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# Induction of Cytochrome P4501A1 by Aryl Hydrocarbon Receptor Agonists in Porcine Aorta Endothelial Cells in Culture and Cytochrome P4501A1 Activity in Intact Cells

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## SUMMARY

Endothelium is a single-cell layer lining blood vessels and constituting capillaries and could be a primary site of chemical effects in the cardiovasculature and systemically. Cytochrome P4501A1 (CYP1A1) is strongly inducible in vertebrate endothelium *in vivo* by aryl hydrocarbon receptor (AhR) agonists [*Mol. Pharmacol.* **36**:723–729 (1989); *Mol. Pharmacol.* **41**:1039–1046 (1992)]. We investigated CYP1A expression and activity in porcine aorta endothelial cells (PAEC) exposed in culture to the AhR agonists 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3,3′,4,4′-tetrachlorobiphenyl (TCB), benzo[*a*]pyrene (BP), or β-naphthoflavone (BNF). Immunoblotting with monoclonal anti-CYP1A1 and polyclonal anti-CYP1A1 and anti-CYP1A2 antibodies showed that CYP1A1 was induced in cultures exposed to TCDD, TCB, BP, or BNF but was not detectable in untreated or dimethylsulfoxide-exposed cultures. CYP1A1 was strongly induced at intermediate concentrations (0.1 μM or 1.0 μM) of TCB, BP, or BNF, but induction was suppressed by higher concentrations, a response not due to general toxicity; cell viability (trypan blue exclusion) was >97% with BNF or TCB at up to 10 μM. CYP1A1 induction by TCDD was maximal at 0.3–1.0 nM. $ED_{50}$ values for induction of CYP1A1 by TCDD, TCB, and BP were 0.016 nM, 3–10 nM, and 180 nM, respectively. Immunohistochemical analysis confirmed CYP1A1 induction in PAEC but also showed that only some cells in the cultures were induced. Subcellular fractionation, marker enzyme analysis, and immunoblot analysis showed that PAEC had a typical complement of microsomal electron-transport components. NADPH-cytochrome P450 reductase showed comparable rates (~40 nmol/min/mg) in induced and control cultures. Cultures maximally induced by 0.1 μM TCB had microsomal CYP1A1 [ethoxyresorufin-*O*-deethylase (EROD)] activity averaging 25 pmol/min/mg. Addition of purified rat reductase to PAEC microsomes increased the EROD rates 3-fold. EROD rates measured in intact cells maximally induced by BP, TCB, or TCDD ranged from 15 to 30 pmol/min/mg of whole-cell protein. Methoxyresorufin *O*-demethylase activity induced by TCDD was 2 pmol/min/mg, i.e., <10% of the EROD activity. In cultures in which CYP1A1 was strongly induced, CYP1A2 was not detectably expressed. The CYP1A2 inducer acenaphthylene did not induce EROD or methoxyresorufin *O*-demethylase in intact cells. The results show that CYP1A1 but not CYP1A2 is strongly induced in mammalian endothelial cells in culture and that CYP1A1 is active in intact cells, although the catalytic rates are low. Endothelial CYP1A1 could alter the outcome of drug therapy, activate promutagens contributing to atherogenesis, alter the metabolism of endogenous compounds in the endothelium, and perhaps act as a binding protein, sequestering and slowly metabolizing AhR agonists/CYP1A substrates.

Endothelium, the single-cell layer lining blood vessels and comprising capillaries, pervades all organs, governs or mediates vascular function, inflammation, edema, thrombosis, and angiogenesis, and may serve as a protective barrier. Endothelium could be a primary site of diverse toxic chemical effects throughout the cardiovasculature and in all organs. Growing evidence implicates the endothelium in cardiovascular toxicity of xenobiotics. Atherosclerosis, which was suggested 15 years ago to result from activation of promutagens in vascular smooth muscle (1), now is thought to involve chemical effects in the endothelium. Endothelium is the probable site of action in the pulmonary toxicity of alkaloids (2). In mammals, birds, and

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**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; NO, nitric oxide; AA, arachidonic acid; PAH, polynuclear aromatic hydrocarbons; AhR, aryl hydrocarbon receptor(s); TCB, 3,3′,4,4′-tetrachlorobiphenyl; AHH, aryl hydrocarbon hydroxylase; BP, benzo[*a*]pyrene; BCIP, 5-bromo-4-chloro-3-indolylphosphate; NBT, nitroblue tetrazolium; BNF, β-naphthoflavone; EROD, ethoxyresorufin *O*-deethylase; PAEC, porcine aorta endothelial cells; MROD, methoxyresorufin *O*-demethylase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAb, monoclonal antibody; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCB, polychlorinated biphenyl; P450 or CYP, cytochrome P450; PBS, phosphate-buffered saline; 3-MC, 3-methylcholanthrene.

fish, TCDD and related compounds produce edema (3), a condition signaling endothelial dysfunction and suggesting a common mechanism of action among vertebrates. Endothelium acts in cardiovascular regulation by transducing flow, pressure, and stretch signals as well as by responding via cell surface hormone receptors (4). Endothelial cells metabolize biogenic amines, reducing their effective tissue concentrations, and produce substances that regulate vascular and cardiac contractility and growth. These include the relaxing factor NO, AA products including the anti-platelet factor prostacyclin, and endothelin, a vasoconstrictor peptide (5). Xenobiotics could alter any or all of these processes, and activation of protoxicants could contribute to endothelial damage and vascular disease.

Mechanisms of chemical effects in the endothelium may involve inducible enzymes in the *CYP1* through *CYP4* gene families. These enzymes are important in the activation or detoxication of lipophilic xenobiotics, including many drugs (6), and in the metabolism of endogenous signaling molecules including AA (7), the precursor for endothelium-derived eicosanoids. *CYP1A* genes are induced in endothelium by PAH and planar halogenated aromatic hydrocarbons (8–12). Inducers act by binding to the AhR, a basic helix-loop-helix protein (13, 14) that with its helix-loop-helix partner, the AhR nuclear translocator protein, activates transcription of *CYP1A* and other genes (15). *CYP1A* and the AhR both could participate in producing toxic effects in the endothelium. CYP1A enzymes activate PAH and amine promutagens, initiating cellular and genetic damage (16), and the AhR apparently mediates the toxicity of agonists such as TCDD independently of CYP1A activity (17). CYP1A induction in endothelium occurs widely in phylogeny from fish to mammals, reflecting the phylogenetic distribution of the AhR (18) and indicating a general significance of induction in this cell type. That induction can be profound, with CYP1A content reaching nearly 5 nmol/mg (25%) of endothelial microsomal protein (10).

The significance of strong CYP1A induction in endothelial function is unclear. In isolated dog coronary artery and rabbit pulmonary artery from animals treated with the CYP1A inducers 3-MC or BNF, endothelium-dependent relaxations elicited by AA and arterial AHH both were increased and both were inhibited by the general P450 inhibitor SKF-525A (19, 20). The CYP1A substrate 7-ethoxyresorufin also can inhibit arterial relaxations (21). Such results suggest the involvement of functional CYP1A, although the 7-ethoxyresorufin effect might stem from interference with electron transfer or even with NO production or action. Moreover, the catalytic function of CYP1A induced in the endothelium could be limited by small amounts of NADPH-P450 reductase, curtailing electron transfer to P450 (11, 22).

Knowledge of the sensitivity of the responses, the nature of the P450 forms induced, and their catalytic capabilities is essential if chemical effects involving endothelium are to be understood. Such studies of intact endothelium are difficult, although endothelia contribute to microsomes from any organ. We have examined the response and sensitivity to diverse AhR agonists of PAEC in culture. PAEC were selected because they are easily grown and porcine aorta has been used extensively in cardiovascular studies. We establish that CYP1A1, but not CYP1A2, is strongly induced in large vessel endothelial cells by BNF, BP, TCB, and TCDD. We also establish that large vessel endothelial cells in culture express functional microsomal electron transport systems, including P450 reductase, and we provide rates of induced CYP1A1 activity in intact endothelial cells.

## Materials and Methods

**Chemicals.** TCB was purchased from Pathfinder Laboratories (St. Louis, MO). The purity of TCB was >99%, as determined by gas chromatography/electrochemical detection and gas chromatography/mass spectrometry analyses. Further analysis by high-resolution (5000 resolution) mass spectrometry (VG Autospec) with selective ion monitoring showed that the potent AhR agonist 3,3′,4,4′,5-pentachlorobiphenyl was not a contaminant of the TCB stock, with a detection limit of 0.001%. TCDD (10 μg/ml; >98% purity) was from Ultra Scientific. BP and acenaphthylene were from Aldrich. Horse heart cytochrome c, BNF (5,6-benzoflavone), NBT, BCIP, BSA, and dicumarol were from Sigma Chemical Co. (St. Louis MO). Ethoxyresorufin, resorufin, and methoxyresorufin were from Molecular Probes (Eugene, OR). Other chemicals were of the highest grade available, from the suppliers indicated below. Livers were obtained from 30-kg female pigs immediately after euthanasia, in connection with an authorized laparoscopy laboratory. Hepatic postmitochondrial supernatants of rats treated intraperitoneally with 500 mg/kg Aroclor 1254 were a generous gift from Dr. John Rundell (Molecular Toxicology Inc., Annapolis, MD). Microsomes from each tissue were prepared using standard procedures.

**Isolation of cells.** PAEC were aseptically prepared from pig thoracic aorta obtained from a local abattoir. Perivascular connective tissue was removed and aortae were opened longitudinally to expose the luminal surface. The endothelial cells were obtained by scraping the luminal surface once with a razor blade, transferring the scraping to a 100-mm-diameter Petri dish (Costar, Cambridge, MA) containing 10 ml of medium (see below), and dispersing the cells by trituration. Cells were grown in Dulbecco's modified Eagle medium (no. 430–1600; GIBCO, Grand Island, NY) supplemented with 3.7 g/liter $NaHCO_3$, 10% fetal bovine serum (Hazelton or Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO). The medium was changed every 2–3 days. Cell cultures were maintained in a humidified incubator at 37°, in the presence of 5% $CO_2$. When individual primary cultures reached confluence, cells from six to 12 pigs were pooled and then divided into aliquots for freezing (−70°), in a solution containing 70% Dulbecco's modified Eagle medium, 20% fetal bovine serum, and 10% DMSO. Before treatment, aliquots were rapidly thawed at 37° and grown in a 75-$cm^2$ flask as the first passage. Cells were subcultured using trypsin (0.05%) diluted 1/2 in calcium- and magnesium-free Dulbecco's PBS (GIBCO). Second-passage cultures were grown in six-well plates, 12-well plates, 48-well plates, or 75-$cm^2$ flasks. Some cultures had HEPES buffer added to the medium at 20 mM, to maintain pH 7.4 when cultures were not in a $CO_2$ incubator.

All cultures used in these studies exhibited the typical endothelial cell characteristics of contact inhibition and a cobblestone appearance at confluence. Cells were examined for uniform uptake of fluorescently labeled acetylated low density lipoprotein (Biomedical Technologies, Stoughton, MA), to establish functional identity. Cultures showing any fibroblast growth were discarded.

**Treatment of cells.** Stock solutions of BNF, BP, acenaphthylene, and TCB (10 mM) were prepared by dissolving crystalline compound in DMSO. TCDD solutions were prepared by drying an aliquot of TCDD in toluene under $N_2$ and dissolving the residue in DMSO. Dilutions were made from these stock solutions in DMSO. TCB or BNF was delivered to larger cultures by replacing medium containing 10% serum with medium containing 1% serum plus test compound at concentrations from 0.01 to 10.0 μM, DMSO alone, or no additive. Alternatively, cultures in 48-well plates were given fresh culture medium with 1% serum and then aliquots of stock solutions of BP, acenaphthylene, TCB, or TCDD, or dilutions thereof, were added directly to the cultures to achieve the desired concentrations from 0.001 nM to 50 μM, depending on the compound. Each dose was applied to

four replicate cultures (wells) of 48-well plates. Control cultures received DMSO alone. DMSO was present at 0.5% (v/v) in all control and treated cultures. Treatment was for 24–48 hr. Cultures were examined microscopically for integrity at the start and at the end of exposures.

Cell viability was assessed by analysis of trypan blue exclusion. Second-passage PAEC were grown in 24-well plates to confluency. Medium with 10% serum was replaced by medium with 1% serum plus either DMSO, TCB, or BNF, or no addition, as described above. After 48 hr, cells were detached with 0.025% trypsin, resuspended, and exposed to 0.2% trypan blue for 5 min, and the percentage of cells excluding dye was determined with a hemacytometer.

**Harvesting and preparation of subcellular fractions.** Cells grown in T-75 flasks or in six-well plates were harvested for analysis. The medium was decanted at the end of the exposure, and the cells were rinsed with cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2PO_4$, 1.5 mM $KH_2PO_4$, pH 7.4) containing EGTA and EDTA (1 mM each). Cells either were harvested directly or were frozen by floating the plates or flasks on liquid nitrogen and then storing the cultures at −80° until use, which was within 2 weeks. Frozen cells were scraped from the flask, with a Teflon spatula, into homogenization buffer (50 mM Tris, 0.15 M KCl, pH 7.4) and were homogenized directly, as described below. Cells harvested freshly were resuspended in a MOPS buffer that contained a mixture of protease inhibitors (20 μM *N*-tosyl-L-lysine chloromethyl ketone, 5 μg/ml leupeptin, 13 μg/ml aprotinin, 7 μg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride), as well as 1 mM EGTA. Cells were lysed by sonication (four 3-sec pulses; Virsonic, power 3) or by a gentler procedure of 10 passes with a Dounce homogenizer, followed by a single 3-sec pulse of sonication. The resulting lysate was analyzed directly or was further fractionated.

Subcellular fractions were prepared from lysates by centrifugation at 750 × *g* for 10 min (nuclei and cell debris), 12,000 × *g* for 10 min (mitochondria), and 100,000 × *g* for 70 min (microsomal and cytosolic fractions). The nuclear and mitochondrial fractions of some preparations were combined, rehomogenized, sonicated, and subjected to differential centrifugation, yielding a second set of subcellular fractions. Particulate fractions were resuspended in a buffer that contained 0.1 M Tris, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol and were frozen in liquid $N_2$. Cytosol was frozen directly in liquid $N_2$. In addition to the analysis of homogenates or fractions of harvested cells, intact cells grown and treated in 48-well plates were analyzed for CYP1A catalytic activity, as described below.

**Enzyme and protein assays.** NADPH-dependent, NADH-dependent, and succinate-dependent cytochrome *c* reductase activities were measured by the reduction of horse heart cytochrome *c* at 37°, using procedures described before (22). The buffer contained 5 μM KCN to inhibit cytochrome oxidase. The DT-diaphorase inhibitor dicumarol was added at 10 μM to some incubations. Relative specific activities of reductases in the subcellular fractions were calculated according to the method of deDuve *et al.* (23). Dithionite-difference spectra of CO-treated microsomes and NADH-difference spectra were obtained as described before (22), with a Shimadzu 210 difference spectrophotometer. Cuvettes contained 0.1–0.25 mg of microsomal protein/ml. Difference spectroscopy of microsomes showed a distinct and typical cytochrome $b_5$ spectrum. Protein content was measured by the method of Smith *et al.* (24), with BSA as the standard.

EROD and MROD activities of homogenates or subcellular fractions were measured fluorometrically using a Cytofluor 2300 (Millipore) multiwell plate reader, as described by Hahn *et al.* (25). Microsomes and other cell fractions were assayed in 48-well plates. Reaction mixtures contained 150 μl of Tris buffer (50 mM Tris, 0.1 M NaCl, pH 7.8), 7-ethoxy- or 7-methoxyresorufin at a final concentration of 2 μM, and 10 μl of enzyme (20–30 μg of protein). Reactions were initiated by the addition of 40 μl of 5 mM NADPH or NADH (final concentration, 1.0 mM) and resorufin production was determined at 2-min intervals, using 530-nm excitation and 590-nm emission filters. Incubations were at instrument temperature (approximately 32°), because the Cytofluor reader lacks a temperature-controlled chamber. Activity was determined by comparison with resorufin standards of 2–150 pmol/well. EROD activity of highly induced scup or rat liver microsomes was assayed as a positive control. Linearity with time under these conditions was established in each assay.

The effect of cytochrome *c* on EROD activity was determined by addition of 90 μM cytochrome *c* to the EROD reaction mixtures. Titration of cytochrome *c* inhibition of EROD activity in liver microsomes showed that 90 μM cytochrome *c* inhibited activity by ≥98%. The limits of detection of resorufin in the presence of cytochrome *c* were empirically determined.

EROD in intact cells grown and treated in 48-well plates was measured using a modification of the methods of Kennedy *et al.* (26). Medium was aspirated and attached cells were gently washed with 0.5 ml of PBS. Tris buffer saline containing ethoxyresorufin or methoxyresorufin at 2 μM was then added to the cells, and resorufin formation was measured at 2-min intervals. Some cultures had NADPH added with the alkoxyresorufin/buffer mixture. At the end of the measurement, the reaction mixture was removed, the cultures were examined microscopically for appearance, and total protein in each well was measured directly using a fluorescamine assay (27), with BSA as the standard.

**Augmentation with rat reductase.** Microsomal NADPH-P450 reductase was purified from livers of untreated rats, as described elsewhere (28). PAEC microsomes (0.5 mg/ml) and purified rat reductase (10 nmol/min/μl) were mixed at a ratio of 9:1 (v/v) and sonicated on ice, and aliquots were assayed for EROD activity. Control reactions contained BSA equivalent to the amount of reductase protein or an equivalent volume of phosphate buffer alone replacing the reductase.

**Antibodies and immunoblotting.** Antibodies to CYP1A used in immunoblotting included MAb 1-12-3, raised against P450E from the marine fish scup (29). Two CYP1A forms occur in mammals, CYP1A1 and CYP1A2. Based on the full length coding sequence, immunological cross-reactivities, and functional and regulatory properties, the scup P450E is ostensibly a CYP1A1 (30, 31). MAb 1-12-3 strongly recognizes mammalian CYP1A1 forms but not CYP1A2 forms (e.g., see Ref. 32). A polyclonal antibody (Peg) raised against mouse Cyp1a-1 and a polyclonal antibody (Rye) against mouse Cyp1a-2 were provided by Dr. Peter Sinclair (Veterans Administration Hospital, White River Junction, VT). The anti-Cyp1a-1 recognizes 1A1 and 1A2 forms with equal intensity, and the anti-Cyp1a-2 recognizes 1A2 forms strongly and 1A1 forms weakly.[1] We confirmed the specificity of these antibodies by immunoblot analysis of PCB-induced rat liver microsomes (see Results) and of human 1A1 and 1A2 expressed in COS cells.[2] Antibodies to rabbit liver NADPH-P450 reductase were generously provided by Dr. Richard Philpot (National Institute of Environmental Health Sciences).

Western blot analyses of cell lysates or subcellular fractions were performed largely as described previously (25, 32). Homogenate protein (70 μg) or microsomal protein (10–30 μg) and a range of standards (0.1–2 pmol of scup CYP1A1) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using an 8–15% acrylamide gradient gel or an 11% acrylamide gel. Proteins were electrophoretically transferred onto 0.2-μm nitrocellulose membranes and were incubated with primary antibodies and then with goat anti-mouse IgG linked to alkaline phosphatase (Bio-Rad). Color was developed with NBT and BCIP or by enhanced chemiluminescence with MAb 1-12-3 at 10 μg/ml and polyclonal antibodies at a 1/5000 dilution, with development as directed for the Schleicher and Schuell Rad-Free chemiluminescence detection kit. Band intensities were quantified by video imaging densitometry. Values for CYP1A equivalents were determined from the integrated absorbance values of the MAb 1-12-3-cross-reactive proteins, relative to those of scup CYP1A1 standards.

**Analysis of dose-response curves.** Data obtained from EROD

[1] P. Sinclair, personal communication.
[2] J. J. Stegeman, unpublished observations.

assays and immunoblots were analyzed by nonlinear regression using the curve-fitting subroutine of SigmaPlot (Jandel). Data were fit to the modified Gaussian equation described by Kennedy *et al.* (26), $y(d) = Y_b + (Y_m - Y_b)\exp[-C[\ln(d) - \ln(d_m)]^2]$, where $C = \ln(2)/[\ln(EC_{50}) - \ln(d_m)]^2$, $y(d)$ is EROD activity or CYP1A1 content at inducer concentration $d$, $Y_b$ is basal EROD activity or CYP1A1 content, $Y_m$ is maximal EROD activity or CYP1A1 content, $d_m$ is the inducer concentration producing maximal EROD activity, and $EC_{50}$ is the inducer concentration producing 50% of maximal EROD activity or CYP1A1 content.

**Immunocytochemistry.** Sheets of control and 0.1 μM TCB-exposed cells were gently scraped from the same 75-cm² flasks from which PAEC microsomes were prepared, and they were transferred to 10% neutral buffered formalin. Pelleted cells were suspended in agar and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin or with MAb 1–12–3, as described previously (12). Sections of thoracic aorta from rabbits given injections of 3-MC (20 mg/kg) and from control rabbits were similarly fixed, embedded, and stained for comparison. Procedural and antibody controls were as described previously (10, 12).

## Results

**CYP1A induction.** In initial experiments, replicate cultures in six-well plates were exposed for 24 or 48 hr to BNF or TCB at 0.1 μM or 10 μM, and whole-cell lysates were analyzed by immunoblotting with MAb 1–12–3. A single cross-reactive protein migrating at about 54 kDa was detected in lysates of each BNF- or TCB-exposed culture but not in lysates of untreated or DMSO-treated cultures (Fig. 1). Microsomes prepared from control cultures also showed no detectable signal, whereas microsomes from TCB-treated cultures showed a strong MAb 1–12–3 signal. The specificity of MAb 1–12–3 for mammalian CYP1A1 but not CYP1A2 proteins indicates the identity of the cross-reacting PAEC protein as a CYP1A1.

The induction of CYP1A1 (MAb 1–12–3-cross-reactive protein) varied with inducer and dose and duration of treatment (Fig. 2). BNF at 0.1 μM elicited an appreciable induction by 24 hr, which declined by 48 hr. BNF at 10 μM produced a greater induction than did 0.1 μM BNF at 24 hr and a slight additional increase in CYP1A content between 24 hr and 48 hr. The CYP1A1 content in cultures exposed to 0.1 μM TCB for 24 hr was similar to that in cultures exposed to 0.1 μM BNF (Fig. 2). With 0.1 μM TCB there was an increase in CYP1A1 content between 24 and 48 hr, rather than a decrease like that seen with BNF. At 24 hr, 10 μM TCB elicited a stronger induction than did 0.1 μM TCB. Induction by 10 μM TCB did not increase between 24 and 48 hr. At 48 hr the response to 10 μM TCB was less than the 48-hr response to 0.1 μM TCB.

CYP1A1 content was examined in six-well cultures exposed to 0.01, 0.1, 1.0, or 10 μM TCB or BNF (at 40 hr), to further identify concentrations eliciting strong induction of CYP1A for use in enzyme studies (see below). With TCB induction was strongest at 0.1 μM and with BNF induction was strongest at 1.0 μM. There was a lesser degree of CYP1A induction at the higher doses of either TCB or BNF, which was not associated with overt toxicity. Trypan blue exclusion showed >97% viability at all doses of BNF or TCB or with DMSO.

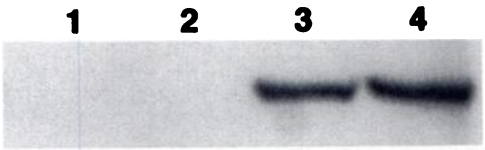


**Fig. 1.** Immunodetection of CYP1A1 in PAEC. Cell lysates of BNF-exposed and control PAEC were blotted and probed with MAb 1–12–3. *Lanes 1* and *2*, 75 μg of cell lysate protein from replicate cultures exposed to DMSO; *lanes 3* and *4*, 75 μg of cell lysate protein from replicate cultures exposed to 0.1 μM BNF for 24 hr. Color development was with BCIP and NBT, as described in Materials and Methods. There were no bands detected at any other location on the gel. Immunoblotting of lysates or microsomes of TCB- or TCDD-exposed cultures gave results similar to those for BNF (see also Fig. 7).

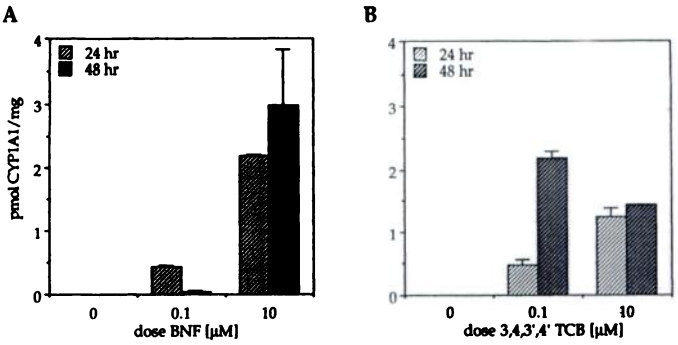


**Fig. 2.** Concentration- and time-dependent induction of CYP1A1 in PAEC by TCB and BNF. PAEC whole-cell lysates were blotted and CYP1A1 was detected as in Fig. 1; NBT and BCIP were used for color development. A, BNF-treated cells; B, TCB-treated cells. Results obtained with duplicate cultures in 9.6-cm² wells are shown, with the range. Quantitation of CYP1A1 content is relative, due to a lack of purified pig CYP1A1 to use as a standard for calibrating the immunoblot results. Comparison with the staining of scup CYP1A1 standards (the immunogen for MAb 1–12–3) gave a value for CYP1A1 equivalents, allowing normalization. Scup CYP1A1 standards were included on each gel.

TABLE 1

**Distribution of cytochrome c reductase activities in PAEC**

The distribution of activity in fractions of untreated cells is shown. Dicumarol inhibited the activity of NADPH-dependent reductase in the nuclear and mitochondrial fractions by about 30–60% but did not affect other activities or fractions. The distribution of dicumarol-insensitive activity is shown. The distribution of reductases was strongly influenced by the homogenization procedures. Upon vigorous sonication without protease inhibitors, as much as 80% of the total NADPH-cytochrome *c* reductase activity occurred in the cytosol, whereas gentler homogenization and sonication with protease inhibitors resulted in about 20% of NADPH-dependent activity in the cytosol, with greater percentages appearing in particulate fractions. Mean values from replicate cultures that were gently homogenized are shown. Variation from the mean was <10% in each case. Relative specific activities near 4.0 define localization in a given fraction (23). Results here are similar to those we determined with subcellular fractions of pig liver (data not shown).

| Activity | Relative specific activity in | | | |
|---|---|---|---|---|
| | Nuclear fraction | Mitochondrial fraction | Microsomal fraction | Cytosolic fraction |
| Succinate-cytochrome *c* reductase | 1.9 | **5.2** | 1.1 | 0.3 |
| NADH-cytochrome *c* reductase | 2.4 | **3.4** | **4.2** | 0.3 |
| NADPH-cytochrome *c* reductase | 2.4 | 1.6 | **4.0** | 0.5 |

**Microsomal enzymes.** Subcellular fractions of PAEC were analyzed to establish the distribution and identity of possible electron transport partners of P450 and the response to inducers. There was a distinct mitochondrial localization of succinate-cytochrome *c* reductase, whereas NADH-cytochrome *c* (cytochrome $b_5$) reductase was localized in mitochondrial and microsomal fractions (Table 1). NADPH-cytochrome *c* reductase was localized in microsomal fractions. NADPH-cytochrome *c* reductase in nuclear and mitochondrial fractions of PAEC was inhibited 30–60% by 10 μM dicumarol (a diaphorase inhibitor), whereas the microsomal activity was inhibited slightly or not at all (three experiments).

The expression of microsomal NADPH-P450 reductase in PAEC, as indicated by catalytic assay, was substantiated by probing of immunoblots with anti-rabbit liver P450 reductase. In microsomes from control and TCB-treated PAEC, there were two cross-reactive proteins, migrating about 10 kDa apart, with the higher molecular mass being near 78 kDa. Identical bands were detected with the same antibody in pig liver microsomes (Fig. 3). Similarly migrating bands were seen in rabbit liver. The two bands in these samples presumably reflect the intact monomer and the large fragment resulting from cleavage of the membrane anchor peptide from P450 reductase. This cleavage probably occurred after the isolation of microsomes, given the robust protease inhibitors in the homogenization buffer.

Rates of NADPH- and NADH-cytochrome *c* reductase were not affected by treatment of PAEC with 0.1 μM TCB. The rate of NADPH-cytochrome *c* reductase in PAEC microsomes was 4–10-fold less than that in pig liver microsomes (Table 2), whereas the NADH-cytochrome *c* ($b_5$) reductase rate was half the rate in liver microsomes (Table 2). The specific content of cytochrome $b_5$ was similar in control and TCB-treated cells and about 10% of that in liver. PAEC microsomal P450 content was at or below the limits of detection. A chromophore of unknown identity, with a strongly absorbing peak at 414–415 nm, appeared upon $Na_2S_2O_4$ reduction of oxidized or CO-bound microsomes. The signal for this chromophore was similar in microsomes of control and TCB-exposed cultures.

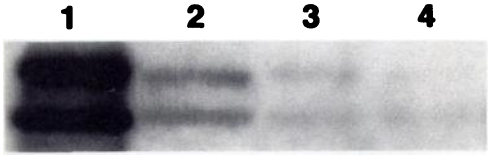


**Fig. 3.** Immunoblot of PAEC microsomes with antibodies to P450 reductase. Microsomal preparations were probed with polyclonal anti-rabbit liver P450 reductase. *Lane 1*, 3-MC-treated rabbit liver; *lane 2*, untreated pig liver; *lane 3*, control PAEC; *lane 4*, 0.1 μM TCB-exposed PAEC. Thirty micrograms of protein were applied to each well. Repeated analyses with and without rabbit liver microsomes on the gel gave similar results for the PAEC. Detection was by enhanced chemiluminescence.

**Immunocytochemistry.** Sheets of cells from confluent PAEC cultures exposed to 0.1 μM TCB showed strong staining by MAb 1-12-3 in the cytoplasm of some cells (Fig. 4). However, there was a heterogeneous response, with alternating patches or individual cells being positive or negative for CYP1A1. In contrast, endothelial cells in the aortae of animals treated with CYP1A inducers *in vivo* typically were uniformly positive (Fig. 4).

**Monooxygenase activity.** Cultures treated with 0.1 μM TCB for 48 hr, freshly harvested, and gently homogenized and sonicated had microsomal EROD activities ranging between 18 and 30 pmol/min/mg and CYP1A1 equivalents of ~5 pmol/mg (Table 2). Neither EROD nor CYP1A1 was detectable in any fraction of control PAEC. Gentle lysis procedures resulted in substantial amounts of CYP1A and EROD activity appearing in nuclear and mitochondrial fractions of TCB-treated cells. Addition of the activity and protein content in all fractions yielded an EROD activity of about 10 pmol/min/mg of total cell protein in PAEC. CYP1A protein in nuclear and mitochondrial fractions could be recovered in a 100,000 × *g* pellet obtained when these fractions were mixed, resuspended, sonicated, and recentrifuged. Nuclear and mitochondrial fractions from that centrifugation retained a small amount of CYP1A1, but NADPH reductase specific activity remaining in the nuclear fractions was like that in microsomes, nearly 40 nmol/min/mg.

Addition of cytochrome *c* at 90 μM to reaction mixtures containing PAEC or pig liver microsomes inhibited EROD activity to below the limits of detection (data not shown). To determine whether P450 activity in PAEC might be limited by the amount of reductase, PAEC microsomes from treated and control cells were augmented with purified rat liver NADPH-cytochrome *c* reductase. Control cell microsomes with added reductase showed no EROD activity, nor did reductase alone. TCB-treated microsomes with added rat reductase showed a 2–5-fold increase in EROD activity. Incubation of PAEC micro-

TABLE 2

**Enzyme activities in PAEC and hepatic microsomes**

| Activity | Control PAEC ($n$ = 4)[a] | 0.1 μM TCB-treated PAEC ($n$ = 4)[a,b] | Untreated pig liver[c] |
|---|---|---|---|
| Total protein yield (mg/T-75 culture) | 1098 ± 79 | 992 ± 119 | |
| Microsomal yield (mg/g of total protein) | 131 ± 19 | 123 ± 39 | 131 |
| P450 (nmol/mg)[d] | 0.015 | <0.007 | 0.49 |
| Cytochrome $b_5$ (nmol/mg)[d] | 0.030 | 0.026 | 0.21 |
| NADPH-cytochrome *c* reductase (nmol/min/mg) | 46 ± 3 | 47± 24 | 371 |
| NADH-cytochrome *c* reductase (nmol/min/mg) | 252 ± 24 | 257 ± 103 | 455 |
| CYP1A1 (pmol equivalents/mg)[e] | ND[f] | 5 ± 1 | ND |
| EROD (pmol/min/mg) | ND | 25 ± 7[g] | 50 |

[a] $n$, number of microsomal preparations analyzed. Two preparations were from single T-75 flasks frozen before harvesting, and two were from cells pooled from four T-75 flasks that were harvested and prepared freshly. EROD values are from the freshly harvested cells only.

[b] Cells were exposed to 0.1 μM TCB for 48 hr before harvesting.

[c] Microsomes prepared from 1 g of liver from the same strain as used for isolation of PAEC.

[d] Single microsomal preparations were analyzed for P450 and cytochrome $b_5$. The P450 content was at or below the limit of detection, which was <0.007 pmol/mg.

[e] Accurate quantitation of putative CYP1A1 content was not possible, due to the lack of a sutable standard (i.e., purified pig CYP1A1) for calibrating the immunoblot results. However, comparison with the staining of CYP1A1 standards from the fish scup (source of the immunogen for MAb 1-12-3) gave a value of 3-5 pmol/mg for CYP1A1 in microsomes from PAEC showing the strongest response to TCB.

[f] ND, not detectable.

[g] For comparison, rat liver microsomes prepared from rats that had been given intraperitoneal injections Aroclor-1254 had EROD and MROD values of 4766 and 594 pmol/min/mg, respectively. The EROD values for untreated rat liver microsomes were 29 pmol/min/mg, similar to those in untreated pig liver.

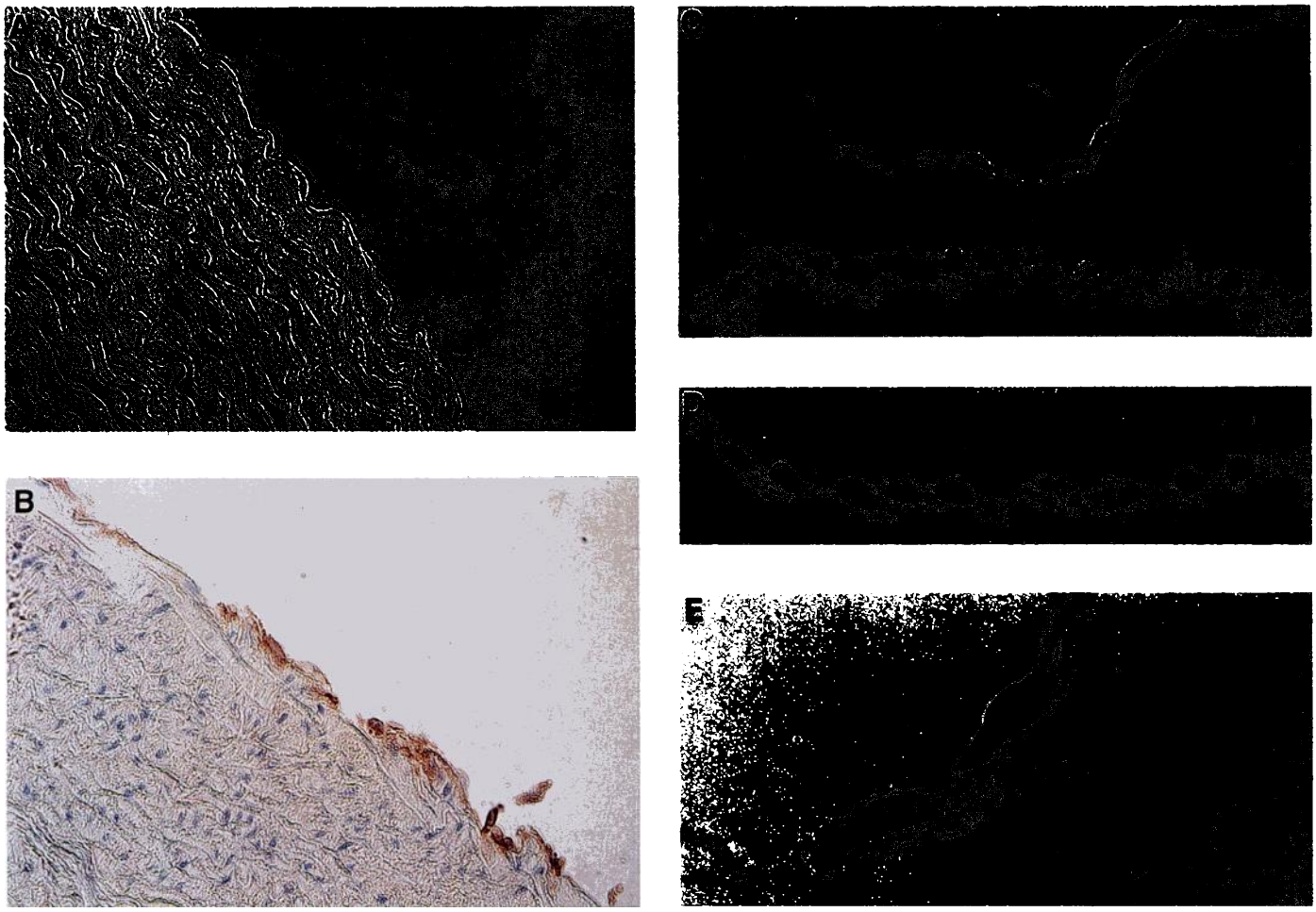


**Fig. 4.** Immunohistochemical demonstration of CYP1A1 in endothelial cells. A, Section through the thoracic aorta of a control (untreated) rabbit. Sections (5 $\mu$m) were stained for CYP1A1 using MAb 1–12–3 and procedures described earlier (31). Aortae from control rabbits receiving carrier only showed no staining in the endothelium. B, Section through the thoracic aorta of a rabbit given subcutaneous injections of 20 mg/kg 3-MC. The aorta was fixed, embedded, and stained as in A. C and D, Stained sections of TCB-treated PAEC. PAEC from replicate 0.1 $\mu$M TCB-exposed and control cultures were fixed in formalin and embedded in paraffin. Replicate TCB-treated cultures were positive; replicate control cultures were uniformly negative. Note the heterogeneity of the response in C and D. Staining of serial sections showed the same patterns of positive and negative cells. E, High magnification of a section through a sheet of PAEC from C.

somes with NADH as cofactor resulted in EROD activity that was only 3% of the activity attained with NADPH (0.5 pmol/min/mg, compared with 15.8 pmol/min/mg in the microsomes assayed), and activity with the two cofactors together was additive.

**Sensitivity of induction and activity in intact cells.** The dose-response curves for induction were determined by measuring rates of EROD in intact cells and CYP1A1 content in lysates of cells exposed to a wide range of TCDD, TCB, and BP concentrations. The patterns of EROD induction by TCDD, TCB, and BP (Figs. 5A and 6A) showed a peak and then a decline of EROD at the higher doses of each compound. That decline was greater with TCB and especially with BP, compared with TCDD. The $ED_{50}$ for induction of EROD by these compounds ranged from 0.015 nM for TCDD to about 180 nM for BP (Table 3). The patterns of MROD induction by TCDD (data not shown) were identical to those for EROD. Acenaphthylene, a preferential inducer of CYP1A2 (33), did not induce either EROD or MROD in these cells. Induction of CYP1A1 protein by TCDD and TCB (Fig. 7) and by BP showed patterns of response like those for EROD induction (Figs. 5B and 6B). In these assays there was a greater plateau in CYP1A1 induction by TCB, whereas the higher doses of TCDD and BP were associated with declines in CYP1A1 content. The $ED_{50}$ values for CYP1A1 induction were similar to those for EROD (Table 3).

To assess whether multiple CYP1A forms are induced in PAEC, lysates from control, TCB-treated, and TCDD-treated cultures were blotted with MAb 1–12–3 or with polyclonal antibodies to mouse Cyp1a-1 and Cyp1a-2. (The specificity of these antibodies is illustrated in Fig. 8A.) MAb 1–12–3 and polyclonal anti-Cyp1a-1 strongly recognized a protein band in the TCDD-treated PAEC, and anti-Cyp1a-2 (which cross-reacts poorly with 1A1) weakly detected a band at the same location (Fig. 8B). Anti-Cyp1a-1 and anti-Cyp1a-2 also reacted with a protein in untreated pig liver microsomes, but there was no MAb 1–12–3-reactive protein in those liver microsomes under any assay conditions.

The maximal rates of EROD activity in intact cells were comparable in cultures treated with BP, TCB, or TCDD, i.e., between 15 and 30 pmol/min/mg (Table 4). MROD activity in the TCDD-treated cells was about one tenth the EROD activity.

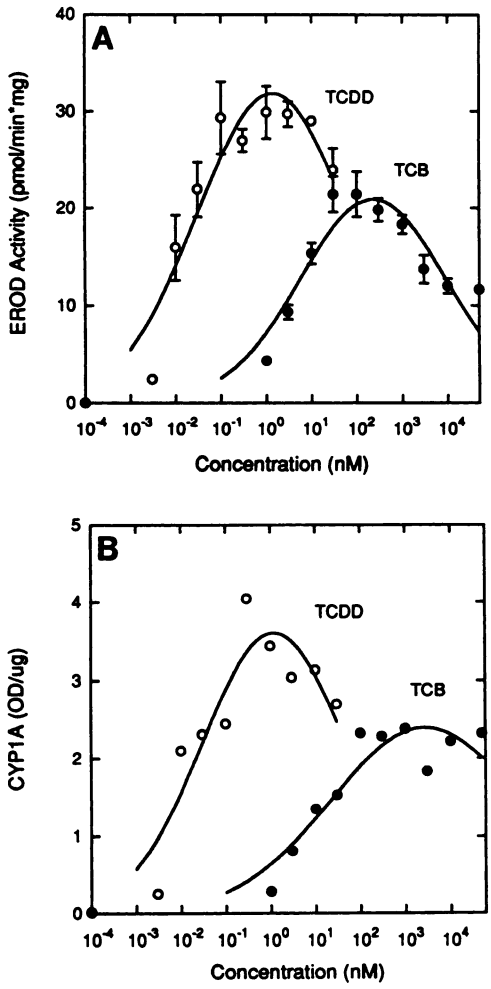


**Fig. 5.** Dose-response curves for CYP1A1 induction in PAEC by TCDD and by TCB. A, Induction of EROD. Rates were measures in intact cells as described in Materials and Methods. Values are mean ± standard error of four cultures. B, Induction of CYP1A1. Values were obtained by immunoblotting of protein pooled from four wells. ○, TCDD; ●, TCB. The results with control (DMSO-treated) cells are placed at the $10^{-4}$ nM position for convenience. The $ED_{50}$ values determined using curve-fitting, as described in Materials and Methods, were 0.016 nM for TCDD and 3–9 nM for TCB. For TCDD this was equivalent to about 1.5 pg/well or about 45 pg/mg of cellular protein.

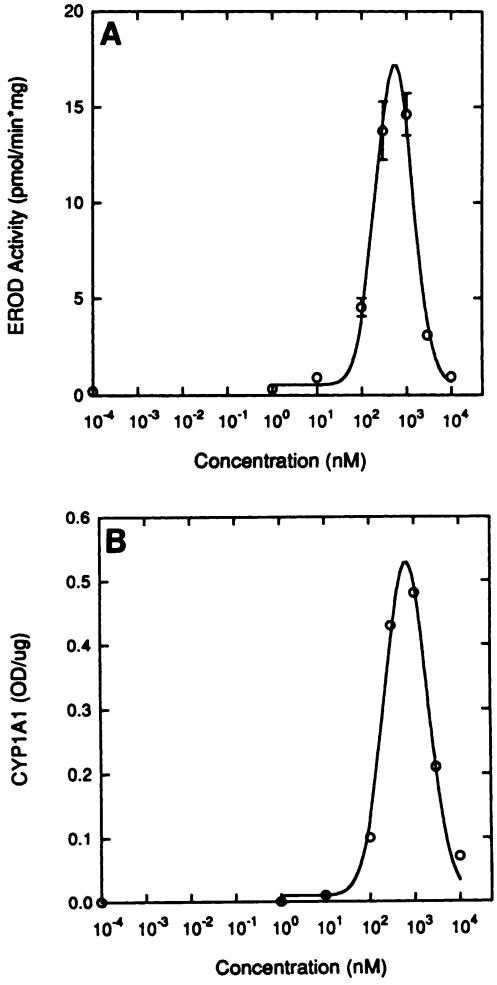


**Fig. 6.** Dose-response curves for CYP1A1 induction in PAEC by BP. A, Induction of EROD, as in Fig. 5. B, Induction of CYP1A1. The $ED_{50}$ values were determined using curve-fitting, as described in Materials and Methods.

TABLE 3

**Induction of P4501A1 in PAEC**

Cells cultured in 48-well plates were exposed to the compounds for 48 hr. The $ED_{50}$ values were calculated according to the equation given in Materials and Methods.

| Inducer | $ED_{50}$, EROD | $ED_{50}$, CYP1A1 | Peak dose, EROD[a] |
|---|---|---|---|
| | nM | nM | nM |
| TCDD | 0.015 | 0.016 | 1.0–3.0 |
| TCB | 3.0 | 8.8 | 30–100 |
| BP | 180 | 182 | 300–1000 |

[a] Peak dose, dose that elicited maximal EROD activity.

Notably, addition of NADPH to the alkoxyresorufin-containing buffer bathing the cells in the deethylase assays did not produce any change in the rates of EROD or MROD. These rates measured in intact cells imply a greater capacity for CYP1A1 activity per cell than that inferred from microsomal rates alone (see Table 2). However, the EROD rate of 22 pmol/min/mg of total protein in cells treated with 0.1 μM TCB was similar to the sum of activities in the subcellular fractions of cells treated with 0.1 μM TCB, i.e., about 10 pmol/min/mg of total cell protein.

## Discussion

**CYP1A proteins induced in PAEC.** The induction of CYP1A1 in PAEC exposed to TCDD, TCB, BNF, or BP shows that mammalian large vessel endothelia in culture retain the capacity for response to toxic and carcinogenic AhR agonists; with antibodies to mouse AhR we recently confirmed the expression of AhR in PAEC in culture.[3] The immunostaining signal achieved with antibodies to CYP1A forms indicates that CYP1A1 can be strongly induced in PAEC, consistent with the immunohistochemical demonstration of strong induction by AhR agonists of CYP1A1 in endothelium of diverse vertebrates *in vivo* (8–12). Significantly, our results also show that NADPH-P450 reductase is expressed in endothelial cells in culture and that the capacity for reducing P450 inherent in intact cells is sufficient to support CYP1A activities.

Unlike CYP1A1, CYP1A2 is induced only weakly or not at

[3] M. E. Hahn and J. J. Stegeman, unpublished observations.

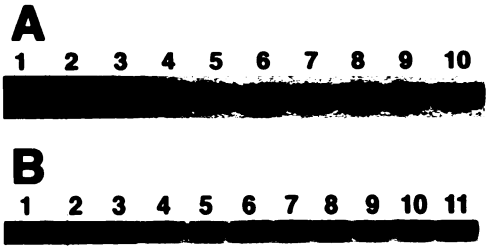


**Fig. 7.** Immunoblot of TCB-exposed and TCDD-exposed PAEC. Cells in 1-cm² cultures were exposed to the compounds. After 48 hr, the cells were solubilized, the entire lysate from two wells at each dose was pooled, protein was determined, and aliquots (50 ± 5 μg) were electrophoresed on a large 12% acrylamide gel. Samples were blotted and analyzed as in Fig. 2B. A, TCDD-treated cells. *Lanes 1–10*, 0 (control), 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 nM TCDD, respectively. B, TCB-treated cells. *Lanes 1–11*, 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 50 μM TCB, respectively. Visualization was by enhanced chemiluminescence.

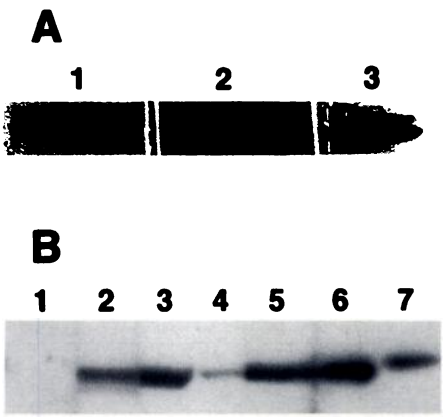


**Fig. 8.** Immunoblot with antibodies to CYP1A1 and CYP1A2 forms. A, Analysis of Aroclor 1254-treated rat liver microsomes, illustrating the specificity of the antibodies used. In each lane, 0.2 μg of microsomal protein from Aroclor-induced rat liver was applied. Primary antibodies were as follows: *lane 1*, MAb 1–12–3; *lane 2*, anti-Cyp1a-1 (Peg); *lane 3*, anti-Cyp1a-2 (Rye). Color development was with NBT and BCIP. B, Pig liver and PAEC microsomes probed with MAb 1–12–3 and the polyclonal antibodies listed in A. Cultures of PAEC that were exposed to 0.3 or 1.0 nM TCDD were lysed, lysates were pooled, and 30 μg of protein from the pool were applied to five-lane mini-gels. Thirty micrograms of untreated pig liver microsomal protein were also used. After transfer, the lanes were split and developed with different antibodies. The lanes and primary antibodies were as follows: *lanes 1-3*, pig liver microsomes, developed with MAb 1–12–3 (*lane 1*), anti-Cyp1a1 (*lane 2*), or anti-Cyp1a2 (*lane 3*); *lanes 4-6*, TCDD-treated PAEC lysates developed with MAb 1–12–3 (*lane 6*), anti-Cyp1a1 (*lane 5*), or anti-Cyp1a2 (*lane 4*); *lane 7*, 0.1 pmol of scup CYP1A1, developed with MAb 1–12–3. Visualization was by enhanced chemiluminescence.

all in PAEC. Polyclonal antibodies to mammalian CYP1A1 and CYP1A2 that recognize both proteins strongly recognized a band in untreated pig liver microsomes, whereas MAb 1–12–3, which is highly specific for CYP1A1, did not. This indicates that CYP1A2 but not CYP1A1 is expressed in liver of untreated pigs, as in other untreated mammals (34). By comparison, the faint recognition by anti-CYP1A2 of a band in PAEC suggests that CYP1A2 is weakly induced or not induced in these cells. In PAEC, TCDD only slightly induced MROD, a preferred but not exclusive 1A2 activity (35), and the 1A2 inducer acenaphthylene was without effect, further indicating little or no induction of 1A2 in these cells. Interestingly, both CYP1A1 and CYP1A2 were detected in a fraction enriched in endothelial cells from liver of PCB- or 3-MC-treated rats (36), but possible

TABLE 4
**P4501A activities in intact PAEC**

| Treatment/ inducer[a] | Dose | *n*[b] | EROD[c] | MROD[c] |
|---|---|---|---|---|
| | | | *pmol/min/mg* | *pmol/min/mg* |
| None | | 20 | ND[d] | ND |
| DMSO | | 20 | ND | ND |
| TCDD | 1.0 nM | 4 | 30 ± 2 | 2.0 ± 0.2 |
| TCB | 0.1 μM | 4 | 22 ± 3 | |
| BP | 0.3 μM | 4 | 15 ± 6 | |
| Acenaphthylene | 0.01–10.0 μM | 4 | ND | ND |

[a] Cell cultures in 48-well plates were exposed to the compounds for 48 hr.
[b] *n*, number of different cultures analyzed at that concentration. The TCDD, TCB, and BP doses shown were those eliciting maximal induction. Four doses of acenaphthylene were tested; all were negative.
[c] Activity per mg of whole-cell protein is given. Medium was removed and buffer containing ethoxy- or methoxyresorufin was added to the wells without lysis of the cells. Reactions were monitored for 10 or 20 min. Incubation of cultures in the absence of cofactor gave results similar to those for cultures with cofactor added.
[d] ND, not detectable. Limits of detection in the assays were <0.3 pmol of resorufin/min/mg of protein.

contamination of that endothelial fraction by liver parenchymal cells, which express both CYP1A forms, cannot be ruled out. Recently, Farin *et al.* (37) reported that transcripts for both CYP1A1 and CYP1A2 were induced by BNF and Aroclor 1254 (mixed PCBs) in human umbilical vein endothelial cells. Translation products were not examined in that study. Thus, whether CYP1A2 might be slightly induced in some endothelia is still an open question.

It is unlikely that we are detecting a novel CYP1 protein in induced PAEC. Analyses to date have not indicated more than two CYP1A genes in mammals (6). Sutter *et al.* (38) described a new dioxin-inducible *CYP1* gene, *CYP1B1*, in humans. The amino acid sequence of CYP1B1 is about 40% identical to those of CYP1A1/1A2, suggesting that there would be limited cross-reactivity with antibodies to CYP1A. Whether induction of CYP1B1 transcript in multiple organs (38) involves the endothelium is an important concern yet to be addressed.

**Dose-response curves.** The $ED_{50}$ values estimated for CYP1A1 induction in PAEC ranged from 180 nM for BP to 0.015 nM for TCDD. The relative sensitivities and structure-activity relationships for induction in different cell types and species are important for the identification of target sites for toxicity and for the derivation of biologically relevant toxic equivalency factors, which have become important in risk assessment. The $ED_{50}$ for TCDD in PAEC is quite similar to the $ED_{50}$ reported for TCDD induction of CYP1A1 activity in the rat liver cell line H4IIE (0.018 nM) (39) or in mouse Hepa-1 cells (0.016 nM) (40). It will be important to determine whether endothelia respond to AhR agonists *in vivo* with the same sensitivity as *in vitro*.

PAEC exposed to higher doses of BP, BNF, TCB, or TCDD showed variable suppression of CYP1A1 and/or EROD induction. High doses of TCB are known to suppress EROD activity in livers of animals exposed *in vivo* and in hepatocytes exposed in culture (25), in large part by competitive inhibition of CYP1A by TCB retained in microsomes. Hahn[4] recently determined a $K_i$ of 0.02 μM for inhibition of EROD by TCB. BP could similarly inhibit and possibly inactivate CYP1A1. A decline in

[4] M. E. Hahn, unpublished observations.

CYP1A1 protein content was observed with higher concentrations of TCDD, BP, BNF, and TCB; further studies are necessary to clarify the extent, mechanisms, and significance of the decrease in CYP1A1 content.

**Catalytic activity.** The degree to which CYP1A1 in the endothelium is catalytically active is crucial to interpretations regarding the significance of induction in this cell type; some have questioned whether it is active at all (11). The induction of EROD in PAEC cultures induced by TCDD, TCB, and BP shows that CYP1A is active in these intact endothelial cells. The metabolism of BP by 3-MC-treated human umbilical vein endothelial cells (41) indicates induction of a functional CYP1A in that endothelial cell type as well. Significantly, rates of EROD or MROD in intact PAEC were unaffected by added NADPH (which should not cross the plasma membrane), indicating that the rates reflect metabolism in cells relying on endogenous cofactor. The metabolic capacity in a given cell could be much greater than that inferred from whole cultures, because only some cells in treated cultures showed induction of CYP1A1. The basis for that heterogeneous response is not known. CYP1A1 activity in intact cells could explain the decrease in CYP1A1 between 24 and 48 hr in PAEC exposed to 0.1 $\mu$M BNF, which could have been metabolized to levels that were no longer effective. Chlorobiphenyls such as TCB are metabolized more slowly by CYP1A1 than is BNF. Consistent with that, there was no temporal decline in induction in PAEC given 0.1 $\mu$M TCB. Likewise, there was no decline in cultures given BNF at 10 $\mu$M, a concentration at which parent compound could be expected to persist at active inducing levels for >48 hr. Studies of CYP1A1 transcription and of metabolism of different inducers over time are required.

Several studies indicate that CYP1A1 is catalytically active also in endothelia induced *in vivo*. Earlier, CYP1A1 protein, EROD, AHH, and the formation of 7,8- and 9,10-dihydrodiols of BP (activities of CYP1A1) all were demonstrated in induced scup heart, where CYP1A1 occurs only in the endothelium (10, 222. Similar studies were performed recently with rat aorta (42), and Brittebo (43) recently reported that tryptophan pyrolysis products (promutagens activated by CYP1A) were activated *in situ* in endothelium of BNF-treated rats. Fractions enriched (88% pure) in endothelial cells from liver of 3-MC- or PCB-treated rats had elevated CYP1A1 and EROD levels (36), suggesting that CYP1A1 is functional in rat liver endothelium, although parenchymal cells could have contributed to the endothelial cell fractions in that study.

Microsomal EROD rates in highly induced PAEC (25 pmol/min/mg) were like the rates in untreated pig liver, in which CYP1A1 was not detectable. Catalytic rates less than expected based on the amounts of CYP1A1 could result from a deficiency in reductase in endothelia (10, 22). Overby *et al.* (11) could not detect expression of P450 reductase in rabbit lung endothelium by immunohistochemistry or by *in situ* hybridization. Our results showing microsomal localization of NADPH-cytochrome *c* reductase and of protein cross-reacting with anti-P450 reductase and the demonstration of microsomal monooxygenase activity indicate that a microsomal P450 reductase is expressed in PAEC. Earlier we showed that NADPH could elicit a P450 spectrum when added to CO-treated cardiac microsomes of induced scup, indicating electron transfer via P450 reductase (22). Thus, the apparent lack of reductase noted in rabbit lung (11) could reflect species differences in reductase expression in endothelium, differences in reductase expression in different types of endothelium (44) (for example, large vessel and microvascular endothelium like that in lung), or differences between intact endothelium and cells in culture. Despite the presence of reductase in PAEC, addition of purified rat liver P450 reductase enhanced PAEC microsomal EROD activity, suggesting that PAEC P450 reductase might not be sufficient to support full function of CYP1A1. Binding of endogenously generated ligands, such as NO, or insufficient heme to occupy all of the apoenzyme synthesized also might result in an apparently less efficient CYP1A1.

Overby *et al.* (11) speculated that endothelial CYP1A1 function might be supported by electron transfer from cytochrome $b_5$ reductase and cytochrome $b_5$ or via hydroperoxides generated endogenously. Rates of NADH-cytochrome *c* ($b_5$) reductase in PAEC were nearly as high as those in liver (Table 2). However, the content of cytochrome $b_5$ in PAEC was slight and NADH only very weakly supported microsomal EROD activity, suggesting that cytochrome $b_5$ reductase and cytochrome $b_5$ do not actively reduce P450 in the endothelium. We did see a dicumarol-sensitive component of NADPH-cytochrome *c* reductase in PAEC nuclear/mitochondrial fractions, implying the presence of a diaphorase. No intermediate electron acceptors were added, suggesting involvement of an endogenous quinone (45). A novel NADPH oxidase has been seen in plasma membranes of endothelium and adventitia (46). Whether the dicumarol-sensitive reductase and NADPH oxidase activities are related or involved with P450 is not known.

**Significance of endothelial CYP1A.** Active CYP1A1 in endothelium could metabolize and alter the action of cardiovascular agents and other drugs and could influence vascular function or health. Vascular neoplasms ostensibly endothelial in origin (47) and atherogenesis could result from CYP1A1 activation of PAH promutagens like those in cigarette smoke. Altered metabolism of endogenous signaling molecules in the endothelium could result from induction of functional CYP1A1, leading to toxicity and/or edema. AA and vasoactive eicosanoids are substrates for CYP1A1, and Rifkind *et al.* (48) have suggested that altered AA metabolism is one avenue by which TCDD is toxic. The cellular location of such toxicity is not known but could involve the endothelium, which, as the source of the AA metabolite prostacyclin, exerts vasoactive and platelet antiaggregatory activity. As we speculated some years ago (10), induced CYP1A content in endothelium also might bind NO, which has a great affinity for heme proteins, possibly affecting the function of CYP1A as well as the availability of NO.

**Function as a binding protein?** Whether catalytically efficient or not, CYP1A1 in endothelium could function in "first-pass" protection of other tissues. Substrate binding is the first step in the catalytic cycle, and catalytically inefficient P450 could serve as a binding protein, sequestering CYP1A substrates and AhR agonists. Such a function has been ascribed to CYP1A2 in liver (49, 50). That does not preclude a similar function for CYP1A1. Slowly metabolized substrates/agonists might be effectively "bound" by endothelial CYP1A1, affecting the concentrations reaching target cells that underlie the endothelium and limiting the penetration of low concentrations of such compounds into distal areas of the organism. There is evidence for the latter possibility. In scup the potent AhR agonist 2,3,7,8-tetrachlorodibenzofuran given at 3 $\mu$g/kg and

the less potent agonist TCB given at 1 mg/kg produced similar responses in liver and other sites proximal to the larger vessels, but 2,3,7,8-tetrachlorodibenzofuran elicited much weaker or no response distally (12). In smokers, elevated AHH activity was seen in placenta but not in umbilical vein (41), which could reflect induction of CYP1A1 and subsequent clearance of inducers by the placental endothelium. Endothelium can constitute between 0.5 and 1.5% of body weight, depending on the species. Endothelium with elevated CYP1A could equal the importance of the liver in the pharmacokinetics of AhR agonists and, we suggest, could affect the shape of dose-response curves at low doses. Additional studies with cells in culture should shed light on the significance of CYP1A1 in endothelial cell functions, the capacity of CYP1A1 to sequester or metabolize inducers, and the use of endothelial cell CYP1A1 induction as a marker of exposure, an approach we are evaluating in fish, marine mammals, and humans.

**Acknowledgments**

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