# PCB 126-INDUCED ENDOTHELIAL TOXICITY IS MODULATED BY CROSS-TALK BETWEEN CAVEOLAE AND NRF2 SIGNALING

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**Introduction**

Exposure to persistent environmental pollutants such as polychlorinated biphenyls has been linked to the induction and/or exacerbation of multiple human pathologies including diabetes and atherosclerosis[1]. Specifically, coplanar polychlorinated biphenyls (PCBs) have been shown to initiate the earliest stages of atherosclerotic plaque formation, e.g., endothelial cell dysfunction and inflammation. Once believed to be an innate barrier, endothelial cells now appear to play an extremely important role in the initiation and progression of atherosclerosis[2]. Coplanar PCBs can further promote endothelial cell inflammation and dysfunction through caveolae lipid micro-domains[2]. Endothelial cell activation can lead to an upregulation of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) which promotes pro-inflammatory leukocyte infiltration and chemokine production that if left unchecked can lead to the formation of foam cells and subsequent arterial blockage. Coplanar PCBs can induce oxidative stress in endothelial cells and in turn cause the upregulation of pro-inflammatory proteins through an NFκB-mediated signaling cascade. Interestingly, it has been shown that PCBs may also be pro-atherogenic by activating other pro-inflammatory pathways such as the lipid signaling domain caveolae. Lipid raft microdomains known as caveolae are flask-shaped invaginations found at the lipid membrane and have been shown to play important roles in endocytosis, atherosclerosis, and environmental pollutant toxicity and. Caveolin-1 (Cav-1), the major structural and signaling protein involved in the caveolae pathway, has been shown through its "Cav-1 binding domain" (CBD) to interact and bind multiple other proteins, many of which are involved in inflammation and atherosclerosis[2]. Coplanar PCBs preferentially sequester in caveolae cellular fractions, and exposure to coplanar PCBs upregulates Cav-1 protein expression and caveolae formation. Silencing Cav-1 via siRNA technology prevents PCB-induced cytochrome P450-mediated superoxide production and subsequent endothelial activation and dysfunction. Importantly, it has been shown that aortic endothelial cells isolated from mice that lack the Cav-1 gene are protected from toxicant-induced cellular dysfunction, but the mechanism of this protection has yet to be elucidated[3]. Physiological systems have evolved multiple signaling pathways to limit the toxicity of xenobiotics such as PCBs. The most significant regulator of redox status and homeostasis, the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant pathway, has been shown to be critical in protecting endothelial cells from PCB toxicity[2, 4]. Nrf2 is primarily regulated by its major inhibitory protein, iNrf2 or Keap1, which promotes Nrf2's ubiquitination and maintains basal levels of Nrf2 activation. Nrf2 also can be inhibited by Fyn kinase, which can promote Nrf2 nuclear exit and degradation. Nrf2 also can be directly activated via phosphorylation by kinases such as Akt and PKC delta. Nrf2 can become activated by xenobiotic electrophiles, reactive oxygen species (ROS), and bioactive phytochemicals found in healthful nutrition[2,5]. Multiple electrophilic bioactive nutrients including components of ginseng, green tea and vegetables such as broccoli have been shown to activate Nrf2, but interestingly, different nutrients may induce Nrf2 through differing mechanisms (e.g., disruption of Keap1/Nrf2 interaction or increased phosphorylation via relevant kinases)[1]. Nrf2 has been shown to be regulated through the cross-talk of multiple signaling cascade pathways such as the aryl hydrocarbon receptor (AhR) and NFκB pathways[1]. Many xenobiotics such as dioxins and coplanar PCBs can activate both AhR and Nrf2 simultaneously, and in fact, this concordant upregulation can be evolutionarily explained since the gene promoter for AhR contains multiple Nrf2 binding elements (AREs) and the promoter for Nrf2 contains AhR binding sites (xenobiotic response elements)[1]. Evidence for direct cross-talk between Nrf2 and NFκB is not as well understood, but multiple studies have shown that activation of Nrf2 leads to a diminished pro-inflammatory NFκB response. Interestingly, we have previously shown that the AhR is a binding partner of Cav-1 which has led us to hypothesize that novel cross-

talk between Cav-1 and Nrf2 could exist and that this cross-talk may help to explain mechanistically the protection from coplanar PCBs observed in Cav-1 −/− animals[1].
Thus, the current study has been designed to investigate mechanistically how the cross-talk between Cav-1 and Nrf2 can modulate PCB-induced cellular dysfunction. Our data provide strong evidence that there are multiple levels of Cav-1/Nrf2 cross-talk and that Cav-1 inhibits the Nrf2 antioxidant response. Thus, reduction or downregulation of endothelial Cav-1 may lead to an upregulated antioxidant response regulated by Nrf2, which could better prime a physiological system prior to toxicological insult.

**Materials and methods**

Materials and chemicals:
3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) was obtained from AccuStandard Inc. (New Haven, CT). VCAM-1 and Keap1 primary antibodies and horseradish peroxidase-conjugated goat secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Actin and GST primary antibodies were purchased from Sigma (St. Louis, MO). The GST antibody used recognized native as well as denatured-reduced forms of GST protein. NQO1 primary antibody was purchased from Abcam (Cambridge, MA). Fyn kinase, P-Akt, Akt, P-PKC delta and Nrf2 primary antibodies and horseradish peroxidase-conjugated rabbit secondary antibody were purchased from Cell Signaling Technologies (Danvers, MA).

Cell culture and experimental media:
Primary vascular endothelial cells were isolated from porcine pulmonary arteries[3]. Cells were cultured in M199 (Gibco, Grand Island, NY), supplemented with fetal bovine serum (FBS; Gibco). EAhy.926 human endothelial cells were cultured as described previously[6]. Endothelial cells were grown to confluence, followed by incubation overnight in medium containing 1% FBS prior to cell treatment. Stock solutions of PCB 126 were prepared in DMSO; control cultures were treated with DMSO vehicle. The levels of DMSO in experimental media were 0.05%. Porcine and human endothelial cells were treated with PCB 126 at 0.25 μM for 16 h or 4 h and mouse pulmonary endothelial cells were treated with 2.5 μM for 24 h, which are established concentrations used previously in our laboratory[3].

Mouse endothelial cell isolation:
Endothelial cells were isolated from Cav-1 deficient (Cav-1 −/−) and wildtype mice (both genotypes were purchased from Jackson Laboratory, Bar Harbor, ME). All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care — certified animal facilities at the University of Kentucky. Age matched C57BL/6 mice were used as controls because Cav-1-deficient mice are backcrossed onto C57BL/6 mice. Briefly, whole lungs were homogenized in culture media containing type II collagenase and dispase. Cells were added to gelatin-coated tissue culture plates in DMEM media containing 20% FBS, heparin, antibiotics, and endothelial cell growth supplement. Endothelial cells were preferentially selected by using an antibody-coated magnetic bead mix (Invitrogen, Carlsbad, CA). Briefly, sheep anti-rat IgG beads were prepared and mixed with rat anti-mouse CD31 PECAM antibodies overnight (BD Biosciences, San Jose, CA). Bead/antibody conjugates were collected via magnetic separation and subsequently were incubated for 1 h with cells isolated from lungs. Cells were then trypsinized, transferred to a magnetic separator, and magnetically bound cells were seeded in 35 mm plates.
Real-time PCR:
The levels of mRNA expression were assessed by real-time PCR using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and SYBR Green master mix (Applied Biosystems) as described earlier[3].
Cav-1 siRNA and transfection studies:
Double stranded small interfering RNA targeting Cav-1 was synthesized as described previously[6]. Porcine endothelial cells and human endothelial cells were transfected with control or Cav-1 siRNA at a final concentration of 80 nM using GeneSilencer transfection reagent (Genlantis, San Diego, CA) in OptiMEM serum free media. Cells were incubated with the transfection mixture for 4 h, followed by the addition of 10% FBS to the cell media. Cells were used for treatments after 48 h incubation.

Immunoblotting:
Western blot analyses for VCAM-1, β-actin, NQO1, Keap1, Fyn kinase, GST, Nrf2, P-PKC delta and P-Akt were performed as described previously[3].

**Results and discussion**

Cav-1 silencing prevents PCB-induced endothelial cell dysfunction by increasing antioxidant gene expression:

Here, we treated primary porcine vascular endothelial cells with a single low dose (0.25 μM) of PCB 126 for 16 h and saw a statistically significant upregulation of VCAM-1 mRNA expression in control siRNA treated cells (Fig. 1). However, this upregulation was not observed in cells treated with Cav-1 siRNA. To possibly explain this observed protection, we also investigated Nrf2 activation and observed increased mRNA expression levels of multiple Nrf2 target genes in cells silenced for Cav-1 (Fig. 2). To investigate possible novel cross-talk mechanisms between Cav-1 and Nrf2 we then investigated the levels of two Nrf2 inhibitory proteins Keap1 and Fyn Kinase in human endothelial cells (Fig. 3). 4 hours of PCB exposure caused a decrease in protein expression of the Nrf2 inhibitory proteins Keap1 and Fyn kinase in both Cav-1 and control siRNA treated cells. Importantly, at basal DMSO conditions, cells silenced for Cav-1 also displayed significantly lower levels of Keap1 and Fyn compared to DMSO-treated control siRNA cells. Exposing PCB to these Cav-1 silenced cells resulted in a statistically significant decrease in Fyn and Keap1 compared to Cav-1 silenced cells treated with DMSO. Also, Keap1 mRNA levels in Cav-1 silenced porcine cells were examined via RT-PCR and similarly significant trends as with human endothelial cell protein data were observe (data not shown). Finally, to confirm the importance of Cav-1/Nrf2 cross-talk in PCB-induced toxicity, lung endothelail cells were isolated from wildtype and Cav-1 KO mice and subsequently exposed to dioxin-like PCB 126 for 24 h. As mirrored in the siRNA experiments utilizing porcine cells, PCB 126 only increased cellular dysfunction (VCAM-1 induction) in cells isolated from C57BL/6 mice and not Cav-1 −/− mice (data not shown). We attribute this protection in Cav-1 −/− mice to the considerably higher observed protein levels of antioxidant enzymes such as NQO1 and GST.

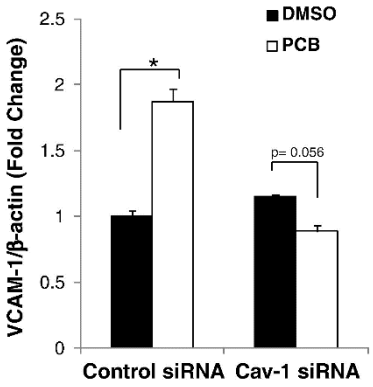



**Fig. 1.** Expression of VCAM-1 mRNA in porcine vascular endothelial cells exposed to coplanar PCB 126. PCB 126 increases VCAM-1 mRNA levels only in endothelial cells with Cav-1 (*p < 0.05).

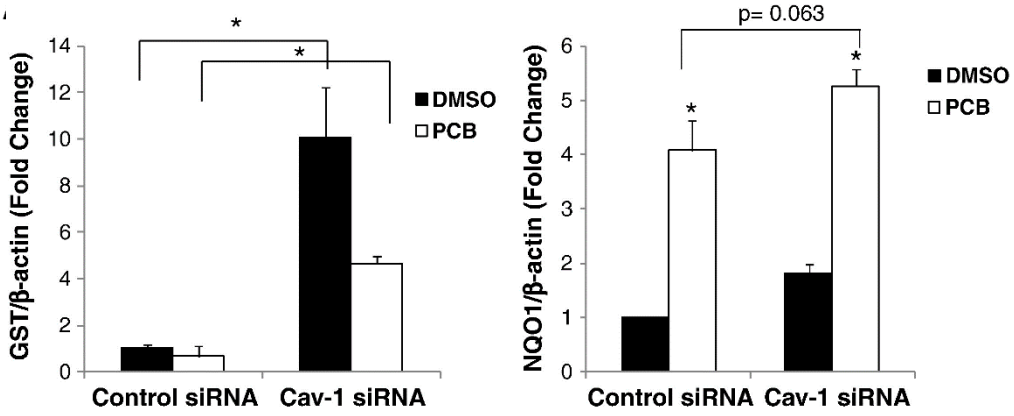
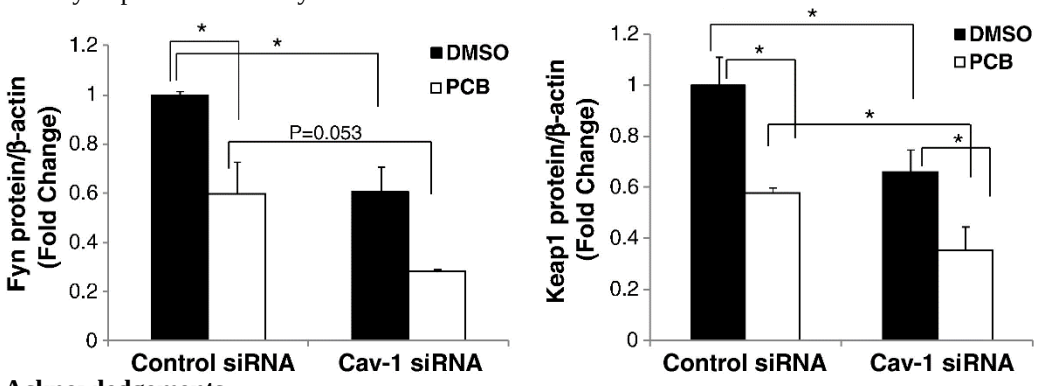



**Fig. 2.** Expression of antioxidant enzymes in cells silenced for Cav-1. Cells silenced for Cav-1 display increased mRNA levels of GST and NQO1 (*p < 0.05).

The importance of caveolae, lipid rafts, and Cav-1 in inflammation and heart disease has become a source of much research, but until recently, cross-talk between caveolae-related proteins and other factors has not been elucidated. Now, evidence points to interactions between Cav-1, AhR, and Nrf2, all proteins involved in inflammation and toxicant-induced disease[1]. Downregulation of endothelial Cav-1 may prove to be an effective modulator of toxicant-induced disease as well as other pathologies hallmarked by chronic inflammation. We have shown previously that healthful nutrition, which is high in bioactive food components such as polyphenols

and omega-3 polyunsaturated fatty acids, can protect against PCB-induced cellular dysfunction[2]. Laboratories have shown that these nutrients may protect through multiple mechanisms including disruption of functional caveolae, increased Nrf2 activity, and decreased NFκB activation. It is plausible to hypothesize that the cross-talk between Cav-1 and Nrf2 pathways is critical for nutritional and/or pharmacological modulation of toxicant-induced disease.

Our data suggest that cross-talk between Cav-1 and Nrf2 exists and may be more complex than described previously. Cav-1 inhibits Nrf2 activation, and decreasing Cav-1 levels increases the expression of Nrf2 target genes. This increased induction of protective genes may help to explain why cells and mice lacking Cav-1 are protected against pro-inflammatory polychlorinated biphenyl exposure. Our studies provide concordant findings from experiments using cellular models from three different mammalian species, i.e., pig, mouse, and human, suggesting some relevance of our findings to human health and risk assessments. Caveolae and Nrf2-related proteins may prove to be important targets for effective nutritional and/or pharmacological protection against the toxicity of pro-inflammatory xenobiotics.

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