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Polychlorinated biphenyl induced ROS signaling delays the entry of quiescent human breast epithelial cells into the proliferative cycle

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Abstract

Polychlorinated biphenyls (PCBs) are environmental chemical contaminants that can produce reactive oxygen species (ROS) by autooxidation of dihydroxy-PCBs and redox-cycling. We investigate the hypothesis that PCB induced perturbations in ROS signaling regulate the entry of quiescent cells into the proliferative cycle. Quiescent MCF-10A human breast epithelial cells were incubated with 0–3 micromolar of 2-(4-chlorophenyl)benzo-1,4-quinone (4-Cl-BQ), 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (PCB 153), and Aroclor 1254 for 4 days. Cells were replated at a lower density and analyzed for cell cycle phase distributions, ROS levels, MnSOD expression, and cyclin D1 protein levels. Quiescent cells incubated with 4-Cl-BQ showed the maximal delay in entering S phase. This delay was associated with a decrease in MnSOD activity, protein and mRNA levels, and an increase in cellular ROS levels. Results from the mRNA turnover assay showed that the 4-Cl-BQ treatment selectively enhanced the degradation of the 4.2 kb MnSOD transcript, while the half-life of the 1.5 kb transcript did not change. Accumulation of cyclin D1 protein levels in replated cells was suppressed in cells treated with 4-Cl-BQ. Pretreatment of quiescent cells with polyethylene glycol-conjugated superoxide dismutase and catalase suppressed 4-Cl-BQ induced increase in ROS levels, which was consistent with an increase in cyclin D1 accumulation, and entry into S phase. These results showed 4-Cl-BQ induced perturbations in ROS signaling inhibit the entry of quiescent cells into S phase.

Keywords

Cyclin D1; MnSOD; catalase; polychlorinated biphenyls; quiescence; cell proliferation; reactive oxygen species; MCF-10A

Introduction

Polychlorinated biphenyls (PCBs) are a group of widely dispersed environmental pollutants. PCBs were used as coolants and lubricants in transformers and as a dielectric in capacitors

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[1]. The persistent and ubiquitous distributions of these compounds cause them to remain potentially serious hazards to human and animal health. PCBs are strongly lipophilic and stable compounds. These characteristics cause them to bioaccumulate in the food chain and concentrate in the fatty tissue, including breast tissues [2–5]. Serum levels of PCBs in Americans average about 10 ppb (~30 nM); and occupationally exposed individuals may have PCB blood levels in the hundreds of ppb [6]. PCB blood levels in individuals living in Anniston, Alabama vary widely, 0.003–6.5 μM [7]. PCB levels in adipose tissue are much higher than those in blood, with levels in low ppm range [8].

The biological effects of individual PCBs depend on the number and position of chlorines in the biphenyl rings, which also determines their chemical and physical properties. Mono- and di-chlorinated biphenyls can be metabolized to hydroquinones, which can be further oxidized to reactive metabolites, notably semiquinones that react with oxygen to form superoxide and quinones [2,9,10]. 2-(4-chlorophenyl)benzo-1,4-quinone (4-Cl-BQ) is a metabolite of 4-chlorobiphenyl (PCB 3) [11]. We have shown recently a higher flux of hydrogen peroxide in 4-Cl-BQ treated MCF-10A human breast epithelial cells, which was accompanied with the formation of a semiquinone radical [12]. Although the mechanisms regulating the biological effects of PCBs are not completely understood, perturbation in cellular redox environment could regulate many of the biological effects of PCB exposures [2,9–12].

Cellular redox environment is a balance between the production of reactive oxygen species (ROS, e.g., superoxide and hydrogen peroxide) and their removal by antioxidants. ROS are produced intracellularly by the univalent reduction of oxygen in the mitochondrial electron transport chain and enzymatic reactions. Enzymatic reactions of superoxide dismutase (SOD) convert superoxide to hydrogen peroxide; catalase and glutathione peroxidases neutralize hydrogen peroxide and organic hydroperoxides. Mammalian cells have three SODs; MnSOD located in the mitochondrial matrix, EcSOD in the plasma membrane and extracellular space, and CuZnSOD in the cytosol, nucleus, peroxisome, and intermembrane space of mitochondria [13–16]. Human MnSOD has two transcripts, 4.2 and 1.5 kb, which differ in the length of their 3'-untranslated region (UTR). A previous report showed a preferential turnover of the 4.2 kb MnSOD transcript in multiple cell types that were treated with TNF α [17], suggesting that specific sequence in the 3'-UTR of the 4.2 kb transcript could regulate MnSOD mRNA levels.

We have shown previously that MnSOD activity regulates cellular ROS levels and growth in mouse and human fibroblasts [18–21]. Cellular growth consists of two distinct states: quiescent (G_0) and proliferative (G_1 , S, G_2 , and M) states. Cellular quiescence is a reversible process essential to prevent aberrant proliferation and protects the capacity of cells to repopulate. MnSOD activity and mitochondria generated ROS signaling are known to regulate both of these properties of quiescent cells [18,19]. We have shown previously that overexpression of MnSOD inhibits age associated increase in cyclin dependent kinase inhibitor (p16) protein levels, and protects the proliferative capacity of normal human skin fibroblasts cultured *in vitro* [18]. In contrast, overexpression of a dominant negative mutant form of MnSOD inhibited the entry of quiescent fibroblasts into the proliferative cycle [19]. MnSOD activity dependent regulation of entry into and exit from the proliferative cycle was associated with changes in cyclin D1 and cyclin B1 protein levels [19]. Cyclin D1 is the first cyclin that responds to mitogenic stimuli. Therefore, an increase in its protein levels is often used as an indicator of reentry of cells from the quiescent to the proliferative growth state.

In general, the majority of the biological effects of PCBs are studied using *in vitro* cell cultures of exponentially growing asynchronous cells. The significance of these results to *in vivo* conditions is not clear because a majority of the proliferation competent cells *in vivo* resides in quiescent growth state. Stem cells are excellent example of cellular quiescence *in vivo*. The present study uses quiescent cultures to better mimic *in vivo* growth state, and determine if

PCB induced changes in ROS signaling perturb the entry of quiescent cells into the proliferative cycle. Quiescent MCF-10A mammary epithelial cells incubated with 4-Cl-BQ decreased MnSOD activity, and increased ROS levels. The increase in ROS levels suppressed cyclin D1 accumulation, and inhibited progression from quiescent to the proliferative cycle. 4-Cl-BQ selectively enhanced the turnover of the 4.2 kb MnSOD transcript, while there was no change in the mRNA levels of the 1.5 kb MnSOD transcript.

Materials and Methods

Chemicals

2-(4-chlorophenyl)benzo-1,4-quinone (4-Cl-BQ), 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (PCB 153), and Aroclor 1254 (commercial mixture of various PCB congeners, [22,23]) were provided by Dr. Hans-Joachim Lehmler, Occupational & Environmental Health, University of Iowa. The synthesis and purity of these PCBs were performed following the previously published methods [11,24–26]. PCB stock solutions were made in dimethyl sulfoxide, and appropriate dilutions of the stock solution were added to cell culture medium where the final concentrations of dimethyl sulfoxide were adjusted to less than 0.5%. Control cultures were adjusted to the same concentrations of dimethyl sulfoxide as the PCB treated cells. Actinomycin D, polyethylene glycol conjugated (PEG)-superoxide dismutase and catalase were purchased from Sigma Chemical Co. DHE (dihydroxyethidium) and CDCFH₂ (5, 6-chloromethyl-2', 7'-dichlorodihydro fluorescein diacetate) were purchased from Molecular Probes (Eugene, Oregon).

Cell culture

MCF-10A human mammary epithelial cells were purchased from the American Tissue Culture Collection (ATCC). MCF-10A cells are spontaneously immortalized cells that possess the characteristics of human normal mammary epithelial cells. Cells were grown in mammalian epithelial growth medium (MEGM, Cell Applications Inc., San Diego, California) supplemented with growth factors and antibiotics following our previously published cell culture protocol [12]. Cells were grown at 37°C, 5% CO₂ and 95% humidity. Cells were subcultured upon confluence with 0.25% trypsin and 1% EDTA. Contact inhibited quiescent growth state was achieved by plating cells at a higher density and culturing for an additional 2 days prior to the PCB treatments. Our experimental design partially mimics *in vivo* quiescence because cells were cultured at 21% instead of *in vivo* concentration of 4% oxygen environment. The percentage of S phase, less than 2%, was considered a quiescent growth state. Control and PCB treated quiescent cells were replated at a lower cell density and cultured for the indicated times in regular growth medium without any PCBs.

Cell population doubling time (Td) was determined by counting cells at the time of replating, and 2, 4, and 6 days post-replating. Td was calculated from the exponential portion of the growth curve using the following equation: $Td = 0.693t / \ln(N_t/N_0)$ where t is time in days, and N_t and N₀ represent cell numbers at time t and initial time, respectively.

Flow cytometry assays: Bromodeoxyuridine (BrdU) labeling

Control and PCB treated quiescent cells were replated and incubated with growth medium containing 10 µM BrdU. Cells were harvested at indicated times and fixed in ethanol. Ethanol fixed cells were washed with PBS containing 0.1% Tween 20, and treated with Pepsin-HCl (0.3 mg pepsin/ml 2 N HCl). Isolated nuclei were incubated with anti-BrdU antibody followed by incubation with FITC conjugated goat anti-mouse IgG secondary antibody. Nuclei were incubated with 1 mg/ml RNase A, propidium iodide (PI, 35 µg/ml), and analyzed by flow cytometry following our previously published protocol [21]. Data were collected from 20,000 events and analyzed by using the FlowJo software (Tree star, Inc., Ashland, Oregon). The

acquired data were analyzed as dual parameter PI vs. log FITC histograms, and three compartments (BrdU positive S phase, and BrdU negative G₁ and G₂ phases) were identified. The number of nuclei in each compartment was calculated and expressed as percentage of the total gated population.

DHE and CDCFH₂ fluorescence

Control and quiescent cells incubated with PCBs were harvested by trypsinizing the monolayer cultures. Cell suspensions were washed with PBS containing pyruvate (5 mM) and incubated with 10 μ M DHE for 40 min at 37°C. DHE fluorescence was analyzed by flow cytometry using the excitation wavelength 488 nm, and emission 585 nm. The mean fluorescence intensity (MFI) of 10,000 cells was recorded. MFI of cells incubated with buffer alone was used to correct for autofluorescence. The fold change in MFI was calculated relative to the MFI of untreated control cells. The specificity of the DHE fluorescence for the measurement of superoxide was determined by incubating the cells with 100 U/ml PEG-SOD or 18 μ M PEG 2 h prior to and during the assay. The fluorescence of cells treated with PCBs and PEG-SOD was subtracted from the fluorescence measured in cells treated with PCBs and PEG, and MFI calculated relative to the difference in untreated control cells that were incubated with PEG and PEG-SOD.

Cellular hydrogen peroxide levels were measured by incubating cells with 5, 6-chloromethyl-2', 7'-dichlorodihydro fluorescein diacetate (CDCFH₂, Molecular Probes, Eugene, Oregon). Monolayer cultures of control and cells incubated with PCBs were washed once with PBS, and incubated with 10 μ g/mL CDCFH₂ for 10 min at 37°C. Cells were harvested on ice, resuspended in PBS, and fluorescence measured by flow cytometry using an excitation wavelength 488 nm and emission 530 nm band-pass filter. An oxidation insensitive chemical, CDCF, was included to account for differences in dye uptake and ester-cleavage. The specificity of the CDCFH₂ fluorescence for the measurements of cellular hydrogen peroxide was determined by incubating cells with PEG-CAT prior to and during the assay. Fluorescence of cells incubated with PEG alone was included in the calculation of MFI as described above.

Antioxidant enzyme activity assays: SOD biochemical activity assay

MnSOD activity was determined by the indirect competitive inhibition assay originally developed by Spitz and Oberley [27]. Sodium cyanide was used to inhibit CuZnSOD activity. The protein levels in each sample were measured using the Lowry protein assay [28]. 4-Cl-BQ by itself does not affect NBT reduction (data not shown). This observation is consistent with earlier reports in the literature [29,30].

Western blotting

Equal amounts of total cellular proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (BIORAD Labs., Hercules, California). Blots were incubated with antibodies to cyclin D1 (PharMingen, San diego, California), and MnSOD (PharMingen, San diego, California). Immunoreactive polypeptide was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection reagents (GE Healthcare, Waukesha, Wisconsin) following manufacturer supplied protocols. Blots were re-probed with antibodies to actin (Santa Cruz Biotechnology, Santa Cruz, California) for comparison of results. The dilutions of antibodies used were as follows: MnSOD, primary: 1:1000, secondary: 1:10,000; cyclin D1, primary: 1:1000, secondary: 1:3000; actin, primary: 1:1000, secondary: 1:3000. Results were quantitated using AlphaImager 2000 (Alpha Innotech, San Leandro, California) and ImageJ software. The images were first corrected for background and then the integrated density value was obtained by selecting the region of interest for each band with fixed area size for all bands. The integrated density values were calculated by using

the sum of the gray values in the selection. Actin levels were used to correct for loading in each sample and fold change calculated relative to control.

Quantitative Real Time PCR analysis

Total cellular RNA was isolated from control and PCB treated quiescent cells using the Trizol reagent (Invitrogen, Eugene, Oregon). RNA was quantified using a ND1000 nanodrop spectrophotometer (Nanodrop, Wilmington, Delaware) and one microgram of RNA from each sample was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster city, California) for 2 hr at 37°C. The cDNA was subjected to Real Time PCR assay using primers specific to MnSOD (ORF, 4.2 and 1.5 kb transcripts), and 18S; MnSOD ORF, forward primer: 5'-GGCCTACGTGAACAACCTGAA-3', reverse primer: 5'-CTGTAACATCTCCCTTGGCCA-3', amplicon size, 70 bp; MnSOD 4.2 kb transcript, forward primer: 5'-GCTTTGGTGGTGGATTGAAAC-3', reverse primer: 5'-CATCCCTACAAGTCCCCAAAGT-3', amplicon size, 187 bp; MnSOD 1.5 kb transcript, forward primer: 5'-TAATGATCCCAGCAAGATAA-3', reverse primer: 5'-TTTTTTTTTTTTTTTGGATGGTTG-3', amplicon size, 184 bp; 18S, forward primer: 5'-CCTTGGATGTGGTAGCCGTTT-3', reverse primer: 5'-AACTTTCGATGGTAGTCGCGG-3', amplicon size, 104 bp. The real time PCR assay was carried out with the 2× Power SYBR Green real time master mix (Applied Biosystems); reverse transcriptase inactivation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (ABI 7000 Sequence Detection System, Applied Biosystems). A threshold of amplification in the linear range of each sample was selected to calculate the cycle threshold (C_T) for each sample. The relative mRNA levels were calculated as follows: ΔC_T (sample) = C_T (mRNA of interest) – C_T (18S); $\Delta\Delta C_T$ = ΔC_T (post-treatment time point) – ΔC_T (control); Relative expression = $2^{-\Delta\Delta C_T}$.

Statistics

Statistical significance was determined by one-way ANOVA with Tukey's post hoc test and Student t-tests using GraphPad Prism, version 4. Results are presented as mean ± standard deviation. Results from at least $n \geq 3$ with $p < 0.05$ are considered significant.

Results

4-CI-BQ delays the entry of quiescent cells into the proliferative cycle

A cumulative BrdU assay was used to determine whether quiescent cells incubated with PCBs maintain their proliferative capacities following reentry into the proliferative cycle. Quiescent MCF-10A cells (< 2% S phase, based on DNA content) were treated with 0–3 μ M PCBs for 4 days. Control and cells treated with PCBs were replated and continued in culture in presence of 10 μ M BrdU. Cells were harvested at indicated times post-replating and analyzed for BrdU positive (cumulative S phase) and BrdU negative (G_1 and G_2 phases) nuclei. Representative FITC and PI histograms of control and cells treated with PCBs are shown in Figure 1A, and the percentage of S phase cells is presented in Figure 1B–D. The percentage of G_1 and S+ G_2 are summarized in Table 1. More than 90% of the cells were in the G_0/G_1 phase in both control and PCB treated quiescent cells at the end of 4 days of treatment, suggesting that the incubation of quiescent cells with PCBs did not result in any redistribution of cells to different cell cycle phases (Fig. 1A and Table 1). Furthermore, cell numbers at the end of the 4 days of incubation were comparable in control and cells treated with PCBs, indicating that the PCB treatments neither stimulated proliferation nor resulted in cell death while the cells were in the quiescent growth state. These results were consistent with the absence of a cell population with sub- G_1 DNA content (data not shown).

Interestingly, cells that were allowed to reenter the proliferative cycle did exhibit significant difference in their entry into S phase. While all PCBs showed a decrease in the percentage of S phase, cells treated with 4-Cl-BQ showed the highest inhibition (~50%) compared to Aroclor 1254 (35%), and PCB 153 (20%) treated cells (Fig. 1D). The percentage of S+G₂ phases in control cells increased to approximately 21 and 23% at 18 and 26 h post replating, respectively (Fig. 1A, and Table 1). The percentage of S+G₂ phases was significantly lower in 3 μ M 4-Cl-BQ treated cells at 18 h post replating, which remained lower at 26 h post replating (Fig. 1A and Table 1). The percentage of S+G₂ phases in cells treated with PCB 153 and Aroclor 1254 were 16 and 14% at 18 h post replating, which increased to 19 and 15% at 26 h post replating (Table 1). The inhibition in entry into S phase correlated with increases in cell population doubling time: control, 37 h; 4-Cl-BQ, 53 h; PCB 153, 47 h; Aroclor 1254, 50 h.

4-Cl-BQ increased cellular ROS levels in quiescent cells

We have previously reported the formation of a semiquinone radical in exponentially growing asynchronous cultures of MCF-10A cells incubated with 3 μ M 4-Cl-BQ, which could be the source of the apparent higher flux of cellular ROS [12]. To determine if PCBs perturb cellular ROS levels in quiescent cultures, control and 3 μ M PCB treated cells were harvested at the end of 4 days, and incubated with DHE and CDCFH₂. Fluorescence was measured by flow cytometry and MFI calculated after correction for autofluorescence; fold change was calculated relative to untreated control. Results (Fig. 2A, upper panel) showed approximately 2 folds increase in DHE fluorescence in 4-Cl-BQ treated cells compared to control. PCB 153 and Aroclor treatments did not show any significant change in DHE fluorescence compared to control. The specificity of the DHE fluorescence for the measurement of superoxide was determined by incubating cells with PEG-SOD 2 h prior to and during the assay; fold change was calculated following the method described in the Methods and Materials section. These results showed approximately 2.5 folds increase in cellular superoxide levels in cells incubated with 4-Cl-BQ (Fig. 2A, lower panel). DHE fluorescence in PEG-alone treated cells did not change compared to untreated control (Supplemental Figure 1). Quiescent cells treated with 4-Cl-BQ showed a similar increase in CDCFH₂ fluorescence (Fig. 2B, upper panel). The increase in CDCFH₂ fluorescence was due to an increase in cellular hydrogen peroxide levels (Fig. 2B, lower panel). These results clearly showed that 4-Cl-BQ perturbs cellular ROS levels, superoxide and hydrogen peroxide, in quiescent MCF-10A cells.

4-Cl-BQ decreased MnSOD expression in quiescent cells

MnSOD activity is known to regulate cellular ROS levels and reentry of quiescent human normal skin fibroblasts into the proliferative cycle [18,19]. To determine if 4-Cl-BQ induced changes in cellular ROS levels could be due to changes in MnSOD activity, quiescent MCF-10A cells were incubated with 3 μ M 4-Cl-BQ for 4 days, and MnSOD activity was measured following the method of Spitz and Oberley [27]. MnSOD activity in control cells was measured to be approximately 150 U/mg (Fig. 3A). MnSOD activity decreased significantly, 68 U/mg, in quiescent cells incubated with 4-Cl-BQ, while PCB 153 and Aroclor treatments did not show any significant difference. The decrease in MnSOD activity in 4-Cl-BQ treated cells was associated with a 50% decrease in MnSOD protein and mRNA levels (Fig. 3B&C).

4-Cl-BQ treatments exhibited a dose dependent decrease in MnSOD protein and mRNA levels (Fig. 4A&B). MnSOD protein levels decreased approximately 60% in quiescent cells incubated with 0.3 μ M 4-Cl-BQ for 4 days, 80% in 3 μ M, and more than 90% in 6 μ M 4-Cl-BQ treated cells (Fig. 4A). MnSOD mRNA levels did not show any change in 0.03 and 0.3 μ M 4-Cl-BQ treated cells compared to control (Fig. 4B). However, higher doses, 3 and 6 μ M 4-Cl-BQ decreased MnSOD mRNA levels approximately 40–50% (Fig. 4B).

To further determine if the decrease in MnSOD mRNA levels could be due to rapid mRNA turnover, MnSOD mRNA half-life was measured. Control and 4-Cl-BQ treated quiescent cells were incubated with actinomycin D to inhibit new transcription, and MnSOD mRNA levels were measured by quantitative RT-PCR at different times during the actinomycin D treatment. The half-life of MnSOD mRNA was calculated to be approximately 22 h in untreated control cells, and 12 h in 3 μ M 4-Cl-BQ treated cells (Fig. 4D). The decrease in MnSOD mRNA levels in actinomycin D treated cells was comparable with the corresponding decrease in MnSOD protein levels (Fig. 4C). These results suggest that a post-transcriptional mechanism could regulate MnSOD mRNA levels in quiescent cells incubated with 4-Cl-BQ.

Preferential turn-over of MnSOD transcripts in 4-Cl-BQ treated quiescent cells

Human MnSOD mRNA has two polyA sites, which results in 1.5 and 4.2 kb transcripts [17, 31]. In order to determine if the 4-Cl-BQ treatment selectively affects turn-over of one transcript compared to the other, quantitative RT-PCR assays were performed using primer pairs designed to specifically amplify the 3'-UTRs of the 4.2 and 1.5 kb MnSOD transcripts (Fig. 5A). Initially, it was determined if the 4-Cl-BQ induced dose dependent decrease in MnSOD mRNA levels could be due to a corresponding decrease in the abundance of the 4.2 kb transcript. Results showed that the abundance of the 4.2 kb transcript was not significantly different in 0.03 and 0.3 μ M 4-Cl-BQ treated cells compared to control (Fig. 5B). However, higher doses of 4-Cl-BQ, 3 and 6 μ M, decreased the levels of MnSOD 4.2 kb transcript approximately 40–50% (Fig. 5B). Interestingly, the same treatments did not affect the abundance of the 1.5 kb transcript (Fig. 5D). To further determine if the decrease in the 4.2 kb MnSOD transcript levels could be due to a decrease in mRNA half-life, mRNA turnover assay was performed. Control and 4-Cl-BQ treated cells were incubated with actinomycin D and total cellular RNA isolated at indicated times (Fig. 5C). Quantitative RT-PCR assay was performed using primer pair specific for the 3'-UTR of the 4.2 kb MnSOD transcript. The half-life of the 4.2 kb transcript in control cells was approximately 22 h, while cells treated with 4-Cl-BQ showed a significantly reduced half-life of 14 h (Fig. 5C). These results suggest that the decrease in MnSOD mRNA levels in 4-Cl-BQ treated quiescent MCF-10A cells could be regulated by a rapid turn-over of the 4.2 kb transcript.

4-Cl-BQ treatments inhibit cyclin D1 protein accumulation during reentry of quiescent cells into the proliferative cycle

We have previously shown that appropriate levels of MnSOD activity and cellular ROS levels are necessary for reentry of quiescent cells into the proliferative cycle [18–21]. These previous reports also showed a correlation between MnSOD activity and cyclin D1 protein levels. Cyclin D1 is the first cell cycle protein that is activated during entry into the proliferative cycle. To determine whether the PCB induced perturbations in MnSOD activity and cellular ROS levels inhibit cyclin D1 protein accumulation during reentry of quiescent cells into the proliferative cycle, control and 3 μ M PCB treated quiescent cells were replated at a lower cell density and total protein extracts prepared at the time of replating (0 h) and 6, 12, and 24 h post-replating. Immunoblotting assay was performed to measure the protein levels of cyclin D1 and actin (Fig. 6). In control cells, cyclin D1 protein levels increased approximately 2.5 folds at 6 h post-replating followed by a decrease at 12 and 24 h post-replating. The time course of cyclin D1 protein accumulation in control cells was comparable to PCB 153 and Aroclor 1254 treated cells. However, cells incubated with 4-Cl-BQ showed a significant inhibition in the accumulation of cyclin D1 protein (Fig. 6). This inhibition is consistent with a significant decrease in the percentage of S phase cells (Fig. 1). Interestingly, quiescent cells incubated with 4-Cl-BQ and PEG-SOD/PEG-CAT reversed the 4-Cl-BQ inhibited accumulation in cyclin D1 protein (Fig. 6, bottom panels).

To further determine if an increase in cellular ROS levels and inhibition in cyclin D1 protein accumulation regulate quiescence to S progression, quiescent MCF-10A cells were incubated simultaneously with 3 μ M 4-Cl-BQ and PEG-SOD/PEG-CAT. Cells were replated at the end of 4 days and cumulative BrdU accumulation measured by flow cytometry following the method described in Figure 1. Representative FITC-PI histograms of cell cycle phase distributions are shown in Figure 7A, and the percentage of S phase distributions is presented in Figure 7B. The percentages of G₁ and S+G₂ are presented in Table 2. The percentage of G₀/G₁ phase in quiescent control, 4-Cl-BQ, and 4-Cl-BQ combined with PEG-SOD and/or PEG-CAT treated cells was approximately 95%, suggesting that none of these treatments perturb cell cycle phase distributions while the cells were in quiescent growth state (Fig. 7, and Table 2). The percentages of S+G₂ was approximately 29% in untreated control cells at 18 h post-replating, and 33% at 26 h post-replating (Table 2). As shown before (Fig. 1 and Table 1), 4-Cl-BQ treated cells exhibited approximately 12% S+G₂ cells at 18 h post-replating, which remained low (13%) at 26 h post-replating. Interestingly, quiescent cells that were treated with PEG-SOD and PEG-CAT abrogate the 4-Cl-BQ induced inhibition in entry into S phase. Cells treated with PEG alone did not override the 4-Cl-BQ induced inhibition in entry into S phase, suggesting that the activities of SOD and CAT are required. Antioxidant treatments may suppress 4-Cl-BQ induced decrease in MnSOD expression. These results indicate that the 4-Cl-BQ induced decrease in MnSOD activity and increase in cellular ROS levels suppressed cyclin D1 protein accumulation, which negatively impacts upon the entry of quiescent cells into the proliferative cycle.

Discussion

A majority of research investigating cellular responses to PCB exposures use *in vitro* cell cultures of exponentially growing asynchronous cells. Although valuable results were obtained using these experimental systems, the *in vivo* significance of these results is not completely understood because a majority of proliferative-competent cells reside in quiescence, e.g. stem cells. Cellular quiescence is a reversible process necessary for cell and tissue renewal as well as inhibiting aberrant proliferation. Therefore, we have used quiescent cultures to partially mimic *in vivo* cell growth conditions. However, since oxygen concentrations are known to influence many of the cellular functions [32–34], our results that were obtained by culturing cells in monolayers and 21% oxygen environment may differ from *in vivo* conditions where oxygen concentration is presumed to be approximately 4% and cells grow in three dimensions.

Our results show quiescent MCF-10A cells incubated with 4-Cl-BQ, PCB 153, and Aroclor 1254 delayed entry into S phase (proliferative cycle) following replating of cells at a lower density. Cells incubated with 4-Cl-BQ exhibited the highest inhibition in progression from G₀/G₁ to S phase (Fig. 1 and Table 1). The number of cells in control and PCB treated quiescent cells was comparable at the time of replating, suggesting that these PCBs did not activate an unscheduled entry into the proliferative cycle while the cells were in contact inhibited quiescent growth state. This observation is also consistent with results obtained from the flow cytometric measurements of cell cycle phase distributions, which demonstrate more than 95% G₀/G₁ cells that were present at the end of the PCB treatment (Fig. 1 and Table 1). Our results differ from an earlier study reporting PCB induced proliferation in quiescent rat liver epithelial cells [35]. Results from this previous study showed an increase in the percentage of S phase in quiescent rat liver epithelial cells treated with “dioxin-like” PCBs (PCB105, PCB126, and 4'-OH-PCB79), which correlated with an increase in cell number. The increase in the percentage of S phase was accompanied with a corresponding increase in cyclin A and cyclin D2 protein levels. The difference in results between this previous and our present studies could be due to the use of different PCBs, species (human vs. rat), cell origin (liver vs. mammary), and experimental design (daily change in medium and addition of PCBs in the earlier study vs. one time addition of the PCBs in our study).

The percentage of cells with DNA content less than G₁ (sub-G₁) and the protein levels of phosphorylated H2AX did not vary between control and PCB treated quiescent cells (data not shown). These results indicate that the dose and duration of the PCB treatments might not cause any DNA double strand break and subsequent toxicity of quiescent MCF-10A cells.

Interestingly, this observation is different to our earlier results demonstrating cytotoxicity of these PCBs in asynchronously growing exponential cultures of MCF-10A [12]. MCF-10A exponential and asynchronous cultures incubated for 3 days with 3 μ M 4-Cl-BQ exhibited a significant increase in the frequency of micronuclei and phosphorylated histone H2AX, indicating that DNA damage could induce cell death in 4-Cl-BQ treated exponential cultures [12]. Our earlier results and results obtained from this study clearly suggest that cellular growth state, quiescence vs. proliferation, could be a significant factor in determining cellular responses to PCB exposures. Quiescent MCF-10A cells are resistant to 4-Cl-BQ induced cytotoxic effects, while proliferating cells are more susceptible.

4-Cl-BQ treatments significantly impaired the proliferative capacity of quiescent MCF-10A cells (Fig. 1, Table 1). Results from the cumulative BrdU assay showed that the percentage of S phase was significantly lower in 4-Cl-BQ treated cells compared to controls at 18 and 26 h post-replating (Fig. 1 and Table 1). These results demonstrate that the proliferative capacity of quiescent MCF-10A cells was severely impaired following incubation with 4-Cl-BQ. The inhibition in entry into S phase was associated with an increase in cell population doubling time, 37 h in control compared to 53 h in 4-Cl-BQ treated cells. PCB 153 and Aroclor 1254 treatments showed an intermediate response. The inhibition in entry into S phase in 4-Cl-BQ treated cells was probably not due to DNA damage because we did not observe any significant difference in the protein levels of phosphorylated histone H2AX between control and 4-Cl-BQ treated quiescent cells (data not shown). These results indicate that certain PCBs could elicit a cytostatic effect independent of DNA damage.

We have previously shown that preservation of appropriate cellular redox environment is necessary to protect the proliferative capacity of quiescent normal human and mouse fibroblasts [18–21,36]. Overexpression of MnSOD protects quiescent normal human skin fibroblasts from age associated loss in proliferative capacity; inhibition in MnSOD activity impairs the ability of quiescent cells to reenter the proliferative cycle [19]. MnSOD activity is also known to regulate cellular ROS levels [19]. Results presented in Figures 2 and 3 showed that 4-Cl-BQ treatments increased cellular ROS levels and decreased MnSOD activity in quiescent MCF-10A cells. Suppression of the DHE and CDCFH₂ fluorescence in PEG-SOD and PEG-CAT treated cells indicated that the 4-Cl-BQ treatment increased cellular superoxide and hydrogen peroxide levels. The increase in cellular ROS levels (superoxide and hydrogen peroxide, Fig. 2) is consistent with our previous results where we have used electron paramagnetic resonance spectroscopy to identify the presence of a semiquinone radical in MCF-10A cells incubated with 4-Cl-BQ [12]. The formation of the semiquinone radical was associated with approximately 4-fold increase in cellular ROS levels [12]. In addition to the semiquinone radical-induced increase in cellular ROS levels, results from the present study suggest that 4-Cl-BQ-induced decrease in MnSOD activity could also contribute to the increase in cellular ROS levels (Figs. 3&4). We have previously shown mitochondrial generated ROS in 4-Cl-BQ treated MCF-10A cells and that the increase in ROS was not due to a change in mitochondrial content [37]. In this previously published work, we used MitoTracker Green and MitoSOX Red oxidation to determine the mitochondrial origin of superoxide production in 4-Cl-BQ treated exponential cultures of MCF-10A cells. Results from the confocal microscopy assay showed a significant increase in MitoSOX Red oxidation in 4-Cl-BQ treated cells colocalizing with MitoTracker Green fluorescence. 4-Cl-BQ treatment did not change MitoTracker Green fluorescence compared to untreated cells. Since MitoTracker Green fluorescence is commonly used to measure mitochondrial content, these previously published results suggest that the 4-Cl-BQ induced increase in ROS levels was not due to an increase in

mitochondrial content. These previously published results and results presented in Figures 3–5 clearly suggest that the 4-Cl-BQ induced decrease in MnSOD expression is not due to a change in mitochondrial content.

A key observation of our study relates to a differential turnover of MnSOD transcripts in 4-Cl-BQ treated quiescent MCF-10A cells (Figs. 4&5). Human MnSOD has two poly(A) sites that result in two transcripts containing the same open reading frame, 1.5 and 4.2 kb. MnSOD expression is regulated both by transcriptional and post-transcriptional mechanisms [17,38]. Results from the mRNA turnover assay showed a significant decrease in MnSOD mRNA half-life in 4-Cl-BQ treated quiescent MCF-10A cells compared to untreated control, 22 h in control vs. 12 h in 4-Cl-BQ treated cells (Fig. 4). These results suggest that a post-transcriptional mechanism regulates MnSOD mRNA levels in 4-Cl-BQ treated quiescent MCF-10A cells.

The decrease in MnSOD mRNA half-life appears to be due to a preferential turnover of the 4.2 kb transcript. The mRNA levels of the 4.2 kb MnSOD transcript show a dose dependent decrease in quiescent cells incubated with 4-Cl-BQ (Fig. 5B). The half-life of the 4.2 kb transcript was calculated to be approximately 14 h in 4-Cl-BQ treated cells compared to 22 h in control (Fig. 5C). Interestingly, the same treatments did not alter the abundance of the 1.5 kb MnSOD transcript (Fig. 5D). Our results are consistent with a previous report where the authors used northern blotting to demonstrate a differential abundance between the 1.5 and 4.2 kb MnSOD transcripts in TNF- α treated cells [17]. The selective use of short and long 3'-UTR containing transcripts is also recently reported for activated immune cells [39]. The abundance of the short vs. long transcripts appears to be related to cell growth states. The abundance of the transcripts with shorter 3'-UTRs is associated with a corresponding increase in their protein levels [39,40]. These studies suggest that the enhanced stability of the transcripts with shorter 3'-UTRs could be due to the loss of the mRNA instability sequence and loss of regulation by microRNAs. The 3'-UTR of the 4.2 kb MnSOD transcript contains both AU-rich mRNA instability sequence and microRNA target sites. While additional studies are necessary to determine the exact mechanisms of MnSOD mRNA turnover in 4-Cl-BQ treated cells, it is possible that redox sensitive binding of RNA-binding proteins to specific sequence in the 3'-UTR could regulate MnSOD mRNA turnover. We have shown previously such a mechanism regulating topoisomerase II- α mRNA levels during the cell cycle and in response to oxidative stress [41].

4-Cl-BQ induced decrease in MnSOD activity and an increase in cellular ROS levels suppressed cyclin D1 protein accumulation during reentry of quiescent MCF-10A cells into the proliferative cycle (Fig. 6). Cyclin D1, a member of the G₁-cyclin family, is believed to be the first cell cycle regulatory protein that responds to mitogenic stimuli facilitating the entry of G₀/G₁ cells into S phase. In control cells, cyclin D1 protein levels peaked at 6 h post-replating and decreased at 12 and 24 h. In 4-Cl-BQ treated cells, cyclin D1 protein levels were not significantly different at 6, 12, and 24 h post-replating compared to 0 h. The suppression in cyclin D1 protein accumulation was associated with a significant decrease in the percentage of S phase (Fig. 1 and Table 1). Interestingly, prior treatment of quiescent cells with PEG-SOD and PEG-CAT abrogated 4-Cl-BQ induced suppression in cyclin D1 protein accumulation and facilitated entry into S phase (Figs. 6&7, Table 2). These results are comparable to a previous study by Smithwick *et al.* [42], where the authors showed decreased levels of cyclin D2 protein levels and an inhibition in progression from G₀/G₁ to S phase in lipopolysaccharide-stimulated and Aroclor 1242 and 2, 2'-chlorobiphenyl treated mice splenocytes.

In summary, we used quiescent MCF-10A human mammary epithelial cultures to demonstrate that 4-Cl-BQ treatments significantly impaired the proliferative capacity of these cells. The loss in proliferative capacity is associated with 4-Cl-BQ induced decrease in MnSOD activity, an increase in cellular ROS levels, and subsequent suppression in cyclin D1 protein

accumulation (Fig. 7C). Furthermore, 4-Cl-BQ treatments resulted in a preferential down regulation of the 4.2 kb MnSOD transcript, while there was no change in the abundance of the 1.5 kb MnSOD transcript. These results are significant because PCB exposures could impair cellular and tissue regeneration in PCB exposed individuals, and applications of antioxidants could be a viable redox-based countermeasure to protect the proliferative capacity of quiescent normal cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BrdU	bromodeoxyuridine
CDCF	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
CDCFH ₂	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
4-Cl-BQ	2-(4-chlorophenyl)benzo-1, 4-quinone
CuZnSOD	copper zinc superoxide dismutase
DHE	dihydroxyethidium
DMSO	dimethyl sulfoxide
EcSOD	extracellular superoxide dismutase
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
MFI	mean fluorescence intensity
MnSOD	manganese superoxide dismutase
ORF	open reading frame
PBS	phosphate buffer saline
PCB	polychlorinated biphenyl
PEG-CAT	polyethylene glycol conjugated catalase
PEG-SOD	polyethylene glycol conjugated superoxide dismutase
PI	propidium iodide
ROS	reactive oxygen species
TNF- α	tumor necrosis factor α
UTR	untranslated region

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Figure 1. 4-Cl-BQ significantly inhibits reentry of quiescent MCF-10A cells into the proliferative cycle

Monolayer cultures of quiescent MCF-10A cells were incubated with 0–3 μ M of 4-Cl-BQ, PCB 153, and Aroclor 1254 for 4 days. Cells were sub-cultured by trypsinizing the monolayer cultures, and replated at a lower cell density. Replated cells were continued in culture in medium containing 10 μ M BrdU and harvested at indicated times. Ethanol-fixed cells were assayed for BrdU-positive (S phase) and negative (G₁ and G₂ phases) nuclei following our previously published protocol [24]. Data were analyzed using FlowJo software. (A) Representative BrdU vs. PI bivariate histograms of cell cycle phase distributions in control and 3 μ M PCB treated cells. (B-D) The percentage of S phase distributions in cells treated with different concentrations of PCBs. Control and PCB treated cells were harvested at 18 h post-plating. Asterisks represent statistical significance relative to untreated control, n=3; *, p<0.05.

Figure 2. 4-Cl-BQ treatment increases cellular ROS levels, superoxide and hydrogen peroxide
Control and 3 μ M PCB treated quiescent MCF-10A cells were assayed for cellular ROS levels at the end of 4 days of treatment. (A) Monolayer cultures were incubated with 10 μ M DHE in 2 ml of PBS containing 5 mM pyruvate at 37°C for 40 min. Cells in replicate dishes were treated with 100 U/ml PEG-SOD or 18 μ M PEG for 2 h prior to and during the DHE labeling. Cells were trypsinized on ice and DHE fluorescence analyzed by flow cytometry. Fold change in MFI was calculated relative to MFI of untreated control cells in top panel. In the bottom panel, the fluorescence of cells treated with PCBs and PEG-SOD was subtracted from the fluorescence of cells treated with PCBs and PEG. The data plotted show fold change in MFI that was calculated relative to the difference in fluorescence of untreated control cells incubated with PEG and PEG-SOD. (B) In a separate series of experiments, control and PCB treated quiescent cultures were incubated with 10 μ g/ml CDCFH₂ in PBS at 37°C for 10 min. Cells in replicate dishes were pretreated with 100 U/ml PEG-CAT or 18 μ M PEG for 2 h prior to and during the CDCFH₂ labeling. Cells were trypsinized on ice and fluorescence analyzed by flow cytometry. Data was plotted as described above. Fold change was calculated relative to untreated control. n=3; * p<0.05; asterisks represent statistical significance relative to untreated control.

Figure 3. 4-Cl-BQ treatment decreases MnSOD activity and expression in quiescent MCF-10A cells Control and 3 μ M PCB treated quiescent MCF-10A cells were harvested at the end of a 4-day treatment and assayed for (A) MnSOD activity. Cells from replicate plates were harvested for (B) immunoblotting and (C) quantitative RT-PCR assays to measure MnSOD protein and mRNA levels. Actin levels were used for loading control in immunoblots. Immunoblots were scanned and quantitated using ImageJ software. Fold change was calculated by first normalizing to actin levels in individual samples and then relative to untreated control. 18S RNA levels were used to calculate the relative fold change in MnSOD mRNA levels. Asterisks represents statistical significance relative to untreated control; n=3, *, p< 0.05.

Figure 4. 4-Cl-BQ decreases MnSOD mRNA stability in quiescent MCF-10A cells

Quiescent MCF-10A cells were treated with 0–6 μ M 4-Cl-BQ for 4 days and harvested for measurements of (A) MnSOD protein and (B) mRNA levels. Actinomycin D was used to measure MnSOD mRNA half-life in control and 4-Cl-BQ treated cells. Quiescent MCF-10A cells were incubated with 3 μ M 4-Cl-BQ for 4 days followed by incubation with 10 μ g/ml actinomycin D. Cells were harvested at indicated times for (C) immunoblot analysis of MnSOD protein levels and (D) quantitative RT-PCR measurements of MnSOD mRNA levels. Asterisks indicate significant difference compared to control, $n = 3$, $p < 0.05$.

Figure 5. Preferential turn-over of the 4.2 kb MnSOD transcript in quiescent cells incubated with 4-Cl-BQ

(A) Schematic illustration of the selection of primer pairs used to amplify total MnSOD mRNA levels (open reading frame, ORF), 1.5 and 4.2 kb transcripts. PAS: polyadenylation sites; triangle and arrows: positions of forward and reverse primers; bp: amplicon size; d(T)₁₅X: anchored primer, X represents sequence specific to the first poly(A) site in the 1.5 kb transcript. (B and D) Quiescent MCF-10A cells were treated with 0–6 μM 4-Cl-BQ for 4 days and harvested for RT-PCR measurements of the 1.5 and 4.2 kb MnSOD transcripts. (C) Control and 4-day 3 μM 4-Cl-BQ incubated quiescent MCF-10A cells were treated with 10 μg/ml Actinomycin D and harvested at indicated times for RT-PCR measurements of the abundance of the 4.2 kb MnSOD transcript. Asterisks represent statistical significance relative to untreated control, n=3; *, p< 0.05. Insets in (B) and (D) represent agarose gel electrophoresis and ethidium bromide staining of PCR amplified products representative of the quantitative RT-PCR assay.

Figure 6. 4-Cl-BQ induced decrease in MnSOD activity and increase in cellular ROS levels inhibit the accumulation of cyclin D1 protein during reentry of quiescent cells into the proliferative cycle Control and 3 μ M PCB treated quiescent MCF-10A cultures were trypsinized and replated at a lower cell density at the end of a 4-day treatment. Total protein extracts were prepared at indicated times post-replating and immunoblotted for cyclin D1. Actin levels were used for comparison. In a separate experiment, cells were incubated simultaneously with 3 μ M 4-Cl-BQ, and 100 U/mL of PEG-SOD and PEG-CAT; cyclin D1 and actin levels were assayed by immunoblotting. Cyclin D1 protein levels were normalized to actin levels in individual samples, and fold change calculated relative to untreated control (right panels). Asterisks represent statistical significance relative to untreated control, $n=3$; *, $p < 0.05$.

Figure 7. PEG-SOD/PEG-CAT abrogates 4-Cl-BQ induced delay in reentry of quiescent cells into the proliferative cycle

Quiescent MCF-10A cells were incubated with 3 μ M 4-Cl-BQ in presence and absence of PEG-SOD/PEG-CAT for 4 days. Cells were replated and continued in culture in presence of 10 μ M BrdU for indicated times. The percentage of cell cycle phase distributions was calculated following the method described in Figure 1. (A) Representative BrdU vs. PI bi-variate histograms; (B) percent S phase; asterisks represent statistical significance relative to untreated control; n=3; *, p< 0.05. (C) Schematic illustration of 4-Cl-BQ induced ROS signaling interfering with reentry of quiescent cells into the proliferative cycle.

Table 1
Percentage of G₁ and S + G₂ phases in MCF-10A cells replated from control and 4 d PCB treated quiescent cells

Treatment (3 μM)	0 h		18 h		26 h	
	G ₁	S + G ₂	G ₁	S + G ₂	G ₁	S + G ₂
Control	93 ± 1.5	7 ± 1.8	75 ± 3.8	21 ± 2.3	73 ± 3.5	23 ± 3.3
4-Cl-BQ	92 ± 2.3	8 ± 1.3	85 ± 3.1	*10 ± 1.5	85 ± 4.5	*11 ± 2.8
PCB 153	93 ± 1.9	7 ± 2.1	79 ± 3.5	*16 ± 1.8	75 ± 4.5	19 ± 1.6
Aroclor	94 ± 1.4	6 ± 1.5	81 ± 3.2	*14 ± 2.9	79 ± 2.4	*15 ± 3.0

Average ± SD, n=3, p<0.05; Asterisks represent statistical significance relative to untreated control

Percentage of G₁ and S + G₂ phases in MCF-10A cells replated from control and 4 d 4-Cl-BQ +/- PEG-CAT/PEG-SOD treated quiescent cells

Table 2

Treat- ment(3μM)	0 h		18 h		26 h	
	G ₁	S + G ₂	G ₁	S + G ₂	G ₁	S + G ₂
Control	95 ± 1.9	5 ± 2.3	71 ± 2.2	29 ± 2.7	67 ± 3.2	33 ± 2.3
4-Cl-BQ	93 ± 1.7	7 ± 2.1	88 ± 2.8	*12 ± 2.5	87 ± 3.0	*13 ± 3.1
4-Cl-BQ + PEG	92 ± 0.9	8 ± 1.1	84 ± 1.3	*16 ± 1.9	82 ± 2.2	*18 ± 1.7
4-Cl-BQ + PEG-CAT	92 ± 2.1	8 ± 1.9	69 ± 2.3	31 ± 2.3	66 ± 3.6	34 ± 2.8
4-Cl-BQ + PEG-SOD	93 ± 1.8	7 ± 1.6	73 ± 3.5	27 ± 3.1	70 ± 3.6	30 ± 2.9
4-Cl-BQ + PEG- SOD/CAT	93 ± 2.4	7 ± 1.8	73 ± 2.9	27 ± 3.0	64 ± 2.8	36 ± 3.3

Average ± SD, n=3, p<0.05; Asterisks represent statistical significance relative to untreated control