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Cytochrome c Adducts with PCB Quinoid Metabolites

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Abstract

PCBs are a group of 209 individual congeners widely used as industrial chemicals. PCBs are found as by-products in dye and paint manufacture and are legacy, ubiquitous and persistent as human and environmental contaminants. PCBs with fewer chlorine atoms may be metabolized to hydroxy- and dihydroxy- metabolites and further oxidized to quinoid metabolites both *in vitro* and *in vivo*. Specifically, quinoid metabolites may form adducts on nucleophilic sites within cells. We hypothesized that the PCB-quinones covalently bind to cytochrome c and thereby cause defects in the function of cytochrome c. In this study synthetic PCB quinones (2-(4'-chlorophenyl)-1,4-benzoquinone, 2-(3', 5'-dichlorophenyl)-1,4-benzoquinone, 2-(3',4', 5'-trichlorophenyl)-1,4-benzoquinone, and 2-(4'-chlorophenyl)-3,6-dichloro-1,4-benzoquinone) were incubated with cytochrome c, and adducts were detected by LC-MS and MALDI TOF. SDS PAGE gel electrophoresis was employed to separate the adducted proteins, while trypsin digestion and LC-MS/MS were applied to identify the amino acid binding sites on cytochrome c. Conformation change of cytochrome c after binding with PCB3-para-quinone was investigated by SYBYL-X simulation and cytochrome c function was examined. We found that more than one molecule of PCB-quinone may bind to one molecule of cytochrome c. Lysine and glutamic acid were identified as the predominant binding sites. Software simulation showed conformation changes of adducted cytochrome c. Additionally, cross-linking of cytochrome c was observed on the SDS PAGE gel. Cytochrome c was found to be in the reduced form after incubation with PCB quinones. These data provide evidence that the covalent binding of PCB quinone metabolites to cytochrome c may be included among the toxic effects of PCBs.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

4-chlorobiphenyl; cytochrome c; protein adducts; quinoid metabolites; post-translational modification; protein cross-linking

Introduction

PCBs are environmental persistent pollutants. They are anthropogenic chemicals of chlorines bonded to biphenyl; PCBs are composed of 209 different configurations with 1 to 10 chlorine atoms on biphenyl (Mullins et al. 1984, Silberhorn et al. 1990). PCB mixtures have been produced for industry use since the 1920s. Because PCBs are resistant to heat and degradation, they were widely used as transformer oil in the electrical industry (Nisbet & Sarofim 1972). They were also utilized as cooling fluids in hydraulic systems, as plasticizers in paints, as gradients in lubricating oils, pesticides, copy paper, sealants, adhesives, and flame retardants (Jensen 1966). Intentional manufacture of PCBs has been prohibited since the 1970s, however, PCBs are still in use in closed system applications, and are found as unintentional by-products in dye and paint manufacture (Hu & Hornbuckle 2010). In 2013, IARC classified all 209 PCB congeners and their metabolites as group 1 carcinogens, carcinogenic to humans (Lauby-Secretan et al. 2013).

As semi-volatile compounds, PCBs are found in the indoor and outdoor air, and humans are exposed to PCBs through inhalation in addition to the traditional dietary route. For instance, PCBs were found in the Chicago air (Hu et al. 2010), and were also identified in several primary school buildings in New York City (Thomas et al. 2012). In most of the samples, the concentrations were higher than the regulatory limit (50 ppm), and the caulks and ballasts were identified as primary sources of PCBs (Thomas et al. 2012). Currently, there are few studies focusing on the toxicities of airborne (lower chlorinated) PCBs compared to dietary exposure, hence, a major challenge and need for risk assessment is to understand their toxicities.

Although higher chlorinated PCBs are persistent, lower chlorinated congeners like those that predominate in air, are metabolized both *in vitro* and *in vivo*. Lower chlorinated congeners are oxidized to hydroxyl or arene oxide metabolites (James 2001). Arene oxide metabolites may be hydrolyzed to dihydrodiol metabolites by epoxide hydrolase (Garner & Matthews 1998, McLean et al. 1996), or rearrange to monohydroxylated PCBs. Hydroxylated PCBs may be conjugated (Dhakal et al. 2012, Tampal et al. 2003), or further oxidized to dihydroxylated PCBs by cytochrome P-450 (Grimm et al. 2015, McLean et al. 1996). Dihydroxylated PCBs, i.e. catechols and hydroquinones, may be further oxidized to quinoid metabolites, semiquinones and quinones, by peroxidases, such as lacto- and myeloperoxidases, and prostaglandin synthase (Oakley et al. 1996, Wangpradit et al. 2009), or by autoxidation or disproportionation reactions (Song et al. 2008b).

Arene oxides and (semi-)quinone metabolites of PCBs are considered highly active compounds (Ludewig et al. 2008). They are reactive towards nucleophiles in DNA, lipids and proteins. In addition to DNA adducts, PCB quinones react slowly with nitrogen nucleophiles, but react instantaneously with some sulfur nucleophiles (Amaro et al. 1996).

Thiol binding of PCB quinone metabolites has been found in glutathione and topoisomerase II, which caused a decrease of topoisomerase II activity (Srinivasan et al. 2002). The covalent binding of different PCB quinones decreased the catalytic activity of human hydroxysteroid sulfotransferase hSULT2A1 (Qin et al. 2013a). Using radioactive compounds, PCB3 and PCB77 were found covalently bound to nuclear proteins in liver *in vivo* (Pereg et al. 2001) and lipids *in vivo* (Morck et al. 2002). PCB3 or PCB3 metabolites can also form hemoglobin adducts *in vivo* (Tampal et al. 2003). Protein adducts of PCB52 quinoid metabolites have been found in liver and brain *in vivo* (Lin et al. 2000).

Cytochrome c is a small globular protein with 104 amino acids (Margoliash et al. 1961) which is highly conserved across species (Dickerson 1971). Cytochrome c plays a key role in the electron transport chain of mitochondria as the electron carrier (Ow et al. 2008). In addition, the release of cytochrome c into the cytoplasm activates caspase-dependent apoptosis (Spierings et al. 2005). PCB3 quinone was reported to increase oxidative stress in the mitochondria of human cells in culture and to inhibit the mitochondrial electron chain; PCB29 quinone caused an increase in mitochondrial oxidative stress, a transfer of cytochrome c from the mitochondria into the cytoplasm, and induced caspase-dependent apoptosis (Xiao et al. 2014, Xiao et al. 2013, Xu et al. 2014, Xu et al. 2015, Zhu et al. 2009). These reports suggest that cytochrome c may play a crucial role in PCB quinone toxicity. Cytochrome c has previously been used as a model protein to develop methods for protein adduction studies of quinones (Fisher et al. 2007, Fisher et al. 2011). We have employed similar methods to investigate the covalent binding of PCB quinoid metabolites to proteins, specifically the important multifunctional cytochrome c, to better understand the mechanisms of toxicity of PCBs. LC-MS and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF) were applied to measure cytochrome c adducts with PCB quinoid metabolites. The binding sites of PCB quinones on cytochrome c were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after tryptic digestion. Separation of the adducted proteins was carried out by SDS PAGE gel electrophoresis followed by NBT staining. SYBYL-X was employed to simulate the conformation change of cytochrome c after binding with PCB3-para-quinone (PCB3-pQ). We observed that the quinoid metabolites of PCBs covalently bind to cytochrome c, particularly lysine and glutamic acid, and cause protein cross-linking. The resulting changes in the protein may cause dysfunction of cytochrome c, in part responsible for the toxicity of PCBs.

Materials and Methods

Chemicals and Reagents

The following materials were obtained from the sources indicated: Horse heart cytochrome c from MP Biomedicals (Solon, OH), trypsin gold (mass spectrometry grade) from Promega (Madison, WI), HPLC-grade solvents from Fisher Scientific (Pittsburg, PA), DMSO from Acros (Fair Lawn, NJ), α -cyano-4-hydroxycinnamic acid (α CHCA) from Sigma-Aldrich (St. Louis, MO), and Amicon ultra centrifugal filters (10K Da membrane) from Millipore Ireland Ltd (Tullagreen, Ireland). 2-(4'-chlorophenyl)-1,4-benzoquinone (PCB3-pQ), 4-(4'-chlorophenyl)-1,2-benzoquinone (PCB3-oQ), 2-(3', 5'-dichlorophenyl)-1,4-benzoquinone,

2-(3',4', 5'-trichlorophenyl)-1,4-benzoquinone, and 2-(4'-chlorophenyl)-3,6-dichloro-1,4-benzoquinone were synthesized, purified and characterized as previously described (Amaro et al. 1996, Song et al. 2008a). All other reagents and solvents were purchased from Fisher Scientific (Pittsburg, PA), unless otherwise stated. Caution: PCB derivatives should be handled as hazardous chemicals in accordance with NIH guidelines.

Adduction of PCB Quinones on Cytochrome c

Cytochrome c was dissolved in 100 mM ammonium bicarbonate buffer, pH=7, at a concentration of 0.1 $\mu\text{mol/mL}$. Quinoid metabolites of PCBs were dissolved in DMSO at a concentration of 10 $\mu\text{mol/mL}$. Cytochrome c and PCB quinones were allowed to react at the ratio of 1:10 at 37 °C for 30 minutes. The mixture was centrifuged at 10,000g for 30 minutes through a centrifuge filter (Amicon ultra centrifugal filters with 10K Da molecular mass cutoff membrane) to remove excess PCB quinone. The concentrated mixture was transferred to a new microcentrifuge tube by reversing the filter device and spinning at 10,000g for 30 minutes. The native cytochrome c and cytochrome c adducted with quinoid PCBs were analyzed by LC-MS and MALDI TOF.

Preparation of Modified Peptides by Tryptic Digestion

Cytochrome c and PCB3-pQ dissolved in DMSO were combined and allowed to react as described above. Control samples consisted of cytochrome c and DMSO alone. The 10K Amicon ultra centrifugal filters were used to remove unreacted PCB3-pQ. The samples were digested with sequencing-grade trypsin at the ratio of 1:50 (w/w) at 37 °C overnight in the dark. The digested samples were then analyzed by LC-MS/MS.

MALDI TOF

The samples of cytochrome c and cytochrome c adducted with PCB quinones were mixed with αCHCA (in water: acetonitrile mixture 1:1 with 0.1% TFA), the matrix for MALDI TOF. One μL of the mixtures was spotted on a 384 sample microtiter plate (MTP) anchorchip. After the crystallization of the sample spots, the anchorchip was loaded on a Bruker UltraFlextreme MALDI TOF. The laser frequency of MALDI was set to 200 Hz, 20% laser power, and 2000 laser shots per click. The MALDI TOF spectra were acquired in positive linear mode with m/z range from 4K to 30K Da and m/z suppress up to 1000 Da.

LC-MS and LC-MS/MS

Native cytochrome c was measured using an LCQ Deca (Thermo, San Jose, CA) interfaced with Dionex Ultimate 3000 LC (Thermo, Sunnyvale, CA). Chromatographic separations were performed at room temperature on a 2.1 mm i.d. $\times$ 15 cm, 5 μM , Supelco Discovery C-18 column coupled to a Supelguard column, 2.1 mm i.d. $\times$ 2 cm, 5 μM (Sigma-Aldrich, MO). The mobile phases used were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Gradient elution was carried out with B from 5% to 60% for the first 40 min, followed by B at 60% for 10 min. The mass spectrometer was used with an electrospray ionization (ESI) source and set to positive mode with spray voltage of 3.5 kV and capillary temperature of 200 °C. The reaction mixture was examined with an LCQ

Deca. The data were analyzed using Xcalibur (Thermo, San Jose, CA). Deconvolution of the mass spectrometry data was performed with MagTran 1.02.

The trypsin digested samples were separated by HPLC as described earlier, and the mass spectrometer acquired a full scan MS in the m/z range of 400–2000. Data-dependent MS/MS and zoom scans were carried out for the most intense ion from a full scan MS. As above, the tandem mass spectrometry data were also analyzed using Xcalibur.

LC-MS/MS Data Analysis and Verification

The protein sequence of cytochrome c was obtained from the NCBI database as CYC_HORSE P00004. The MS/MS spectra raw data files were converted to mzXML data files by Trans-Proteomic Pipeline software package (Seattle Proteome Center). X!Tandem (The Global Proteome Machine) was used as the open-source search engine to match protein sequences with mass spectra (Craig & Beavis 2004). The mzXML data were uploaded and analyzed to identify peptide sequences by X!Tandem. *E. caballus* was chosen for taxon, unmodified was chosen for complete modifications, oxidation was selected, and 216 Da increase on lysine and glutamic acid was set for potential modifications, oxidation on methionine and tryptophan was selected, and 216 Da increase on lysine, histidine and glutamic acid was set for potential modifications. All other parameters were left as default. Manual validation of MS/MS spectra was carried out to confirm the peptide sequence and adducted amino acid sites according to the protocol reported (Tabb et al. 2006).

Circular Dichroism (CD) Spectroscopy

Cytochrome c (100 μ M) in phosphate buffered saline (pH=7) was incubated with PCB3-pQ or PCB3-oQ at the ratio of 1:10 at 37 °C for 1 hour. Samples of cytochrome c incubated with different concentrations of PCB3-pQ or PCB3-oQ, and with different incubation times were also prepared. Circular dichroism spectra measurements were obtained from native and adducted cytochrome c. Circular Dichroism (Jasco-815 Spectropolarimeter) was applied using a quartz cell of 1.0 mm optical path length over a wavelength range of 190–250 nm. CDpro (<http://lamar.colostate.edu/~sreeram/CDPro/>) was used to analyze the original data from CD using CDSSTR algorithm (Greenfield 2007). Each of the CD measurements was the average of three scans at room temperature. The α -helical contents of the adducted cytochrome c with PCB3-pQ or PCB3-oQ were compared with the native cytochrome c using two-tailed t-test with the significant level of 0.05.

Cross-linked Cytochrome c Separation by SDS PAGE

Cytochrome c was incubated with PCB3-pQ or PCB3-oQ at the same conditions mentioned above. Different incubation ratios of cytochrome c to PCB quinones (10:1, 1:1, 1:5, and 1:10) and different incubation pHs (pH=6, 7, 8) were applied to examine formation of protein adducts. Ten percent SDS-PAGE gels were prepared to separate native and adducted cytochrome c products and potential cross-linked cytochrome c. Gel electrophoresis was carried out in Tris-glycine running buffer at a 120 V voltage for 1 hour. The protein bands were transferred to PVDF membranes at 4 °C overnight. A nitro blue tetrazolium solution (0.24 mM NBT in 2 M potassium glycinate, pH 10) was prepared freshly and the PVDF membranes were immersed into the NBT staining solution for 1 hour (Paz et al. 1991). The

membranes were washed in ddH₂O to remove debris from membranes. A Ponceau S staining solution (0.1% w/v) in 5% acetic acid was used to stain the NBT-stained membranes.

Molecular Modeling

Protein Data Bank entry 1HRC was employed as the structural protein model for cytochrome c. Sybyl X 1.3 (Tripos) was used to carry out the molecular modeling studies. PCB quinone adducts were built on the appropriate residues of cytochrome c. The modified proteins were fixed side chain bumps, and Powell's minimization performed to obtain the lowest-potential-energy structure. The structures of adducted proteins were compared with the original structure of cytochrome c.

NADPH - Cytochrome c Reductase Activity Assay

Cytochrome c acts as the electron acceptor for NADPH-cytochrome c reductase. Electron transfer from the reductase reduces ferric cytochrome c into ferrous cytochrome c, which has absorbance at 550 nm. To evaluate the function of PCB-adducted cytochrome c as electron acceptor the NADPH-Cytochrome c Reductase Activity assay was adapted from a protocol described previously (Wu et al. 2009) using liver microsomes as source of NADPH-cytochrome c reductase. The microsomes were prepared as published previously (Lai et al. 2012, Lai et al. 2013). Microsomes were obtained from liver homogenate of male Sprague-Dawley rats (Harlan, Indianapolis, IN) treated with 400 µmol of phenobarbital (PB, saline) and 100 µmol β-naphthoflavone in soybean oil per kilogram body weight for three consecutive days. Liver homogenate was centrifuged at 10,000g for 20 min (Sorvall, rotor SS-34) and the resulting supernatant was then centrifuged (Beckman, L7 Ultracentrifuge, rotor SW41) at 100,000g for 1 h. The microsomal pellet was resuspended in sucrose/EDTA. Protein concentration was determined by the Lowry method using bovine serum albumin as standard. The microsomal suspensions were frozen in aliquots at -80 °C until use. The rate of reduction of cytochrome c was determined with a LAMBDA 650 UV/Vis Spectrophotometer (Perkin-Elmer, Norwalk, CT). Briefly, 80 µL of 0.5 mM cytochrome c, 10 µL of 100 mM KCN, 10 µL of 100 mM MgCl₂, 10 µL microsomes (0.2 mg protein/mL), and 880 µL of 300 mM potassium phosphate buffer were added to a 1-ml cuvette (path length of 10 mm). The cuvette was inverted several times to mix the solution well and the absorption at 550 nm was measured for 3 minutes. Then, 10 µL of 10 mM NADPH was added to the cuvette and everything mixed well after which the UV absorption was continuously measured for another 3 minutes. Samples of adducted cytochrome c were prepared for this assay by incubating cytochrome c with PCB3-pQ at a 1:1 ratio with or without the addition of 10 mM ferric chloride to keep the adducted cytochrome c in the ferric state before the assay. The ratio of cytochrome c to Fe(III) was 1:1 or 1:5, as indicated. After incubation at 37 °C for 30 minutes, the UV/Vis spectra of the samples were obtained by scanning the absorbance over wavelengths from 400 nm to 600 nm. Native cytochrome c incubated with ferric ions alone was used to test for possible interference of ferric ions in the cytochrome c reduction assay. All NADPH-cytochrome c reductase activity assays were repeated at least twice.

Results

PCB Quinones Adducted to Cytochrome c

Because ESI source allows multiple charging for proteins, deconvolution is a necessary step to obtain the molecular weight of the target protein. The ESI charge-state envelopes can be used as an indicator of proteins' spectra.

TIC chromatographs of cytochrome c and cytochrome c adducted with different quinoid metabolites of PCBs were achieved. The charge-state envelopes of cytochrome c were deconvoluted to obtain the molecular weight 12,360 Da of cytochrome c (Figure S1A). The same process was applied to mass spectra of adducted cytochrome c samples. From Figure S1B the cytochrome c peak was recognized, and additional charge-state envelopes were identified with the molecular weights of 12,576 Da and 12,791 Da. The peak of 12,576 indicated one molecule of PCB3-pQ bound to cytochrome c, and 12,791 Da showed two molecules of PCB3-pQ bound. Different PCB quinones displayed different binding patterns on cytochrome c (Figure S1). These different binding patterns are summarized in Table S1.

Binding Sites of PCB3-pQ on Cytochrome c

The PCB3-pQ binding sites on cytochrome c were determined by LC tandem MS after trypsin digestion. The preferable binding sites on cytochrome c were lysine and glutamic acid. The binding sites of lysine 27 and lysine 39 are showed in Figure 2. In Figure 2A the b3 ion had a molecular weight increase of 216 Da, but the y11 ion did not have a weight increase. These results indicate that lysine 27 is the binding site for PCB3-pQ. In Figure 2B the b2 ion shows the molecular weight increase of PCB3-pQ, but the y12 ion does not indicate a weight increase. Because threonine was not the binding site for the quinones, the only binding site would be lysine 39. Both glutamic acid 61 and 62 are binding sites. Due to a lack of sufficient fragmentation information, the exact binding site between lysine 54 and 56, lysine 73 and 74, and glutamic acid 61 and 62 could not be determined. The binding sites are summarized in Table 1.

Software Simulation of Cytochrome c Conformation Changes

The conformation of cytochrome c after modification at lysine 27 or 39 with PCB3-pQ was simulated by Sybyl X. The PCB3-pQ binds to lysine covalently through the ϵ -amino group. The covalent binding of PCB3-pQ did not change the conformation much as compared with the conformation of cytochrome c without adduction (Figure 3). However, the simulation only included one binding site. The actual reaction of PCB quinones showed more than one quinone may be bound, which would likely change the conformation of cytochrome c.

Secondary Structure Change of Cytochrome c

Circular dichroism spectroscopy was applied to measure the secondary structural change of modified cytochrome c. The α -helix ratio of proteins can be determined by the negative absorption at 208 and 222 nm. Both PCB3-pQ and PCB3-oQ decreased the α -helical content of cytochrome c significantly (Table 2). The higher concentrations of PCB3-oQ significantly changed the α -helix ratio of cytochrome c (Figure S2). The α -helix is the conserved

secondary structure of cytochrome c, change of α -helical content may impair the function of cytochrome c.

Cross-linking of Cytochrome c

After incubation with PCB3-pQ or PCB3-oQ, cross-linking of cytochrome c was detected by separating the molecules using SDS-PAGE gel followed by NBT and Ponceau S dual staining. With native cytochrome c alone only the band at about 12,000 daltons, the molecular weight of the cytochrome c monomer, was visible (Figure 4). Incubation of cytochrome c with PCB3-Q produced additional bands. Those bands located between the 15K Da and 25K Da marker indicate dimers of cytochrome c. The other bands with molecular weights higher than 25K Da depict polymers of cytochrome c. These higher molecular weight products were found in all lanes of cytochrome c incubated with quinoid metabolites of PCB3. Thus both PCB3-pQ and PCB3-oQ caused cross-linking of cytochrome c. Different pHs (pH=6, 7 and 8) did not have a significant effect on cross-linking of cytochrome c. Higher concentrations of quinones increased the bands of the higher molecular weight section of the SDS-PAGE gel and decreased monomeric cytochrome c indicating increased dimer and polymer formation of cytochrome c via crosslinking.

Cytochrome c as Electron Acceptor for NADPH-Cytochrome c Reductase

As shown in Figure 5A, incubation of cytochrome c with PCB3-pQ reduced the ferric ion in the heme group of cytochrome c resulting in an absorbance increase at 550 nm. By addition of ferric ions into the incubation mixture, the absorbance of the adducted cytochrome c at 550 nm did not increase compared to native cytochrome c, indicating that the adducted cytochrome c was in the oxidized, ferric form. In the NADPH-cytochrome c reductase assay (Figure 5B), no significant increase of reduced cytochrome c was observed in the sample of cytochrome c incubated with PCB3-pQ 1:1 (red line) after adding NADPH. This adducted cytochrome c had already a higher absorbance at 550 nm, indicating that cytochrome c was in the reduced state. For native cytochrome c (blue line) and adducted cytochrome c incubated with ferric ions (green and purple lines), the addition of NADPH started the reduction reaction. The slope increase of adducted cytochrome c was much lower compared to the native cytochrome c, which indicates the reaction rate was slower. Incubating native cytochrome c with ferric ions at the ratio of Cyt c: Fe(III) of 1:1 or 1:5 did not change the rate of reduction reaction of cytochrome c as shown in Figure S3.

Discussion

Airborne PCBs are widely detected both in indoor and outdoor air; the major components of airborne PCBs are PCBs with lower number of chlorines (summarized in the Table 1 in (Grimm et al. 2015). Lower chlorinated biphenyls are more rapidly metabolized. For example, PCB3 (4-chlorobiphenyl) displays a short half-life time of hours in rats (Dhakal et al. 2014). Lower chlorinated congeners are metabolized to the hydroxyl PCBs and then conjugated or further metabolized to reactive compounds such as quinoid metabolites, the latter ones being most likely the ultimate carcinogens (Espandiar et al. 2004).

Quinones participate in Michael addition reactions in which they react with the nucleophilic groups in proteins and DNA (Bolton et al. 2000). PCB quinones also undergo substitution reactions in which a chlorine atom in the quinone ring is displaced (Song et al. 2009). The results of MALDI TOF and LC-MS examinations in this study show that all tested PCB quinoid metabolites formed adducts with cytochrome c. The MS results also indicate that more than one molecule of PCB quinone can be bound to one molecule of cytochrome c. Although cytochrome c is a small globular protein, it contains more than one potential binding site, i.e. 2 cysteines, 19 lysines, and 3 histidines. 4-Hydroxy-2-nonenal, a reactive product of lipid peroxidation, was found to react with multiple binding sites in cytochrome c and up to 4 molecules were bound per cytochrome c molecule (Isom et al. 2004). We used PCB paraquinones with 1 to 3 chlorines in different positions to gain insight into structure-activity relationships for protein binding. The MALDI TOF spectra revealed that different PCB quinones have differing capacities to form adducts with cytochrome c (Figure 1). At a molar ratio of 10:1, quinone to cytochrome c, multiple adductions per cytochrome c molecule were dominant with the monochlorinated quinone, while these products were less prominent with additional chlorines in the non-oxygenated ring. Similarly, increasing chlorination decreased the rate of DNA adduction by PCB quinones with 1-3 chlorines (Zhao et al. 2004). Interestingly, we found that chlorines in the quinone ring resulted in adduct formation by a substitution reaction, not Michael addition as with the previous compounds, with very diverse multiple-binding/multiple chlorines-loss patterns (Table S1). More compounds with similar structures need to be tested to identify generalizable structure-activity patterns and mechanisms.

The amino acid binding sites of PCB3-pQ were identified from tandem mass spectra. Previous research showed that compared to all the other amino acids, the sulfur group of cysteine is most favorable for binding of PCB quinones (Amaro et al. 1996), but we did not observe any binding to cysteines. There are two cysteines in cytochrome c, but they are covalently bound to the heme group (Banci et al. 1997, Bushnell et al. 1990), making them inaccessible for PCB quinone binding. Instead lysine and glutamic acid were the predominant binding sites, i.e. K27, K39, K54 and/or K56, E61 and/or E62, and K73 and/or K74. 4-Hydroxy-2-nonenal was found to bind to H33, K87, and R38 (Isom et al. 2004), suggesting compound specific preferences for specific protein residues. p-Benzoquinone was reported to bind to Lys and His (H26) residues in cytochrome c and a novel cyclized diquinone adduct between adjacent lysines at residue 23-27 or 86-87 at two locations of the protein surface were identified (Person et al. 2003). Similarly adduction of PCB quinones on the preferred exocyclic N2 of deoxyguanine was followed by cyclization and dehydration (Zhao et al. 2004). We did not attempt to identify the cyclized products. The fact that two identified binding sites have adjacent lysines suggest that such a reaction could happen and thereby create very stable adducts. Experiments with the N-acetyl cysteine (NAC)-adduct of pBQ found adducts on cytochrome c, not only on lysine-rich regions of the protein, but also on specific (E62, E92) glutamic acid residues (Fisher et al. 2007). Residues K7, K25, K39, 62ETLM65, and K72 are involved in the complex formation of cytochrome c with Apaf-1, a prerequisite of caspase-3 activation and apoptosis induction by cytosolic cytochrome c (Yu et al. 2001). Also, K27 and K72 or K86 and K87 were discussed as participants in the complex formation between cytochrome c and cytochrome b5 during electron transfer

(Northrup et al. 1993). Based on the preferred binding sites of PCB3 pQ, it may be assumed that binding may impact the function of cytochrome c in apoptosis induction and/or mitochondrial electron transfer.

The secondary structure of native cytochrome c contains 5 α -helices (Bushnell et al. 1990), and the α -helical content of cytochrome c is over 40% (Oellerich et al. 2004). The binding of PCB3-pQ or PCB3-oQ changed the secondary structure of cytochrome c. It is noteworthy that cytochrome c has a highly conserved secondary structure, and a less highly conserved amino acid sequence among species, pointing towards high functional importance of the secondary structure (Dobbs et al. 1996). The decrease of the α -helical content may therefore have a significant effect on the function of cytochrome c.

We observed cross-linking formation of cytochrome c molecules on SDS PAGE gels after incubation with PCB3-pQ or PCB3-oQ. This effect was faster and more pronounced with the *para*- compared to the *ortho*-quinone. Un/low-substituted quinones have more than one electrophilic site. Both 1,4-benzoquinone (Hill et al. 1993) and PCB quinoid metabolites (Amaro et al. 1996, Song et al. 2009, Srinivasan et al. 2002) were found to form conjugates with multiple glutathiones. Benzoquinone was also reported to non-enzymatically form cross-linking of proteins (Yamauchi et al. 2009). Native cytochrome c in the electron chain is a monomer and even though it is known to produce polymers, it loses its function as electron donor for cytochrome c oxidase (Hirota et al. 2010). Thus crosslinking of cytochrome c molecules by PCB quinones can be expected to have profound influence in cytochrome c function in mitochondria.

The reduction of cytochrome c can be observed by measuring its absorbance at 550 nm. The wavelength scanning spectra suggest that the adducted cytochrome c was reduced during incubation with PCB3-pQ. One possibility is that the reduction may be caused by PCB3 hydroquinone or semiquinone radicals, as described with benzoquinones (Williams 1963, Winterbourn 1981). Interestingly, after adduct formation with sulfhydryl groups in peptides/proteins like N-acetyl cysteine (Wangpradit et al. 2009) and human hydroxysteroid sulfotransferase hSULT2A1 (Qin et al. 2013b) only hydroquinone derivatives of PCBs were identified, while the adducts we found with cytochrome c were all quinoid derivatives of PCBs. This may be caused by the oxidation of a hydroquinone or semiquinone adduct by the ferric heme group in cytochrome c resulting in reduced cytochrome c and a PCB quinone adduct. Lysine 27 was reported to surround the heme crevice (Geren & Millett 1981) and to interact with the heme group in cytochrome c (Rackovsky & Goldstein 1984). Thus the adduct formation of PCB3-pQ on lysine 27 may lead to an interaction with the heme group. The adducted quinone, in turn, may react with other cytochrome c molecules causing cross-linking. The reduced adducted cytochrome c lost its function as electron acceptor. Addition of ferric ions kept adducted cytochrome c in the oxidized, ferric form which was reduced in the cytochrome reductase assay after addition of NADPH, although only partially and at a slower rate than native cytochrome c. The addition of ferric ions to the incubation did not alter cytochrome c's ability to act as an electron acceptor for the reduction reaction, as the results show. Thus the spectrophotometric results indicate that PCB quinone-adducted cytochrome c completely lost its function due to the reduction of cytochrome c during the

adduction and even if adducted cytochrome c is kept in its ferric form, it partially lost its function to act as an electron acceptor due to the adduct formation.

Conclusions

In conclusion, PCB quinones covalently bind to specific lysine and glutamic acid residues on the surface of cytochrome c, and PCB quinones can form multiple bonds with cytochrome c. The covalent binding of PCB quinones change the secondary structure of cytochrome c and, in addition, cross-linking of cytochrome c was observed. The blocking of lysine residues that are involved in apoptosis induction and the change of secondary structure, cross-linking, reduction, and adduction of cytochrome c which may influence its electron transfer activity in the mitochondrial respiratory chain, suggest that lower chlorinated PCBs that are metabolically activated to quinones may interfere with important cellular functions through covalent protein adduction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The MALDI TOF spectra of cytochrome c and cytochrome c adducted with different PCB quinoid metabolites, (A) at the 1:1 ratio, (B) at the 1:10 ratio

I) The spectrum of cytochrome c binding with PCB3-pQ. The molecular weight difference between two nearby peaks is 216, indicating that the PCB3-pQ binds to cytochrome c. II) The spectrum of cytochrome c binding with 2-(3',5'-dichlorophenyl)-2,5-benzoquinone. The

molecular weight increase between two nearby peaks is 250. III) The spectrum of cytochrome c binding with 2-(3',4',5'-trichlorophenyl)-2,5-benzoquinone. The molecular weight difference of 284 shows the binding of 2-(3',4',5'-trichlorophenyl)-2,5-benzoquinone to cytochrome c. IV) The spectrum of cytochrome c binding with 2-(4'-chlorophenyl)-3,6-dichloro-1,4-benzoquinone. The molecular weight difference of 284 shows the binding of 2-(4'-chlorophenyl)-3,6-dichloro-1,4-benzoquinone. The molecular weight difference of 71 indicates the loss of 2 atoms of chlorine. The bars indicate the molecular weight increase between two peaks.

Figure 2. Tandem mass spectra of the PCB3-pQ binding sites on cytochrome c

The tandem mass spectrum shows that PCB3-pQ binds to cytochrome c (A) on lysine 27 and (B) on lysine 39.

Figure 3. The predicted conformation change of cytochrome c following adduction of PCB3-pQ Adduction on (A) lysine 27, and (B) lysine 39 were simulated. The blue ribbons indicate the conformation of native cytochrome c, and the red ribbons represent the conformation of cytochrome c after binding with PCB3-pQ. The black arrows in these figures indicate the conformation changes after cytochrome c binding with PCB3-pQ.

Figure 4. Cross-linking of cytochrome c molecules by PCB3-pQ and PCB3-oQ at (A) different pHs, and (B) different molar ratios of cytochrome c to quinone
SDS PAGE separation shows the cytochrome c monomer, dimer and polymers as indicated by black arrows. Abbreviations: CytC = cytochrome c; Q = PCB3 pQ or oQ, PQ = PCB3-pQ, OQ = PCB3-oQ.

Figure 5. NADPH-cytochrome c reduction assay with native and adducted cytochrome c (A) Wavelength scans of samples from 400 to 600 nm. The product of cytochrome c adduction with PCB quinone is reduced cytochrome c. Potassium phosphate buffer was used as blank. (B) 550 nm scan of NADPH induced cytochrome c reduction. Quinone adduction of cytochrome c (Cyt c: PCB3-pQ = 1:1) yields the fully reduced product. Presence of equimolar or an excess of Fe(III) preserves the oxidized status. The rate of cytochrome c reduction shown by the increasing slope after adding NADPH into the mixture is lower with adducted cytochrome c.

Table 1

Binding sites of PCB3-pQ on cytochrome c.

Adducts (MW Increase)	PCB3-pQ Binding Sites	MW of Peptides	Ions (m/z)
216	H K ²⁷ TGPNLHGLFGR	1649.808	(550.96+3H) ⁺³
216	K ³⁹ TGQAPGFTYTDANK	1814.794	(906.72+2H) ⁺²
512	W K ⁶¹ E ⁶² TLMEYLENPK	2241.900	(745.57+3H) ⁺³
216	GITW K ⁶¹ E ⁶² TLMEYLENPKK [*]	2425.152	(809.55+3H) ⁺³
216	TGQAPGFTYTDANK ⁵⁴ N K ⁵⁶	1928.837	(963.97+2H) ⁺²
216	GITWKEETLMEYLENPK ⁷³ K ⁷⁴ [*]	2425.152	(1211.50+2H) ⁺²

The binding sites were marked in bold.

* Either of the bold and italic amino acids may be the binding site, but the exact binding sites cannot be determined.

Table 2

CD spectroscopy associated with modification of cytochrome c by PCB3-oQ or PCB3-pQ

α -Helical Content	Native Cytochrome C	CytC with PCB3-pQ	CytC with PCB3-oQ
Average (n=3)	0.539	0.495 [*]	0.492 [*]
Standard Error	0.011	0.008	0.010

CytC stands for cytochrome c

^{*} indicates $p < 0.05$ vs. cytochrome c.