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Maternal Smoking Increases Xenobiotic Metabolism in Placenta but Not Umbilical Vein Endothelium

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ABSTRACT. It is unclear whether placental xenobiotic metabolism can protect the human conceptus. In particular, the role of placental metabolism of toxic components of cigarette smoke such as polycyclic aromatic hydrocarbons (PAHs) is poorly understood. We hypothesized that increased aryl hydrocarbon hydroxylase (AHH) activity observed in placentas from smokers might help clear PAHs from maternal circulation and thereby prevent transplacental induction of AHH by PAHs. Our studies of AHH activity in human placentas and umbilical vein endothelium support this premise. While AHH activity was significantly increased in placentas from smokers compared with activity in placentas from nonsmokers, AHH activity in umbilical vein endothelium from these same pregnancies was unaffected by maternal smoking and remained low. In order to confirm that AHH present in endothelium was inducible, we also demonstrated dose-dependent increases in AHH activity in primary cultures of human umbilical vein endothelial cells exposed to PAHs. These findings may indicate first pass protection of the fetus by placental xenobiotic metabolism, or that endogenous factors suppress AHH induction in the fetus but not placenta. (*Pediatr Res* 18:1071–1075, 1984)

Abbreviations

AHH, aryl hydrocarbon hydroxylase
PAH, polycyclic aromatic hydrocarbon
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Maternal cigarette smoking increases risks for spontaneous abortion, premature delivery, and fetal growth retardation and

may contribute to the development of certain birth defects (4, 16, 17). Although complex mixtures of chemicals contained in cigarette smoke have been described, little is known about the mechanisms by which these compounds may be embryopathic or about how the human conceptus can be protected from such chemical toxicity.

In order to approach these problems, we have been studying the effects of maternal smoking on xenobiotic metabolism in human placenta, concentrating on AHH, a cytochrome P-450 monooxygenase system which increases its activity when mothers smoke (14, 20, 24, 29, 30). AHH catalyzes the first step in the metabolism of several of the toxins absorbed from cigarette smoke including PAHs. These compounds, which are concentrated in cigarette smoke, have been found to be carcinogenic, mutagenic, and teratogenic in animals (5). The consequences of increased placental AHH activity in response to maternal smoking are unknown. On the one hand, microsomes from placentas from smokers have been shown to metabolize certain PAHs to mutagenic products *in vitro* (15). On the other hand, AHH participates in the clearance of toxins such as PAHs *in vivo* (18, 23), and increased placental activity may reduce levels of toxic PAHs in fetal tissues. Hence, increased placental AHH, coupled with a toxic load of PAHs from maternal smoking, could theoretically protect or endanger the developing fetus.

Because we are unable to measure placental clearance of PAHs directly in humans, we decided to approach the question of the role of placental PAH metabolism indirectly by comparing effects of cigarette smoking on placenta and a fetal tissue immediately distal to it, umbilical vein endothelium. We hypothesized that if increased placental AHH activity were involved in effectively decreasing levels of PAHs in distal tissues, then we would expect distal tissues to be less affected than the placenta by maternal cigarette smoke. We used AHH activity itself as a measure of PAH exposures and measured activity in placentas and umbilical vein endothelium from smoking and nonsmoking women. We report that while AHH activity in placenta is markedly increased by maternal cigarette smoking, activity in umbilical vein endothelium is not.

MATERIALS AND METHODS

Procurement of tissues. Placentas were collected at the time of delivery. Several representative samples of villous tissue were immediately removed and frozen at $-70°$ C. Umbilical cords were removed and refrigerated no longer than 8 h prior to use. Cords were used only if they contained untraumatized segments greater than 18 cm in length.

Maternal smoking was determined by review of medical records and personal interview. Women were considered to be smokers if they used cigarettes daily up to admission for delivery. Nonsmokers had not used cigarettes at all during the pregnancy. Pregnancies were uncomplicated and mothers received no medications other than analgesics and anesthetics during labor. All pregnancies studied delivered at term (38–42 wk).

Isolation of endothelial cells. All cords were studied on the day of delivery and endothelial cells from individual cords were treated separately. The umbilical veins were cannulated with straight plastic tubing connectors (Cobe Laboratories, Lakewood, CO) attached to three-way stopcocks and rinsed with 0.9% saline or with calcium- and magnesium-free Puck's Saline G until free of blood. A solution of HEPES-buffered (2.5 mM, pH 7.4) tissue culture medium (M199, Gibco, Grand Island, NY) containing 0.4 mg/ml collagenase (Worthington, Freehold, NJ) was then instilled with a syringe, the distal stopcock was closed, and the cord was incubated at 37° C for 15 min. Following incubation, the solution containing endothelial cells was removed from the vein and the collagenase was inactivated by addition of an equal volume of tissue culture medium containing 20% fetal bovine serum (Biocell, Carson, CA).

Culture and identification of endothelial cells. Endothelial cells were cultured from single umbilical cords according to the methods of Gimbrone *et al.* (8). Cells were harvested as described above, washed in calcium- and magnesium-free Puck's Saline G, and resuspended in complete growth medium (M199) containing HEPES (pH 7.4, 25 mM), thymidine (10^{-5} M), antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 0.25 μg/ml from Gibco; and gentamicin, 40 μg/ml from Schering, Kenilworth, NJ) and 20% fetal bovine serum. Suspended cells were cultured in 25-cm^2 flasks at 37° C in humidified room air containing 5% CO_2. Medium was replaced after 24 h and the remaining adherent cells were cultured until confluent (10 to 14 days) with media replacement twice weekly.

The identity of these cells as endothelial cells was confirmed by their cobblestone monolayer morphology when viewed by phase contrast microscopy (8, 13) and by the presence of factor VIII antigen detected by indirect immunofluorescence microscopy (11, 12).

Measurement of AHH activity. AHH activity was measured in placental microsomes as activity toward benzo(*a*)pyrene according to the procedure described by Vaught *et al.* (29). Within 3 days of delivery, microsomes were prepared from homogenates of the frozen placental samples as previously described (20). Metabolites of benzo(*a*)pyrene were quantitatively estimated by comparing their relative fluorescence with that of a standard 3-OH-benzo(*a*)pyrene solution (a gift from Dr. Leland Chung, University of Colorado, Boulder) in a Perkin-Elmer model 203 fluorescence spectrophotometer. Activity was expressed as picomoles phenolic benzo(*a*)pyrene metabolites formed/min/mg protein. Protein was measured by the method of Lowry *et al.* (19).

Umbilical vein endothelial cells were harvested as described above and washed in 0.05 M Tris-HCl buffer, pH 8.5, containing 3 mM $MgCl_2$ and 0.2 M sucrose. They were then resuspended in 1 ml of the same buffer containing an NADPH-generating system. Final concentrations of cofactors were 1 mM NADP, 10 mM glucose 6-phosphate, and 2 units/ml glucose 6-phosphate dehydrogenase. The reaction mixtures in 15-ml screw-top tubes were placed in a 37° C shaking water bath (Dubnoff) and benzo(*a*)pryene (Aldrich, Milwaukee, WI) was added in acetone (50 μl) to a final concentration of 100 μM. These mixtures were incubated for 16 h and the reaction then was stopped by addition of 4 ml acetone:hexane (1:3). The phenolic products of benzo(*a*)pyrene metabolism were extracted and measured according to the procedure of Gurtoo *et al.* (9) again using 3-OH-benzo(*a*)pyrene as standard. Activity was expressed as picomoles metabolites formed in 16 h/μg DNA. DNA was measured according to the method described by Paigen *et al.* (25). Cells harvested from individual umbilical veins were incubated for 16 h in order to get sufficient reaction products to avoid having to pool cells from different individuals in order to detect activity. We validated this approach by demonstrating that activity measured after 16 h was dependent upon cell protein and NADPH concentration. In addition, we compared AHH activity in intact cells with that observed in cells sonicated prior to incubation. Activities were comparable in these preparations, indicating that induction, which requires intact cells, had not occurred.

AHH in cultured endothelial cells. Confluent cultures of umbilical vein endothelial cells (more than 90% pure) were refed with complete growth medium containing known inducers of AHH activity. 3-Methylcholanthrene (1 μM, Sigma, St. Louis, MO), β-naphthoflavone (10 μM, Aldrich) or vehicle (5 μl, acetone) was added and the cells incubated for 24 h at 37° C in 5% CO_2 in air. Control and treated cells from the same cord were incubated in parallel. After 24 h the endothelial cells were harvested by treatment with trypsin (0.05%)-EDTA (0.02%, Gibco), washed, and resuspended in the Tris:sucrose:$MgCl_2$ buffer described above. AHH activity was determined as activity toward benzo(*a*)pyrene in reaction mixtures containing 2–3 ×
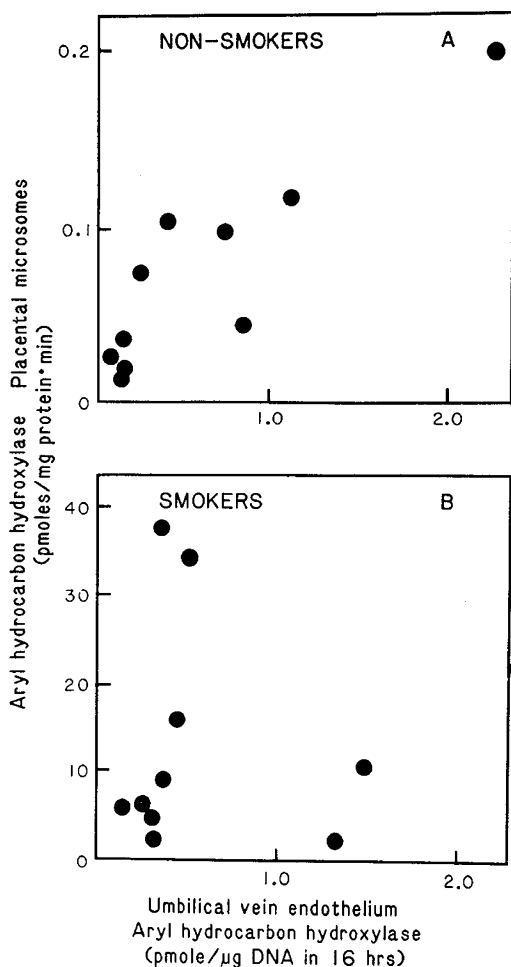
Fig. 1. AHH activities in placental microsomes and umbilical vein endothelium. Placentas were collected from 10 nonsmokers and 10 smokers. AHH activity was measured in placental microsomes and in

10^6 cells by the assay of Gurtoo *et al.* (9) as described above. Cells were incubated for 30 min and AHH activity is expressed as picomoles phenolic metabolites formed/10^6 cells. Cell counts were determined microscopically.

RESULTS

AHH activities in placenta and umbilical vein endothelium. We compared placental and umbilical vein endothelial cell AHH activities in material from 10 smoking and 10 nonsmoking women. AHH activity in cells from umbilical veins correlated well with AHH activity in placental microsomes from the same individuals when mothers did not smoke ($r = 0.823$, $p < 0.01$; Fig. 1*A*), consistent with the fact that placenta and umbilical vein both express fetal genotype. However, when mothers smoked, AHH activity in placental microsomes was much higher and the correlation with endothelial AHH activity was lost ($r = -0.06$, $p =$ NS Fig. 1*B*). Comparison of mean AHH activities (Fig. 2) indicated that while maternal cigarette smoking was associated with significantly higher ($p < 0.01$; *t* test) AHH activity in placental microsomes, it had no effect on AHH activity in umbilical vein endothelium.

Induction of AHH in cultured endothelial cells. Since maternal smoking did not alter AHH activity in umbilical vein endothelium, it became necessary to determine whether it is possible to induce AHH activity in endothelial cells. We therefore exposed cultured endothelial cells to 3-methylcholanthrene and β-naphthoflavone *in vitro*. We found that AHH activity was increased 2- to 10-fold in cell cultures from five cords following such treatments. As shown in Figure 3, AHH activity increased as a function of the concentration of inducing agent present.

endothelial cells obtained from the same individual. *A* compares activities from nonsmokers. A significant correlation ($r = 0.823$, $p < 0.01$) is seen between placental and endothelial AHH activities. *B* compares activities from smokers. Placental AHH activity is markedly increased and the correlation with endothelial AHH activity is lost ($r = -0.06$).
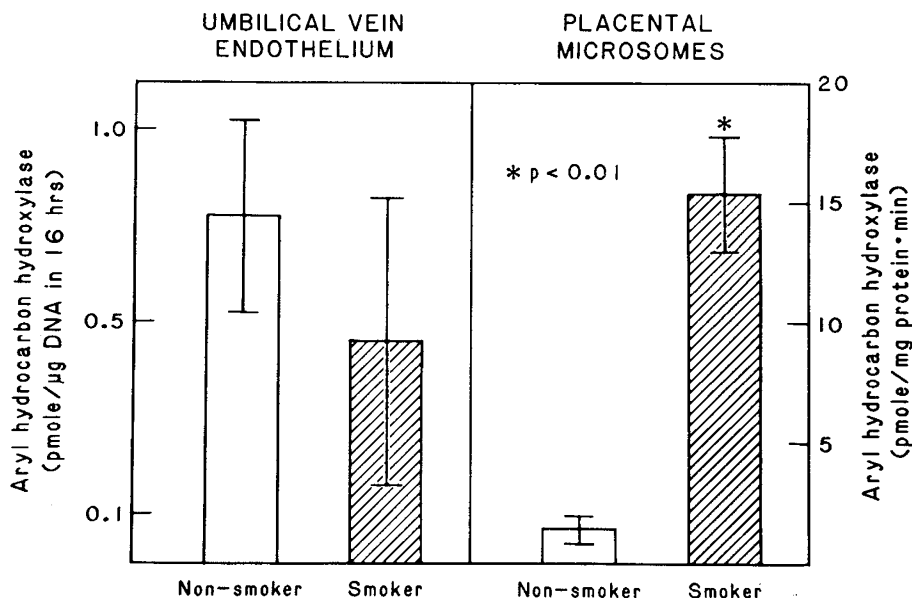

Fig. 2. Mean AHH activities in umbilical vein endothelium and placental microsomes. Mean AHH activity (0.717 ± 0.20, nonsmokers *versus* 0.445 ± 0.32 pmol/μg DNA in 16 h, smokers) was not significantly different in endothelial cells harvested from umbilical vein from nonsmokers and smokers, but mean AHH activity in placental microsomes from smokers (15.5 ± 4.4 pmol/mg protein/min) was significantly higher than mean AHH activity in placental microsomes from nonsmokers ($0.09 \pm .02$ pmol/mg protein/min).
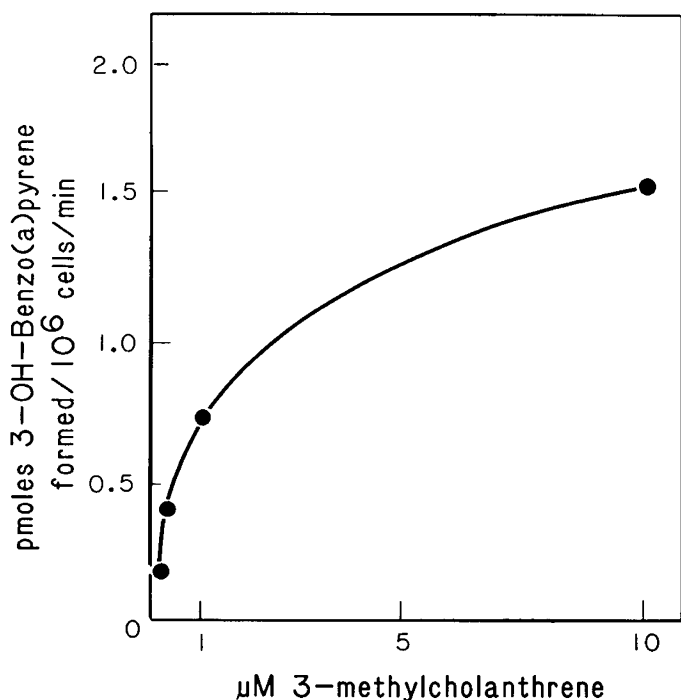
Fig. 3. Effect of PAH exposure on AHH activity in cultured human umbilical vein endothelial cells. Four primary cultures of endothelial cells from a single umbilical vein were exposed for 24 h to various concentrations of 3-methylcholanthrene. A dose-dependent increase in AHH activity was observed.

DISCUSSION

The results of this study indicate that while human umbilical vein endothelial cells are capable of increasing AHH activity in response to *in vitro* exposures to PAHs, AHH activity in umbilical vein endothelium does not increase *in vivo* under conditions which readily induce placental AHH activity. Therefore, since both of these tissues express fetal genotype, they appear to be exposed to markedly different toxicologic environments in smokers but not in nonsmokers.

These results are interesting because they represent the first correlation of AHH activities in paired placentas and fetal tissues from viable newborns. This approach allows the simultaneous evaluation of effects of maternal chemical exposures on tissues from maternal and fetal circulations. In addition, this approach can be applied to virtually any *in utero* xenobiotic exposure. In the case of maternal cigarette smoking, we have focused on responses of metabolic detoxication systems to PAHs and found that the tissue (placenta) which is perfused by the maternal circulation demonstrated AHH induction while the tissue (umbilical vein endothelium) which is perfused by the fetal circulation demonstrated no *in utero* AHH induction.

In previous studies, we and others have shown that placental AHH activity is quite sensitive to maternal smoke exposures (21, 22). In contrast, Pelkonen *et al.* (26) and Rifkind *et al.* (27) found that AHH levels in several tissues from medically aborted human fetuses do not appear to vary with maternal smoking. Our study confirms these findings and extends them by demonstrating lack of AHH induction in fetal tissue immediately distal to the induced placenta.

The mechanisms underlying these findings are unknown. However, the results are consistent with the hypothesis that increased placental AHH activity may participate in the clearance of PAHs from maternal circulation (6) and thereby lower the concentration of these compounds in fetal circulation. Such low PAH concentrations would then be insufficient to induce AHH activity in umbilical vein endothelium, consistent with the

data in Figure 3. In support of this concept, Rowland *et al.* (28) have recently presented data suggesting that placental clearance of cortisol contributes to reduced levels of this potential teratogen in rat fetuses. This finding may explain why the rat is relatively resistant to the teratogenic actions of maternally administered cortisol.

Alternative explanations of our findings include the possibility that factors unique to fetal circulation may inhibit induction of AHH in fetal, but not placental, tissues. It is known, for instance, that a number of hormones may influence induction of AHH activity (10, 22). This possibility appears unlikely since none of these hormones are known to be specific to fetal circulation. Furthermore, since the placenta is also perfused with blood from the fetal circulation, such inhibitors might be expected to influence AHH induction in the placenta as well as umbilical vein. Another possibility is that the ability of specific organs to respond to PAH exposures may be under control of temporal genes (3). If this were the case here, some fetal tissues such as endothelium might be unable to respond to PAHs during gestation and early postnatal life while AHH activity in another tissue such as placenta might be induced by PAH exposure. Delayed postnatal development of metabolism of caffeine in human neonates (1) could reflect action of such temporal genes. This observation is particularly relevant because caffeine may be a substrate for AHH (2). While our data do not exclude this possibility, the ability of endothelial cells cultured from fetal circulation to respond to PAHs *in vitro* but not *in vivo* would require selective metabolic changes (loss of temporal gene effect) to occur in cells which are otherwise known to maintain many of their *in vivo* characteristics in tissue culture (7).

Our observation that maternal smoking induces AHH activity in placental, but not fetal, tissues may have important toxicologic consequences. Animal studies indicate that induction of AHH activity can contribute both to metabolic activation of xenobiotics and to their clearance (18). The relationship between the adverse effects of chemical exposures such as those associated with maternal smoking and xenobiotic metabolism by specific maternal and fetal tissues is likely to be very complex. Further understanding of the mechanisms responsible for differential effects of maternal smoking on placental and fetal tissues may provide better insight into the susceptibility of developing humans to toxic chemical exposures.

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Anticholeraic Effect of Methylated Casein in Rat Jejunum

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ABSTRACT. To explore the antisecretory effect of methylated casein (MC) cholera toxin was placed in isolated jejunal loops, and in vivo water fluxes were measured 3 h later in the presence or absence of MC. Secretion was observed in the loops filled with Ringer's solution only, but net absorption was observed in all 10 loops to which MC was added. Its actions was evident within 20 min, and was exerted directly on the luminal side of the epithelium. This response was dose-dependent and the antisecretory effect vanished after boiling MC and after ultrafiltration. In vitro, the antisecretory effect of MC consisted of reversing net Na and Cl fluxes from secretion to absorption (ΔJ_{net}^{Na} = 6.18 ± 1.25 and ΔJ_{net}^{Cl} = 5.10 ± 1.66 μEq·h^{-1}·cm^{-2}). This change was due to the enhancement of mucosal to serosal flux. Transepithelial potential difference and tissue conductance did not alter. Interestingly, MC did not interfere with intestinal function in the absence of stimulation by cholera toxin. In the presence of cholera toxin, MC and glucose both stimulated ionic absorption by different mechanisms, MC stimulating neutral NaCl absorption, and glucose stimulating electrogenic Na absorption. MC did not alter basal adenylate cyclase activity but it inhibited the cholera toxin-stimulated increase in activity. The present results indicate that methylated casein inhibits water and electrolyte secretion induced by cholera toxin in rat jejunum. Its availability, low cost, and curative effect from the luminal side constitute compelling indications for further investigation. (Pediatr Res 18:1075–1079, 1984)

Abbreviations

CT, cholera toxin
I_{sc}, short circuit current (μEq·h^{-1}·cm^{-2})
PD, transepithelial potential difference (mV)
G, tissue conductance (mS·cm^{-2})
J_{ms}, mucosal to serosal flux (μEq·h^{-1}·cm^{-2})
J_{sm}, serosal to mucosal flux (μEq·h^{-1}·cm^{-2})
MC, methylated casein