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HEMOPEXIN AND ARYL HYDROCARBON HYDROXYLASE INDUCTION IN THE MOUSE AND IN CELL CULTURE

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Abstract—Plasma hemopexin levels in mice treated with polycyclic aromatic compounds such as 3-methylcholanthrene or β-naphthoflavone are increased 71–160 per cent in three inbred strains known to be "responsive" at the Ah locus (C57BL/6N, C3H/HeN and Balb/cAnN) and ranged from 14 to 60 per cent in three inbred strains known to be "nonresponsive" at the Ah locus (DBA/2N, NZB/BLN and NZW/BLN). Phenobarbital treatment causes increases in plasma hemopexin of similar magnitude in both aromatic hydrocarbon responsive and nonresponsive mice. In F_2 progeny from crosses between the C57BL/6N and NZW/BLN progenitor strains, a correlation between inducible hepatic hydroxylase and plasma hemopexin concentrations could not be demonstrated, principally because of the small magnitude in hemopexin induction. Data in this study show that plasma hemopexin rises later than the initiation of the aryl hydrocarbon hydroxylase induction process and, therefore, presumably in response to increased heme synthesis—whether it is the genetically regulated hemoprotein P_1-450 induction by polycyclic aromatic compounds or hemoprotein P450 induction by phenobarbital. An association between hemopexin and hydroxylase induction is not seen in H-4-II-E Reuber rat hepatoma cultures. Hemopexin synthesis and secretion into the growth medium occurs in H-4-II-E cells but is not detectable in HTC or Hepa-1 cultures. The rate of hemopexin appearance in the culture medium is the same in benz[a]anthracene-treated as in control H-4-II-E cells, whereas the hydroxylase induction occurs primarily in benz[a]anthracene-treated and not in control cultures. Daily changes in growth medium result in greater intracellular hemopexin levels and a greater rate of hemopexin secretion into the medium, compared with cells whose medium was changed less frequently or not at all.

Cytochrome P450-mediated membrane-bound mono-oxygenases require NADPH and molecular oxygen during the first step in the metabolic conversion of numerous endogenous hydrophobic substrates and of various drugs, carcinogens, and other environmental pollutants to more polar products [1–6]. The induction of at least ten of these mono-oxygenase activities and cytochrome $P_1$450 in mice* treated with various polycyclic aromatic compounds is regulated by the Ah locus (reviewed in [6]).

None of the factors controlling this induction process are in fact understood. The process presumably requires new heme synthesis, which occurs in the mitochondrion [1, 12]. The mechanism of heme transport from the mitochondrion to the endoplasmic reticulum site at which heme combines with its apo-enzyme(s) is also not understood.

One candidate for intracellular heme transport is hemopexin [13]. Although having no known intracellular function, hemopexin is synthesized in the liver and aids in the serum transport and distribution of heme throughout the body [13]. Moreover, hemopexin levels increase after treatment of the animal with various inducers of drug-metabolizing enzyme activities [14–16]. Plasma hemopexin concentrations vary among several inbred strains of mice, and hemopexin synthesis in these animals appears to be stimulated most when the basal hemopexin level is low [13].

* The nomenclature for various forms of cytochrome P450 is currently inadequate, in view of the four or more forms distinguishable by electrophoretic [7] or immunochemical [8] techniques. Eventually, a better understanding of biochemical and catalytic properties should permit a more suitable nomenclature to be devised. In this report, "P450" in the general sense denotes all forms of CO-binding hemoproteins associated with membrane-bound NADPH-dependent mono-oxygenase activities. "$P_1$450" is defined as that form(s) of cytochrome increasing during polycyclic aromatic inducer treatment and concomitantly associated with induced aryl hydrocarbon hydroxylase activity. Whether this inducible form(s) of hemoprotein in nonhepatic tissues of the intact animal [9] or in tissue culture [10] is identical to the inducible form(s) of hemoprotein in liver of the intact animal [7, 8] remains to be determined. Recent data [11] indicate that $P_1$450 in rabbit liver is distinctly different from the polycyclic aromatic-inducible form(s) causing the spectral shift to about 448 nm when reduced and combined with CO (i.e. cytochrome "P448").

The purpose of this study was to determine whether an association exists between hemopexin induction and the Ah allele (as determined by AHH† induction). If such an association exists, the reasons for hemopexin induction could be either simply a secondary response to changes in heme synthesis or an event of central importance in the regulation of the AHH induction process. Therefore, we examined hemopexin and AHH induction among inbred mouse strains and the offspring derived from appropriate genetic crosses and hemopexin and AHH levels in several hepatoma-derived established cell culture lines.

EXPERIMENTAL PROCEDURE

Materials. The polycyclic hydrocarbon BA was obtained from Sigma Chemical Co., St. Louis, MO; BNF from Aldrich Chemical Co., Milwaukee, WI; MC from Eastman Kodak Co., Rochester, NY; and sodium phenobarbital from Merck & Co., Inc., Rahway, NJ. NADH and NADPH were obtained from either CalBiochem, Los Angeles, CA or Sigma. Bovine serum albumin was purchased from Armour Pharmaceutical Co., Chicago, IL. Tissue culture supplies were bought from Falcon Plastics, Oxnard, CA.

All strains of mice were obtained from the Veterinary Resources Branch of the National Institutes of Health, Bethesda, MD. H-4-II-E, derived from Reuber (rat) hepatoma H-35 [17], and HTC, derived from a Morris minimal deviation (rat) hepatoma [18], were generously provided in 1971 by Dr. E. Brad Thompson, National Cancer Institute, Bethesda, MD. Hepa-1, a mouse cell line derived from the transplantable hepatoma BW 7756 originally produced in the C57/LJ mouse [19], was generously given to us in 1971 by Dr. Gretchen Darlington, Department of Biology, Yale University, New Haven, CT.

Cell culture technique. The established cell lines were grown in culture as previously described [20]. During the logarithmic phase of growth, cells were either treated with 13 μM BA previously dissolved [20, 21] in the appropriate medium or were maintained in control medium. Control or BA-containing medium was changed every 24 hr when cells were maintained in culture for longer than that period of time, unless indicated otherwise. Medium (3 ml) from duplicate 60-mm dishes containing logarithmically growing cells was collected at specific time intervals and stored at 20°C for hemopexin determinations. The washed cell pellet was frozen for subsequent AHH assay, as previously described [21]. In certain experiments hemopexin determinations were also performed on frozen cell pellets. Viability was checked by light microscopy, total protein per dish, and by [^{14}C]leucine and [^{3}H]thymidine 30-min pulses, as previously detailed [21].

Treatment of the mice. Several inbred strains of mice received a single intraperitoneal dose (80 mg/kg) of MC or BNF dissolved in corn oil. Appropriate controls received corn oil. With regard to phenobarbital treatment, the drug was dissolved at a concentration of 0.05% (w/v) in the drinking water and administered for as long as 14 days. Mice about 6–8 weeks of age and weighing about 20 g were used throughout this study.

Collection of blood for hemopexin determinations. At appropriate times blood from an individual mouse was collected into a heparinized beaker after carotid artery and jugular vein laceration. Typically 0.5 to 1.5 ml of heparinized blood was obtained per 20 g of mouse. The blood was subsequently transferred to a small test tube and centrifuged at 1000 *g* for 10 min. The plasma layer was carefully transferred to a plastic vial with cap, and 10 μl of 1% sodium merthiolate was added/1 ml of plasma. The plasma samples were either shipped immediately or after storage at 20°.

Preparation of microsomes. After exsanguination of the animal, the liver was removed and placed in ice-cold 0.15 M KCl–0.25 M potassium phosphate buffer, pH 7.25. Liver homogenates were made and microsomal pellets were prepared as described [22].

Aryl hydrocarbon hydroxylase assay. Liver microsomal and tissue culture hydroxylase activities were determined as previously described [22]. One unit of aryl hydrocarbon hydroxylase activity is defined as that amount of enzyme catalyzing the formation/min at 37° of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene.

Hemopexin determinations. Plasma hemopexin concentrations (in mg/ml) were measured by radial immunodiffusion [23], whereas the hemopexin content (in μg/ml) in the cell culture medium was determined by the radioimmunoassay utilizing polyethylene glycol [24]. Intracellular hemopexin levels were performed by the same method after the cells were freeze-thawed in 1% Triton X-100.

RESULTS AND DISCUSSION

Six inbred strains screened. Induction of cytochrome $P_1$450 and AHH activity and several other associated mono-oxygenase activities associated with the Ah locus can be accomplished by such polycyclic aromatic inducers as BNF, MC or BA (reviewed in [6]). Table 1 shows the plasma hemopexin concentrations of six control and BNF-treated inbred strains of mice. Whereas the three aromatic hydrocarbon responsive strains displayed increases in hemopexin levels ranging from 71 to 160 per cent, the aromatic hydrocarbon nonresponsive strains displayed increases of 14–60 per cent. Since non-responsive strains possess some inducible AHH activity and cytochrome $P_1$450 in their non-hepatic tissues [6], though considerably less than that in responsive strains, it is perhaps not surprising to see some increase in hemopexin levels even in the nonresponsive strains. The maximal increase seen in Table 1 (2.6-fold) is not as striking as the 5-fold increases found in rabbits treated with MC

† Abbreviations used include: AHH, aryl hydrocarbon (benzo[a]pyrene) hydroxylase; BNF, β-naphthoflavone; MC, 3-methylcholanthrene; BA, benz[a]anthracene; B6, the C57BL/6N inbred strain; and NZW, the NZW/BLN inbred strain.

Table 1. Plasma hemopexin levels in control and BNF-treated mice of six inbred strains*

Inbred strain	Control (mg/ml)	BNF (mg/ml)	Per cent increase	P†	Ah locus‡
C57BL/6N	1.18 ± 0.11	3.12 ± 0.49	160	< 0.001	Responsive
Balb/cAnN	1.30 ± 0.42	2.59 ± 0.87	100	< 0.02	Responsive
C3H/HeN	1.63 ± 0.91	2.79 ± 0.52	71	< 0.05	Responsive
DBA/2N	2.00 ± 0.24	3.22 ± 0.42	60	< 0.001	Nonresponsive
NZB/BLN	2.70 ± 0.15	3.09 ± 0.66	14	> 0.05	Nonresponsive
NZW/BLN	1.53 ± 0.30	1.94 ± 0.22	27	< 0.05	Nonresponsive

* Six animals per group were treated with corn oil or BNF 48 hr before sacrifice, and the plasma was obtained as described in Experimental Procedure. The C57BL/6N, DBA/2N and C3H/HeN mice were females; the remaining three inbred strains were males. Values are expressed as means ± S.D.

† Confidence level for differences between values in control and BNF-treated mice of each inbred strain.

‡ Inbred strain judged to be responsive or nonresponsive to aromatic hydrocarbon inducers such as BNF, as recently reviewed [6].

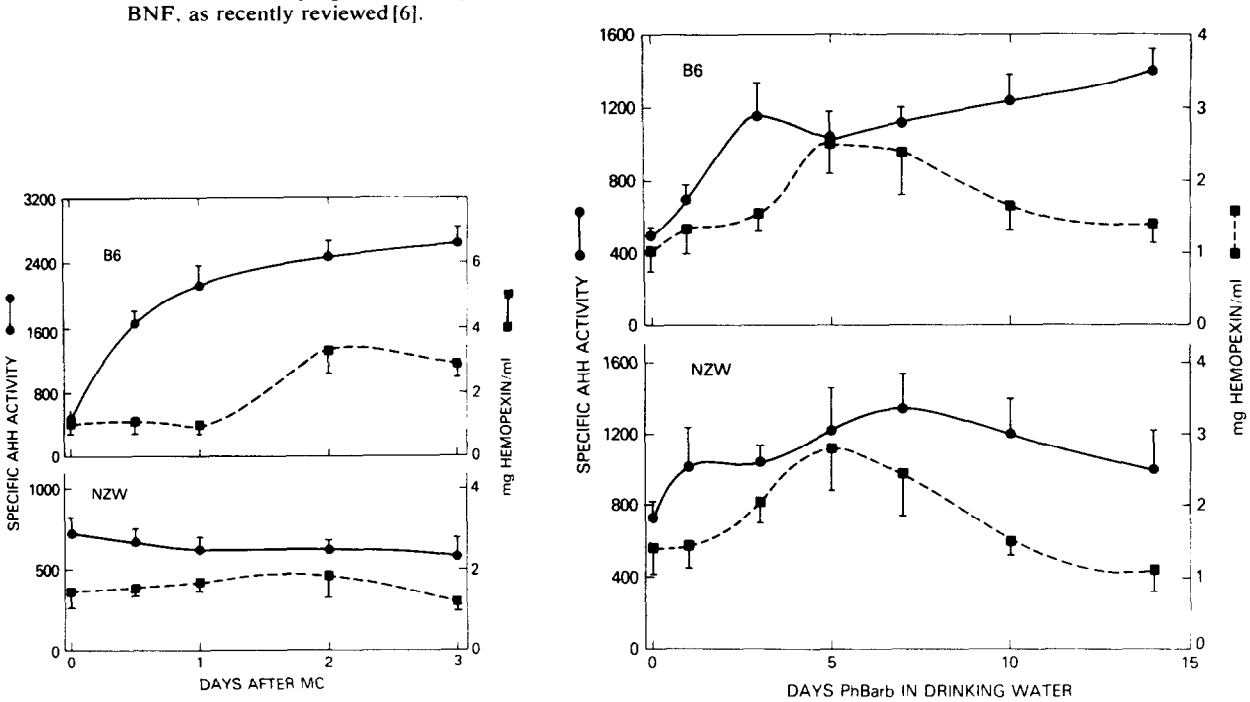

Fig. 1. Hepatic specific AHH activity and plasma hemopexin concentration in B6 (top) and NZW (bottom) inbred strains as a function of time after A, a single i.p. dose of MC, or B, continuous administration of phenobarbital (PhBarb) in the drinking water, as described in Experimental Procedure. Each symbol and bracket represent the mean ± S.D. in which N = 3 or 4 for each group.

or allylisopropylacetamide [14] or the 8-fold increases found in mice treated with streptococcal vaccine [25].

Kinetics of hemopexin induction. From the data in Table 1 we chose for further studies B6 as the strain having the most inducible hemopexin and NZW as the strain having a low amount of inducible hemopexin. NZB/BLN was not studied further because this strain could not be readily obtained from the Veterinary Resources Branch in adequate number. The rate of hemopexin induction after MC administration was examined (Fig. 1A). In B6 mice the rise in hemopexin concentration occurred almost completely between 24 and 48 hr after giving the inducer MC; no rise occurred in NZW mice. In contrast, the rise in MC-inducible AHH activity occurs principally during the first 24 hr. The increase in plasma hemopexin, therefore, appears to be subsequent to, rather than preceding or concomitant with, the genetically controlled process of AHH induction by MC.

Phenobarbital induces AHH activity to similar levels in both aromatic hydrocarbon responsive and nonresponsive mice [22, 26]. The major rise in AHH activity occurred during the first 24 hr and a peak developed between 2 and 7 days of continuous daily phenobarbital administration (Fig. 1B). Increases in hemopexin occurred to a similar extent in both the responsive B6 and the nonresponsive NZW strains. Again, the increase in hemopexin followed by 1 or more days the rise in AHH induction by phenobarbital. In both strains hemopexin was not significantly elevated 24 hr after phenobarbital treatment had begun and hemopexin levels were highest at 5 days.

Hemopexin response to MC among (B6 X NZW) F_2 progeny. From the results in Table 1 and Fig. 1, it appeared that genetically controlled hepatic AHH

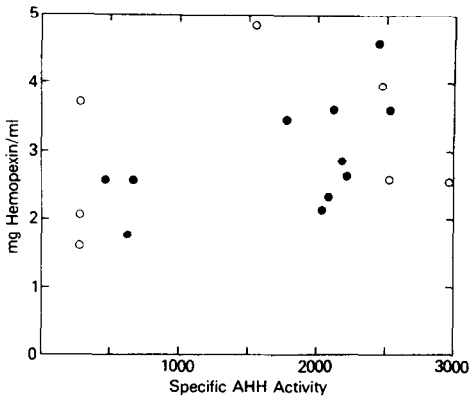

Fig. 2. Plasma hemopexin levels as a function of hepatic specific AHH activity in MC-treated F_2 offspring in which B6 and NZW are the progenitor inbred strains. Experiment 1 (○) and Experiment 2 (●) were performed on different days. MC was given 48 hr prior to sacrifice. The correlation coefficient $r = 0.43$ for Experiment 1 and $r = 0.50$ for Experiment 2 ($P > 0.05$ for both experiments). Each symbol represents an individual animal.

induction after MC treatment was associated with the subsequent rise in plasma hemopexin. However, because inbred strains differ at thousands of genetic loci, we sought to prove this correlation among offspring in the same litter. Figure 2 shows plasma hemopexin concentrations as a function of AHH activity in MC-treated F_2 offspring from the responsive B6 and the nonresponsive NZW progenitor strains. In the two litters examined in two different experiments, AHH was clearly not inducible in six out of a total of eighteen progeny; this result is not significantly different from the expected 25 per cent incidence. Although the mean plasma hemopexin level for the six nonresponsive mice was less than that for the twelve responsive siblings, the correlation coefficient is not statistically significant.

Hence, because of large individual variations in hemopexin levels and a magnitude of hemopexin induction of, at best, 3-fold, an association of plasma hemopexin concentrations with the Ah locus could not be demonstrated experimentally. The hemopexin level in the same individual mouse, before and after MC treatment, would be the ideal experiment. However, the amount of blood required for a single hemopexin determination necessitates exsanguination of the animal, resulting in death. It is, for example, possible that the nonresponsive sibling having the plasma hemopexin concentration of 3.7 mg/ml after MC treatment (Fig. 2) had a basal hemopexin concentration of 3.0 or 3.5 mg/ml and that the responsive siblings having the plasma hemopexin levels of 2.1 and 2.3 mg/ml after MC treatment had control hemopexin levels of about 1.0 mg/ml.

AHH and hemopexin induction in tissue culture. We found that hemopexin production occurred in H-4-II-E cultures but did not occur in HTC or Hepa-1 cell cultures. The kinetics of AHH and hemopexin induction in H-4-II-E cells during the same experiment, therefore, were studied further (Fig. 3). AHH activity was maximally induced by BA at 48 hr, and basal AHH activity increased only slightly in cultures receiving fresh control medium alone. The rate of hemopexin secretion into the growth

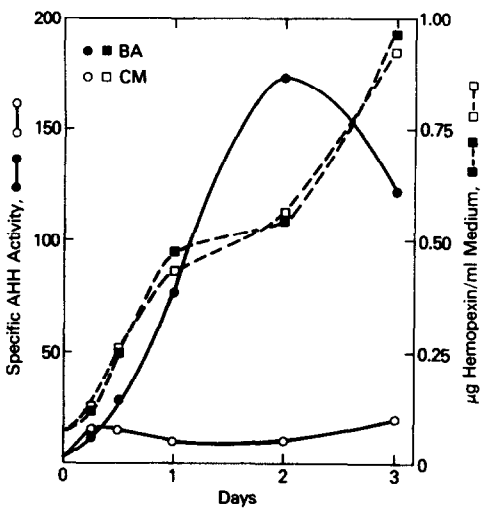

Fig. 3. Specific AHH activity in H-4-II-E cells and hemopexin levels in the growth medium after treatment with 13 μM BA or control medium alone (CM). The values for AHH activity are the averages of two samples. Medium from the same tissue culture dishes as those used for the AHH assay was combined from two samples for hemopexin determinations. For samples collected on day 2 and day 3, the medium was collected daily and combined with that from the preceding day(s), and fresh medium was added to the cultures.

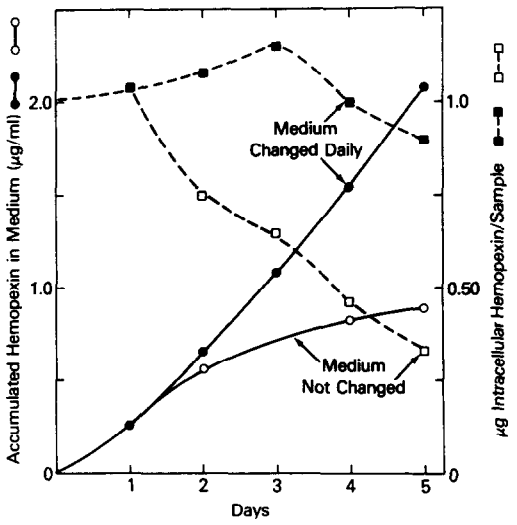

Fig. 4. Accumulated hemopexin in the growth medium and H-4-II-E intracellular hemopexin concentration as a function of days in culture. The medium was changed daily (closed symbols) or not changed (open symbols) during the 5-day experiment. Intermediate values were found when the medium was changed every other day (data not illustrated). When collected daily, the medium was combined with that from the preceding day(s), and fresh medium was added to the cultures. Each symbol denotes the results from two 60-mm dishes (containing approximately 6×10^6 cells) and represents one sample. The total protein/dish, [^{3}H]thymidine pulse, and [^{14}C]leucine pulse in cell cultures with the medium not changed for 4 days was 76, 81 and 140 per cent, respectively, of that found in cell cultures with the medium changed daily. In cultures not receiving fresh medium daily, therefore, we cannot explain the observed decrease in excretion of hemopexin on the basis of a loss of viability.

medium, however, was identical in both BA-treated and control cells. Thus, any relationship between inducible hepatic AHH activity and plasma hemopexin concentrations in the intact animal could not be demonstrated in H-4-II-E cultures.

Intracellular vs extracellular hemopexin in tissue culture. During the course of our experiments, it became apparent that frequent removal of the tissue culture medium resulted in an increased secretion rate of intracellular hemopexin into the culture medium (Fig. 4). If the medium was not changed daily, intracellular as well as extracellular hemopexin concentrations were proportionately less, compared with cultures undergoing daily changes of medium.

The reason for this effect is not understood, but we can suggest two possible explanations. First, an equilibrium is developed by mass action, the higher extracellular hemopexin concentration thereby impeding the passive transfer of any more of the intracellular hemopexin into the growth medium. A similar intracellular/extracellular equilibrium was shown [27] for the passive excretion of BA metabolites into the medium from secondary fetal cultures derived from whole mouse fetuses. Second, an absence of freshly exchanged medium leads to alterations in pH [28], macromolecular turnover [28–31], accumulation of various secretory products in the growth medium, intracellular cyclic adenosine and guanosine monophosphate concentration [32, 33], and accumulation of various secretory products in the growth medium. These alterations (mentioned in the second possible explanation) appear more likely to affect hemopexin secretion into the culture medium than would an equilibrium between intracellular and extracellular hemopexin, because hemopexin is a protein whose secretion from the cell most likely involves an active transport process. Any one of these alterations could account for the inhibition of hemopexin secretion into the growth medium. A decreased rate of hemopexin synthesis, combined with a constant rate of hemopexin degradation, for example, could account for the decreased intracellular hemopexin concentration. Further studies to help understand the inhibitory effect illustrated in Fig. 4 are underway.

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