]-styrene glycol formed in 5 min per milligram of nitrogen; nitrogen content was determined by the micro-Kjehldahl method. All enzyme specific activities are given as means ± standard deviations, with a minimum of 10 mice assayed individually in each group.

Inbred or hybrid mouse	Proposed genotype	Tumors per total mice in group	Aryl hydrocarbon hydroxylase specific activity				Epoxide hydrase specific activity	
			Liver microsomes		Skin		Liver microsomes	
			Control[a]	MC	Control[a]	MC	Control[a]	MC
C57BL/6N	*AhAh*	0/13	610 ± 100	2570 ± 510	1.1 ± 0.23	8.9 ± 2.1	38 ± 2.8	42 ± 2.0
DBA/2N	*ahah*	19/21	560 ± 110	540 ± 90	1.0 ± 0.17	1.8 ± 0.28	49 ± 3.2	44 ± 2.9
D2 × B6D2F$_1$ or B6D2F$_2$	*AhAh* or *Ahah*[b] *ahah*	20/34 16/25	600 ± 98[c]	2750 ± 480 550 ± 98	1.1 ± 0.18[c]	9.9 ± 2.3 2.2 ± 0.48		44 ± 3.6 46 ± 3.5

[a] The specific activities for the control enzymes were determined on separate groups of appropriate control mice, i.e., of similar age and genotype, treated with corn oil intraperitoneally 24 hr prior to assay of liver microsomes, or with 50 μl of acetone topically 24 hr prior to assaying aryl hydrocarbon hydroxylase activity in skin.

[b] We cannot distinguish between mice homozygous or heterozygous for the *Ah* allele.

[c] For the control enzyme activities, we cannot distinguish among *AhAh*, *Ahah*, or *ahah* mice (23–25).

ylase activity in the skin of these D2 mice. B6 or D2 mice receiving phorbol ester alone developed no tumors. We also found that repeated topical applications of phorbol ester depressed the baseline aryl hydrocarbon hydroxylase activity in the skin of B6 or D2 mice by 30–70%. Perhaps sensitivity of the skin to phorbol ester, leading to inflammation, is a genetic factor in D2 mice not found in B6 mice. In any event, this factor was canceled in the hybrids between the B6 and D2 inbred strains.

In offspring from the back-cross between the D2 and the B6D2 F_1 mice and in the F_2 offspring, aryl hydrocarbon hydroxylase activity was genetically inducible by aromatic hydrocarbons in 34 and not inducible in 25 animals. Comparing these two groups, we found that 20 of the 34 and 16 of the 25 animals had tumors, i.e., about 60% of either group. The mean specific activities of the hepatic microsomal or skin constitutive aryl hydrocarbon hydroxylase were not significantly ($p > 0.05$) different from those in the B6 or D2 inbred mice. Likewise, the mean aryl hydrocarbon hydroxylase activity in the MC-treated hybrids having the inducible enzyme was not significantly ($p > 0.05$) different from that in the MC-treated B6 mice, and the mean enzyme activity in the MC-treated but genetically nonresponsive hybrids was not significantly different from that in the MC-treated D2 mice or from the hepatic or skin enzyme activity in the control hybrids.

Among these offspring, we also found mice with a greater sensitivity to the repeated phorbol ester treatments in both the tumor-bearing and non-tumor-bearing groups, with about the same frequency. We thus conclude there is no absolute correlation between the magnitude of phorbol ester produced inflammation and skin tumorigenesis.

Are differences in the rates of metabolism of polycyclic hydrocarbon epoxides more important than the relative level of aryl hydrocarbon hydroxylase activity in these mice? As shown in Table 1, no significant differences were found in the hepatic epoxide hydrase activities of control and MC-treated inbred or hybrid mice. Epoxide hydrase activity was not detected in the skin of these mice, even with a modified, more sensitive assay; i.e., the specific epoxide hydrase activity with [^{3}H]styrene oxide as substrate was less than 0.10 nmole of diol formed per 5 min per milligram of nitrogen, which is less than 0.20% of that found in the livers of these mice. This sensitive radiometric assay for epoxide hydrase[4] is still only about $\frac{1}{30}$ as sensitive as the spectrophotometric assay for aryl hydrocarbon hydroxylase (1, 23–25).

We found that the AHH system in the skin of D2 mice is consistently induced by MC to levels significantly ($P < .05$) greater than the constitutive enzyme activity. This finding is altogether different from that in liver, kidney, bowel, and lung where the specific activities after MC treatment are not greater than the control levels in *ahah* mice (25). Thus, it appears that whereas AHH induction by aromatic hydrocarbons is completely repressed in liver, kidney, bowel, and lung, this "repression" is not complete in the skin of these same mice. This finding would also explain why AHH activity in secondary cultures of fetal DBA/2N mouse cells, which are comprised of skin fibroblasts as well as cells derived from liver, kidney, bowel, and lung, is always slightly inducible by polycyclic hydrocarbons in the growth medium (1, 24).

In summary, under the conditions of these experiments, genetic differences in the extent of aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons are unrelated to skin tumorigenesis initiated by DMBA and promoted by repeated applications of phorbol ester. Several interpretations exist. (a) If an aryl hydrocarbon hydroxylase-mediated metabolite of DMBA is the active carcinogen, the constitutive activity of this enzyme in the skin of these inbred and hybrid mice may be sufficient to initiate the cancer. (b) The parent DMBA molecule itself may be the carcinogenic agent. (c) DMBA may be metabolically activated through mechanisms not mediated by the aryl hydrocarbon hydroxylase system, either at methyl groups (26, 27) or at other positions (28–30). Experiments similar in design, but with the use of non-

[4] The earlier assay for epoxide hydrase (13) with [^{3}H]styrene oxide was modified as follows. The incubation time for the 0.4-ml reaction mixture at pH 9 was 60 min with 0.8 μmole of [^{3}H]styrene oxide (1.4×10^6 dpm). The extracted [^{3}H]styrene glycol, along with carrier, was purified by thin-layer chromatography (silica gel GF; benzene–chloroform–ethyl acetate, 1:1:1), extracted from the silica gel with methanol, and assayed spectrophotometrically for recovery and radiometrically for conversion. The sensitivity of the assay is limited by nonenzymatic conversion of oxide to glycol.

methylated carcinogenic aromatic hydrocarbons, are also in progress. (d) The activity of epoxide hydrase or of a possible coupled aryl hydrocarbon hydroxylase–epoxide hydrase system[5] (cf. ref. 32) in the skin of these inbred and hybrid mice may differ widely at a level below the sensitivity of the enzyme assay; thus removal of the epoxide—by formation of the *trans*-dihydrodiol relative to the rates of phenol formation or covalent interaction with cellular macromolecules—may be more important than the extent of aryl hydrocarbon hydroxylase induction. Further studies with the use of genetic models such as these inbred and hybrid mice would be helpful in evaluating these possibilities.

Note Added In Proof: Preliminary data indicate that there also exists no correlation between the genetic differences in the extent of aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons and skin tumorigenesis initiated by 20 μg of benzo[a]pyrene and promoted by repeated applications of phorbol ester.

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[5] The hepatic microsomes and the skin homogenates from control or MC-treated mice were incubated as usual for the aryl hydrocarbon hydroxylase assay *in vitro* (1, 23–25) in the presence of trichloropropene oxide, an inhibitor of epoxide hydrase (31) that does not affect aryl hydrocarbon hydroxylase activity (unpublished observations). Formation of the phenol 3-hydroxybenzo[*a*]pyrene was not measurably increased in the presence of this inhibitor, indicating that conversion of the arene oxide to the *trans*-dihydrodiol by epoxide hydrase in C57BL/6N or DBA/2N mouse liver and skin is a relatively minor pathway. Thus, if trichloropropene oxide is able to block epoxide hydrase activity in a tightly coupled aryl hydrocarbon hydroxylase–epoxide hydrase membrane-bound system, it would appear that such a system either does not exist or exists in undetectable amounts in the liver and skin of these mice.

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