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POLYCHLORINATED BIPHENYL (PCB)-INDUCED OXIDATIVE STRESS AND CYTOTOXICITY CAN BE MITIGATED BY ANTIOXIDANTS FOLLOWING EXPOSURE

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Abstract

PCBs and PCB metabolites have been suggested to cause cytotoxicity by inducing oxidative stress but the effectiveness of antioxidant intervention following exposure is not established. Exponentially growing MCF-10A human breast and RWPE-1 human prostate epithelial cells continuously exposed for 5 days to 3 μ M PCBs [Aroclor 1254, PCB153, and the 2-(4-chlorophenyl)-1,4-benzoquinone metabolite of PCB3 (4ClBQ)] were found to exhibit growth inhibition and clonogenic cell killing, with 4ClBQ having the most pronounced effects. These PCBs were also found to increase steady-state levels intracellular $O_2^{\cdot-}$ and H_2O_2 (as determined by dihydroethidium, MitoSOXTM red and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate oxidation). These PCBs also caused 1.5- to 5.0-fold increases in MnSOD activity in MCF-10A cells and 2.5- to 5-fold increases in CuZnSOD activity in RWPE-1 cells. Measurement of MitoSOXTM red oxidation with confocal microscopy coupled with co-localization of MitoTracker green in MCF-10A and RWPE-1 cells, supported the hypothesis that PCBs caused increased steady-state levels of $O_2^{\cdot-}$ in mitochondria. Finally, treatment with either N-acetyl-cysteine (NAC), or the combination of polyethylene glycol (PEG) conjugated CuZnSOD and PEG-catalase added 1 hour after PCBs, significantly protected these cells from PCB toxicity. These results support the hypothesis that exposure of exponentially growing human breast and prostate epithelial cells to PCBs causes increased steady-state levels of intracellular $O_2^{\cdot-}$ and H_2O_2 , induction of MnSOD or CuZnSOD activities, as well as clonogenic cell killing that could be inhibited by a clinically relevant thiol antioxidant, NAC, as well as by catalase and superoxide dismutase following PCB exposure.

Keywords

Oxidative stress; mitochondria; cytotoxicity; PCBs; N-acetylcysteine; superoxide dismutase

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Introduction

Polychlorinated biphenyls (PCBs) are a group of 209 chemically related compounds, which differ in both number and position of the chlorines on the biphenyl moiety (Fig 1) [1–3]. PCBs were discovered over 100 years ago and first commercially manufactured in 1930s. Because of their remarkable electrical insulating properties and their flame resistance, PCBs were widely used in many industrial applications such as in dielectric fluids, in transformers and capacitors, hydraulic fluids, and as sealants. For several decades, they were also routinely used in the manufacturing of a wide variety of common products such as plastics, adhesives, paints and varnishes [1,4,5]. From 1929 to 1977, it is estimated about 1.1 billion pounds of PCBs were produced in the world, roughly 4 million pounds of which are estimated to be environmentally available. Because of their persistent presence in the environment and growing concern with health effects, commercial production of PCBs in North America was banned in 1977 [6,7]. However, due to their continued use and their persistence in the environment, PCB exposure in human populations continues to be a significant health hazard, especially in highly contaminated areas. Although PCBs were initially thought to be biologically benign, chronic exposure to PCBs and their metabolites have now been suggested to lead to a variety of human health effects including hepatotoxicity, neurotoxicity, immunotoxicity, hormonal disruption, and cancer development including malignant melanoma as well as breast and prostate cancers [6,8–10].

Recent results suggest that PCBs can induce increases in steady-state levels of reactive oxygen species [5]. It is known that the formation of the ROS can play a role in causing oxidative stress leading to cell injury, mutagenesis, carcinogenesis, and cell death [11–13]. Therefore, metabolic oxidative stress caused by PCBs and their metabolites may play an important role in cytotoxicity in mammalian cells. Although there is mounting evidence that PCBs cause oxidative stress [14], the precise identity of the ROS (i.e., $O_2^{\cdot-}$ and H_2O_2) and the intracellular source and identity of ROS responsible for PCB toxicity remain incompletely understood. Precise information regarding the identity (i.e., $O_2^{\cdot-}$ and H_2O_2) and target organelles responsible for ROS production could be invaluable in designing interventions to mitigate the effects of PCBs in human populations.

Since PCBs and quinone metabolites of PCBs are potentially able to alter electron transfer in mitochondrial electron transport chains [15], it is logical to hypothesize that mitochondria from PCBs-exposed cells could provide a source for excess ROS production during PCB exposure in epithelial cells. In the current study PCBs are shown to lead to steady-state increases in superoxide originating from mitochondria as well as inhibition of cell growth and clonogenic cell killing in exponentially growing MCF-10A human nonmalignant breast and RWPE-1 human nonmalignant prostate epithelial cells. The same PCBs were also found to cause increases in the activity corresponding to the mitochondrial form of superoxide dismutase (MnSOD) in MCF-10A cells and induce increases in the activity corresponding to the cytosolic form of superoxide dismutase (CuZnSOD) in RWPE-1 cells. Furthermore treatment of cells with antioxidants (i.e., PEG-superoxide dismutase and PEG-catalase or NAC) one hour following exposure to PCBs was able to significantly diminish the toxicity associated with PCBs in human breast and prostate epithelial cells. These results support the hypothesis that PCBs or their metabolites can enhance steady-state levels of ROS originating from mitochondrial metabolism which significantly contribute to PCB-induced alterations in cell proliferation and cytotoxicity. Furthermore, since NAC is a commonly used clinically relevant antidote for acetaminophen intoxication [16,17], these results suggest that NAC could also be useful in mitigating the biological effects of PCBs if given following exposure.

Materials and Methods

Cells and PCBs used in the experiments

The non-malignant MCF-10A human breast epithelial cells were cultured in the MEBM serum free medium (Lonza Group Ltd, Switzerland) with additives [recombinant human epidermal growth factor (rhEGF), bovine pituitary extract (BPE), recombinant human insulin, hydrocortisone, and gentamicin]. The non-malignant RWPE-1 human prostate epithelial cells were cultured in the Keratinocyte-SFM (KSFM) serum free medium (Gibco Invitrogen, American) with additives [Epidermal Growth Factor 1-53 (EGF 1-53) and Bovine Pituitary Extract (BPE)]. Cells were maintained and experiments were accomplished in a humidified 37°C with 5% CO₂. All experiments were done using exponentially growing cell cultures at 50 % confluence. DMSO was used as the vehicle control in all experiments.

The PCBs used in the experiments (Fig 1) were synthesized and characterized as previously described [18–21]. The purity of each PCB congener was greater than 99%, as assayed by gas-chromatography with flame ionization detection. The PCB congener profile of the Aroclor 1254 mixture has been reported elsewhere [22].

Growth Curve and Clonogenic Survival

To determine whether the PCB exposure alters the cell proliferation in MCF-10A and RWPE-1 cells, a growth curve was constructed. 50,000 cells/dish cells were plated in 60 mm tissue culture dishes. After 48 hours, the cells were treated with PCBs every day for 5 days. Media in all the dishes were changed daily and fresh 3 µM PCBs were added. The cell numbers were counted from day 1 through day 5 and used to construct the growth curve. At the same time, cells were re-plated using appropriate dilutions, and clonogenic survival evaluated after 14 days in regular growth medium in order to test the effects of PCBs on the reproductive integrity of the cell populations [23–26]. Cells were stained with Coomassie blue, and colonies of more than 50 cells counted and utilized to calculate clonogenic survival as described [23–26].

Transduction of Antioxidant Enzymes

Replication incompetent adenoviral vectors, AdCMV Bgl II (AdBglII), and AdCMV MnSOD (AdMnSOD) were purchased from Viraquest (North Liberty, Iowa). They were prepared by inserting the gene of interest into the E1 region of an Ad5 E1/particle E3 deleted replication deficient adenoviral vector. The cDNAs were all under the control of a CMV promotor. The adenovirus constructs were originally prepared by Dr. John Engelhardt, University of Iowa [27]. Cells were plated the day before virus administration. The desired amount of viral particles was added for 24 h, and then the media was changed to fresh media and left for another 48 h prior to each experiment with PCB treatment.

Estimation of intracellular superoxide levels using Dihydroethidium (DHE) oxidation

Steady-state levels of superoxide were estimated using the fluorescent dyes, dihydroethidium (DHE) purchased from Molecular Probes (Eugene, Oregon). Cells were plated and treated for 5 days with PCBs as described earlier. On day 6, the cells were trypsinized and washed with 5 mM/L pyruvate containing PBS once then labeled with DHE (10 µM, in 0.1% DMSO, 40 min) at 37°C. After labeling, cells were kept on ice. Samples were analyzed using a FACScan flowcytometer (Becton Dickinson Immunocytometry System, INC., Mountain View, CA) (excitation 488 nm, emission 585 nm band-pass filter). The mean fluorescence intensity (MFI) of 10,000 cells was analyzed in each sample and corrected for autofluorescence from unlabeled cells. The MFI data was normalized to control levels [28,29]. The identity of superoxide as being responsible for any increase in MFI was confirmed by suppressing the signal using 2 hr pretreatment with 100 U/mL PEG-CuZnSOD (Sigma Chemical Co., St. Louis MO) or PEG

alone (18 μ M). The differences between PEG-SOD and PEG alone groups was plotted as “normalized SOD inhibitable MFI”.

MitoSOXTM red oxidation to estimate mitochondrial superoxide production

To determine if PCBs can alter steady-state levels of superoxide originating from mitochondria in MCF-10A and RWPE-1 cells, the cationic superoxide sensitive dye, MitoSOXTM red (Molecular Probes), was used. Cells were plated and treated with PCBs for 5 days as described above. On day 6, the cells were trypsinized and washed with 5 mM/L pyruvate containing PBS once then labeled with MitoSOXTM red (2 μ M, in 0.1% DMSO, 20 mins) at 37°C. After labeling, cells were kept on ice. Samples were analyzed using a FACScan flowcytometer (Becton Dickinson Immunocytometry System, INC., Mountain View, CA) (excitation 488 nm, emission 585 nm band-pass filter). The mean fluorescence intensity of 10,000 cells was analyzed in each sample and corrected for autofluorescence from unlabeled cells. The MFI data was normalized to control levels.

To visualize the mitochondrial localization of superoxide production in MCF-10A and RWPE-1 cells, confocal microscopy was used. Cells were plated onto microscope slides and incubated with 100 plaque forming units per target cell [multiplicity of infection (MOI)] AdMnSOD to confirm any change was mediated by superoxide or 100 MOI empty vector control for 24 hours then allowed to recover for 48 hrs as described above. Slides were washed with PBS and monolayers were then labeled with MitoSOXTM red (2 μ M), and MitoTracker green (100 nM) in 0.11% DMSO for 20 mins in 5 mM/L pyruvate containing PBS at 37°C. PCBs were then added 1 minute before analysis using a laser scanning Zeiss LSM 510 confocal system, with the excitation wavelength set at 488 nm and 543 nm. Images were acquired with a 63 $\times$ 1.4 NA Apochromat objective (Zeiss). For quantification of fluorescence intensities, non-saturated images were taken with a full-open pinhole. For multichannel imaging, each fluorescent dye was imaged sequentially in the frame-interlace mode to eliminate cross-talk between the channels. All image processing was performed using the Zeiss LSM 5 image examiner software with identical background and gain settings. Six to eight areas were randomly picked, and representative ones are presented [30,31]. Only signal that was inhibited by MnSOD over expression was considered to originate from superoxide.

Measurement of Intracellular Hydroperoxides

Steady-state levels of hydroperoxides were estimated using the oxidation sensitive {5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, (CDCFH₂)} and oxidation-insensitive {5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, (CDCF)} fluorescent dyes purchased from Molecular Probes. Cells were plated and treated with PCBs for 5 days as described above. On day 6, the cells were trypsinized and washed with PBS once then labeled with CDCFH₂ or CDCF (10 μ g/mL, in 0.1% DMSO, 15 mins) at 37°C. After labeling, cells were kept on ice. Samples were analyzed using a FACScan flowcytometer (Becton Dickinson Immunocytometry System, INC., Mountain View, CA) (excitation 488 nm, emission 530 nm band-pass filter). The MFI of 10,000 cells was analyzed in each sample and corrected for autofluorescence from unlabeled cells. The MFI data was normalized to control levels [29]. The identity of the hydroperoxide responsible for any increase in MFI as H₂O₂, was confirmed by suppressing the signal using 100 units/mL PEG-catalase as described in the legend of Figure 6. The differences between PEG-CAT and PEG alone (18 μ M) groups plotted as “normalized CAT inhibitable MFI”.

Thiol analysis

Cells were grown to 70–80% confluency on 100 mm dishes and scraped in PBS at 4°C, centrifuged, and the cell pellets were frozen at –20°C until analysis. Samples were thawed and whole homogenates were prepared as described [23,32]. Total glutathione (GSH + GSSG) was

determined using a recycling method [23,32]. All biochemical determinations were normalized to the protein content using the method of Lowry et al [33].

SOD activity

Total SOD activity and MnSOD activity was determined by an indirect competitive inhibition assay [34]. Superoxide is generated from xanthine by xanthine oxidase and detected by recording the rate of reduction of nitroblue tetrazolium (NBT). SOD scavenges superoxide and competitively inhibits the reduction of NBT. One unit of SOD activity is defined as the amount of protein required to inhibit 50% of the maximal NBT reduction. To obtain the amount of MnSOD activity 5 mM sodium cyanide was added to inhibit the CuZnSOD enzyme activity [34]. The protein levels in each sample were measured using the Lowry protein assay [33].

Western blot analysis

To determine MnSOD immunoreactive protein, western blot analysis was used with equal protein loading as determined by the Bradford method [35]. Denatured protein (10–15 µg) was resolved on 12% SDS-PAGE and electroblotted into nitrocellulose membranes (Bio Rad, Hercules, CA, USA). The membrane was incubated with monoclonal antibody diluted at 1:1000 in 5% non-fat dry milk for 1 h at room temperature. After washing, the membrane was incubated with a secondary antibody at a 1:10000 dilution for 1 h at room temperature. After washing, signals were detected on an X-ray film using an enhanced ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA)

Survival experiments using PEG-CAT/PEG-CuZnSOD and NAC Treatments

In order to test for a possible causal relationship between the biological effects of PCBs and PCB metabolites, as well as the observed increases in parameters indicative of oxidative stress, a clonogenic assay with PCBs and antioxidant treatments was performed. Cells were plated at a density of 50,000 cells/60 mm dish, and first treated with 3 µM PCBs after 48 hours. One hour after PCB treatment PEG alone (18 µM) or PEG-CAT combined with PEG-CuZnSOD (50 U/mL each) or NAC (5 mM) were added into the cell culture media on target cells. This protocol was repeated with a fresh media change every 24 hours for 3 days. On day 4, cells were trypsinized, counted, and re-plated in complete control media using appropriate dilutions, and clonogenic survival evaluated.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data were expressed as mean SEM unless otherwise specified. One-way ANOVA analysis with Tukey's post-analysis was used to study the differences among three or more means. Significance was determined at $p < 0.05$ and the 95% confidence interval.

Results

In order to demonstrate the biological effects of PCBs on cell growth and clonogenic survival, the MCF-10A and RWPE-1 human breast and prostate epithelial cells were incubated continuously with 3 µM PCBs for 5 days. This dose was chosen as representative of the upper limit of doses measured in the circulation of people living in the Anniston, Alabama flood plain which represents a heavily PCB contaminated site in the United States (Dr. Allan Silverstone, State University of New York, personnel communication). Following treatment, cells were trypsinized and cell numbers as well as clonogenic survival were determined. The results in Figure 2A and 2C showed that Aroclor 1254 and PCB153 significantly inhibited cell growth whereas 4CIBQ completely halted the cell growth in both breast and prostate epithelial lines.

Interestingly in this model system, PCB3 and PCB77 did not significantly alter the rate of cell growth compared to the vehicle control cells (DMSO). Both MCF-10A and RWPE-1 cell doubling times (Td) were calculated by using the equation: $Td = 0.693t / \ln(Nt/N0)$, where Nt and N0 represent cell number at time t and time zero respectively. The doubling times of MCF-10A and RWPE-1 cell lines treated with various PCBs were as follows (PCB: MCF-10A, RWPE-1). Control: 23 h, 22h; PCB3: 26 h, 23h; PCB77: 30 h, 31h; PCB153: 49 h, 59h; Aroclor 1254: 57 h, 68h. The doubling time in the 4CIBQ group couldn't be calculated due to the complete suppression of the cell growth. The results of doubling time experiments suggested that, relative to MCF-10A, RWPE-1 cells seem to be more sensitive to the PCB 153 and Aroclor 1254 induced growth inhibition. It also appeared that the RWPE-1 cells treated with PCB153 and Aroclor began to grow slowly in the first 3 days, however, during the last two days, the treatment caused cell growth inhibition and then a decrease the cell number per dish (Figure 2).

In the next series of experiments the ability of PCB treated MCF-10A or RWPE-1 cells to continue to undergo mitosis was assayed using the clonogenic cell survival assay, which quantifies irreversible cytotoxic responses [24–26]. The results of the clonogenic cell survival assays also showed that 5 days exposure to 4CIBQ reduced the plating efficiency of the MCF-10A cells from 30% to 10% (Figure 2B) and RWPE-1 cells from 32% to 9% (Figure 2D) clearly demonstrating irreversible PCB-induced clonogenic cell killing. Aroclor 1254 and PCB153 also caused significant decreases in clonogenic cell survival in both cell lines but not as dramatic as 4CIBQ (Figure 2B and Figure 2D). Furthermore, it again appeared that Aroclor 1254 and PCB153 affected the clonogenic survival of RWPE-1 cells more severely than that of MCF-10A cells, suggesting differential toxicity of various PCBs towards cells from different tissue origins. Consistent with the growth curve results, PCB3 and PCB77 did not show any significant clonogenic cell killing compared to the control cells in both breast and prostate cell lines. Overall, the results in Figure 2 demonstrate that PCBs and their metabolites can disrupt cell growth in human mammary and prostate epithelial cells as well as causing irreversible loss of reproductive integrity.

When MCF-10A and RWPE-1 cells were treated with PCBs as in Figure 2, steady-state levels of intracellular $O_2^{\cdot-}$ (as determined by DHE oxidation) were found to be significantly higher in PCB153 (1.5-fold), Aroclor 1254 (2-fold), and 4CIBQ (4-fold) treated groups, compared to vehicle controls (Figure 3A). Similar increases of the DHE oxidation were also obtained from PCB-exposed RWPE-1 prostate cells (Figure 3C). Since DHE oxidation following PCB exposure was measured in the absence of PCBs and the analysis was done using flow cytometry, where no remaining extra-cellular probe was possible, these data strongly suggest that the increase in DHE oxidation occurred inside the cells. Furthermore, these results were confirmed when DHE oxidation was also found to be significantly higher in PCB153 (8-fold in MCF-10A, 3-fold in RWPE-1), Aroclor (9-fold in MCF-10A, 5-fold in RWPE-1), and 4CIBQ (20-fold in MCF-10A, 9-fold in RWPE-1) treated cells when PEG-SOD inhibitable DHE oxidation was determined, relative to PEG-alone vehicle controls (Figure 3B and Figure 3D). Since CuZnSOD is believed to be very specific for $O_2^{\cdot-}$, these results provide compelling evidence that PCB exposure results in significantly increased intracellular steady-state levels of $O_2^{\cdot-}$ in human breast and prostate epithelial cells.

Because it was previously suggested that PCBs and their metabolites could affect mitochondrial metabolism [36,37], the mitochondrial localization of $O_2^{\cdot-}$ in PCB-treated MCF-10A breast and RWPE-1 prostate cells was determined using an $O_2^{\cdot-}$ sensitive, fluorescent dye that localizes in mitochondria (i.e., MitoSOX™ red). The flow cytometry results in Figure 4A demonstrate that exposures to 3 μ M PCBs for 5 days significantly increased intracellular MitoSOX™ red oxidation [PCB153 (1.5-fold), Aroclor (3.5-fold) or 4CIBQ (9-fold)], in MCF-10A cells. In addition, Figure 4B, shows that, relative to MCF-10A, RWPE-1 prostate

epithelial cells appeared to have significantly higher steady-state levels of MitoSOX™ red oxidation in PCB153 (6-fold), Aroclor 1254 (10-fold), and 4CIBQ (19-fold) treated groups. To further support the conclusion that these increases in MitoSOX™ red oxidation are truly representative of mitochondrial $O_2^{\cdot-}$, confocal microscopy was used to visualize the intracellular co-localization of MitoSOX™ red oxidation and MitoTracker Green fluorescence. In this experiment the cells were pre-labeled in the dark with MitoSOX™ red and MitoTracker green for 20 mins to allow the fluorophores to be localized in the mitochondria, the attached cells were then washed with PBS (to remove extra-cellular MitoSOX™ red and MitoTracker green), and the PCBs were added for 1 minute. When this was done with 4CIBQ treated MCF-10A and RWPE-1 cells, the MitoSOX™ red oxidation image (Figure 5A and Figure 5G) showed much brighter red color compared to the vehicle control MitoSOX™ red oxidation image (Figure 5D and Figure 5J). The overlay of the MitoSOX™ red oxidation image (Figure 5A and Figure 5G) with the MitoTracker Green image (Figure 5B and Figure 5H) showed nearly exact concordance in staining as evidenced by the yellow to orange color seen in Figure 5C and Figure 5I. In a similar experiment PCB153 and Aroclor also showed mitochondrial localization of MitoSOX™ red oxidation using the same methodology (data not shown) in both cell lines. To identify the signal from MitoSOX™ red oxidation was truly representing mitochondrial superoxide, superoxide dismutase (MnSOD) was over expressed in the cells prior to PCBs exposure using the AdMnSOD vector (A'B'C' and G'H'I'). Figure 5A' and Figure 5G' show that the oxidation of MitoSOX™ red oxidation after 4CIBQ exposure, was nearly completely suppressed to control levels by AdMnSOD treatment (compare Figure 5A' to Figure 5D' and Figure 5G' to Figure 5J'). Furthermore, the overlay image (Figure 5C' and Figure 5I') looked identical to the MitoTracker Green image (Figure 5B' and Figure 5H') confirming that the increased levels of superoxide being detected did originate from mitochondria (compare Figure 5C to Figure 5C' and Figure 5I to Figure 5I'). In a similar experiment PCB153 and Aroclor also showed suppression of MitoSOX™ red oxidation following AdMnSOD treatment (data not shown). These results provide compelling evidence supporting the hypothesis that mitochondria are a major site for increased steady-state levels of $O_2^{\cdot-}$ in exponentially growing MCF-10A and RWPE-1 cells during and following PCB exposure.

Once it is generated in the cell, $O_2^{\cdot-}$ could either spontaneously (or through the action of superoxide dismutase enzymes) undergo dismutation to form H_2O_2 which also could adversely affect cell growth and proliferation. To determine if the intracellular steady-state levels of hydroperoxides, particularly H_2O_2 , were also increased in MCF-10A breast and RWPE-1 prostate cells treated with PCBs and their metabolites, cells were labeled with the oxidation-sensitive probe CDCFH₂ after 5 days of treatment with 3 μ M PCB3, PCB77, PCB153, Aroclor, or 4CIBQ. CDCFH₂ crosses cellular membranes and is enzymatically hydrolysed by "intracellular" esterases, and then can be further oxidized to produce a green fluorescent compound (CDCF) trapped inside of the cell for detection by flow cytometry. In PCB treated MCF-10A cells, there was a significant 3-fold increase in CDCFH₂ oxidation (Figure. 6A) in cells treated with 4CIBQ, however, other PCBs did not show any increase in probe oxidation, compared to the control. When the experiment was repeated using PEG-CAT inhibitable CDCFH₂ oxidation as described in the methods section (by determining the differences in fluorescence between PEG-CAT and 18 μ M PEG alone treated cells for each treatment group), similar results were obtained clearly demonstrating that most (if not all) of the increased probe oxidation caused by 4CIBQ was mediated by H_2O_2 (Figure. 6B). Interestingly, unlike MCF-10A cells, the steady-state level of hydroperoxides detected using the aforementioned methodology in PCB-exposed RWPE-1 cells were found to be significantly increased in PCB 153 (5-fold), Aroclor 1254 (7-fold) and 4CIBQ (15-fold) treatment groups (Figure 6C). To further confirm PCB-induced increases in steady-state levels of hydroperoxides, the experiments in Figure 6D showed similar increases in PEG-CAT inhibitable CDCFH₂ oxidation in PCB exposed RWPE-1 cells. To further confirm that changes in CDCFH₂ oxidation were detecting only changes in probe oxidation, the previous experiments were also

repeated using the oxidation insensitive analog (CDCF) which measures changes in dye uptake, ester cleavage, and efflux (independent of oxidation). The results showed that there was no significant difference in CDCF labeling between any treatment groups compared to control (DMSO treated) cells (data not shown).

Overall, the data obtained from the studies measuring DHE, MitoSOXTM red, and CDCFH₂ oxidation (Figures 3–6) clearly demonstrate that treatment with PCBs and their metabolites cause increases in intracellular steady-state levels of ROS (i.e., O₂^{•-} and H₂O₂) in MCF-10A breast and RWPE-1 prostate epithelial cells. In addition, the studies using MitoSOXTM red and MnSOD over expression also strongly support the hypothesis that mitochondria represent a major source for increased steady-state levels of O₂^{•-} in these PCB-treated human breast and prostate epithelial cell lines.

To determine if PCB-induced increases in steady-state levels of ROS were accompanied by alterations in cellular antioxidants associated with O₂^{•-} and H₂O₂ metabolism, intracellular GSH level, CuZnSOD activity, and MnSOD activity were analyzed. The results in Table 1 demonstrate significantly decreased levels of total GSH in cells treated daily dose of 3 μM for 3 days with PCB153, Aroclor 1254, or 4CIBQ, relative to vehicle controls. Furthermore, treatment with 3 μM PCB153, Aroclor 1254, or 4CIBQ for 3 days also induced very significant 3–4 fold increases in MnSOD activity (but not CuZnSOD), relative to vehicle controls in MCF-10A cells (Figure 7A). Interestingly, when western blot analysis was performed using antibodies against MnSOD there were no significant changes in immunoreactive protein levels among any treatment or control groups, relative to α-actin loading controls (Figure 7B). However, similar exposures to PCBs were found to slightly decrease the MnSOD activity while inducing CuZnSOD activity (3–5 folds) in RWPE-1 cells (Figure 7C).

To determine if the biological effects of PCBs are causally related to PCB-induced increases in steady-state levels of ROS, MCF-10A and RWPE-1 cells were treated with PCBs for 1 hour followed by addition of antioxidant enzymes (i.e. 50 U/mL PEG-SOD and PEG-CAT) or a non-specific thiol antioxidant (5 mM, N-acetylcysteine; NAC) for the next 23 hours. This treatment sequence was repeated for 3 consecutive days using 3 μM PCB153, Aroclor 1254, and 4CIBQ and then the cells were assayed for clonogenic cell survival. The results in Figure 8A and 8B show that the combination of PEG-CAT and PEG-SOD (50 U/ml each) added one hour after PCBs during each day of exposure for three days can partially rescue MCF-10A and RWPE-1 cells from the cytotoxicity mediated by PBC153, Aroclor, and 4CIBQ. Since PEG not conjugated to the antioxidant enzymes was incapable of rescuing the cells from PCB-induced toxicity, these results strongly support the hypothesis that PCB-induced increases in steady-state levels of O₂^{•-} and H₂O₂ play a significant role in the loss of cellular reproductive integrity seen during PCB exposure. Furthermore treatment with the thiol antioxidant, NAC added one hour after PCBs during each day of exposure for three days, completely rescued the MCF-10A cells from the toxicity associated with PCB 153 and Aroclor as well as partially rescuing cells from the toxicity of 4CIBQ. In RWPE-1 cells, treatment with NAC after PCB exposure could also significantly rescue the cells from PCB-induced clonogenic inactivation (Figure 8); however, it was also found that NAC treatment of RWPE-1 decreased the plating efficiency of untreated cells by 50% (data not shown). Overall these results strongly support the hypothesis that antioxidant treatments targeting ROS scavenging and/or enhancing the thiol capacity of the cell following PCB exposure can significantly protect human breast and prostate epithelial cells from PCB-induced cytotoxicity. This novel finding suggests that manipulation of antioxidants (following exposure to PCBs and their metabolites) in breast and prostate epithelial cells could mitigate the deleterious biological effects of PCBs.

Discussion

The current human exposure to PCBs in Europe, Japan, and the USA is being driven by residual PCBs in the diet, air, water, and soil, especially those PCBs historically used as dispersants in pesticides, lubricants in electrical transformers, and other industrial usages [1,9,38]. PCBs remain in adipose tissue and blood lipids for extended periods of time leading to gradual release and chronic exposures. Occupational exposure to PCBs, such as that found among farmers and industrial workers, has also been associated with increased incidence of prostate or breast cancer [8,38].

PCB exposure has been suggested to cause alterations in cell growth and toxicity via disruptions in oxidative metabolism leading to oxidative stress mediated by increased intracellular steady-state levels of reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_2 [7,14,38]. Under physiological circumstances where a primary route of PCB metabolism is via liver cytochrome P-450 enzymes, primary phenolic metabolites of PCBs can undergo a second hydroxylation and become further oxidized to yield reactive hydroquinones and quinones which could generate semi-quinone radicals via redox cycling [39]. It has been suggested that this route of PCB metabolism could form semi-quinone radicals which could mediate cytotoxicity by leading to increased intracellular steady-state levels of $O_2^{\cdot-}$ and H_2O_2 [39]. Since ROS are known to play a role in both mitogenesis and genomic instability [12], which may contribute to carcinogenesis, it has been proposed that PCB-induced metabolic oxidative stress contributes to neoplastic transformation [7,8,40,41] and other deleterious effects of PCBs and their metabolites [5,7,8,14]. However, despite the interest in PCB-induced oxidative stress there is very little direct evidence in living cells identifying the specific ROS involved in mediating PCB induced cell injury or identifying the intracellular sites of ROS production. Furthermore it is not known if intervening with antioxidants following PCB exposure is capable of mitigating the toxic biological effects, which is the situation most often faced in human environmental exposures [42,43]. In the current study, human breast and prostate epithelial cell treated with PCBs and PCB metabolites exhibited inhibition of cell growth as well as clonogenic cell killing that was most pronounced when using the 4CIBQ metabolite of PCB3 but was also significant with PCB153, and Aroclor 1254. These results are consistent with previous studies where PCBs were shown to play an important role in cytotoxicity by disrupting the cell growth and cell proliferation [6,8–10,44].

In addition to perturbations in cell growth, the current study clearly demonstrated that intracellular steady-state levels of $O_2^{\cdot-}$ and H_2O_2 could be detected as SOD inhibitable DHE oxidation in human breast and prostate epithelial cells treated with PCB153, Aroclor 1254, and 4CIBQ (Fig 3B and Fig 3D). Interestingly, different levels of increases in steady-state levels of H_2O_2 (Figure 6) were found in the two cells lines especially in PCB153 and Aroclor 1254 treated groups. In RWPE-1 cells, increased levels of H_2O_2 were detected in PCB153, Aroclor 1254 and 4CIBQ treated groups (20–35 fold). However, in PCB-exposed MCF-10A cells, the increased steady-state levels of H_2O_2 were only found in cells treated with 4CIBQ (Fig 6B) suggesting $O_2^{\cdot-}$ was being converted to H_2O_2 which was being efficiently removed in PCB153 and Aroclor 1254 treated breast epithelial cells. At this time, it is not possible to specify the mole to mole relationship between the increased levels of $O_2^{\cdot-}$ and H_2O_2 . We speculate that the different levels of increases in steady-state levels of H_2O_2 could be related to differential metabolism of H_2O_2 by the cells being studied.

Consistent with the idea that an adaptive response involving antioxidants may be induced by PCBs, chronic exposure to both Aroclor 1254 and 4CIBQ caused significant 2–4-fold increases in the activity of the mitochondrial superoxide scavenging enzyme, MnSOD, but interestingly this was not accompanied by similar changes in MnSOD immuno-reactive protein (Fig 7A and Fig 7B) in MCF-10A cells. It can be speculated that the post-translational modification of the

MnSOD protein via phosphorylation/dephosphorylation might be involved in the up regulation of MnSOD activity [45] in MCF-10A cells as an adaptive response to PCB-induced oxidative insult. Interestingly, in RWPE-1 cells CuZnSOD activities were found to demonstrate 3–5 fold increases, while MnSOD activity demonstrated a decrease when exposed to PCB153, Aroclor 1254 and 4CIBQ (Figure 7C). Since our results also suggested that PCB 153 and Aroclor 1254 may have more profound effects on the reproductive integrity of RWPE-1 cells, the differences seen in the activity of different isoforms of SODs in MCF10-A and RWPE-1 cells may further indicate that a differential coping mechanism against PCBs and their metabolites in cells from different tissue origins. Finally total GSH levels were also significantly reduced by exposure to PCBs in MCF-10A cells (Table 1) supporting the hypothesis that the increased oxidative stress may significantly contribute to the toxicity caused by these PCBs.

The fact that the alteration of MnSOD (the mitochondrial form of superoxide dismutase) activity by PCBs exposure, suggested that mitochondria may represent a significant source for increased $O_2^{\cdot-}$ in PCB exposed cells. To test this hypothesis as well as to demonstrate intracellular localization of probe oxidation, MitoSOX™ red oxidation was used to determine if the superoxide production was mitochondrial in origin (Figure 4) and co-localization with mitotracker green as detected by confocal microscopy (Figure 5) was utilized to confirm the mitochondrial origin of the signals that were detected. The results of the flow cytometric and confocal microscopic analysis demonstrated that PCB153, Aroclor 1254, and 4CIBQ increased intracellular $O_2^{\cdot-}$ production and since the fluorescent signal in all groups was co-localized with MitoTracker Green, it was evident that the mitochondria were the primary site for $O_2^{\cdot-}$ production in both MCF-10A and RWPE-1 cells during PCB exposure. In addition, when MnSOD was over expressed prior to PCB exposure using an adenoviral vector, MitoSOX™ red oxidation was nearly completely suppressed to control level. These results provide the first clear evidence in living human breast and prostate epithelial cells that PCB exposure causes increases in steady-state levels of mitochondrially derived superoxide.

To establish a causal link between increased steady-state levels of specific ROS (i.e., $O_2^{\cdot-}$ and H_2O_2) and PCB-induced cytotoxicity, Both MCF-10A and RWPE-1 cells were exposed to cell permeable forms of superoxide dismutase and catalase (PEGSOD/CAT) 1 hour following addition of PCBs to the cell culture media (to scavenge $O_2^{\cdot-}$ and H_2O_2) followed by clonogenic survival assay after 3 days of exposure. Figure 8 shows that treatment of MCF-10A and RWPE-1 cells with PGSOD/CAT was capable of partially rescuing cells from PCB-induced cytotoxicity, strongly supporting the hypothesis that $O_2^{\cdot-}$ and H_2O_2 play an important role in the process of PCB-induced cytotoxicity. Furthermore, a clinically significant non specific thiol antioxidant used in the mitigation of Tylenol-induced cytotoxicity (NAC) [16], added 1 hour following PCBs each day for 3 days was capable of completely rescuing MCF-10A and RWPE-1 cells from cell killing mediated by PBC153 and Aroclor 1254 exposure as well as significantly protecting against 4CIBQ-induced toxicity (Figure 8). Overall, these results strongly support the hypothesis that PCB and PCB metabolites exert their toxic effects on human breast epithelial cells through increased levels of intracellular ROS as well as oxidative stress that can be mitigated by treatment with antioxidants.

To the best of our knowledge, this is the first study showing that the PCBs and their metabolites can increase steady state levels of $O_2^{\cdot-}$ and H_2O_2 through mitochondrial metabolism in human breast and prostate epithelial cells. Furthermore, the data clearly demonstrate that PCB-induced perturbations in cell growth and clonogenic survival can be mitigated using specific antioxidant enzymes targeting $O_2^{\cdot-}$ and H_2O_2 or a clinically relevant non-specific thiol antioxidant added following PCB exposure. These results provide very significant proof-of-principle evidence supporting the potential efficacy of pharmacological interventions aimed at mitigating metabolic oxidative stress following exposure in PCB contaminated populations.

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Abbreviations

4CIBQ	2-(4-chlorophenyl)-1,4-benzoquinone
CDCFH₂	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
DHE	dihydroethidium
DMSO	dimethyl Sulfoxide
ETCs	electron transport chains
GSH	glutathione
H₂O₂	hydrogen peroxide
MFI	mean fluorescence intensity
MnSOD	Manganese superoxide dismutase
MOI	multiplicity of infection
NAC	N-acetyl-cysteine
NBT	nitroblue tetrazolium
O₂^{•-}	superoxide
PCB	polychlorinated biphenyl
PBS	phosphate buffered saline
PEG-CAT	polyethylene glycol conjugated catalase
PEG-SOD	polyethylene glycol conjugated CuZn superoxide dismutase
ROS	reactive oxygen species
SOD	superoxide dismutase

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A: PCB 3 (4-Chlorobiphenyl) **B: PCB 77 (3,3',4,4'-Tetrachlorobiphenyl)** **C: PCB 153 (2,2',4,4',5,5'-Hexachlorobiphenyl)** **D: 4ClBQ (2-(4-chlorophenyl)-1,4-benzoquinone)** **Figure 1. Chemical structure of PCBs and their metabolites used in the study**

PCBs consist of a biphenyl (two benzene rings with a carbon to carbon bond between carbon 1 on one ring and carbon 1' on the second ring) with a varying number of chlorines. Chlorines can be attached to any of the carbons by removing the hydrogen from that carbon and substituting the chlorine in its place. A: PCB3, B: PCB77, C: PCB153, D: 4ClBQ

Figure 2. PCB-induced perturbations in MCF-10A cell growth and clonogenic survival

In Panel A (MCF-10A) and C (RWPE-1), cells were given fresh complete media daily containing 3 μ M PCBs for 5 days. Monolayer cultures were trypsinized, and cell numbers counted from day 1 to day 5 (panel A or C) and in panel B (MCF-10A) and D (RWPE-1) cells were plated for clonogenic survival. In panel A and C each data point represent $n=6$ treated dish from 2 separate experiment where 3 dishes per group were treated (± 1 SD). * $p < 0.001$ as compared to Control. In panel B and D, error bars represent mean ± 1 SD of $n=4$ treatment dishes done in 2 separate experiments where each treatment dish was used to prepare 3 replicate cloning dishes for analysis. * $p < 0.001$ as compared to Control Day 1, † $p < 0.001$ as compared to Control Day 2, ¶ $p < 0.001$ as compared to Control Day 3, § $p < 0.001$ as compared to Control Day 4, ♂ $p < 0.001$ as compared to Control Day 5.

Figure 3. Steady-state levels of $O_2^{\bullet-}$ in PCB exposed cells as determined by DHE oxidation

Asynchronously growing cultures of MCF-10A (panel A) and RWPE-1 (panel C) cells were incubated with 3 μ M PCBs daily for five days as in Figure 2. Monolayer cultures were harvested and trypsinized, washed once with PBS and labeled 40 mins with 5 μ M DHE (in 0.1% DMSO) in PBS containing 5 mM pyruvate at 37°C. Each sample was then analyzed for the Mean fluorescence Intensity (MFI) of 10,000 cells by flow cytometry (panels A and C). Samples were assayed in triplicate; mean $\pm$ 1 SEM of 3 separate experiments containing 3 treatment dishes per group (n=9), * p < 0.001 as compared to Control. Panels B (MCF-10A) and D (RWPE-1) show the data from the third replicate of the experiments shown in panels A and C, where the DHE labeling was done in the presence (100 U/ml PEG-SOD) or absence (18 μ M PEG alone) of polyethylene glycol conjugated CuZnSOD given 2 hours before and during DHE labeling. PEG-SOD inhibitable DHE oxidation was obtained by calculating the differences in DHE oxidation between PEG-SOD and PEG alone pretreated cells for each treatment group. The PEG-SOD inhibitable DHE oxidation data were then normalized to the control (non-PCB treated cells) and plotted in panel B (MCF10-A) and panel D (RWPE-1); mean $\pm$ 1 SD (n=3), * p < 0.001 as compared to Control.

Figure 4. Steady-state levels of $O_2^{\cdot -}$ as determined by Mitosox oxidation via flow cytometry Asynchronously growing cultures of MCF-10A (panel A) and RWPE-1 (panel B) were incubated with 3 μ M PCBs daily for five days as in Figure 2. Monolayer cultures were harvested, trypsinized, cells washed once with the PBS, and labeled in PBS containing 5 mM pyruvate with 2 μ M Mitosox (in 0.1% DMSO, 20 mins) at 37°C. Mean fluorescence intensity (MFI) of 10,000 cells was analyzed by flow cytometry. Samples were assayed in triplicate; Mean $\pm$ 1 SEM of 3 separate experiments, each containing 3 treatment dishes (n=9), * $p < 0.001$ as compared to Control.

Figure 5. Mitochondrial localization of $O_2^{\bullet-}$ as determined by Mitoxox oxidation and Mitotracker colocalization using confocal microscopy in MCF-10A cells

Asynchronously growing cultures of MCF-10A and RWPE-1 cells on microscope slides were incubated with 100 MOI AdMnSOD (or 100 MOI empty vector control) for 24 hours then allowed to recover for 48 hrs and then washed with PBS. Monolayer cultures were then incubated in PBS (containing 5 mM pyruvate) with Mitoxox (2 μ M), and Mitotracker (100 nM) in 0.2% DMSO for 20 mins at 37°C. PCBs were then added for 1 minute at 37°C, cells were then washed in PBS, and placed on ice before analysis using a laser scanning Zeiss confocal microscopy system. Results are representative of fields from five randomly picked areas on each slide. The results confirm that the PCB-induced increases in steady-state levels of

superoxide were located in the mitochondria based on the co-localization of Mitosox oxidation with Mitotracker green (see yellow color in panel C or I and the inhibition of yellow color seen in Panel C' or I' by overexpressing MnSOD in MCF-10A and RWPE-1 cells. Panel A-C, A'-C': 4ClBQ treated MCF-10A cells, Panel D-F, D'-F': MCF-10A vehicle control, Panel G-I, G'-I': 4ClBQ treated RWPE-1 cells, Panel J-L, J'-L': RWPE-1 vehicle control.)

Figure 6. Steady-state levels of H₂O₂ as determined by catalase inhibitable CDCFH₂ oxidation
 Asynchronously growing cultures of MCF-10A (panel A) and RWPE-1 (panel C) were incubated with 3 μ M PCBs daily for five days as in Figure 2. Monolayer cultures were harvested and trypsinized, washed once with PBS then labeled in PBS with CDCFH₂ (10 μ g/mL, in 0.1% DMSO 15 mins) at 37°C. Mean fluorescence intensity (MFI) of 10,000 cells was analyzed by flow cytometry. Samples were assayed in triplicate; Mean $\pm$ 1 SEM of 3 separate experiments containing 3 treatment dishes per group (n=9), * p < 0.001 as compared to Control (panel A and panel C). In the third replicate experiment shown in panel A and panel C, the CDCFH₂ labeling was done in the presence (100 U/ml PEG-CAT) or absence (18 μ M PEG alone) of polyethylene glycol conjugated Catalase given 2 hours before and during CDCFH₂ labeling. Only the normalized PEG-CAT inhibitable MFI of the CDCFH₂ oxidation signal is plotted (panel B: MCF10-A and panel D: RWPE-1); mean $\pm$ 1 SD (n=3), * p < 0.001 as compared to Control.

Figure 7. MnSOD activity is induced in PCB-exposed MCF-10A cells

Asynchronously growing cultures of MCF-10A (panel A) and RWPE-1 (panel B) were incubated with 3 μ M PCBs daily for 3 days as in Figure 2. Cells were harvested, scraped and pelleted in PBS at 4°C, and whole cell homogenates prepared from the pellets re-suspended in 50 mM potassium phosphate buffer, pH 7.8. Homogenates were used for biochemical analysis of total SOD, CuZnSOD and MnSOD activities in MCF-10A and RWPE-1 cells (panel A and panel B). The data from three independent experiments is shown. Data represent the mean of each group $\pm$ 1SD (n=3), * p < 0.001 as compared to Control total SOD, † p < 0.001 as compared to Control MnSOD in MCF-10A or Control CuZnSOD in RWPE-1. Panel C shows the MCF-10A Western blot analysis and relative immuno-reactive protein levels normalized to actin loading control. PCB-exposed MCF-10A samples were first assayed for MnSOD activity. The same samples were then used for Western blotting to detect the MnSOD immuno-reactive protein.

Figure 8. MCF-10A cells treated with PEGCAT+PEGSOD or 5 mM NAC 1 hour following PCB exposure are protected from PCB-induced toxicity
 Asynchronously growing cultures of MCF-10A (panel A) and RWPE-1 (panel B) were incubated with a daily dose of 3 μ M PCBs for three days as in Figure 2. One hour after PCB treatment each day, PEG alone (18 μ M) or PEGCAT/SOD (50 U/mL each) or NAC (5 mM) was added to the dishes. Following three days of treatment with PCBs, cells were re-plated for clonogenic survival assay in the absence of PCBs. In MCF-10A cells (panel A) and in RWPE-1 cells (panel B), Errors represent $\pm$ 1SEM of 3 separate experiments, each experiment had 1 treatment dish and 3 replicate cloning dishes ($n=3$). * $p < 0.001$ as compared to Control PEG alone, $^{\dagger} p < 0.01$ as compared to Control PEGSOD/CAT, $^{\P} p < 0.01$ as compared to each respective PCB + PEG alone. $^{\S} p < 0.01$ as compared to each respective PCB + PEG alone.

Table 1

Intracellular total GSH is decreased in PCB-exposed human MCF-10A cells.

Group	Total GSH (nmol GSH/mg protein)
Control	3.2 ± 0.2
PCB153	2.3 ± 0.1*
Aroclor	1.9 ± 0.1*
4CIBQ	2.2 ± 0.2*

Asynchronously growing cultures of MCF-10A were incubated with 3 μ M PCBs daily for 3 days. Cells were harvested and scraped in PBS at 4°C. Whole cell homogenates were used for biochemical analysis of total GSH levels. Errors represent Mean $\pm$ 1 SEM of 9 samples from three separate experiments (n=9).

*
p < 0.05 as compared to Control.