Published in final edited form as:

Chem Biol Interact. 2008 November 25; 176(2-3): 71–78. doi:10.1016/j.cbi.2008.08.007.

Coplanar polychlorinated biphenyl-induced CYP1A1 is regulated through caveolae signaling in vascular endothelial cells

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Abstract

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants that can induce inflammatory processes in the vascular endothelium. We hypothesize that the plasma membrane microdomains called caveolae are critical in endothelial activation and toxicity induced by PCBs. Caveolae are particularly abundant in endothelial cells and play a major role in endothelial trafficking and the regulation of signaling pathways associated with the pathology of vascular diseases. We focused on the role of caveolae and their major protein component, caveolin-1 (Cav-1), on aryl hydrocarbon receptor (AhR)-mediated induction of cytochrome P450 1A1 (CYP1A1) by coplanar PCBs. Endothelial cell exposure to PCB77 increased both caveolin-1 and CYP1A1 levels in a time-dependent manner in total cell lysates, with a maximum increase at 6 h. Furthermore, PCB77 accumulated mainly in the caveolae-rich fraction, as determined by gas chromatograph-mass spectrometry. Immunoprecipitation analysis revealed that PCB77 increased AhR binding to caveolin-1. Silencing of caveolin-1 significantly attenuated PCB77-mediated induction of CYP1A1 and oxidative stress. Similar effects were observed in caveolin-1 null mice treated with PCB77. These data suggest that caveolae may play a role in regulating vascular toxicity induced by persistent environmental pollutants such as coplanar PCBs. This may have implications in understanding mechanisms of inflammatory diseases induced by environmental pollutants.

Keywords

caveolin-1; aryl hydrocarbon receptor; polychlorinated biphenyls; persistent organic pollutants; endothelial cell activation; atherosclerosis

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Introduction

Polychlorinated biphenyls (PCBs) are industrial chemicals that were being produced and sold in the U.S. for approximately 50 years. Even though PCBs are no longer commercially produced in the United States, high levels of the chemicals remain in various parts of the country due to their persistence. Thus, humans and animals continue to be exposed to these persistent organic pollutants via airborne particle matter or oral ingestion of contaminated food products [1]. Circulating environmental contaminants like PCBs in blood are in direct contact with the vascular endothelium. Endothelial cells are vulnerable to chemical insult, which can lead to severe endothelial dysfunction [2–4]. Dysfunction of endothelial cells is a critical underlying step in the initiation of cardiovascular diseases such as atherosclerosis. One functional change in atherosclerosis is the activation of the endothelium, which is manifested as an increase in the expression of specific cytokines and adhesion molecules that mediate the inflammatory aspects of the disease by regulating the vascular entry of leukocytes [5]. We have demonstrated previously that coplanar as well as non-coplanar PCBs can cause endothelial cell dysfunction as determined by markers such as expression of cytokines and adhesion molecules [6].

The mechanisms by which environmental chemicals induce endothelial cell activation are not fully understood. Oxidative stress-induced transcription factors, which regulate inflammatory cytokine and adhesion molecule production, play critical roles in the regulation of inflammatory responses. Oxidative stress can be induced by interaction of specific environmental contaminants, i.e., coplanar PCBs or 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD), with the aryl hydrocarbon receptor (AhR) and subsequent activation of the cytochrome P450 1A subfamily [3,7]. Our earlier work indicates that coplanar PCBs that function as AhR agonists may be proinflammatory and proatherogenic by activating nuclear factor- κ B (NF- κ B) in vascular endothelial cells [8]. There is evidence linking AhR activation with mechanisms associated with cardiovascular diseases [9,10], and AhR ligands may be atherogenic by disrupting the functions of endothelial cells in blood vessels. In fact, from epidemiological studies, there is substantial evidence that atherosclerosis and related cardiovascular diseases are linked to exposure to environmental pollution [1,6]. Most importantly, a recent study reported increased hospitalization rates for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants [11].

There is increasing evidence that membrane domains called caveolae play a critical role in the pathology of atherosclerosis [12] and that the lack of the caveolin-1 (Cav-1) gene may provide protection against the development of atherosclerosis [13]. 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 [14,15], possibly including lipoprotein- and albumin-associated persistent organic pollutants. The lipid/lipoprotein receptors CD36 and scavenger receptor class B1 (SR-B1) have been reported to be associated with caveolae [15,16]. Caveolin-1 is also a structural component in lipid body formation and thus cellular lipid uptake [17]. Besides their possible role in cellular uptake of lipophilic substances, caveolae contain an array of cell signaling molecules that are involved in endothelial cell dysfunction and inflammation [18].

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 demonstrate that PCB77 accumulates in caveolae-rich fractions of endothelial cells and that caveolae may provide a regulatory platform for PCB-induced cytochrome P450 1A1 (CYP1A1) via AhR signaling.

Materials and Methods

Chemicals and antibodies

PCB77 (3,3',4,4'-tetrachlorobiphenyl, PCB126 (3,3',4,4',5-pentachlorobiphenyl), and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) were kindly provided by Dr. Larry W. Robertson, University of Iowa, or purchased from AccuStandard (New Haven, CT). Rabbit polyclonal anti-caveolin-1 and mouse monoclonal anti-AhR antibodies were obtained from Affinity BioReagents (Golden, MO); rabbit polyclonal anti-actin antibody from Sigma (St. Louis, MO), and goat polyclonal anti-CYP1A1, as well as anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and PCB treatment

Primary endothelial cells were isolated from porcine pulmonary arteries and cultured as described previously [19]. The basic culture medium consisted of M199 (GIBCO Laboratories, Grand Island, NY) containing 10% (v/v) fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). PCB77, PCB126, and PCB153 were solubilized in dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO) and the final concentration of DMSO in the cell culture media did not exceed 0.1% (v/v). PCBs were used at a concentrations not exceeding 2.5 μ M, as it falls within serum concentrations after acute exposure [20]. For the experiments, endothelial cells were grown until confluent, synchronized by being maintained for 16 h in M199 containing 1% (v/v) FBS, and treated with PCBs or vehicle in M199 containing 1% (v/v) FBS.

Animals

All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facilities at the University of Kentucky. Low density lipoprotein receptor null (LDL-R^{-/-}) mice and caveolin-1 (Cav-1^{-/-}) null mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were cross-bred at the University of Kentucky to generate LDL-R/caveolin-1 double null mice (LDL-R^{-/-}Cav-1^{-/-} mice). At 8 weeks of age, mice (4 females and 8 males for each genotype) were placed on a standardized diet containing 20% calories from fat (89 g safflower oil and 1.5 g cholesterol per kg diet; Dyets Inc., Bethlehem, PA). After 2 weeks, mice were injected intraperitoneally with PCB77 (170 μ mol/kg body weight) or the vehicle (olive oil). This treatment was repeated after 6 days. In our previous studies, 170 μ mol/kg of PCB77 was sufficient to induce VCAM-1 up-regulation in mouse aorta [8], and repeated administration of PCB77 led to increased atherosclerosis in aortic root sections [21]. Mice were sacrificed 24 h after the last treatment. Then, liver tissue samples were obtained and frozen in liquid nitrogen for further analysis.

Western blot

Endothelial cells were treated with either vehicle (0.1% DMSO) or PCB77 for indicated time periods. Cells were washed twice, scraped in ice-cold PBS and centrifuged. Appropriate amounts of boiling lysis buffer (1% SDS, 1 mM Na₂VO₄, 10 mM Tris, pH 7.4) were added to the cell pellets. The samples were boiled for 5 min and passed several times through a 26-gauge needle. The mouse livers were homogenized in ice-cold lysis buffer (50 mM Tris, 0.5% Triton X-100, 0.5% NP-40, 1 mM Na₂VO₄, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mg/l leupeptin, 10 mg/l pepstatin, 10 mg/l aprotinin, 100 mg/l PMSF, 2 mM DTT) and incubated on ice for 30 min. After centrifugation, protein concentrations were determined in the supernatants using BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of proteins were resolved by SDS-PAGE (10% gel), and equal loading was verified by Ponceau-S staining of membranes prior to probing. The membranes were then immunoblotted with the respective primary antibodies. After immunoblotting, the bands were visualized by incubation

of membranes with HRP-conjugated secondary antibody followed by ECL (Amersham Life Science, Birmingham, UK). The results were normalized to actin (loading control).

Detergent-free purification of caveolae-enriched membrane fractions

Detergent-free purification of caveolin-rich membrane fractions was performed as described previously [22] with minor modifications. Briefly, after treatment with PCB77 or vehicle for 6 h, endothelial cells were washed with ice-cold PBS and lysed in ice-cold MES-buffered saline (MBS; 25 mM MES [morpholineethanesulfonic acid, pH 6.5], 150 mM NaCl) containing 500 mM Na₂CO₃. The cell suspension was homogenized with a loose-fitting Dounce homogenizer and subjected to sonication. The homogenate was centrifuged at 1,800 g for 10 min, 4°C. The supernatant was adjusted to 45% sucrose by addition of 90% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube, overlaid with a discontinuous sucrose gradient (5 ml of 35% sucrose and 3 ml of 5% sucrose), and subjected to centrifugation at 39,000 rpm for 16 h using SW41 rotor (Beckman Instruments, Palo Alto, CA). A light-scattering band consistent with membrane caveolae was observed at the 5–30% sucrose interface. Twelve 1-ml fractions were collected, and aliquots of each fraction were subjected to SDS-PAGE and immunoblotting to assess caveolin-1.

Transmission electron microscopy (TEM)

TEM was performed as previously described [23]. Primary endothelial cells were grown in 6-well tissue culture plates for electron microscopy studies. After treatment with PCB77 or vehicle for 6 h, cells were removed with a scraper, washed with ice-cold PBS, and fixed in 4% buffered formalin over night. Cells were pelleted and post-fixed with 2% OsO₄, then dehydrated in graded ethanol, and embedded in Araldite 502 resin. Polymerized blocks were sectioned, collected on copper grids and stained before examination in a Philips CM 10 electron microscope (Philips Electron Optics B.V) operated at 60 Kv.

Analysis of PCB77 in caveolae-enriched membrane fractions

Endothelial cells were treated with PCB77 or vehicle for 6 h, followed by purification of caveolin-rich membrane fractions. PCB content in the caveolae-enriched membrane fractions was analyzed with a gas chromatograph-mass spectrometer (GC-MS) as previously reported [24]. The percent recovery of PCB77 was validated using mouse liver tissue and was over 95%. PCB77 levels in all samples were quantified using response relative to the internal standard (IS). Ten microliters of 1mg/l PCB209 was added as an IS. In addition, standard of PCB77 in hexane was used to prepare calibration plots.

Immunoprecipitation

Endothelial cells were treated with PCB77 or vehicle for 1 h, extensively washed with PBS, harvested, pelleted by centrifugation, resuspended in OG buffer (60 mM OG, 50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 2 mM dithiothreitol, 50 µM EGTA, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and sonicated as described previously [25]. The homogenates were incubated with a rabbit anti-caveolin-1 antibody (Affinity BioReagents, Golden, MO) at a final concentration of 5 µg/ml. After overnight at 4 °C, protein G-Sepharose beads (50 µl of 50 % slurry) were added to the supernatant for further 1 h incubation at 4 °C. Bound immune complexes were washed three times with OG buffer and then once with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. In some experiments, the supernatant fraction (remaining following pelleting of the protein G-Sepharose immune complexes) was precipitated by addition of trichloroacetic acid and buffered with a Tris-HCl solution, pH 7.4. The immunoprecipitates and/or the corresponding supernatant precipitates were then eluted by boiling in Laemmli sample buffer. Bands for AhR or caveolin-1 were identified using Western blot.

siRNA design and transfection

Caveolin-1 gene silencer was designed as previously described [26]. Cells were transfected with control siRNA or caveolin-1 siRNA at a final concentration of 40 nM using GeneSilencer (Genlantis, San Diego, CA) in OptiMEM I medium (Invitrogen, Carlsbad, CA). Cells were incubated with transfection mixtures for 4 h and then replaced with regular medium. 48 h after transfection, cells were treated with PCB77 or vehicle for 6 h and analyzed for protein levels and oxidative stress.

Measurement of oxidative stress

Oxidative stress was assessed as the conversion of dichlorofluorescein diacetate (DCF-DA) into the fluorescent product 2',7'-dichlorofluorescein (DCF). After transfection with control or caveolin-1 siRNA, cells were treated with vehicle control (DMSO) or PCB77 (2.5 μ M) for 6 h, and DCF fluorescence was measured as described previously [27].

Statistical analysis

The statistical analysis of the data was performed by SigmaStat software (Systat Software Inc., Richmond, CA, USA). 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 induces caveolin-1 and CYP1A1 in endothelial cells

Caveolae are particularly abundant in endothelial cells, which was confirmed in our cell culture model as demonstrated by TEM (Figure 1). Endothelial cell exposure to PCB77 increased caveolin-1 protein expression in total cell lysate in a time dependent manner (Figure 2). Similar to the induction of caveolin-1, exposure to PCB77 increased the expression of CYP1A1, a marker of AhR activation, with maximum induction at 6 h (Figure 2). Maximal up-regulation of both caveolin-1 and CYP1A1 was reached at the concentration of 2.5 μ M (data not shown). In addition to PCB77, PCB126 also increased caveolin-1 expression in our endothelial cell model. For example, concentrations as low as 0.1 μ M up-regulated caveolin-1 expression 2-fold (control: 0.46 ± 0.05 ; PCB126: 0.94 ± 0.03 ; expressed as caveolin-1/actin). However, the non-coplanar PCB153 did not increase caveolin-1 expression at up to 2.5 μ M.

PCB77 accumulates in caveolae fraction of endothelial cells

Caveolae fractions were isolated using sucrose gradient isolation, and caveolin-1 protein levels were determined by Western blotting. The majority of caveolin-1 accumulated in fraction 4 (Figure 3A) that was recognized as a highly specific caveolae fraction in previous studies [22]. After treatment with PCB77, levels of caveolin-1 in caveolae fractions were increased suggesting increased caveolae formation (Figure 3A). Fractions from the gradient were analyzed for PCB77 by direct analytical quantification using GC-MS, and PCB77 content was highest in fraction 4 (Figures 3B and 3C). As demonstrated in Figure 3A, fraction 4 contains the majority of cellular caveolin-1 and represents the caveolae fraction. These findings suggest that PCBs accumulate in membrane caveolae of vascular endothelial cells where they might interact with caveolae-associated receptors and signaling pathways.

PCB77 induces AhR binding to caveolin-1 in endothelial cells

We observed that PCB77 localizes mainly to the caveolae-rich fraction in endothelial cells. Therefore, we tested the hypothesis that caveolae are the place of interaction between PCB77 and its main molecular target, AhR. To examine the localization of AhR to caveolae and its association caveolin-1, protein extracts were immunoprecipitated with antibodies against

caveolin-1 or AhR and then Western blotted (Figure 4A). It was demonstrated that AhR binds caveolin-1 by probing reciprocally against either AhR or caveolin-1. Using this approach, it was also shown that the association of AhR with caveolin-1 increased after the treatment with PCB77 (Figure 4B). These data demonstrate that AhR binds caveolin-1 and suggest that accumulation of PCB77 in caveolae promotes this interaction.

Caveolin-1 silencing decreases the induction of CYP1A1 levels and oxidative stress by PCB77

To demonstrate that induction of CYP1A1 by PCB77 through AhR requires functional caveolae, endothelial cells were transfected with caveolin-1 siRNA. The ability of siRNA targeted for caveolin-1 to specifically silence caveolin-1 protein is shown in Figure 5A. By Western blotting, a reduction in caveolin-1 protein level was detected at 48 h after transfection of siRNA caveolin-1 compared to control scrambled siRNA. Silencing of the caveolin-1 gene also markedly decreased CYP1A1 induction in vehicle, as well as in PCB77-treated cultures (Figure 5B). Figure 5A also shows that this effect is not mediated by decreased levels of AhR in absence of caveolin-1, since AhR levels remained unchanged. It has been shown that PCB77 uncouples CYP1A1 leading to the production of reactive oxygen species [28]. We previously observed an increased oxidative stress after PCB77 treatment in primary endothelial cells [2]. Silencing of the caveolin-1 gene markedly decreased oxidative stress induced by PCB77 in our endothelial cell cultures (Figure 5C). These data confirm that caveolin-1 is required for activation of AhR-mediated transcriptional up-regulation, as well as downstream adverse effect, such as oxidative stress. Separate studies with LDL-R-deficient mice revealed that hepatic CYP1A1 induction by PCB77 exposure (110 fold increase) is significantly reduced in LDL-R^{-/-} mice which also lack the Cav-1 gene (19 fold increase).

Discussion

The vascular endothelium is involved in the regulation of the structure and function of blood vessels. Endothelial cells not only form a barrier protecting the underlying vascular tissue, but these cells also generate signaling molecules, which serve diverse autocrine and paracrine functions. Endothelial cell activation and dysfunction and subsequent inflammatory events are considered early events in the etiology of vascular diseases such as atherosclerosis [5].

There is substantial evidence that cardiovascular diseases are linked to environmental pollution. For example, there was a significant increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to PCBs for at least five years [29], and most excess deaths were due to cardiovascular disease in power workers exposed to phenoxy herbicides and PCBs in waste transformer oil [30]. Most importantly, a recent study reported increased hospitalization rates for coronary heart disease in populations residing near areas contaminated with persistent organic pollutants [11]. Many environmental pollutants interact with the AhR to initiate xenobiotic metabolizing activity linked to an increase in cellular oxidative stress and onset of a variety of pathologies [31]. It has been suggested that AhR activation is a critical participant in mechanisms associated with cardiovascular diseases [9,10] and that AhR ligands may be atherogenic by disrupting the functions of endothelial cells in blood vessels.

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 [32]. Caveolae are a subset of lipid rafts that are abundant within the plasma membrane of endothelial cells [33]. Caveolae compartmentalize signal transduction molecules which regulate multiple endothelial functions. For example, the mechanisms underlying eNOS localization in caveolae and associated activity have been studied extensively [33]. Based on their lipid composition and biophysical features, caveolae are capable of endocytosis [34]. Thus, caveolae also appear to be important in

internalization and intracellular trafficking of proteins, receptors and possibly lipophilic compounds such as persistent organic pollutants. Caveolin-1, the primary structural protein required for the formation and functional activity of caveolae, is also important in regulating transcytotic pathways and cellular trafficking and thus endothelial permeability [35].

We provide novel data suggesting that endothelial activation and proatherogenic activities of persistent organic pollutants, such as PCBs, are in part regulated via caveolae signaling. As expected, PCB77 induced CYP1A1 protein expression, a characteristic metabolic outcome mediated by the interaction with the AhR [4,36]. We also demonstrated that endothelial cell exposure to coplanar PCBs such as PCB77 can increase caveolin-1 protein expression in total cell lysate in a concentration-dependent manner. Similar results were observed with PCB126, but not with PCB153. Current and past studies by our group provide evidence of “cross-talk” between activation of pro-inflammatory cell signaling pathways (e.g. AhR, NFκB), caveolin-1 expression and CYP1A1 induction upon exposure to PCB77 or other AhR agonists. For example, we have demonstrated a role of caveolin-1 in PCB77-induced eNOS phosphorylation in human-derived endothelial cells. Exposure to PCB77 induced caveolin-1 and eNOS, and caveolin-1 silencing abolished PCB77-stimulated eNOS phosphorylation [37]. We also demonstrated that caveolin-1 silencing blocked DNA binding of NF-κB, a critical transcription factor in endothelial inflammation (e.g., induction of adhesion molecules) [37]. Previous studies from our laboratory have shown that dietary flavonoids (e.g., EGCG and quercetin) can down-regulate PCB-induced oxidative stress, CYP1A1 induction, and AhR-DNA binding activity in vascular endothelial cells [27]. Finally, in a recent study [38], we demonstrated in human endothelial cells (HUVEC) that benzo[a]pyrene can induce adhesion molecule (e.g., ICAM-1) expression through a caveolae and AhR mediated pathway. Silencing of either caveolin-1 or AhR reduced ICAM-1 expression associated with exposure to benzo[a]pyrene, suggesting cross-talk between caveolin-1 and AhR.

Caveolin-1 is an essential protein in the formation of caveolae [34], and in caveolin-1 knockout mice, the complete lack of caveolae invaginations is readily seen in endothelial cells [39]. In addition to PCB-induced upregulation of the caveolin-1 gene, we provide evidence that this persistent organic pollutant can also induce caveolae formation, as quantified by sucrose gradient isolation and qualitative demonstration via TEM imaging. Our data provide further evidence that the proinflammatory and proatherogenic properties of coplanar PCBs may occur or be initiated at the level of caveolae signaling. Previous studies by our group demonstrated that coplanar PCBs, including PCB77, PCB126 and PCB169 (3,3',4,4',5,5'-hexachlorobiphenyl), can induce endothelial dysfunction by increasing oxidative stress, activation of pro-inflammatory transcription factors such as NF-κB and expression of adhesion molecules and cytokines. In contrast, the non-coplanar PCB153 did not exhibit these effects [8]. Because coplanar PCBs induce both caveolin-1 expression and inflammatory responses in endothelial cells, our results strongly suggest that caveolae play a role in endothelial dysfunction by coplanar PCBs.

Caveolae have been linked to the regulation of cholesterol homeostasis and cellular sterol transport [40]. Caveolae may also serve as critical transport vesicles involved in transcytosis of solutes, membrane components, proteins, and viruses, extracellular ligands [41] and possibly lipophilic compounds such as PCBs. We were able to demonstrate selective accumulation of PCB77 in the caveolae-rich fraction of total cell lysate which also contained the PCB-inducible caveolin-1 protein. These findings are significant, because they suggest that caveolae may be a critical site of cellular uptake of lipophilic environmental pollutants. Indeed, we recently demonstrated that PCB77 increases phosphorylation of caveolin-1 in endothelial cells [37], which has been linked to an increase in the rate of caveolae-dependent endocytosis [42]. These data also suggest a direct link between PCB accumulation in caveolae and its induction of

caveolin-1 gene and the trigger of mechanisms involved in endothelial cell activation and proinflammatory events [8].

Lipid rafts like caveolae provide sites of dynamic regulatory events in receptor-induced signal transduction. For example, mediators of vascular function, including G-protein coupled receptors, Src family tyrosine kinases, receptor tyrosine kinases, protein phosphatases and nitric oxide synthase, are concentrated within caveolae microdomains [43]. Previous studies by others have shown that nuclear receptors can interact with caveolae, and ligand dependent activation can promote binding to caveolin-1 [44,45]. Interaction with caveolin-1 and the ligand binding domain of the androgen receptor have been reported. This interaction was shown to be necessary for dihydrotestosterone dependent activation of the androgen receptor [46]. We provide evidence of colocalization of the AhR with caveolin-1 and that PCB77 treatment can increase AhR binding to caveolin-1. We also demonstrated that PCB77-induced CYP1A1 requires functional caveolae and that silencing the caveolin-1 gene can markedly decrease CYP1A1 induction by PCB77. Preliminary *in vivo* studies (data not shown) demonstrated that only PCB77 but not PCB153 increased caveolin-1 protein expression in mouse lungs. Because PCB77 but not PCB153 is a AhR ligand, this provides further evidence of AhR binding to caveolin-1 as a prerequisite for subsequent gene induction. Furthermore, our *in vivo* studies demonstrate that PCB77-induced up-regulation of hepatic CYP1A1 protein levels is markedly reduced in LDL-R-deficient mice that lacked the caveolin-1 gene (data not shown). LDL-R-deficient mice have become a preferred model for atherosclerosis studies because they mimic human atherosclerosis. We previously demonstrated that PCB77 increases aortic adhesion molecule expression in LDL-R-deficient mice [1].

In summary, we show that coplanar PCBs like PCB77 can induce caveolin-1 and caveolae formation in vascular endothelial cells. We also provide evidence that interaction between AhR and caveolin-1 can influence induction of CYP1A1 expression. These results demonstrate the involvement of caveolae as a regulatory platform in endothelial cell effects of coplanar PCBs. Because caveolae and caveolins have been implicated in several human diseases and in particular in vascular diseases, our data may have implications in understanding mechanisms of inflammatory diseases induced by exposure to environmental pollutants.

Acknowledgements

This study was supported by the National Institute of Environmental Health Sciences/National Institutes of Health (P42ES07380); and the Kentucky Agricultural Experimental Station.

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Figure 1. Endothelial cells exhibit caveolae formation

Cells were fixed for TEM analysis (Figure 1). Plasma membranes were scanned for caveolae (defined as uniform 50 to 100 nm flask-shaped membrane invaginations). After PCB exposure, cells appeared to exhibit an increase in caveolae formation (arrows).

Figure 2. PCB77 induces caveolin-1 (Caveolin-1) and CYP1A1 protein expression in endothelial cells

Cells were incubated with PCB77 (2.5 μ M) or vehicle control (DMSO) for the times indicated. Total cell proteins were resolved using SDS-PAGE, and caveolin-1, CYP1A1 and actin protein levels were detected by Western blot. Densitometry results represent mean $\pm$ SEM of three independent experiments. *A significant difference compared with control cultures (0 time or 0 concentration in Figure 1A) ($p < 0.05$).

Figure 3. PCB77 accumulates in caveolae fraction of endothelial cells

Cells were treated with PCB77 (2.5 μ M) or control (vehicle) for 6 h. Cell fractions were obtained by sucrose gradient centrifugation and analyzed for caveolin-1 by Western blot (Figure 3A). Fraction 3A represents the caveolae-rich fraction as described previously [47]. PCB77-treated fractions from sucrose gradient centrifugation were quantified by GC-MS. PCB77 accumulated predominantly in the caveolin-rich fraction (Figure 3B). This increase of PCB77 level in fraction 4 was highly significant (Figure 3C). The means $\pm$ SEM of PCB77 concentrations for 3 different experiments are shown as ng/ μ l per fraction in (Figure 2C). *A significant difference compared with all other cell fractions ($p < 0.05$).

Figure 4. PCB77 induces AhR binding to caveolin-1 (Cav-1) in endothelial cells

Cells were treated with PCB77 (2.5 μ M) or vehicle (Control) for 1 h and whole cell lysates were harvested. Immunoprecipitates [24] were resolved by SDS-PAGE and subjected to Western blot analysis with anti-caveolin-1 and anti-AhR. The association of AhR with caveolin-1 was demonstrated by immunoprecipitation of either AhR or caveolin-1, followed by Western blot analysis (Figure 4A). The association of AhR with caveolin-1 was increased in the presence of PCB77 (Figure 4B).

Figure 5. Caveolin-1 silencing decreases CYP1A1 induction and oxidative stress by PCB77

Endothelial cells were transfected with control siRNA or with siRNA for caveolin-1 (Cav-1) (Ctr-siRNA or Cav-1-siRNA, respectively) and treated with vehicle or PCB77 (2.5 μ M) for 6 h. Total lysates were probed with anti-Cav-1, anti-actin or anti-AhR antibodies (Figure 5A) or anti-CYP1A1 antibody (Figure 5B). Densitometry results represent mean $\pm$ SEM of three independent experiments. DCF-fluorescence was determined after the cells were treated with or without PCB77 for 6 h (Figure 5C). *A significant difference compared with control cultures ($p < 0.05$). **A significant difference compared with the respective control-siRNA for CYP1A1 induction or oxidative stress measurement ($p < 0.05$).