Presence of Cytochrome P-450–Dependent Monooxygenase in Intimal Cells of the Hog Aorta

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SUMMARY **Cytochrome P-450-dependent mixed function oxidase activity is present in vascular tissue; however, as far as we could determine, the distribution of monooxygenase activity across the blood vessel wall has not previously been assessed. The aryl-hydrocarbon hydroxylase activity was examined by metabolism of benzo[*a*]pyrene in microsomes prepared from intimal and smooth muscle cell scrapings of the hog thoracic aorta. Microsomes of intimal cells comprising 95% endothelial cells showed an approximately 2.5-fold increase in aryl-hydrocarbon hydroxylase activity compared with that in microsomes prepared from medial smooth muscle cells. Michaelis-Mentin kinetics for the intimal enzyme yielded an apparent K_m value of 11.11 μM and an apparent V_{max} of 3-OH benzo[*a*]pyrene of 40 pmol/mg protein/10 min. Aryl-hydrocarbon hydroxylase activity was dependent on nicotinamide adenine dinucleotide phosphate and was inhibited by 7,8 benzoflavone, SKF 525A, and carbon monoxide. The localization of cytochrome P-450-dependent mixed function oxidase primarily to the intimal surface of the aorta may indicate a role for this enzyme system in vasoregulation and the pathogenesis of atherosclerosis. (Hypertension 7: 899–904, 1985)**

KEY WORDS • aryl-hydrocarbon hydroxylase • benzo[*a*]pyrene • endothelium • media • arachidonic acid • aortic microsome

CYTOCHROME P-450-dependent enzymes catalyze the oxidation of a great variety of foreign compounds and participate in the detoxification of chemicals and the biotransformation of environmental mutagens.[1] Enzymatic reactions mediated through cytochrome P-450 enzyme systems also contribute to the metabolism of endogenous substrates and to the oxidative transformation of fatty acids, such as arachidonic acid[2] and its cyclooxygenase and lipoxygenase products.[3] Since cytochrome P450-dependent enzymes play a dominant role in the metabolism of drugs, attention has centered primarily on the hepatic microsomal cytochrome P-450-containing system.[4] However, various extrahepatic tissues, including lung, kidney, intestine, bone marrow, and adrenal glands, contain substantial amounts of cytochrome P-450 mixed function oxidase (MFO) activity.[5–7]

Ullrich and Graf[8] have shown the presence of cytochrome P-450 in hog aorta microsomes. In their study, they were unable to demonstrate monooxygenase activity using typical substrates for microsomal cytochrome P-450 reactions. In contrast, Juchau et al.[9] demonstrated the presence of cytochrome P-450 monooxygenase in homogenates of aorta based on hydroxylation of benzo[*a*]pyrene; the latter oxidation is known to be catalyzed by P-450-dependent enzyme. Aryl-hydrocarbon hydroxylase (AHH) activity subsequently was demonstrated in cultured endothelial cells isolated from bovine aorta.[10] Further, using immunohistofluorescence techniques, Dees et al.[11] reported that pulmonary arteries and veins and renal arteries contained two isozymes of cytochrome P-450. Although control tissue showed no fluorescent activity, the isozymes were inducible with the polycyclic hydrocarbon 2, 3, 7, 8 - tetrachlorodibenzo - *p* - dioxin, which gave rise to intense fluorescent staining localized to the endothelium.

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The evidence to date does not permit a quantitative assessment of the distribution of cytochrome P-450-dependent monooxygenase activity within the blood vessel wall. This is particularly important in view of the recent report that inhibitors of cytochrome P-450 antagonize endothelium-dependent vasodilation induced by methacholine, A23187, or arachidonic acid.[12] Such a role for cytochrome P-450 MFO in the control of vascular tone would require a specific localization in the endothelium. Consequently, the objective of this study was to determine the relative activity of the cytochrome P-450 MFO system in two different zones of the vascular wall, the intimal and medial layers. Experiments were also conducted to characterize the enzyme in terms of the kinetics of the AHH reaction and its susceptibility to inhibition or activation by compounds known to interact with the cytochrome P-450 system.

Materials and Methods

Segments of thoracic aorta were obtained from mature hogs within 15 minutes of slaughter and transported at 4°C to the laboratory. Experiments were conducted with the entire thoracic segment, extending from the aortic arch to the diaphragm. At least 12 complete segments (approximately 450 g of cleaned tissue) were used to prepare each batch of intimal cells; in the two experiments in which apparent K_m determinations were made, the microsomes were obtained with intimal cells pooled from 18 and 25 animals respectively.

The tissue composing the tunica intima and tunica media was sampled as follows. Each vessel was opened longitudinally and pinned, intimal surface up, to a Styrofoam board. The endothelial lining was gently rinsed with cold Krebs-Ringer bicarbonate buffer (1.0 g/L dextrose, pH 7.4); small blood clots were removed with a cotton tip applicator, and areas with firmly attached clots were excised. In most of the experiments, intimal cell suspensions were obtained by light scraping with a rubber policeman; however, in initial experiments either a cotton tip applicator or a scalpel blade was used to collect the cells. (Essentially identical results with regard to the fluorimetric assay of AHH activity were obtained irrespective of the collection method.) The initial harvest of intimal cells, suspended in 30 to 50 ml of Krebs buffer containing 4.5% bovine serum albumin, was filtered through two plys of cotton gauze, and an aliquot was taken for light microscopic observation and cell counting with a hemocytometer. At least four complete aortic segments were used in the preparation of microsomes from the tunica media. Medial layer scrapings were obtained, after removal of the internal elastic membrane, by roughly scraping the luminal margin of the muscular coat. Identical procedures were used for the subcellular fractionation of intimal or medial samples. After brief centrifugation (15 min, 1500 *g*, 20°C) with a Beckman I2-21 centrifuge (Palo Alto, CA, USA), the supernatant was discarded and the cells were washed by resuspending the pellet in approximately 30 ml of Krebs buffer without bovine serum albumin. The washing procedure was repeated twice, after which the intimal cells or medial scrapings were subjected to a fourth centrifugation step (900 *g*) and treated with 5 ml of NH_4Cl-$KHCO_3$-Tris (pH 7.4) buffer for 5 minutes to hemolyze contaminating erythrocytes. The resultant pellet was homogenized in ice-cold distilled water, and the pH of the homogenate subsequently was adjusted to 7.6 by adding KH_2PO_4 buffer to a final concentration of 0.1 M. Microsomal preparations were obtained as previously described.[13]

The method used to determine benzo[*a*]pyrene hydroxylase activity was adapted from procedures described by Nebert and Gelboin[14] and Alvares et al.[15] with spectrophotofluorimetric determination of 3-OH benzo[*a*]pyrene after an alkaline extraction of the organic phase. Microsomes or intimal cells were incubated for 10 minutes at 37°C in a reaction mixture containing 50 μM benzo[*a*]pyrene, 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM reduced nicotinamide adenine dinucleotide (NADH), 1 mM MgCl, and 0.1 mg bovine serum albumin. Volume was adjusted to 0.6 ml with 0.1 M KH_2PO_4 buffer, pH 7.8. The reaction was stopped by the addition of 3 ml of ice-cold acetone/hexane (3:1). The mixture was vortexed vigorously, and hydroxylated benzo[*a*]pyrene was extracted into 1 M NaOH. The concentration of hydroxylated benzo[*a*]pyrene in the alkali phase was determined by measuring the fluorescence at 522 nm during excitation at 396 nm using an Hitachi-Perkin Elmer spectrophotometer (Model MPF-3; Wilton, CT, USA). Duplicate analyses were carried out on all samples, and the amount of 3-OH benzo[*a*]pyrene generated was determined by referring to a standard curve. The results were obtained after subtracting values for blanks, which were incubated under conditions identical to control incubations but lacked microsomes or cells. Zero-time blanks, prepared with microsomes or cells, showed slight activity; in these incubations the reaction was stopped immediately after addition of benzo[*a*]pyrene. Protein concentrations were determined by the method of Lowry et al.[16]

Preliminary experiments with intact cells, lysates, and microsomes established that reaction velocity was dependent on cell protein and was linear for at least 10 minutes. The apparent K_m for hydroxylation of benzo[*a*]pyrene by intimal microsomes was determined by incubating different amounts of benzo[*a*]pyrene substrate with 0.2 mg of microsomal protein under the aforementioned reaction conditions. The 7,8 benzoflavone was dissolved in dimethylsulfate. The SKF 525A was dissolved in buffer and added to the reaction mixture at a final concentration of 200 μM.

The initial pellet of freshly harvested intimal cells or medial scrapings was used to obtain toluidine blue smears for light microscopic examination. The tissue specimen was heat fixed, stained with 0.1% toluidine blue, rinsed in distilled water, and directly examined without embedding or sectioning. To obtain some assessment of viability, intimal cells were scraped with a scalpel blade into sterile Krebs buffer with bovine se-

rum albumin and then seeded into tissue culture flasks containing Dulbecco's modified Eagle medium with 10% fetal calf serum. The cells were cultured using methods and materials similar to those described by Ryan et al.,[17] and the cultures were observed microscopically at 4 days and at 14 days, when they reached confluency.

Results

Light scraping of the intimal surface yielded large numbers of cells, approximately 20 million per aorta. On light microscopic examination, the toluidine blue–stained specimen (Figure 1) exhibited a uniform appearance and the primary cell type was represented by the endothelial cell, as judged by the morphological resemblance to similarly stained preparations removed by electrophoretic cellulose acetate paper.[18] Moreover, when examined by phase-contrast microscopy, the cells grew as a monolayer with the characteristic "cobblestone" appearance, although these primary cultures contained at least one other contaminating cell type. In particular, another adherent cell, much larger than an endothelial cell, was found in preconfluent and confluent cultures; these composed roughly 5% of the total, as judged by phase microscopy. Scrapings obtained from the tunica media showed a heterogeneous appearance, with variable numbers of elongate ("cigar-shaped") nuclei and large amounts of particulate material.

Microsomes prepared from aortic tissue, particularly from the intimal zone, contained considerable AHH activity. To prepare each batch of microsomes we used aortic tissues pooled from approximately 20 hogs. In the five batches studied, AHH activity ranged from 6 pmol 3-OH benzo[*a*]pyrene per milligram of protein per 10 minutes to 13 pmol/mg protein/10 min. Table 1 shows that incubation of intimal microsomes with benzo[*a*]pyrene resulted in a level of AHH activity 2.5-fold greater than that achieved by incubation of medial microsomes. It is clear that AHH was present in greater specific activity in the intimal zone (mainly endothelial cells) than in the medial, muscular zone.

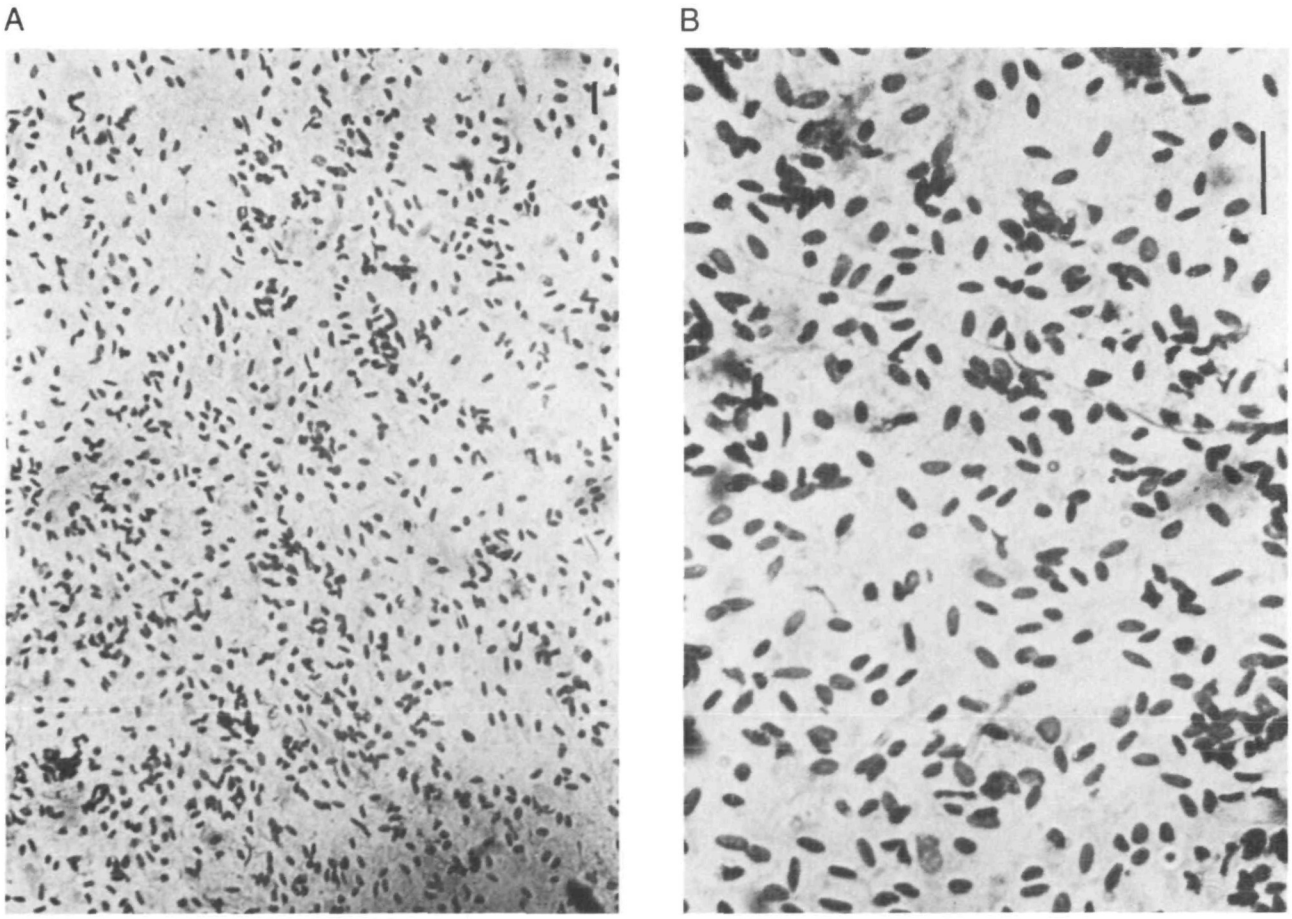

FIGURE 1. *Toluidine blue–stained smear of freshly harvested intimal cells obtained by gentle scraping of hog thoracic aorta. Low (A, × 125) and high power (B, × 315) photomicrographs indicate that these intimal scrapings were comprised almost entirely of endothelial cells. The smear was made from a pellet of cells isolated from a large number of aortae by scraping with a rubber policeman into Krebs buffer containing 4.5% bovine serum albumin. (The original magnification stated above has been reduced. The bar in each figure [upper right] represents 50 μm.)*

TABLE 1. *Comparison of Aryl-Hydrocarbon Hydroxylase Activity Present in Microsomes from Intimal and Medial Layers of Hog Thoracic Aorta*

Layer	Specific activity*	Relative specific activity*
Intimal	6.5	2.5
Medial	2.9	1.0

Values are the means of triplicate determinations.
*Measured as picomoles of 3-OH benzo[*a*]pyrene formed per milligram of protein per 10 minutes.

TABLE 2. *Requirements for Aryl-Hydrocarbon Hydroxylase Activity and Effects of Specific Inhibitors of Monooxygenases*

Incubation system	Activity (pmol/mg protein/10 min)
Complete system*	8.2
− NADPH	2.4
+ Carbon monoxide†	2.6
+ SKF 525A	3.4
+ 7,8 Benzoflavone	5.1

Reaction mixtures contained 100 μmol potassium phosphate, pH 7.5, 1 μmol NADPH, 1 μmol MgCl, 0.8 mg of microsomal protein, and 50 μM benzo[*a*]pyrene (in 20 μl of acetone) in a total volume of 1.0 ml. Incubation was in open air in a 12-ml tube for 10 minutes at 37°C. The reaction was stopped as described in Materials and Methods.
NADPH = reduced nicotinamide-adenine dinucleotide phosphate.
*Results of duplicate determinations were always within 10% of each other.
†The reaction mixtures were bubbled with carbon monoxide for 2 minutes before the substrate (benzo[*a*]pyrene) was added, after which time the incubation was continued in a covered water bath.

A series of experiments was conducted to corroborate the assay results indicating the presence of cytochrome P-450-dependent monooxygenase activity within the aortic intima. As shown in Table 2, the AHH activity exhibited an absolute requirement for NADPH or its generating system. Exposure of the microsomes to carbon monoxide inhibited 3-OH benzo[*a*]pyrene formation by 69%. Ethylenediaminetetraacetic acid, of which 0.1 mM has been reported to inhibit AHH activity in hamster fetus cell cultures,[14] had no effect on AHH activity in our preparation of intimal microsomes. Addition of 7,8 benzoflavone and SKF 525A inhibited the reaction by 37% and 59% respectively. The NADPH dependency and the inhibition caused by 7,8 benzoflavone, SKF 525A, and carbon monoxide all indicate that a form of cytochrome P-450 is involved in the hydroxylation reaction. The reaction catalyzed by the AHH was apparently linear for 10 minutes (Figure 2). A Lineweaver and Burk double reciprocal plot for the hydroxylation of benzo[*a*]pyrene by intimal microsomes is shown in Figure 3. It can be seen that the reaction conformed to Michaelis-Menten kinetics, yielding an apparent K_m value of 11.11 μM and an apparent V_{max} of 40 pmol 3-OH benzo[*a*]pyrene formed per milligram of protein per 10 minutes, which is comparable to the constants reported for the rabbit kidney but higher than that reported for rabbit liver.[18]

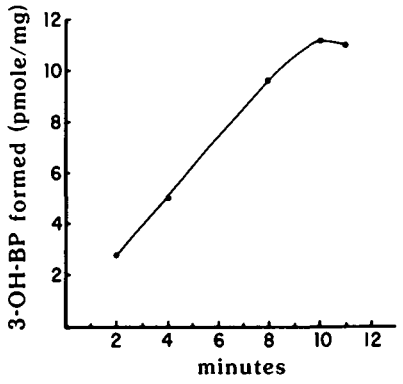

FIGURE 2. *Time course of aryl-hydrocarbon hydroxylase activity present in microsomes prepared from hog aortic intima. The microsomal fraction was obtained by differential centrifugation following homogenization and hypotonic lysis of a suspension of intimal cells obtained as in Figure 1. Intimal microsomes were incubated with benzo[a]pyrene (BP) as substrate for the indicated time intervals. The 3-OH benzo[a]pyrene was measured as described in Materials and Methods.*

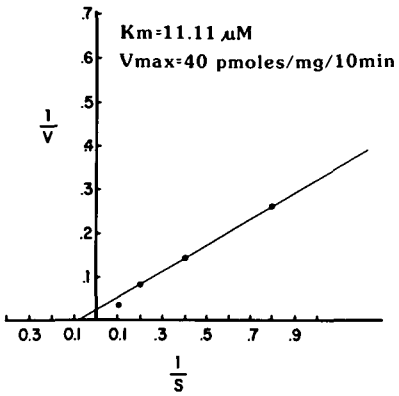

FIGURE 3. *Double reciprocal plot of aryl-hydrocarbon hydroxylase activity in intimal microsomes prepared from hog thoracic aorta. The formation of 3-OH benzo[a]pyrene was determined by incubating different concentrations of benzo[a]pyrene substrate with 0.35 mg of microsomal protein under reaction conditions described in the text (V = velocity; S = substrate).*

The results shown in Figure 4 were obtained by repetitive scanning of incubation medium containing 0.35 mg of microsomal protein (from aortic intima) and 80 μM benzo[*a*]pyrene. The excitation wavelength maximum was 396 nm, which allowed maximal fluorescence emission of 3-OH benzo[*a*]pyrene (λ maximum emission = 522 nm) but not of benzo[*a*]pyrene (λ maximum emission = 427 nm). Spectra were recorded 10 minutes after benzo[*a*]pyrene had been added to the incubation medium. Maximum fluorescence emission of 3-OH benzo[*a*]pyrene at 522 nm was dependent on enzyme protein and the presence of NADPH.

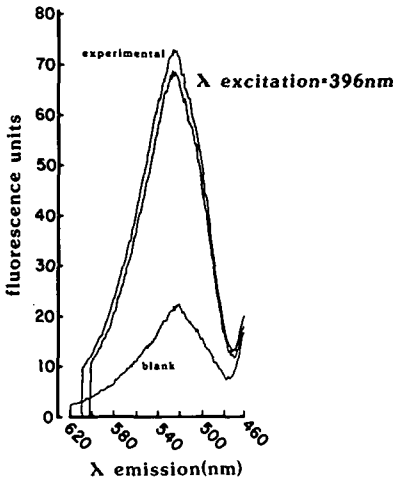

FIGURE 4. *Spectral analysis of aryl-hydrocarbon hydroxylase activity in hog aortic intima. The enzyme assay was performed using a microsomal preparation under conditions described in Materials and Methods. The results of duplicate analyses are shown as well as data obtained in a concurrently incubated blank.*

Discussion

These experiments demonstrate that microsomes prepared from intimal scrapings of the hog aorta contain a low but readily detectable level of cytochrome P-450 MFO activity as assessed by the metabolism of benzo[*a*]pyrene. In contrast, microsomes of the medial layer were virtually devoid of activity. Juchau et al.[9] reported the metabolism of benzo[*a*]pyrene in homogenates of whole aorta in 1976. Other studies indicate activity in cultured aortic endothelial cells[10] and cytochrome P-450-specific immunofluorescent staining of the endothelium of blood vessels.[11] However, none of these studies addressed the distribution of this MFO system across the vascular wall. The present study specifically attempted to quantitatively analyze AHH activity in the intimal and medial layers of the blood vessel. Our isolation technique resulted in a preparation of intimal cells composed almost exclusively of endothelial cells, which indicates that endothelial cells are the source of the intimal AHH activity. The level of activity was comparable to that previously measured in the outer medulla of the rabbit kidney using similar assay methods (unpublished data). Our results indicate that the AHH activity is clearly a cytochrome P-450-dependent reaction, since it was NADPH dependent and inhibited by 7,8 benzoflavone and SKF 525A, which are both potent inhibitors of cytochrome P-450-containing enzymes.

The relative absence of cytochrome P-450-dependent AHH activity in smooth muscle cells of the medial layer is of interest since it has recently been suggested that products of arachidonic acid formed by a cytochrome P-450-dependent mechanism mediate endothelium-dependent vasorelaxant responses to methacholine, A23187, and arachidonic acid.[12] The localization of AHH activity found in the present study lends support to such a proposal, since endothelium-dependent responses have also been observed in the pig aorta.[19] Moreover, cells of the medulla of the kidney[20] as well as polymorphonuclear leukocytes[21] contain cytochrome P-450-dependent enzymes capable of generating vasodilator products from arachidonic acid.

The ability of the intima to serve as an oxygen sensor is evident from the work of Busse et al.,[22] who showed that removal of endothelial cells blocked the vasodilatory response of the rat tail artery to intraluminal hypoxia. The vascular cytochrome P-450 MFO system may be implicated in hypoxic pulmonary vasoconstriction, since inhibitors of cytochrome P-450 such as carbon monoxide and metyrapone block the vasoconstrictor response.[23, 24] Constriction of the ductus arteriosus of the lamb on exposure to oxygen is also thought to be mediated by a cytochrome P-450-dependent mechanism.[25] It therefore appears that both vasoconstrictor and vasodilator metabolites may be formed and that the metabolism is governed by tissue-specific differences or oxygen tension, or both. It also should be noted that, while aortic tissue has been used extensively for studies of endothelium-dependent vascular responses, endothelial cells of large and small blood vessels show major differences in morphology and function.[26] The findings of the present study may not be indicative of the cytochrome P-450 activity in resistance or capacitance vessels.

In summary, the present results indicate that the vascular cytochrome P-450 MFO system is localized in the intima; very little activity was detected in cells from the medial layer. The disposition of this enzyme system is consistent with a role in endothelium-dependent vasodilation. Moreover, because of its strategic location, this MFO may also be involved in the pathogenesis of atherosclerosis and the production of cytotoxic mutagens, as suggested by Juchau et al.[9]

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