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Polychlorinated biphenyl quinone induces endothelial barrier dysregulation by setting the cross talk between VE-cadherin, focal adhesion, and MAPK signaling

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Zhang P, Feng S, Bai H, Zeng P, Chen F, Wu C, Peng Y, Zhang Q, Zhang Q, Ye Q, Xue Q, Xu X, Song E, Song Y. Polychlorinated biphenyl quinone induces endothelial barrier dysregulation by setting the cross talk between VE-cadherin, focal adhesion, and MAPK signaling. Am J Physiol Heart Circ Physiol 308: H1205–H1214, 2015. — Environmental hazardous material polychlorinated biphenyl (PCB) exposure is associated with vascular endothelial dysfunction, which may increase the risk of cardiovascular diseases and cancer metastasis. Our previous studies illustrated the cytotoxic, antiproliferative, and genotoxic effects of a synthetic, quinone-type, highly reactive metabolite of PCB, 2,3,5-trichloro-6-phenyl-[1,4]benzoquinone (PCB29-pQ). Here, we used it as the model compound to investigate its effects on vascular endothelial integrity and permeability. We demonstrated that noncytotoxic doses of PCB29-pQ induced vascular endothelial (VE)-cadherin junction disassembly by increasing the phosphorylation of VE-cadherin at Y658. We also found that focal adhesion assembly was required for PCB29-pQ-induced junction breakdown. Focal adhesion site-associated actin stress fibers may serve as holding points for cytoskeletal tension to regulate the cellular contractility. PCB29-pQ exposure promoted the association of actin stress fibers with paxillin-containing focal adhesion sites and enlarged the size/number of focal adhesions. In addition, PCB29-pQ treatment induced phosphorylation of paxillin at Y118. By using pharmacological inhibition, we further demonstrated that p38 activation was necessary for paxillin phosphorylation, whereas extracellular signal-regulated kinases-1/2 activation regulated VE-cadherin phosphorylation. In conclusion, these results indicated that PCB29-pQ stimulates endothelial hyperpermeability by mediating VE-cadherin disassembly, junction breakdown, and focal adhesion formation. Intervention strategies targeting focal adhesion and MAPK signaling could be used as therapeutic approaches for preventing adverse cardiovascular health effects induced by environmental toxicants such as PCBs.

PCB; quinone; VE-cadherin; focal adhesion; adherens junction; paxillin

POLYCHLORINATED BIPHENYL (PCB) is a class of ubiquitous environmental pollutants, which has been widely used in different industrial applications in the last century. Even though most countries banned PCB production in the late 1970s, it persists globally as a consequence of improper transport, storage, and disposal (49). PCB exposure has been suggested to cause several adverse effects in humans, for instance, endocrine, immunologic, and developmental disorders (51). Although accumulating evidences indicated that PCB exposure is highly linked with the dysfunction of vascular endothelial cells (ECs) (17, 25, 58), the underlying molecular mechanisms need further investigation.

Vascular endothelium works as a semipermeable barrier between blood and the interstitial space in the regulation of macromolecule transport, leukocyte trafficking, and tumor metastasis. Thus the maintenance of endothelial barrier integrity plays an essential role in homeostasis (61, 64). The integrity of endothelial junction is maintained by the balance between contractility and cell-cell adhesive interactions (26, 28). Vascular endothelial (VE)-cadherin homophilic bindings maintain endothelial cell-cell interactions, sealing the adherens junctions (12, 13). VE-cadherin has significant functions in the processes of angiogenesis, tumor transmigration, and atherosclerosis (11, 37, 50). At resting states, VE-cadherin is localized to the adherens junctions, associated with α-catenin, β-catenin, p120-catenin, and plakoglobin via its cytoplasmic domains. The phosphorylation of VE-cadherin tyrosine residues results in the dissociation of p120 and β-catenin complex from cytoplasmic tails of VE-cadherin, which in turn disrupts endothelial junction integrity. VE-cadherin phosphorylation entails an increase in VE-cadherin internalization and endothelial permeability (19). Cytoskeleton rearrangement, such as cortical actin dissolution and stress fiber formation, and actomyosin-mediated cell contractility also play important roles in the events of endothelial retraction and gap formation (4, 29, 48, 69).

PCB can be metabolized into its hydroxylated and dihydroxylated (hydroquinone) metabolites with cytochrome P-450 catalysis (44). Hydroquinone can undergo further oxidation to turn into its corresponding oxidized quinone form (60). Interestingly, previous studies also indicated that quinone-type compounds increase the permeability of the vascular endothelium (42, 43). In the current study, we investigated the effect of PCB quinone derivative, 2,3,5-trichloro-6-phenyl-[1,4]benzoquinone, namely PCB29-pQ, on vascular endothelial integrity. We found that at noncytotoxic doses, PCB29-pQ induced endothelial gap formation and VE-cadherin phosphorylation. Furthermore, PCB29-pQ promoted actin stress fiber formation.

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and focal adhesion assembly. Additionally, p38 and extracellular signal-regulated kinase-1/2 (ERK1/2) mitogen-activated protein kinases (MAPKs) were involved in focal adhesion formation and VE-cadherin phosphorylation.

MATERIALS AND METHODS

Reagents. PCB29-pQ was synthesized and characterized as previously described (59). Stock solution (50 mM) of PCB29-pQ was prepared in dimethyl sulfoxide (DMSO) before use. F12-K medium was obtained from Gibco (Gibco, Grand Island, NY). Thiolutin, 3-(2-aminoethoxy)-5-[4(4-ethoxyphenyl)methylene]-2,4-thiazolidinedione hydrochloride (CAS No. 294675-79-9), SB-203580, and 3-(4,5-dimethyl-2-thiazolylo)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). Antiphosphorylated VE-cadherin (Y658) antibody was purchased from Chemicon (Temecula, CA). Anti-paxillin antibody was obtained from Santa Cruz (Dallas, TX). Antiphosphorylated paxillin (Y118) antibody was purchased from Abcam (Eugene, OR). Anti-p38 and ERK1/2 antibodies were purchased from Proteintech Group (Wuhan, China). Anti-VE-cadherin, phosphorylated p38, and phosphorylated ERK1/2 antibodies were purchased from Cell Signaling Technology.

Cell culture. Human umbilical vein ECs (HVECs) were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained in F12-K medium with 10% FBS, 50 μg/ml of EC growth supplement, 50 μg/ml heparin (Mallinckrodt Baker), and 100 U/ml of penicillin-streptomycin (Biofluids). Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Cell viability. Cell viability was determined using a MTT assay. HVECs were seeded onto 96-well culture plates at a density of 0.5 × 10⁴ cells/well and incubated overnight at 37°C. After being treated with PCB29-pQ at different doses, cells were incubated with 5 mg/ml MTT for 4 h. The MTT-containing growth medium was then replaced with 100 μl of DMSO and mixed thoroughly for 10 min. The optical density readings of each well were determined at 570 nm using a microplate reader (BioTek ELX800). The effect of PCB29-pQ on cell viabilities was expressed as the percentage of viable cells in treated groups compared with DMSO control. Values (means ± SE) from five independent experiments.

Western blot analysis. HVECs were collected and rinsed with PBS and then lysed with radioimmunoprecipitation assay lysis buffer, consisting of 20 mM Tris, 5 mM MgCl₂, 1 mM PMSF, 20 mg/ml aprotonin, 10 mg/ml leupeptin, 1 mM Na₃VO₃, and 20 mM β-glycerophosphate. The lysates were centrifuged at 14,000 rpm for 15 min. Protein concentrations across samples were checked by Bradford method. The samples were denatured by adding SDS running buffer consisting of 0.2% bromophenol blue, 4% SDS, 100 mM Tris (pH 6.8), and 20% glycerol and β-mercaptoethanol. The samples were analyzed by SDS-PAGE on~10–12% gels. After the proteins were transferred to nitrocellulose membrane, VE-cadherin, phosphorylated VE-cadherin (Y658), paxillin, phosphorylated paxillin (Y118), p38, ERK1/2, phosphorylated p38, and phosphorylated ERK1/2 were detected with corresponding primary monoclonal antibodies (1:1,000, diluted in blocking buffer), followed by horseradish peroxidase-conjugated secondary antibody. The labeled proteins were visualized using a chemiluminescence kit (Thermo Scientific, Waltham, MA).

Fluorescent staining. HVECs were grown on coverslips coated with fibronectin (1 μg/ml) before being treated with a variety of doses of PCB29-pQ. Cells were then washed twice with PBS and fixed with 5% paraformaldehyde for 10 min. Cells were permeabilized with 0.3% Triton X-100 in PBS and blocked for 30 min with 5% BSA. Subsequently, coverslips were incubated with anti-VE-cadherin or anti-paxillin for 1 h at room temperature. This was followed by staining with Alexa 488-conjugated anti-rabbit IgG. To image actin filaments, rhodamine-phalloidin (1:40; Invitrogen) was incubated with cells for 40 min. Finally, fluorescent staining was visualized with IX71 Olympus inverted microscopy (Olympus) with ×40 magnification. Colocalization of paxillin and actin was processed by ImageJ. To analyze the size and number of paxillin-containing focal adhesions, images were background subtracted before thresholding and segmentation were conducted to detect the edges of focal adhesions. The mean size (in pixels) and number of focal adhesions in each cell were then calculated. Twelve cells were analyzed for each treatment condition.

Disruption of VE-cadherin was identified from analysis of discontinuity of green fluorescence at VE-cadherin junctions between HVECs. Gap area within disrupted VE-cadherin junctions was determined from six images. Gap area was quantified as the ratio of pixels within all the gaps to the total number of pixels of an image. Results were representative of three independent experiments.

Pharmacological inhibition. To inhibit focal adhesion, cells were treated with 1 μg/ml thiolutin for 1 h before PCB29-pQ exposure. To inhibit p38 and ERK1/2, cells were treated with 10 μM p38 inhibitor SB-203580 or 25 μM ERK1/2 inhibitor, 3-(2-aminoethyl)-5-[(4-ethoxyphenyl)methylene]-2,4-thiazolidinedione hydrochloride for 1 h.

Transendothelial electrical resistance. Transendothelial electrical resistance (TER) with respect to time was measured with Millicell ERS-2 Voltohmmeter (Millipore, Billerica, MA). HUECs were seeded onto fibronectin-coated transwell insert membrane (0.4 μm pore). Confluent endothelial monolayers were incubated in DMEM, containing 10 mM HEPES (pH 7.4). PCB29-pQ-induced endothelial TER changes were then measured. The final TER values were calculated (as Ω × cm²) by multiplying it with surface area of transwell insert. The resistance values were normalized as the ratio of measured resistance to baseline value and are plotted as a function of time.

Statistical analysis. All data were obtained from at least three independent experiments and expressed by means ± SE. Statistical significance was determined using Student’s t-test or ANOVA. Tukey’s test was used in post hoc analysis for ANOVA. A probability value of P < 0.05 or P < 0.01 was considered to be statistically significant.

RESULTS

PCB29-pQ at a dose of <25 μm does not exert antiproliferative effect on HVECs. PCB29-pQ was previously reported to mediate apoptosis of human hepatocellular liver carcinoma (HepG2) cells (70). To examine the cytotoxicity of PCB29-pQ on HVECs, we measured the dose and time effect of PCB29-pQ on HVEC viability by MTT assay. Doses of PCB29-pQ between ~0.2–12.5 μM did not significantly alter the viability of HVECs (P > 0.05) (Fig. 1). The viabilities of HVECs following 25 and 50 μM PCB29-pQ exposure for 3 h were decreased to 77.3 and 46.3%, respectively. To exclude the possibility that the EC permeability-promoting effects of the compound could be attributed to its antiproliferative effects, nontoxic doses (<25 μM) of PCB29-pQ were used in the following studies.

PCB29-pQ mediates VE-cadherin dimer disassembly and junction breakdown. Endothelial permeability is dependent on the integrity of VE-cadherin-based adherens junctions. Since PCB 104 was shown to increase EC permeability (12), we hypothesized that PCB quinone may regulate the integrity of adherens junction. By fluorescently staining VE-cadherin, we demonstrated that VE-cadherin junction was disrupted upon PCB29-pQ treatment (Fig. 2A). In addition, PCB29-pQ induced endothelial VE-cadherin dimer dissociation and gap formation in a dose-dependent manner. VE-cadherin staining became absent along the periphery of HVECs, suggesting that VE-cadherin might be internalized. A quantitative analysis...
of gap size indicated that the percentage of gap was elevated with increasing doses of PCB29-pQ exposure for 6 h. PCB29-pQ treatment (10 μM) increased EC gap area by twofold compared with DMSO control (Fig. 2A). PCB29-pQ-induced gap formation was also a function of time with a plateau reached after 6 h (Fig. 2C). PCB29-pQ treatment caused a dose-dependent increase in endothelial permeability (Fig. 2D). PCB29-pQ treatments (1 μM, 5 μM, and 10 μM) decreased EC monolayer electrical resistance within 6 h by 10, 35, and 56%, respectively. Concomitantly, there was an increase in the phosphorylation of VE-cadherin at Y658 in a concentration- and time-dependent manner (Fig. 3), which has been shown to mediate the dissociation of β-catenin from VE-cadherin and the loss of barrier function upon PCB29-pQ exposure (50, 57).

Focal adhesion formation is required for PCB29-pQ-induced junction breakdown. Focal adhesion sites are associated with actin stress fibers, which may serve as holding points for cytoskeletal tension and be responsible for the regulation of cell contractility (69). Enhanced cell contraction may trigger EC junction breakdown. Thiolutin was suggested to induce focal adhesion kinase inactivation and paxillin degradation, leading to reduced focal adhesion assembly (45). To determine whether PCB29-pQ-induced adherens junction breakdown was dependent on focal adhesion formation, we incubated HUVEC monolayers with thiolutin. Our result indicated that thiolutin treatment significantly reduced PCB29-pQ-mediated EC gap formation (P < 0.05) (Fig. 4), suggesting that focal adhesion is required in PCB29-pQ-mediated junction breakdown.

PCB29-pQ promoted actin stress fiber formation and focal adhesion assembly. We next analyzed the effect of PCB29-pQ on actin cytoskeleton remodeling and focal adhesion dynamics. In control cells, filamentous actin assembled around cell periphery, with only a few thin stress fibers located within cell body (Fig. 5A). The actin morphology in cells treated with 1 μM PCB29-pQ was comparable with that in control group. In a sharp contrast, 5 μM PCB29-pQ-treated cells exhibited thick stress fibers that traversed the cell body. In 10 μM PCB29-pQ-treated cells, the stress fibers became more robust and organized. This result implied that PCB29-pQ mediated the rearrangement of actin cytoskeleton. Paxillin is a multidomain scaffold adaptor protein that recruits structural and signaling molecules to focal adhesions. It plays an important role in transducing signals to regulate cell adhesion, cytoskeletal re-organization, and gene expression (6). By staining paxillin, we demonstrated that in control cells, focal adhesion stainings were dim. Small focal adhesions were visible at cell periphery and almost disengaged with thin stress fibers. PCB29-pQ-treated HUVECs exhibited bright punctate focal adhesions that were colocalized with the end of thick stress fibers. PCB29-pQ increased the size and number of focal adhesions in a dose-dependent manner (Fig. 5, B and C).

PCB29-pQ mediated the phosphorylation of paxillin. The phosphorylation of paxillin is critical for the recruitment of adaptor and kinase proteins to focal adhesion complexes (5). To investigate whether PCB29-pQ treatment regulates the phosphorylation states of paxillin, HUVECs were treated with PCB29-pQ at a dose range of ~1–10 μM before being subject to Western blot analysis of paxillin phosphorylation at Y118. PCB29-pQ triggered paxillin phosphorylation with a peak level at 10-μM concentration (Fig. 6). In addition, paxillin phosphorylation level was increased with longer PCB29-pQ exposure durations. This result implies that paxillin phosphorylation may contribute to PCB29-pQ-mediated gap formation.

p38 and ERK1/2 differentially participated in PCB29-pQ-induced paxillin and VE-cadherin phosphorylation. Previous studies indicated that MAPKs, including p38 and ERK1/2, play critical roles in regulating VE-cadherin redistribution and endothelial permeability (4, 14, 48). More importantly, p38 and ERK1/2-controlled junction breakdown was critical for tumor extravasation (32, 63). Therefore, we investigated the effect of PCB29-pQ exposure on MAPK activities in ECs. PCB29-pQ stimulation induced p38 and ERK1/2 phosphorylation in a dose-dependent manner (Fig. 7A). The effect of PCB29-pQ exposure on MAPK phosphorylation was time dependent, as p38 and ERK1/2 activations were biphasic with an initial increase (peaking at 6 h), followed by a slight drop of the phosphorylation levels (Fig. 7B). PCB29-pQ-induced MAPK activation had a kinetic profile comparable with those observed for paxillin and VE-cadherin. This observation prompted us to study the roles of p38 and ERK1/2 in regulating paxillin and VE-cadherin phosphorylation. Pharmacological inhibition of p38 with SB-203580 considerably attenuated paxillin phosphorylation induced by PCB29-pQ (Fig. 7C). On the contrary, ERK1/2 inhibitor 3-[(2-aminoethyl)-5-[(4-ethoxyphenyl)methylene]-2,4-thiazolidinedione hydrochloride reduced VE-cadherin phosphorylation without affecting paxillin phosphorylation (Fig. 7C). Taken together, these results provided evidence that in PCB29-pQ-treated ECs, ERK1/2 modulates VE-cadherin phosphorylation at Y658, whereas p38 MAPK is required for paxillin phosphorylation and focal adhesion assembly.

DISCUSSION

There is only limited information available regarding the toxicity of PCB metabolites, although a wide spectrum of adverse effects of PCB parent individuals on human health have been investigated (54). Upon absorption, PCB is metabolically activated by cytochrome P-450 and oxidized into
arene oxide or radical intermediates, which can form covalent adducts with cellular components, like DNA and proteins (44). Hydroxylated PCB metabolites have been identified as the most abundant metabolites (18), and their oxidized forms, PCB quinones, have been suggested to induce oxidative DNA damage, genotoxicity, and mitochondria-mediated cell apoptosis in our previous studies (15, 39, 70). In the current study, we assessed the effect of PCB29-pQ on HUVEC permeability and junction disassembly. PCB29-pQ was selected as our model compound, since quinone-type PCB may be more toxic than its parental compound and the effect of quinone-type PCB on endothelial dysfunction has not be studied. Our main findings are as follows: 1) noncytotoxic doses of PCB29-pQ induced VE-cadherin phosphorylation at tyrosine residue 658 and endothelial junction breakdown; 2) focal adhesion formation and cytoskeletal rearrangement were required for PCB29-pQ-mediated junction breakdown; and 3) p38 was involved in regulating focal adhesion protein (paxillin) phosphorylation, whereas ERK1/2 participated in mediating VE-cadherin phosphorylation (Fig. 8).

Although PCB was banned 40 years ago, many old transformers and capacitors containing PCBs are still being used. People in industrial countries have been exposed to PCBs to certain extents through various routes. It was reported that average daily intake of PCBs by human beings via ambient air is about 100 ng, and average daily intake via drinking water is <200 ng (7). The average serum PCB levels for general population is about 1 to 2 parts/billion (ppb) (30, 33), whereas in occupationally exposed populations the PCB levels are more than 4 ppb (30, 33). Roya et al. (52) detected blood PCB concentration in women with endometriosis and found that women with stage IV endometriosis had a blood PCB29

Fig. 2. PCB29-pQ mediates vascular endothelial (VE)-cadherin disassembly and gap formation. A: Immunofluorescence staining of VE-cadherin junctions after HUVECs were treated with various doses of PCB29-pQ for 6 h. Scale bars = 10 µm. Arrowheads show the disruption of VE-cadherin homodimers. B: percentage of endothelial gap formation induced by PCB29-pQ exposure or 100 ng/ml TNF-α treatment for 6 h. *P < 0.05 and **P < 0.01 compared with DMSO. C: percentage of endothelial gap formation induced by 10 µM PCB29-pQ exposure for indicated time periods. Values are means ± SE; n = 3 replicates. D: dose effect of PCB29-pQ exposure or 100 ng/ml TNF-α treatment on endothelial permeability (temporal changes of transendothelial electrical resistance) was determined. Values are means ± SE; n = 3 replicates.
Fig. 3. PCB29-pQ mediates VE-cadherin phosphorylation (p) at tyrosine-658 in a dose- (A) and time-dependent (B) manner. β-Tubulin served as a loading control. Relative VE-cadherin phosphorylation levels for densitometric analysis are shown. All results are representative of at least 3 independent experiments.

Concentration of 0.99 ± 0.54 ppb. In the current study, the experimental concentrations of PCB29-pQ used were 1–10 μM (equivalent to 0.25–2.5 ppb), which falls in the lower range of PCB levels found in blood of people exposed to this toxicant, suggesting that similar mechanism found with HUVECs may occur in vivo (68). It can also be envisioned that in some individuals experiencing intense occupational exposure (up to 200 ppb), vascular endothelial leakage tends to be pathologically exacerbated. Several epidemiological studies indicated a strong link between PCB exposure at such dosage and cardiovascular diseases (2, 24). In addition, it was reported that PCB alters the expression of the tight junction proteins, like claudin-5, occludin, and zonula occludens-1 in brain capillaries (56). These alterations are associated with increased permeability of the blood-brain barrier, leading to the formation of blood-borne metastasis in mouse models. The propensity of malignant tumor and leukocyte to extravasate is dependent on their interactions with the postcapillary veins (1, 31, 71). Thus the leaky vascular endothelium due to PCB-induced alterations of adherens junctions may facilitate transcapillary transfer of tumor cells and leukocytes and contribute to the development of metastasis and inflammation. Increasing evidence suggests that exposure to PCB can lead to cancer progression (21) and cardiovascular diseases. For instance, there was a significant increase in mortality from cardiovascular diseases among Swedish capacitor manufacturing workers exposed to PCBs for at least five years (20), and cardiovascular diseases in power workers exposed to PCBs in waste transformer oil caused most excess death (24). In addition, the studies on the Seveso population (as a result of the industrial accident that occurred in the town of Seveso, Italy, in 1976) reported an increase in cardiovascular diseases (2).

Endothelial adherens junctions play vital roles in the control of vascular permeability. Intact endothelial monolayer is maintained by VE-cadherin homophilic associations which function as zippers to prevent barrier leakage (12). When endothelial layer is damaged and cells are retracted, the resultant increase in endothelial permeability is associated with obvious loss of vascular barrier function. Tyrosine phosphorylation of VE-cadherin is highly linked with changes in the integrity of the endothelial monolayer. Although there is a controversy with respect to which tyrosine residues of VE-cadherin are phosphorylated by different stimulations (12, 50), it is generally accepted that tyrosine-658 is a critical phosphorylation sites within the tail of VE-cadherin, whose phosphorylation leads to the disruption of cell-cell junctions. It was suggested that VE-cadherin, through the regulation of Y658 phosphorylation, competes for junctional localization with N-cadherin, regulating vascular permeability (23). Here, our results clearly demonstrated that tyrosine-658 serves as one of the targets in response to PCB29-pQ exposure. As the consequence of VE-cadherin phosphorylation, endothelial gap was induced upon the treatment of PCB29-pQ. Our data are comparable with previous findings that suggested that PCB104 induces endothelial hyperpermeability and increases transmigration of breast cancer cells (10, 17). Since the endothelium is in direct contact with the blood, ECs are susceptible to insults caused by circulating environmental pollutants and their metabolites. Sipos et al. (58) demonstrated that proinflammatory adhesion molecules, i.e., intercellular cell adhesion molecule-1 and vascular EC adhesion molecule-1, facilitate PCB-mediated enhancement of brain metastasis formation. In addition, caveolin-1 was found to play a role in PCB77-induced endothelial dysfunction by phosphorylating endothelial nitric oxide synthase (38). Another in vivo study demonstrated that exposure to different PCB individuals (PCB126, PCB118, and PCB153) altered the integrity of brain capillary endothelium and facilitated the formation of bloodborne metastases (56). VE-cadherin is linked to a large number of intracellular partners. Thus it is of interest to investigate the roles of other junction proteins, like α-catenin, β-catenin, and p120, in regulating junction breakdown mediated by PCB quinone treatment in the future studies. PCB29-pQ has been shown to upregulate reactive oxygen species (ROS) in cells (36). Since vascular endothelial integrity can be altered by ROS, PCB29-pQ may increase endothelial permeability by promoting ROS generation (16).

Focal adhesions are central regulatory sites for modulating EC barrier function. Focal adhesions contain focal adhesion

Fig. 4. Focal adhesion integrity is required for PCB29-pQ-mediated VE-cadherin disassembly. HUVEC monolayer was treated with or without 1 μg/ml thiolutin in endothelial permeability and increases transmigration of breast cancer cells (10, 17). Since the endothelium is in direct contact with the blood, ECs are susceptible to insults caused by circulating environmental pollutants and their metabolites. Sipos et al. (58) demonstrated that proinflammatory adhesion molecules, i.e., intercellular cell adhesion molecule-1 and vascular EC adhesion molecule-1, facilitate PCB-mediated enhancement of brain metastasis formation. In addition, caveolin-1 was found to play a role in PCB77-induced endothelial dysfunction by phosphorylating endothelial nitric oxide synthase (38). Another in vivo study demonstrated that exposure to different PCB individuals (PCB126, PCB118, and PCB153) altered the integrity of brain capillary endothelium and facilitated the formation of bloodborne metastases (56). VE-cadherin is linked to a large number of intracellular partners. Thus it is of interest to investigate the roles of other junction proteins, like α-catenin, β-catenin, and p120, in regulating junction breakdown mediated by PCB quinone treatment in the future studies. PCB29-pQ has been shown to upregulate reactive oxygen species (ROS) in cells (36). Since vascular endothelial integrity can be altered by ROS, PCB29-pQ may increase endothelial permeability by promoting ROS generation (16).

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Fig. 5. PCB29-pQ exposure enhanced stress fiber formation and focal adhesion assembly. A: HUVECs were treated with DMSO or PCB29-pQ at a dose range of 1–10 μM for 6 h. Cells were stained with rhodamine-phalloidin and paxillin antibodies. A, right: magnified views of the boxed area in the merged images. Scale bar = 10 μm. F-actin, red; paxillin, green. B and C: quantification of the average number and size (in μm²) of paxillin-containing focal adhesions in PCB29-pQ-treated cells using ImageJ software. Twelve cells were analyzed per condition in each experiment. Results are expressed as means ± SE. *P < 0.05 compared with DMSO control.
molecules, like paxillin, focal adhesion kinase, talin, and vinculin, regulating cell adhesion and migration (46). Previous studies showed that focal adhesions contribute significantly to EC tethering, through mediating the attachment of actin cytoskeleton to extracellular matrix (69). Besides, focal adhesion dynamics also affects cytoskeletal organization and cell migration (41, 55). Using immunofluorescent staining assay, we showed that PCB29-pQ-induced focal adhesion formation and cytoskeleton remodeling were inhibited by focal adhesion inhibitor thiolulin. Since thiolulin inhibits HUVEC adhesion to vitronectin by reducing paxillin in HUVECs (45), focal adhesion dynamics also affects cytoskeletal organization and cell migration (41, 55). Using immunofluorescent staining assay, we showed that PCB29-pQ-induced focal adhesion formation and cytoskeleton remodeling were inhibited by focal adhesion inhibitor thiolulin. Since thiolulin inhibits HUVEC adhesion to vitronectin by reducing paxillin in HUVECs (45), focal adhesion formation and paxillin phosphorylation may play essential roles in regulating PCB29-pQ-induced junction breakdown. The downstream events from focal adhesion dynamics include MAPK activation plays an important role in the regulation of endothelial permeability (4, 47). Inhibition of endothelial MAPK activities was shown to enhance endothelial barrier function. A previous study reported that tumor transmigration was dependent on endothelial ERK1/2 and p38 activation, as well as a myosin light chain phosphorylation-mediated formation of stress fibers (63). The differential roles of these two MAPKs in regulating endothelial permeability were evident, as p38 initiates stress fiber formations, whereas ERK1/2 contributes to the dissociation of VE-cadherin/β-catenin complexes. Indeed, in consistent with the study, we found that p38 and ERK1/2 play different roles in junction disassembly with p38 and ERK1/2 mediating phosphorylation of paxillin and VE-cadherin, respectively. The downstream kinase of p38, heat shock protein 27, has been implicated in the oxidative stress-induced formation of actin stress fibers and cytoskeletal reorganization (27). Interestingly, our previous studies strongly suggested that oxidative stress plays important roles in PCB29-pQ-induced toxicity, which can be eliminated by antioxidant treatments (15, 39). However, the effects of antioxidants on PCB29-pQ-induced EC dysfunction is worth further investigation. The importance of ERK1/2 in vascular endothelial growth factor (VEGF)-induced barrier permeability in en-
dothelium has been established (65). VE-cadherin negatively regulates EC growth by inhibiting VEGF signaling pathways mediated by ERK1/2 activation, which in turn regulates the stability of cell-cell junctions and vascular permeability. Our result clearly showed that ERK1/2 inhibitor abolished the phosphorylation of VE-cadherin. In this regard, Lee et al. (35) reported that angiopoietin-1 promotes endothelial integrity through the dephosphorylation of VE-cadherin through integrin/ERK/caspase-9 axis. Haidari et al. (22) reported that proinflammatory stimuli, such as monocyte adhesion or IL-1β treatment, elevate VE-cadherin tyrosine-731 phosphorylation via ERK signaling and the inhibition of VE-cadherin tyrosine-Y731 phosphorylation protected adherens junction from disruption. Their results, along with our current data, further supported ERK as a central player in the regulation of EC integrity.

To demonstrate the relevance of our in vitro study with an in vivo scenario, in vivo vascular permeability assay and staining of phosphorylated proteins on mouse vessels can be conducted. To this end, PCB29-pQ can be administrated intraperitoneally. We hypothesize that vascular endothelial permeability ($K_{f,c}$) value may increase since PCB29-pQ can induce endothelial dysregulation in vitro. To demonstrate the effect of PCB exposure on mouse endothelial paxillin and VE-cadherin phosphorylation, future experiments are needed. For instance, VE-cadherin pY658 and paxillin pY118 can be stained on paraffin-embedded mouse blood vessel samples as previously described (29). The tissue samples can be analyzed by combined staining for ECs (CD31) and VE-cadherin (pY658) or paxillin (pY118).

To assess the roles of p38 and ERK1/2, p38 or ERK1/2 inhibitor can be injected intraperitoneally before PCB29-pQ administration. VE-cadherin (pY658) or paxillin (pY118) can then be stained on endothelium-outlined (CD31 staining) mouse tissues.

**Conclusions.** In conclusion, our current study demonstrated, for the first time that PCB29-pQ exposure could mediate loss of endothelial junction integrity by inducing phosphorylation of ERK1/2 and p38, which thereby regulates the phosphorylation of VE-cadherin and focal adhesion assembly. Our findings provide new mechanistic insights into the toxic effect of PCB29-pQ on vascular endothelium and associated pathogenesis of cardiovascular diseases and cancer metastasis.

PCBs are chlorinated aromatic hydrocarbons that consist of 209 congeners (53). Usually, PCBs exist as mixture and are marketed with various names by the percentage of chlorination. For example, the most common mixtures of PCB congeners are named as Aroclor 1254, Aroclor 1242, and Aroclor 1016, which represent 54, 42, and 41% chlorine in their weight, respectively (9). However, the composition of PCB individuals may vary from commercial products to real environmental/biological samples due to the metabolism of low chlorinated PCB. Thus, by using the relative abundances in real environmental/biological samples as criteria, those congeners that reflect the best mimics of PCB bioaccumulation profiles are often used in different studies. For instance, Aroclor 1260 is most suitable for simulating PCB bioaccumulation profile in human adipose tissues (67). For the same reason, it is also popular to carry out studies with reconstituted mixture of “indicator congeners” PCB, which is most abundant in biological matrices (3). The most obvious drawback for picking “remaining” or “high abundant” PCB individuals is the omission of toxic contribution from PCB metabolites.

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**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**Author Contributions**

P. Zhang and Y.S. conception and design of research; P. Zhang, H.B., P. Zeng, F.C., Y.P., Qin Zhang, Qiuyao Zhang, Q.X., and X.X. performed experiments; P. Zhang, S.F., H.B., C.W., Y.P., Qin Zhang, Q.Y., and E.S. analyzed data; P. Zhang and S.F. interpreted results of experiments; P. Zhang and P. Zeng prepared figures; P. Zhang and S.F. drafted manuscript; P. Zhang, S.F., and Y.S. edited and revised manuscript; P. Zhang, S.F., and Y.S. approved final version of manuscript.
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