

## Proinflammatory Properties of Coplanar PCBs: *In Vitro* and *In Vivo* Evidence

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So-called coplanar polychlorinated biphenyls (PCBs), as well as other environmental contaminants that are aryl hydrocarbon receptor (AhR) agonists, may compromise the normal functions of vascular endothelial cells by activating oxidative stress-sensitive signaling pathways and subsequent proinflammatory events critical in the pathology of atherosclerosis and cardiovascular disease. To test this hypothesis, porcine endothelial cells were exposed to PCB 153 and to three coplanar PCBs (PCB 77, PCB 126, or PCB 169). In contrast to PCB 153, which is not a ligand for the Ah receptor (AhR), all coplanar PCBs disrupted endothelial barrier function. All coplanar PCBs increased expression of the CYP1A1 gene, oxidative stress (DCF fluorescence), and the DNA-binding activity of nuclear factor  $\kappa$ B (NF- $\kappa$ B). PCB-induced oxidative stress was concentration-dependent, with PCB 126 exhibiting a maximal response at the lowest concentration (0.5  $\mu$ M) tested. The increase in NF- $\kappa$ B-dependent transcriptional activity was confirmed in endothelial cells by a luciferase reporter gene assay. In contrast to PCB 153, coplanar PCBs that are AhR ligands increased endothelial production of interleukin-6. At 3.4  $\mu$ M, expression of the adhesion molecule VCAM-1 was most sensitive to PCB 77 and 169. We also provide *in vivo* evidence, suggesting that binding to the AhR is critical for the proinflammatory properties of PCBs. Twenty hours after a single administration of PCB 77, VCAM-1 expression was increased only in wild-type mice, while mice lacking the AhR gene showed no increased staining for VCAM-1. These data provide evidence that coplanar PCBs, agonists for the AhR, and inducers of cytochrome P450 1A1, produce oxidative stress and an inflammatory response in vascular endothelial cells. An intact AhR may be necessary for the observed PCB-induced responses. These findings suggest that activation of the AhR can be an underlying mechanism of atherosclerosis me-

diated by certain environmental contaminants. © 2002 Elsevier Science (USA)

**Key Words:** polychlorinated biphenyls; environmental contaminants; aryl hydrocarbon receptor; oxidative stress; endothelial cells; atherosclerosis; disease.

Dysfunction of endothelial cells is a critical underlying cause of the initiation of cardiovascular diseases, such as atherosclerosis. In addition to endothelial barrier dysfunction, another functional change in atherosclerosis is the activation of the endothelium that is manifested as an increase in the expression of specific cytokines and adhesion molecules. These cytokines and adhesion molecules are proposed to mediate the inflammatory aspects of the disease by regulating the entry of leukocytes, both macrophages and lymphocytes, into the vascular wall. There is evidence that the CYP1A subfamily is present in most and possibly all animal and human systems. For example, CYP1A has been detected in endothelial linings of fish and rodents, as well as in cultured human and porcine endothelial cells (Smolowitz *et al.*, 1992; Stegeman *et al.*, 1995; Toborek *et al.*, 1995; Celander *et al.*, 1997; Zhao *et al.*, 1998; Hennig *et al.*, 1999; Annas *et al.*, 2000; Schlezinger and Stegeman, 2001).

There is increasing evidence that exposure to polycyclic aromatic hydrocarbons can lead to cardiovascular toxicity and atherosclerosis. For example, there was a significant increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to PCBs for at least 5 years (Gustavsson and Hogstedt, 1997), and most excess deaths were due to cardiovascular disease in power workers exposed to phenoxy herbicides and PCBs in waste transformer oil (Hay and Tarrel, 1997). Furthermore, the latest studies on the Seveso population (as a result of the industrial accident that occurred in the town of Seveso, Italy, in 1976) now detect an increase in cardiovascular disease (Bertazzi *et al.*, 1998). Finally, a recent report by Tokunaga *et al.* (1999) confirms many other studies with chronic Yusho patients (accidental ingestion

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of rice-bran oil contaminated with PCBs), which showed in this population that elevated serum levels of triglycerides and total cholesterol were significantly associated with the blood PCB levels.

Certain environmental chemicals, such as PCBs or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), can cause vascular endothelial cell dysfunction (Stegeman *et al.*, 1995; Toborek *et al.*, 1995; Hennig *et al.*, 1999). Several studies suggest that a critical underlying mechanism of PCB-mediated endothelial cell activation and dysfunction is an increase in cellular oxidative stress (Smith *et al.*, 1995; Oakley *et al.*, 1996; Machala *et al.*, 1998; Narayanan *et al.*, 1998; Hennig *et al.*, 1999). There is also evidence which suggests that the oxidative stress induced by specific environmental contaminants, i.e., aromatic hydrocarbons like PCB 77 or TCDD, as well as endogenous compounds such as arachidonic metabolites (Schaldach *et al.*, 1999), is due to the interaction of these compounds with the AhR and activation of the cytochrome P450 1A subfamily (Nebert, 1989; Alsharif *et al.*, 1994; Safe and Krishnan, 1995; Toborek *et al.*, 1995; Hennig *et al.*, 1999). Induction of CYP1A1 or 1A2 may lead to oxidative stress as a result of excessive generation of reactive oxygen species (Morehouse *et al.*, 1984; Stohs, 1990; Shertzer *et al.*, 1998; Schlezinger and Stegeman, 2001), which can result in an imbalance in the cellular oxidative stress/antioxidant balance and thus cause cell injury. Recently, Schlezinger *et al.* (1999) have demonstrated that PCB 77 can uncouple the catalytic cycle of cytochrome P4501A1, resulting in the formation of reactive oxygen species within the active site. Thus, heme iron may undergo cycles of oxidation and reduction and hence act as a Fenton catalyst, generating hydroxyl radicals and other reactive oxygen species. There is strong evidence that the vascular endothelium may be one of the major sites of PCB-mediated induction of CYP1A1 (Farin *et al.*, 1994; Stegeman *et al.*, 1995), and that these enzymes play important roles in determining the metabolic fates of circulating toxic substances. Furthermore, a strong correlation was demonstrated between cytochrome P450 1A and endothelial cell activation or injury in TCDD-mediated pericardial edema and cardiovascular dysfunction (Cantrell *et al.*, 1996; Guiney *et al.*, 1997; Hornung *et al.*, 1999).

Transcriptional regulation of metabolic events leading to endothelial cell dysfunction and an inflammatory response induced by AhR agonists, such as coplanar PCBs, is not well understood. Recent evidence suggests that the AhR agonist TCDD can activate NF- $\kappa$ B and AP-1, and it was proposed that CYP1A1-dependent and AhR complex-dependent oxidative signals are in part responsible for the observed activation of these transcription factors (Puga *et al.*, 2000). Furthermore, a dose-dependent activation of NF- $\kappa$ B was observed in fish exposed to coplanar PCBs (Schlezinger *et al.*, 2000). Our present data indicate that coplanar PCBs that function as AhR agonists may be proinflammatory and atherogenic by activating NF- $\kappa$ B in vascular endothelial cells. We also provide *in vivo* evidence using an AhR-deficient mouse model that a

functional AhR is critical for the proinflammatory events mediated by coplanar PCBs.

## MATERIALS AND METHODS

**Synthesis of the PCBs.** PCB 77 and PCB 153 were synthesized as described by Schramm *et al.* (1985). PCBs were purified by Florisil (Macherey-Nagel, Düren, Germany) and Alumina (Aluminiumoxid 90, Merck, Darmstadt, Germany) chromatography and recrystallization from methanol. Structural assignments were confirmed by nuclear magnetic resonance spectrometry and mass spectroscopy. PCBs 126 and 169 were purchased from Ultra Scientific (North Kingstown, RI).

**Cell culture and experimental media.** Endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described (Hennig *et al.*, 1984). Cultures were verified as endothelial cells by uniform cobblestone morphology and by quantitative determination of angiotensin-converting enzyme activity or by their uptake of fluorescent labeled acetylated low-density lipoprotein (LDL; 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc., Eugene, OR). The basic culture medium consisted of M199 (GIBCO Laboratories, Grand Island, NY) containing 10% bovine calf serum (BCS; HyClone Laboratories, Inc., Logan, UT). The experimental media were composed of M199 enriched with 5% FBS and the coplanar PCBs, PCB 77 (3,3',4,4'-tetrachlorobiphenyl), PCB 126 (3,3',4,4',5-pentachlorobiphenyl), or PCB 169 (3,3',4,4',5,5'-hexachlorobiphenyl) or the non-coplanar PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl). In most experiments, PCBs were solubilized in DMSO and used at a concentration of 3.4  $\mu$ M. This level was chosen because it reflects serum concentrations after exposure to PCBs (Jensen 1989; Wassermann *et al.*, 1979). Optimal specific time points for gene expression used in the present experimental design were characterized and reported previously (Toborek *et al.*, 1995; Hennig *et al.*, 1999; Slim *et al.*, 1999).

**Endothelial barrier function (albumin transfer studies).** Endothelial barrier function was measured as transendothelial albumin transfer using polystyrene chambers with a 0.8- $\mu$ m pore size polycarbonate membrane (Millipore Corporation, Bedford, MA) according to the method of Hennig *et al.* (1984). After approximate confluence, endothelial monolayers were exposed to control or PCB-enriched media. Following treatments, chambers with endothelial cells attached to the membranes were washed with M-199 and exposed to 200  $\mu$ M bovine serum albumin (fatty acid-free, Sigma Chemical Company, St. Louis, MO) in M199 for 1 h. After incubation with albumin, the albumin transferred across endothelial monolayers was determined using bromocresol green (Sigma) and recorded spectrophotometrically at 630 nm.

**Measurement of oxidative stress.** The effect of exposure to PCBs on cellular oxidation was determined by dichlorofluorescein (DCF) assay (Mattson *et al.*, 1995) and modified for use by a fluorescent microplate reader. Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) passively diffuses into cells, is deacetylated (DCFH<sub>2</sub>), and subsequently oxidized by reactive oxygen species to yield fluorescent 2',7'-dichlorofluorescein. After treatment of endothelial cells with PCBs (153, 77, 126, or 169) for up to 3 h, cells were incubated in the presence of 10  $\mu$ M H<sub>2</sub>DCFDA for 30 min at room temperature. Before analysis, cells were washed twice with Hanks and once with 10 mM Hepes buffer (pH 7.4), and the fluorescence within the dye loaded cells was measured using a Spectramax GeminiXS (Molecular Devices, Sunnyvale, CA) microplate spectrofluorometer with excitation and emission wavelengths of 490 and 510 nm, respectively.

**Electrophoretic mobility-shift assays.** Nuclear extracts from endothelial cells were prepared according to the method of Dignam *et al.* (1983). Binding reactions were performed in a 20- $\mu$ L volume containing 7  $\mu$ g of nuclear protein extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 0.5  $\mu$ g of poly[dI-dC] (nonspecific competitor) and 40,000 cpm of <sup>32</sup>P-labeled specific oligonucleotide probe. Double-stranded oligonucleotides containing a tandem duplicate of the NF- $\kappa$ B binding site (underlined) (5'-AGTTGAGGGGACTTCCAGGC-3') were radiolabeled

with [ $\gamma$ - $^{32}$ P]ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase. Resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using  $0.25 \times$  TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were performed by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction. Rabbit polyclonal anti-NF- $\kappa$ B p65 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and employed in supershift experiments. Densitometric quantitations of these and other gels were performed using Un-Scan-It software (Silk Scientific, Inc., Orem, UT).

**Measurement of cytosolic I $\kappa$ B.** Endothelial cells were grown until confluent on 60-mm plates, and cytosolic extracts were prepared and then electrophoresed as described by Sambrook *et al.* (1989). Briefly, cell monolayers were harvested by scraping, washed in cold PBS, and incubated in 200  $\mu$ L lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL pepstatin, 1  $\mu$ g/mL leucine thiol, 0.1% Nonidet P-40) for 15 min on ice. The crude nuclei were then pelleted by centrifugation, and the supernatants (cytosolic extracts) were collected and stored at  $-70^{\circ}\text{C}$ . Cytosolic extracts were electrophoresed on 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, 20% methanol at 37 mA for 1.0 h ( $\kappa$ B) at  $4^{\circ}\text{C}$ . Western blots were analyzed for I $\kappa$ B- $\alpha$ . Anti-I $\kappa$ B- $\alpha$  rabbit polyclonal antibodies (IgG isotypes, Santa Cruz Biotechnology) were diluted 1:500 in blocking buffer. Then, immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (ECL Western blotting detection reagents; Amersham Life Sciences, Piscataway, NJ) using 1:2000 horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody.

**Reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was extracted by the use of TRI reagent (Sigma, St. Louis, MO) and reverse-transcribed at  $42^{\circ}\text{C}$  for 60 min in 20  $\mu$ L of 5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/ $\mu$ L of recombinant RNasin ribonuclease inhibitor, 15 U/ $\mu$ g of AMV reverse transcriptase, and 0.5  $\mu$ g of oligo(dT)<sub>15</sub> primer. Specific primer sequences were designed using the software package Oligo 6.0 (National Biosciences Inc., Plymouth, MN) and were synthesized by IDT Technologies, Inc. (Coralville, IA). The primer combinations used for VCAM-1 were: sense, 5'-GTTTACCCGGTT-GAAAAGTTGGAG-3'; antisense, 5'-CACCGTGTCGCCTGTCTCT-3'; and for CYP1A1: sense: 5'-TGGAGAGGCAAGAGTAGTTGG-3'; antisense: 5'-GGCACAACGGAGTAGCTCATA-3'. Oligonucleotide primers to amplify the porcine housekeeping gene  $\beta$ -actin were used according to Barchowsky *et al.* (1998). The PCR mixture consisted of a Taq PCR Master Mix (Qiagen, Valencia, CA), 2  $\mu$ L of the reverse-transcriptase enzyme, and 20 pmol of primer pairs in a total volume of 50  $\mu$ L. Thermocycling was performed with annealing temperatures of  $57^{\circ}\text{C}$  for CYP1A1 and  $61^{\circ}\text{C}$  for VCAM-1 for 30 and 27 cycles, respectively. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Eugene, OR), and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

**Transfection and dual luciferase assays.** Transient transfections of endothelial cells were performed using pF $\times$ -7 (Invitrogen, Carlsbad, CA) as described earlier (Lee *et al.*, 2001). Cells were transfected with 10  $\mu$ g of the firefly luciferase reporter plasmids containing the human NF- $\kappa$ B promoter sequences. The cells were cotransfected with 0.5  $\mu$ g of the *Renilla* luciferase control vector (pRL-SV40; Promega, Madison, WI) to normalize for transfection efficiency. Following transfections, cultures were maintained in normal growth medium for 24 h and then exposed to PCBs for an additional 18 h in M199 media enriched with 10% FBS. All detection of firefly and *Renilla* luciferase activities was performed using the Dual-Luciferase reporter assay system (Promega). Briefly, the cells were washed with phosphate-buffered saline and lysed with Passive lysis buffer (Promega). Cell lysates were mixed with Luciferase Assay Reagent II, and the firefly luminescence was measured using a luminometer with dual automatic injector (Turner Designs, Sunnyvale, CA). The samples were then mixed with the Stop & Glo reagent, and the *Renilla* luciferase activity was measured as an internal control.

**Interleukin-6 production.** After experimental treatments, media were removed from the wells and frozen immediately at  $-80^{\circ}\text{C}$  until interleukin-6 (IL-6) analysis. The remaining cells were trypsinized and washed with phosphate-buffered saline (PBS) twice and resuspended in 0.2% SDS with 0.2 M NaOH for protein analysis (Lees *et al.*, 1972) or washed with PBS and stained with trypan blue to determine cell viability. Each experimental group was done in triplicate, and total protein as well as cell viability were measured. IL-6 production and release into the medium were determined using the murine hybridoma cell line B9 (kindly supplied by Dr. L. A. Aarden, Emeryville, CA) as described by Helle *et al.* (1988). Viability of the B9 cell line is IL-6 dependent. Thus, the incorporation of [ $^3\text{H}$ ]thymidine by growing cells was a reflection of the quantity of IL-6 produced by endothelial cells. The final IL-6 concentration was determined from the standard curve, where 8 pg of IL-6 per mL media corresponds to  $12.6 \times 10^7$  units/mg recombinant IL-6.

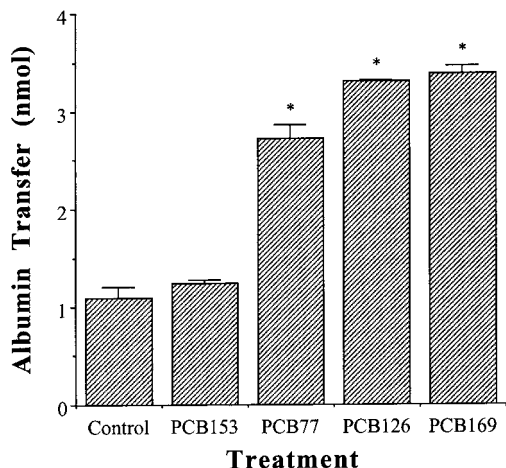
**Animal studies.** The AhR-deficient mice used in this study have been characterized recently (Vorderstrasse *et al.*, 2001). The AhR $+/+$  and AhR $-/-$  mice were derived from a heterozygous cross ( $+/-$ ) from founder mice initially provided by P. Fernandez-Salguero and F. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD). These mice are of a mixed genetic background (129/SV $\times$ C57B1/6N). Homozygous  $-/-$  and  $+/+$  mice were maintained by brother-sister mating for more than four generations. Because of restricted availability of the AhR-deficient mice, only a limited number of animals (three mice per treatment group) were used in this study. Mice were injected intraperitoneally with PCB 77 (170  $\mu$ mol/kg mouse) or the vehicle (stripped corn oil; Acros Chemical Company, Pittsburgh, PA) and 20 h later, aortic tissue was obtained for analysis. According to our combined experience with several animal species, long-term intraperitoneal injections of 100 to 300  $\mu$ mol/kg body weight per injection are sufficient to initiate disease states such as tumor production. In our preliminary studies we saw adhesion molecule expression at 170  $\mu$ mol/kg mouse per injection, with the assumption of an initial 100% absorption into the circulation. This amount of PCB was based on calculated values from our *in vitro* experiments which were based on levels that are usually found in humans after acute exposure.

**Immunostaining of aortic tissue.** Aortic tissue from the thoracic regions was dissected free, immersed in OCT embedding media, frozen at  $-20^{\circ}\text{C}$ , and 8- $\mu$ m sections were cut on a cryostat. Immunocytochemistry was performed as described previously (Daugherty *et al.*, 1997). Briefly, endogenous peroxidase was abolished using hydrogen peroxide (3%) in methanol. Samples were blocked in the serum of the secondary antibody host. Primary antibodies for VCAM-1 (Catalog No. 01811D; PharMingen, San Diego, CA) were detected using biotinylated secondary antibodies and peroxidase ABC kits (Vectastain, Burlingame, CA). Aminoethylcarbazole was used as chromogen, and sections were counterstained with hematoxylin.

**Statistical analysis.** The data were analyzed using SYSTAT 7.0 (SPSS, Inc., Chicago, IL). Comparisons between treatments were made by one-way ANOVA with post hoc comparisons of the means made by Fischer's least significance difference method (Snedecor *et al.*, 1974). Statistical probability of  $P < 0.05$  was considered significant.

## RESULTS

Time points of the different experiments were chosen according to the following rationale. Our previous DCF fluorescence studies (oxidative stress) suggested that PCB-mediated formation of oxidative stress is not instantaneous. We found maximum changes at about 2 to 3 h after exposure, with minimum change at earlier (less than 2 h) or later (after 6 h) time points. Thus, a 3-h treatment regimen was selected to investigate whether an early upregulation in the expression of the CYP1A1 gene may act as an initiating event in the overall scenario in induction of oxidative stress and endothelial cell



**FIG. 1.** Effect of cellular incubation with PCB 153 or the coplanar PCBs 77, 126, or 169 on endothelial barrier function. Cultures were exposed to control media or treated with PCB (3.4  $\mu$ M) for 24 h. Values are means  $\pm$  SEM ( $n = 6$ ). \*Significantly different from control cultures.

activation. We have observed activation of NF- $\kappa$ B in endothelial cells at 3 h, which peaked at 6 h, after treatment with PCB 77, but not with the non-coplanar PCB 153 (Hennig *et al.*, 1999; Slim *et al.*, 1999). During our preliminary studies, we found that endothelial cells treated with PCB 77 for 2, 4, 8, 16, and 24 h showed a maximum increase in mRNA levels for VCAM-1 at 8 h when compared to vehicle control. However, longer exposure times are required to study protein expression. Therefore, endothelial cells were treated for 12 h to determine IL-6 expression or for 18 h to measure luciferase activity.

Figure 1 shows the effect of cellular incubation with the coplanar PCBs 77, 126, and 169 or PCB 153 (a non-AhR ligand) on endothelial barrier function. Barrier function was assessed as the transendothelial flux of albumin. Compared to control cultures, all coplanar PCBs significantly increased albumin transfer across endothelial monolayers. In contrast, PCB 153 (3.4  $\mu$ M) caused no changes in endothelial barrier function.

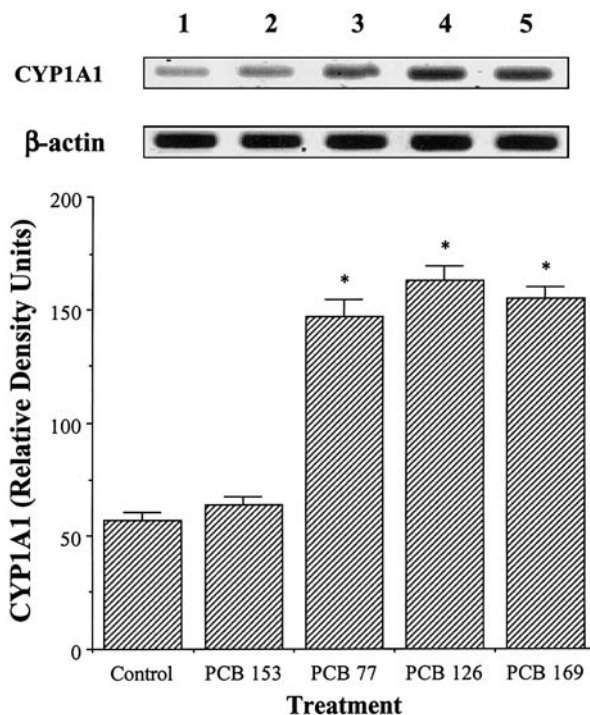
Expression of cytochrome P450 1A (ethoxyresorufin-*O*-deethylase) is shown in Fig. 2. After a 3-h exposure of endothelial cells to PCBs, all coplanar PCBs, but not PCB 153, increased expression of the CYP1A1 gene.

The effect of PCBs on oxidative stress was determined by DCF fluorescence. Figure 3 demonstrates that at 3.4  $\mu$ M concentrations all coplanar PCBs, but not PCB 153, contributed to increased cellular oxidative stress. In addition, concentration-dependent effects of the coplanar PCBs on oxidative stress were studied (Fig. 4). All three coplanar PCBs induced cellular oxidative stress in a concentration-dependent manner, but the concentration(s) needed for maximal induction of oxidative stress differed among the coplanar PCBs tested. PCB 126 exhibited maximal oxidative stress already at 0.5  $\mu$ M (Fig. 4B), whereas the other two PCBs required 2.5  $\mu$ M for maximal induction of cellular oxidative stress (Figs. 4A and 4C).

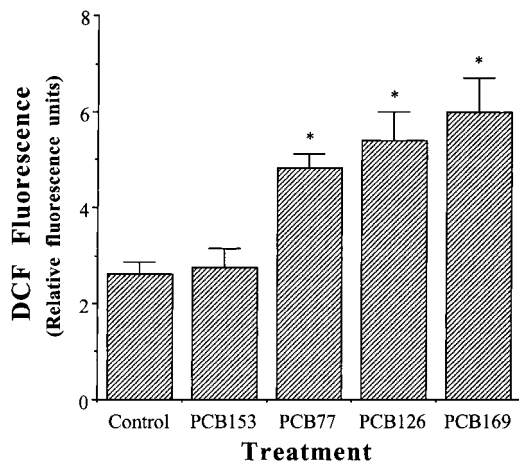
Evidence suggests that oxidative stress can alter gene expression via the activation of transcription factors like NF- $\kappa$ B. To test whether coplanar PCBs, independent of degree of chlorination, can activate NF- $\kappa$ B, cells were exposed to PCBs 77, 126, and 169, as well as to PCB 153, for 6 h. Compared to control cultures, all coplanar PCBs activated NF- $\kappa$ B (Fig. 5). In contrast, the capacity of NF- $\kappa$ B binding was similar in control and PCB 153 treated cultures.

I $\kappa$ B- $\alpha$  specifically binds to the p65 subunit of NF- $\kappa$ B, thereby sequestering this transcription factor in the cytoplasm, and rendering it transcriptionally inactive. Consequently, the presence of I $\kappa$ B- $\alpha$  in the cytoplasm can be indicative of inactivation of NF- $\kappa$ B. In contrast, decreased levels of I $\kappa$ B- $\alpha$  may suggest activation and nuclear translocation of NF- $\kappa$ B. Figure 6 shows that I $\kappa$ B- $\alpha$  levels were lower in cells treated with coplanar PCBs as compared to control cultures or cultures that were treated with PCB 153.

To further determine that coplanar PCBs activate NF- $\kappa$ B and alter gene transcription, endothelial cells were transfected with the NF- $\kappa$ B-responsive plasmid containing firefly luciferase as the reporter gene. In addition, cells were cotransfected with an internal control plasmid, which contained *Renilla* luciferase as the reporter gene. Results of the transfection studies are shown



**FIG. 2.** Effect of a 3-h exposure to PCBs on upregulation of CYP1A1 mRNA expression as analyzed by RT-PCR. The concentration of each PCB was 3.4  $\mu$ M. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphorimaging. Lane 1, control; lane 2, PCB 153; lane 3, PCB 77, lane 4, PCB 126; lane 5, PCB 169. Experiments were repeated 3 times, and this figure is a representative of one of the experimental outcomes. The lower panel shows densitometric quantitation of RT-PCR gels. Values are means  $\pm$  SEM ( $n = 3$ ). \*Significantly different from control cultures.



**FIG. 3.** Effect of endothelial cell incubation with PCB 153 or the coplanar PCBs 77, 126, or 169 on cellular oxidative stress as measured by DCF fluorescence in live or viable cultures. Cultures were exposed to control media or treated with PCB (3.4  $\mu$ M) for 3 h. Values are means  $\pm$  SEM ( $n = 6$ ). \*Significantly different from control cultures or cultures treated with PCB 153.

in Fig. 7. In contrast to control cultures or cells treated with PCB 153, all coplanar PCBs were able to induce NF- $\kappa$ B-dependent transcription in cultured endothelial cells.

Figure 8 shows IL-6 production in endothelial cells during PCB exposure. These data show that all coplanar PCBs have inflammatory properties by promoting endothelial cell-mediated production of IL-6. In contrast, treatment with the non-AhR ligand PCB 153 had no effect on IL-6 production.

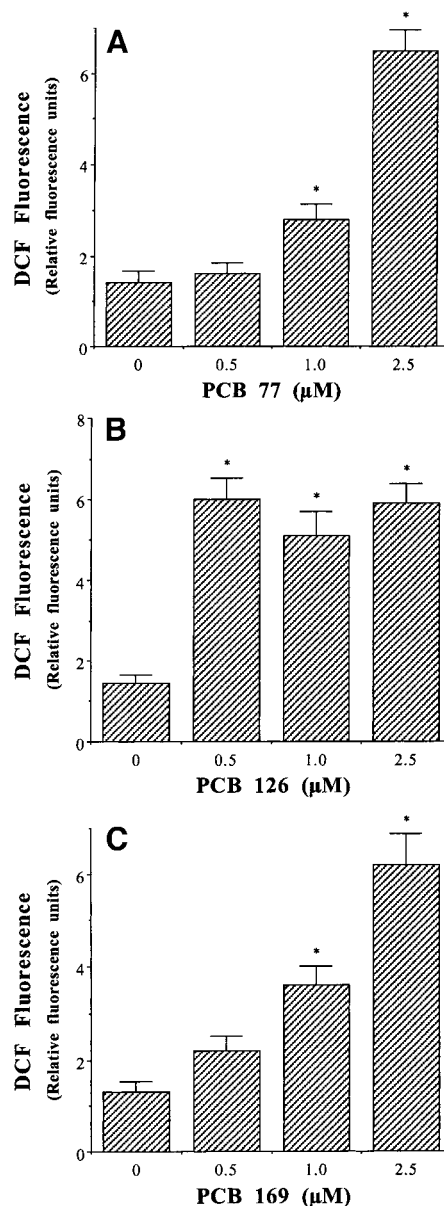
In addition to IL-6, vascular adhesion molecules such as VCAM-1 also are sensitive to activation of NF- $\kappa$ B. Expression of adhesion molecules facilitates recognition and transendothelial migration of leukocytes, critical events of an inflammatory response and early pathology of atherosclerosis. Endothelial cell exposure to culture media supplemented with all coplanar PCBs for 8 h resulted in an increase in the expression of the VCAM-1 gene (Fig. 9). Even though a slight but nonsignificant increase in the expression of VCAM-1 was observed after exposure to PCB 153, all coplanar PCBs increased mRNA levels most markedly compared to control cultures. Of the three coplanar PCBs tested, changes in VCAM-1 expression were most sensitive to PCBs 77 and 169 at 3.4  $\mu$ M concentration.

The role of the AhR in the coplanar PCB-mediated induction of VCAM-1 expression was confirmed in wild-type or genetically altered mice after injection with PCB 77. Twenty hours after a single administration of PCB 77, VCAM-1 expression was increased only in wild-type mice (Fig. 10C), while PCB 77-treated mice lacking the AhR gene showed no increased staining for VCAM-1 (Fig. 10D).

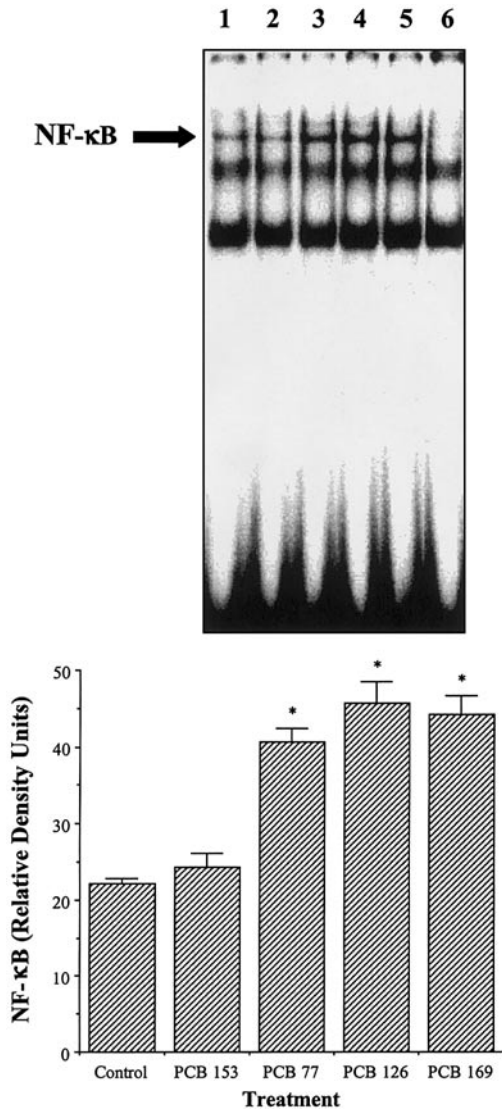
## DISCUSSION

There is evidence in humans that exposure to certain environmental contaminants, such as polyhalogenated aromatic

hydrocarbons, e.g., PCBs (and in particular coplanar PCBs that are aryl hydrocarbon receptor (AhR) ligands) or TCDD, can be implicated in the development of atherosclerosis. There is also evidence that exposure to PCBs can contribute to cardiovascular diseases such as atherosclerosis by promoting vascular endothelial cell dysfunction which predisposes the endothelium to inflammatory reactions (reviewed in Hennig *et al.*, 2001). However, specific mechanisms by which coplanar PCBs cause endothelial cell activation and dysfunction and thus contribute to atherosclerosis are still unclear. We and others

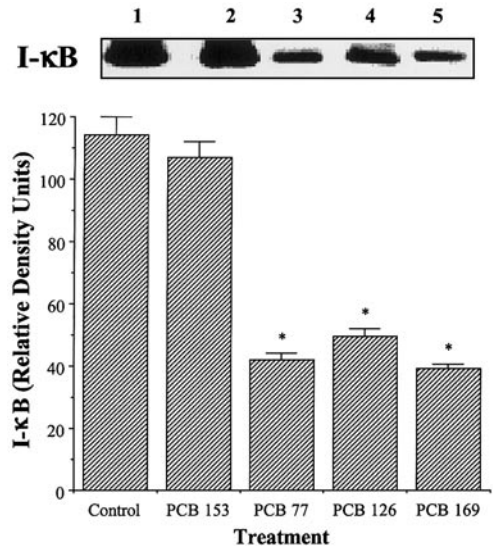


**FIG. 4.** Concentration-dependent effects of coplanar PCBs on cellular oxidative stress as measured by DCF fluorescence. Cultures were exposed to control media or treated with increasing concentrations of PCB 77 (A), PCB 126 (B) or PCB 169 (C) for 3 h. Values are means  $\pm$  SEM ( $n = 6$ ). \*Significantly different from control cultures.



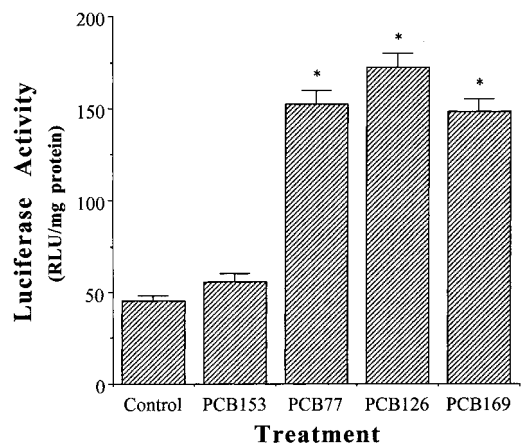
**FIG. 5.** Effect of cellular incubation with PCBs on activation of NF- $\kappa$ B. Cells were exposed to PCBs ( $3.4 \mu\text{M}$ ) for 6 h. Lane 1, control; lane 2, PCB 153; lane 3, PCB 77; lane 4, PCB 126; lane 5, PCB 169; lane 6, supershift. The NF- $\kappa$ B band was confirmed by supershift assay to be the transcriptionally active p65/p50 heterodimer (lane 6). Experiments were repeated 3 times, and this figure is a representative of one of the experimental outcomes. The lower panel shows densitometric quantitation of the NF- $\kappa$ B binding affinity within each treatment group. Values are means  $\pm$  SEM ( $n = 3$ ). \*Significantly different from control cultures.

have demonstrated that certain environmental chemicals, such as PCBs, can cause vascular endothelial cell dysfunction (Stegeman *et al.*, 1995; Toborek *et al.*, 1995; Hennig *et al.*, 1999). We also found that oxidative stress was induced only by the coplanar PCB 77 and not by the diortho-substituted PCB 153 (Toborek *et al.*, 1995; Hennig *et al.*, 1999). Presumably, this is due to the interaction of PCB 77 with the AhR and activation of the CYP1A subfamily (Nebert, 1989; Alsharif *et al.*, 1994; Safe and Krishnan, 1995; Toborek *et al.*, 1995; Hennig *et al.*, 1999). In the present study we present evidence

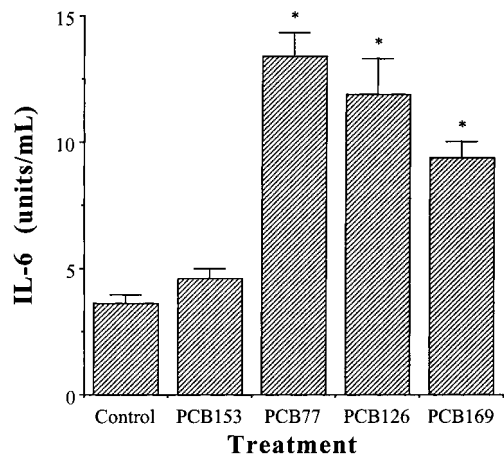


**FIG. 6.** Effect of cellular incubation with PCBs on I $\kappa$ B levels. Cells were exposed to PCBs ( $3.4 \mu\text{M}$ ) for 6 h. Lane 1, control; lane 2, PCB 153; lane 3, PCB 77; lane 4, PCB 126; lane 5, PCB 169. Experiments were repeated 3 times, and this figure is a representative of one of the experimental outcomes. The lower panel shows densitometric quantitation of the I $\kappa$ B levels as determined by Western blotting. Values are means  $\pm$  SEM ( $n = 3$ ). \*Significantly different from control cultures.

that, in addition to PCB 77, other coplanar PCBs, i.e., PCB 126 and PCB 169, also can induce oxidative stress in vascular endothelial cells. Our data also suggest that all three coplanar PCBs tested can induce cellular oxidative stress in a concentration-dependent manner, but that the concentration(s) needed for the apparent maximal induction of oxidative stress differs among these coplanar PCBs tested. Interestingly, PCB 126 exhibited maximal oxidative stress already at  $0.5 \mu\text{M}$ , whereas



**FIG. 7.** Effects of PCBs on NF- $\kappa$ B gene transcription. Endothelial cells were transfected with the NF- $\kappa$ B-responsive plasmid containing firefly luciferase as the reporter gene. Cells were exposed to PCBs for 18 h. Data are expressed as luciferase activity per milligram cell protein. Values are means  $\pm$  SEM ( $n = 6$ ). \*Significantly different from control cultures.



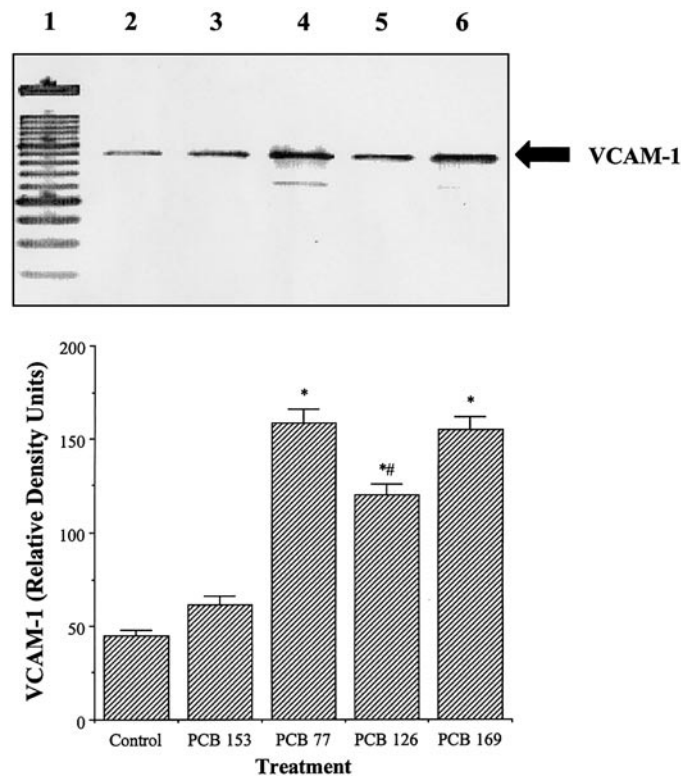
**FIG. 8.** Effect of cellular incubation with PCBs on IL-6 production in endothelial cells. Cultures were exposed to control media or media enriched with PCBs (3.4  $\mu$ M) for 12 h. Values are means  $\pm$  SEM ( $n = 6$ ). \*Significantly different from control cultures.

the other two PCBs required 2.5  $\mu$ M for maximal induction of cellular oxidative stress. These results may be explained by the fact that PCB 126 has comparatively the highest binding affinity for the AhR (Bandiera *et al.*, 1982). We also found that oxidative stress actually decreased after exposure to high concentrations of coplanar PCBs (data not shown). There is evidence that much of the oxidative stress is generated as a result of uncoupling of CYP1A1 and that excessive oxidative stress in fact down-regulates CYP1A1 (Schleizinger and Stegeman, 2001). This suggests that chronic exposure to low concentrations of coplanar PCBs may be even more detrimental in promoting inflammatory diseases than previously thought.

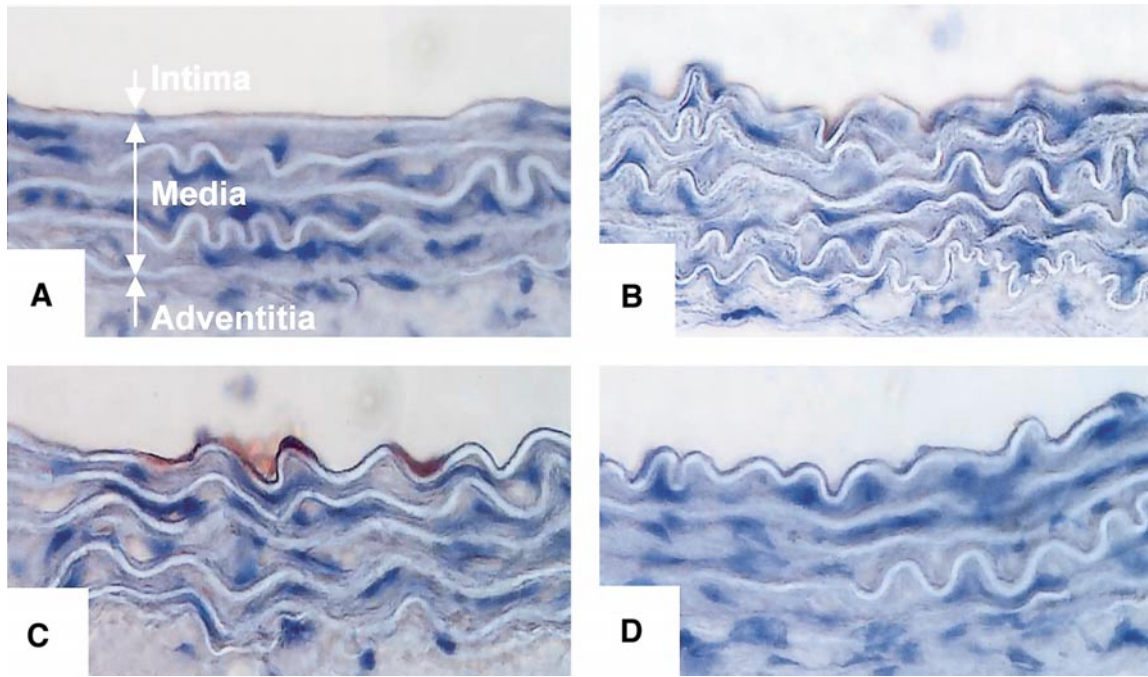
Some aspects of mechanisms by which environmental chemicals alter endothelial cell metabolism have been investigated. We have previously shown that PCB 77, a AhR ligand, but not PCB 153 (not a AhR ligand) significantly disrupted endothelial barrier function (Toborek *et al.*, 1995). PCB 77, but not PCB 153, also contributed markedly to cellular oxidative stress, which was accompanied by increased activity and content of CYP1A and by a decrease in the vitamin E content in the culture medium. We also demonstrated that vitamin E and  $\alpha$ -naphthoflavone (an AhR antagonist and inhibitor of CYP1A1) can markedly reduce PCB 77-mediated oxidative stress, activation of NF- $\kappa$ B and production of inflammatory cytokines (e.g., IL-6), as well as PCB-mediated endothelial barrier dysfunction (Slim *et al.*, 1999). Furthermore, we studied the cellular glutathione redox status as a modulator of the endothelial defense against PCB toxicity (Slim *et al.*, 2000), and we demonstrated that PCB 77 can induce a cellular stress response which is reflected by the activation of c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK). These results suggest that AhR ligands, such as PCB 77, cause vascular endothelial cell activation and dysfunction by modulating intracellular glutathione, which subsequently leads to

induction of stress-specific kinases. In our previous studies, we have demonstrated that an increase in cellular oxidative stress is a critical underlying mechanism of PCB 77-mediated endothelial cell activation and dysfunction (Toborek *et al.*, 1995, Hennig *et al.*, 1999) and that antioxidants can block the proinflammatory properties of PCB 77 (Slim *et al.*, 1999). Similar mechanistic phenomena of coplanar PCBs were recently reported in PCB 126-induced toxicity using the chick embryo as a model (Jin *et al.*, 2001).

In these earlier studies we employed PCB 77 as a model AhR agonist. However, PCB 77 is also known to be a substrate for the induced CYP1A enzymes (Toborek *et al.*, 1995). A lingering question was whether the metabolic activation of PCB 77 also played a role in the associated oxidative stress events. Thus, our present study was extended beyond one coplanar PCB by including a series of coplanar PCBs, i.e., PCBs 77, 126, and 169. With increasing chlorination, the rate of metabolism would be greatly diminished. Should metabolic activation of PCBs markedly contribute to their biological effects, we could expect a marked decrease in proinflammatory



**FIG. 9.** Effect of a 8-h exposure to PCBs on upregulation of VCAM-1 mRNA expression as analyzed by RT-PCR. The concentration of each PCB was 3.4  $\mu$ M. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphorimaging. Lane 1, control; lane 2, PCB 153; lane 3, PCB 77, lane 4, PCB 126; lane 5, PCB 169. Experiments were repeated 3 times, and this figure is a representative of one of the experimental outcomes. The lower panel shows densitometric quantitation of RT-PCR gels. Values are means  $\pm$  SEM ( $n = 3$ ). \*Significantly different from control cultures. #Significantly lower compared with cultures treated with PCB 77 or PCB 169.



**FIG. 10.** Immunoreactivity of VCAM-1 antiserum against sections of mouse aorta. Primary antibodies for VCAM-1 were detected using biotinylated secondary antibodies and peroxidase ABC kits. The red color as seen in panel C reflects positive chromogen development for VCAM-1 immunostaining on the endothelial surface. The magnification was 400 $\times$ . (A) AhR $^{+/+}$  control mice; (B) AhR $^{-/-}$  control mice; (C) AhR $^{+/+}$  injected with PCB 77; (D) AhR $^{-/-}$  mice injected with PCB 77.

properties of these coplanar PCBs relative to an increase in chlorination. However, this was not the case, and PCBs 126 and 169 were as efficacious as PCB 77 in disrupting endothelial barrier function, in causing oxidative stress and activation of NF- $\kappa$ B, in production of IL-6 and expression of the VCAM-1 gene in cultured cells.

In the present study we showed that coplanar PCBs as a general class of PCBs can contribute to an inflammatory response. Endothelial cell exposure to all coplanar PCBs, but not to the non-AhR agonist PCB 153, resulted in activation of the oxidative stress-sensitive transcription factor NF- $\kappa$ B and increased the production of IL-6. Expression of VCAM-1 also was affected by endothelial cell exposure to PCBs. However, at 3.4  $\mu$ M concentration, changes in VCAM-1 expression appeared to be most sensitive to PCBs 77 and 169. Adhesion molecules such as VCAM-1 play an important role in the migration of leukocytes and their adherence to the endothelium, one of the initial steps in the pathogenesis of atherosclerosis (Osborn *et al.*, 1989; Elices *et al.*, 1990; Cybulsky and Gimbrone, 1991). NF- $\kappa$ B is involved in the regulation of adhesion molecule expression in endothelial cells after activation by inflammatory cytokines, and the activation at the protein level is accompanied by induction of NF- $\kappa$ B p65 mRNA expression (Wagner *et al.*, 1997). Results of our studies are consistent with literature data suggesting that activation of NF- $\kappa$ B is critical for subsequent expression of inflammatory genes in endothelial cell activation and atherosclerosis (Col-

lins, 1993; Berliner *et al.*, 1995; Ross, 1999). We report in the present study that activation of NF- $\kappa$ B by coplanar PCBs can lead to expression of inflammatory genes involved in endothelial cell activation.

Recent evidence suggests that the AhR agonist TCDD can activate NF- $\kappa$ B and AP-1, and it was proposed that CYP1A1-dependent and AhR complex-dependent oxidative signals are in part responsible for the observed activation of these transcription factors (Puga *et al.*, 2000). We now also provide *in vivo* evidence using an AhR-deficient mouse model that a functional AhR is critical for the proinflammatory events mediated by coplanar PCBs and possible other AhR agonists. Using mice lacking the AhR, Shimizu *et al.* (2000) have demonstrated that an intact AhR is a critical component in benzo[*a*]pyrene-induced carcinogenicity. Other *in vivo* studies with AhR knockout mice provide evidence that AhR activation is involved in the inflammatory response to TCDD hepatotoxicity (Thurmond *et al.*, 1999). Furthermore, a recent study demonstrated that induction of xanthine oxidase/xanthine dehydrogenase activity by TCDD and enhanced lipid peroxidation in livers of mice treated with TCDD were not found in AhR-deficient mice (Sugihara *et al.*, 2001).

In summary, our data suggest that coplanar PCBs can be atherogenic by producing an endothelial cell inflammatory response. We provide evidence that this inflammatory response is dependent on a functional AhR and that induction of CYP1A1 and activation of NF- $\kappa$ B are critical mechanistic

mediators. Thus, our findings suggest that activation of the AhR is an underlying mechanism of endothelial cell stimulation mediated by certain environmental contaminants and that exposure to coplanar PCBs, and possibly other AhR agonists, may potentiate the pathology of cardiovascular diseases.

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