The role of caveolin-1 in PCB77-induced eNOS phosphorylation in human-derived endothelial cells

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FROM EPIDEMIOLOGICAL studies, there is substantial evidence that cardiovascular diseases are linked to environmental pollution. For example, increased hospitalization rates have been reported for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants (45). Polychlorinated biphenyls (PCBs) are widespread persistent environmental contaminants, which have been widely used for various industrial applications. There is evidence linking the aryl hydrocarbon receptor (AhR) with mechanisms associated with cardiovascular diseases (42) and that AhR ligands such as coplanar PCBs may be atherogenic by disrupting the functions of endothelial cells in blood vessels. We have demonstrated previously that coplanar as well as noncoplanar PCBs can cause endothelial cell dysfunction as determined by an induction of inflammatory markers, such as the expression of cytokines and adhesion molecules (15). Because the endothelium is in immediate contact with the blood, endothelial cells are particularly susceptible to insult by circulating environmental contaminants and their metabolites (16). The mechanisms by which PCBs induce endothelial cell activation and dysfunction, oxidative stress, and inflammation are not fully understood. Oxidative stress-induced transcription factors, such as nuclear factor-κB (NF-κB), play a significant role in regulating inflammatory cytokine and adhesion molecule production (6). Binding sites for NF-κB and related transcription factors were identified in the promoter regions of a variety of inflammatory genes (20, 31), such as interleukin-6, vascular cell adhesion molecule-1 (VCAM-1), or cyclooxygenase-2 (COX-2), all of which are upregulated during PCB toxicity (5, 14, 21, 49).

Endogenous nitric oxide (NO) is generated from arginine by a family of three calmodulin-dependent NO synthase enzymes (3). NO produced from the endothelial isoform endothelial NO synthase (eNOS) regulates vital functions associated with vascular tone and local blood flow (36). Abnormalities of NO production by vascular endothelial cells, and in particular a decrease in bioavailable NO, can result in endothelial dysfunction and accelerated atherosclerosis (18). Excessive production of NO can mediate cellular toxicity by damaging critical metabolic enzymes and by reacting with superoxide to form peroxynitrite, a potent oxidant. Thus the beneficial protective effects of NO (e.g., anti-inflammatory) are lost after reaction with the superoxide anion (36). There are a number of kinases and phosphatases that can continuously associate or dissociate from the eNOS-signaling complex and thus provide a platform for regulatory processes (8). For example, eNOS is activated by several growth factors through the phosphatidylinositol 3-kinase (PI3K)/Akt pathways. (4, 9, 48, 50). Furthermore, caveolin-1 regulates NO signaling in the endothelium by binding to and inhibiting eNOS (30). eNOS activity is regulated by Ca2+-calmodulin, phosphorylation, and interactions with caveolin-1 (26, 46). Activation of kinases such as Akt can lead to eNOS activation and its dissociation from caveolin-1 (54). Thus the regulation of eNOS by substrate and cofactor dependence, phosphorylation, and interaction with caveolin might all affect the level of bioavailable NO.

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There is increasing evidence that membrane domains called caveolae play a critical role in the pathology of atherosclerosis (23) and that the lack of the caveolin-1 gene may provide protection against the development of atherosclerosis (10). Caveolae are particularly abundant in endothelial cells, where they are believed to play a major role in the regulation of endothelial vesicular trafficking as well as the uptake of lipids and related lipophilic compounds (28, 38), possibly including lipoprotein- and albumin-associated persistent organic pollutants. Besides their possible role in cellular uptake of lipophilic substances, caveolae contain an array of cell signaling molecules. In fact, numerous genes involved in endothelial cell dysfunction, inflammation, and PCB toxicity are associated with caveolae (11).

We and others have shown that persistent organic pollutants (such as PCBs) can induce certain cell signaling pathways leading to the activation of proinflammatory transcription factors such as NF-kB, which control inflammatory genes in endothelial cells, including COX-2 and VCAM-1 (reviewed in Ref. 15). However, mechanisms, and in particular intracellular signaling pathways, responsible for the regulation of PCB-mediated endothelial cell activation are not well understood. In the present investigation, we hypothesized that caveolae play a critical role in endothelial activation and associated vascular toxicity induced by persistent environmental pollutants such as PCBs. Our data suggest that PCB77 induces proinflammatory parameters through eNOS signaling and that caveolae may play a critical role in regulating vascular endothelial cell activations and toxicity induced by persistent environmental pollutants such as PCB77.

**MATERIALS AND METHODS**

*Materials.* Anti-caveolin-1 antibody was obtained from Affinity BioReagents (Golden, MO). Anti-phospho-caveolin (Tyr14), anti-eNOS, anti-phospho-eNOS (Ser1177), anti-Akt, anti-phospho-Akt (Ser473), and anti-rabbit Ig horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-p65 NF-kB antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin was purchased from Sigma (Saint Louis, MO). Supplies and reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). Both the PI3K inhibitors (LY-294002 and wortmannin) and the Src inhibitor (PP2) were purchased from Calbiochem (San Diego, CA). 3,3,4,4'-Tetrachlorobiphenyl (PCB77) was purchased from AccuStandard (New Haven, CT).

*Cell culture.* Primary endothelial cells were isolated from porcine pulmonary arteries and cultured as previously described (17). EA.hy926 cells (a kind gift from Dr. C. S. Edgell, University of North Carolina) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics. Cell cultures were grown until confluent and then synchronized by maintaining them in 1% serum for 16 h before treatment for various times periods.

The EA.hy926 line was derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549 (7). EA.hy926 cells are contact inhibited in growth, show reduced growth factor requirements, express von Willebrand factor, and upregulate ICAM-1, VCAM-1, and E-selectin expression upon stimulation with...
TNF-α (2). EA.hy926 cells were used as a model for endothelial cells because, like endothelial cells, EA.hy926 cells endogenously express eNOS and caveolin-1.

Caveolin-1 small-interfering RNA and transfection. The caveolin-1 gene silencer was designed as previously described (37). Cells were transfected with control small-interfering (si)RNA or caveolin-1 siRNA at a final concentration of 80 nM using GeneSilencer (Genlantis, San Diego, CA) in Optimem I medium (Invitrogen). Cells were incubated with transfection mixtures for 4 h and then replaced with regular medium. Forty-eight hours after transfection, cells were synchronized overnight and then treated with PCB77 (2.5 μM) or vehicle.

Immunoblot analysis. Cells were treated with either vehicle (0.1% DMSO), PCB77 (2.5 μM), PI3K inhibitors (LY-294002 and wortmannin), or Src inhibitor (PP2) in DMEM with 1% FBS. Cells were washed twice, scraped in ice-cold PBS, and centrifuged. Appropriate amounts of boiling lysis buffer (1% SDS, 1 mM sodium orthovandate, 10 mM Tris, pH 7.4) were added to the cell pellets. The

Fig. 2. PCB77 induces eNOS phosphorylation through a Src/phosphatidylinositol 3-kinase (PI3K)/Akt-dependent mechanism. Cells were treated with PCB77 (2.5 μM) for the indicated times, and phosphorylation of Akt was determined by Western blot analysis using anti-p-Akt antibody (A). Cells were treated with PCB77 in the absence or presence of PI3K inhibitors (wortmannin, 200 nM; or LY-294002, 50 μM; 2 h pretreatment). Phosphorylation of Akt and eNOS was determined by Western blot analysis using specific antibodies (B). To test whether Src also can phosphorylate eNOS, cells were treated with PCB77 for 5 min with or without the Src inhibitor (PP2, 1 μM; 2 h preincubation). Total cell lysates were subjected to Western blot analysis (C). Densitometry results shown in parallel represent means ± SE of 3 independent experiments. *Significantly different compared with vehicle control. **Significantly different compared with PCB77.
samples were boiled for 5 min and passed several times through a 26-gauge needle. After centrifugation, protein concentrations of supernatants were determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Cellular proteins were resolved by SDS-PAGE (12–15% acrylamide) and transferred to nitrocellulose membranes. Blots were incubated overnight in Tris-buffered saline (pH 7.6) containing 0.05% Tween 20 (TBST) containing 5% milk powder. After three washes with TBST, membranes were incubated overnight with the primary antibody (1,000-fold diluted in TBST containing 5% bovine serum albumin) and for 1 h with HRP-conjugated secondary antibody (5,000-fold diluted). Bound antibodies were detected using enhanced chemiluminescence (ECL) (Amersham Life Science, Birmingham, UK).

Measurement of total NO. The accumulation of nitrite and nitrate (total NO) in culture media after PCB77 treatment was measured as described previously (32). The cell culture supernatants were centrifuged at 7,740 g for 5 min at 4°C with anti-nitrotyrosine antibody. The beads were recovered by centrifugation at 1,500 g for 5 min at 4°C and washed four times with 1 ml of PBS. Bound proteins were eluted by boiling in loading buffer. Immunoprecipitates were separated by 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane. After being blocked with 5% milk, the membrane was incubated for 2 h with mouse monoclonal anti-nitrotyrosine antibody in a 1:1,000 dilution. The bands were visualized by incubation of membranes with HRP-conjugated secondary antibody followed by ECL (Amersham Life Science).

Electrophoretic mobility shift assays. Nuclear extracts from EA.hy926 cells were prepared as described previously (24). Synthetic 5′-biotinylated complementary oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Nuclear extracts were incubated for 20 min with biotin-labeled oligonucleotide probes containing the enhancer DNA element for NF-κB (5′-AGTTGAGGGGACTTTCGCCAGGC-3′). Gel mobility shift assay was carried out using a LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL) (41).

Statistical analysis. Images were quantified, and data were analyzed using the Scion Image and Sigma Stat software, respectively. Comparisons between treatments were made by one- or two-way ANOVA with post hoc comparisons of the means made by Tukey’s tests. A statistical probability of \( P < 0.05 \) was considered significant.

RESULTS

PCB77 increases eNOS phosphorylation and NO production in endothelial cells. eNOS is an enzyme that generates NO and is activated by phosphorylation of a serine residue at position 1177 in endothelial cells. eNOS activation was tested in both primary porcine endothelial cells and in EA.hy926 cells. Both cell types were incubated with PCB77 for 5, 10, or 30 min. PCB77 markedly induced eNOS phosphorylation between 5 and 10 min in both cell types (Fig. 1, A and B). In EA.hy926 cells, densitometric analysis of phospho-eNOS bands showed that PCB77 treatment increased eNOS phosphorylation by sevenfold after 5 min and fourfold after 10 min (Fig. 1B).

To investigate whether PCB77-induced eNOS phosphorylation results in an increase in NO production and may be involved in PCB77-induced endothelial dysfunction, we measured NO production and nitrotyrosine formation. Treatment with PCB77 rapidly increased NO production, with close to a threefold increase as early as 5 min after exposure. EA.hy926 cell-derived NO returned to near control levels after 10 min of PCB exposure (Fig. 1C). This increase in NO production was followed by a marked increase in nitrotyrosine formation 30 min after PCB77 treatment (Fig. 1D).

PCB77 induces eNOS phosphorylation through a Src/PI3K/Akt-dependent mechanism. Several protein kinases, including the serine threonine kinase Akt (PKB), have been shown to be localized upstream of eNOS and can phosphorylate eNOS. Therefore, the effect of PCB77 on Akt phosphorylation was investigated. PCB77 caused maximal Akt Ser473 phosphorylation 5 min after PCB exposure (Fig. 2A).

To investigate whether PI3K is involved in PCB77-induced eNOS phosphorylation, cells were preincubated for 2 h with the PI3K inhibitors LY-294002 or wortmannin. Both of these PI3K inhibitors decreased PCB77-stimulated Akt and eNOS activation while not affecting total protein levels (Fig. 2B). These data indicate that PI3K is critical for PCB77-induced serine-1177 eNOS. Src family tyrosine kinases have been

Fig. 3. PCB77 increases NF-κB DNA binding through the Src/PI3K/Akt pathway. EA.hy926 cells were treated with PCB77 (2.5 μM) for 2 h in the absence or presence of PI3K inhibitors [wortmannin (Wort), 200 Nm; or LY-294002 (LY29), 50 μM; 2 h preincubation] or the Src inhibitor (PP2, 1 μM; 2 h preincubation). NF-κB binding was determined by electrophoretic mobility shift assay with nuclear proteins extracted from treated cells. NF-κB DNA binding complexes are identified by arrows. Binding intensity was quantified by densitometry. Densitometry results shown in parallel represent means ± SE of 3 independent experiments (A). *Significantly different compared with vehicle control. **Significantly different compared with PCB77. Gel shift assay using p65 antibody and competition assay were used to confirm NF-κB-DNA binding activity. Lane 1, free probe; lane 2, nuclear extract; lane 3, nuclear extract plus 200× excess of unlabeled NF-κB probe; lane 4, nuclear extract plus anti-p65 antibody (B).

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known to be localized upstream of PI3K/Akt pathway (19). To test whether Src can also phosphorylate eNOS via the PI3K/Akt pathway, we determined the effect of the Src inhibitor PP2 on eNOS activation by PCB77. EA.hy926 cells were preincubated with or without PP2 for 2 h before PCB77 stimulation. PP2 significantly decreased eNOS phosphorylation (Fig. 2C). These results suggest that PCB77 stimulates Src, which in turn can activate the PI3K/Akt pathway to ultimately lead to eNOS phosphorylation.

**PCB77 increases NF-κB DNA binding through the Src/PI3K/Akt pathway.** NF-κB is a critical oxidative stress-sensitive transcription factor, which regulates inflammatory genes in endothelial cells. Therefore, we tested whether the Src/PI3K/Akt pathway can also be involved in PCB77-induced activation of NF-κB DNA binding. As shown in Fig. 3, treatment with PCB77 for 1 h markedly induced NF-κB activation. Both the Src inhibitor PP2 and the PI3K inhibitors LY-294002 and wortmannin decreased the PCB-induced activation of NF-κB, suggesting a regulatory involvement of the Src/PI3K/Akt pathway (Fig. 3A). The specificity of NF-κB binding to the consensus oligonucleotide was demonstrated by a decrease of the NF-κB band by adding an excess of unlabeled oligonucleotide and antibody against the p65 subunit of NF-κB (Fig. 3B).

**PCB77 induces caveolin-1 phosphorylation.** It has been suggested that phosphorylation of caveolin-1 is required for the engagement of the signaling machinery responsible for caveolae-mediated endocytosis (47). To examine whether PCB77 can induce phosphorylation of caveolin-1, cells were incubated with PCB77 for up to 10 min. PCB77 markedly induced caveolin-1 phosphorylation at 5 min and then returned to near control levels by 10 min (Fig. 4).

**Caveolin-1 silencing reduces PCB-induced eNOS and Akt phosphorylation.** To determine the role of caveolin-1 in PCB77-dependent phosphorylation of eNOS, we used siRNA to specifically silence caveolin-1. Caveolin-1 siRNA reduced caveolin-1 expression by ~80% compared with that in control cells (Fig. 5A). However, β-actin levels, total protein levels of
Fig. 6. Cav-1 silencing reduces the activation of NF-κB by PCB77. Cells were transfected with siRNA for Cav-1 or with control siRNA and treated with PCB77 (2.5 μM) for 5 min. The efficiency and specificity of silencing were determined by Western blot analysis with Cav-1 and β-actin antibodies. Electrophoretic mobility shift assay for NF-κB was performed with nuclear proteins extracted from endothelial cells. NF-κB DNA binding complexes are identified by arrows. Binding intensity was quantified by densitometry. Densitometry results shown in parallel represent means ± SE of 3 independent experiments. *Significantly different compared with vehicle control (Con). **Significantly different compared with PCB77.

eNOS, and Akt were unchanged in caveolin-1 siRNA-transfected cells, indicating the specificity of the silencing (Fig. 5, A–C). Subsequently, we measured the effect of PCB77 on phosphorylation of eNOS and Akt in caveolin-1-silenced EA.hy926 cells. Caveolin-1 silencing significantly decreased the PCB77-induced phosphorylation of eNOS Ser1177 (Fig. 5B) and Akt Ser473 (Fig. 5C). Caveolin-1 silencing did not affect Akt phosphorylation and expression in cells without PCB treatment (Fig. 5C). Similar results were observed with the eNOS data (Fig. 5B).

Caveolin-1 silencing reduces the activation of NF-κB by PCB77. To determine the role of caveolin-1 in PCB77-dependent NF-κB activation, siRNA technology was used to specifically silence caveolin-1. Similar to the experiments presented in Fig. 5, caveolin-1 siRNA reduced caveolin-1 expression by ~80% compared with that in control cells without affecting β-actin levels. As illustrated, caveolin-1 silencing significantly decreased the PCB77-induced activation of NF-κB (Fig. 6).

**DISCUSSION**

There is substantial evidence that the etiology of cardiovascular diseases is linked to the exposure of environmental pollution. For example, the mortality from cardiovascular disease was increased among Swedish capacitor manufacturing workers exposed to PCBs for at least 5 yr (12). Also, in workers exposed to phenoxy herbicides and PCBs in waste transformer oil, deaths were due primarily to complications from cardiovascular disease (13). Most importantly, a recent study reported increased hospitalization rates for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants (43). Many environmental pollutants, including coplanar PCBs such as PCB77, interact with the AhR to initiate xenobiotic metabolizing activity linked to an increase in cellular oxidative stress (1). It has been suggested that AhR activation is a critical participant in mechanisms associated with cardiovascular diseases (42, 53) and that AhR ligands may be atherogenic by disrupting the functions of endothelial cells in blood vessels.

The vascular endothelium serves a critical role in the regulation of both the structure and function of blood vessels. Endothelial cells not only form a barrier protecting the underlying vascular tissue but also generate signaling molecules, which serve diverse autocrine and paracrine functions. Endothelial cell activation and dysfunction and subsequent inflammatory events are considered critical in the etiology of vascular diseases such as atherosclerosis (39). Environmental toxicants, once absorbed, distribute themselves to tissues, especially adipose, where they are in dynamic equilibrium with the blood. Thus risk factors of such pollutants, such as PCBs, are chronic and can continuously amplify pathologies of diseases that are associated with endothelial dysfunction. Data from our present study confirm that endothelial exposure to PCB77 provides a prooxidative cellular environment sufficient to induce oxidative stress-sensitive transcription factors such as NF-κB.

We have shown previously that coplanar PCBs cause endothelial cell dysfunction, which was associated with an increase in activity of cytochrome P-4501A and cellular oxidative stress (52). Subsequent in vitro and in vivo studies demonstrated a strong link between exposure to PCBs and induction of inflamm...
matory markers characteristic of early events in the pathology of atherosclerosis (14). Using mice lacking the AhR gene, we also showed that the PCB-induced proinflammatory properties were dependent on an intact AhR (14).

Mechanisms of PCB-induced oxidative stress and inflammation are not clear; however, eNOS dysfunction, resulting in peroxynitrite formation and protein tyrosine nitration, has been proposed to be an independent marker of cardiovascular disease (35). In our current study we demonstrated that exposure to PCB77 can increase phosphorylation of eNOS, with a subsequent increase in peroxynitrite formation and induction of NF-κB. There is evidence that coplanar PCBs can markedly stimulate reactive oxygen species production through uncoupling of cytochrome *P*-4501A (43). These data and results from our own work (14, 40) strongly support our hypothesis that exposure to coplanar PCBs can lead to endothelial dysfunction and induction of inflammatory markers through eNOS signaling. In the current study we demonstrated that exposure to PCB can increase phosphorylation of eNOS in 5 and 10 min, with a subsequent increase in peroxynitrite formation at 30 min and induction of NF-κB at 1 h.

We also present evidence that kinase signaling pathways upstream of eNOS may be critical regulatory mechanisms involved in the toxicity of persistent organic pollutants such as PCBs. Using appropriate inhibitors, we were able to demonstrate that PCB77 can induce eNOS phosphorylation in endothelial cells through a Src/Pi3K/Akt-dependent signaling pathway. Our data are supported by mechanistic studies of AhR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), where it was observed that TCDD was able to directly activate cellular Src tyrosine kinase (34). Activated Src can recruit the p85 subunit of Pi3K, resulting in Pi3K/Akt activation (19).

There is evidence that caveolae play a critical role in the pathology of atherosclerosis and that the lack of the caveolin-1 gene reduces an atherogenic outcome (44). Caveolae compartmentalize signal transduction molecules that regulate multiple endothelial functions. For example, the mechanisms underlying eNOS localization in caveolae and associated activity have been studied extensively (29). For optimal activation, eNOS is targeted to caveolae, and caveolin-1, the major coat protein of caveolae, regulates eNOS activity (27). We provide novel data, demonstrating that the silencing of the caveolin-1 gene can markedly downregulate PCB-induced eNOS phosphorylation, peroxynitrite formation, and activation of NF-κB. Our data strongly support our hypothesis that caveolae present a critical platform in regulating inflammatory signaling pathways induced by environmental pollutants such as PCBs. As mentioned above, an important mechanism that is regulated by caveolae is eNOS signaling, and cross talk between caveolin-1 and eNOS may be an important factor in mechanisms of the pathology of atherosclerosis. In human blood vessels, eNOS was significantly increased in the endothelium overlying intimal thickening and atherosclerotic plaques compared with the adjacent endothelium overlying normal media (55). Interestingly, the endothelial caveolin-1-to-eNOS ratio increased fivefold only in endothelium overlying plaque, suggesting a close relationship between increased caveolin-1 and atherosclerotic vessels (55).

Caveolae may also serve as critical transport vesicles involved in transcytosis of solutes, membrane components, proteins, viruses, extracellular ligands (22), and possibly lipophilic compounds such as PCBs. Thus, in addition to serving as a critical platform during inflammatory signaling, caveolae also may facilitate and regulate cellular entry of PCBs. It has been shown that PCBs in plasma are associated primarily with albumin and low-density lipoproteins (33). Since the albumin acceptor gp60 (51) and the lipid/lipoprotein receptor CD36 (26) are enriched in caveolae, membrane caveolae likely mediate the first contact of endothelial cells with PCBs, suggesting that caveolae may have implications in PCB toxicity.

In summary, we have described a new mechanism of PCB77-induced activation of endothelial cells that involves a rapid Pi3K-dependent activation of Akt and subsequent serine phosphorylation of eNOS. The mechanisms by which PCB77 induces Pi3K/Akt/eNOS activation appear to be regulated by and dependent on functional caveolin-1 (Fig. 7). Our data provide an understanding of how cross talk between caveolin-1 and eNOS signaling can lead to an PCB77-mediated induction of proinflammatory genes in vascular endothelial cells. These mechanisms include eNOS as a potential source of reactive oxygen species and peroxynitrite. Because caveolae and caveolins have been implicated in several human diseases and, in particular, vascular diseases, our data may have implications in understanding the mechanisms of inflammatory diseases induced by an exposure to environmental pollutants.

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REFERENCES


