Published in final edited form as:

Environ Int. 2010 November ; 36(8): 924–930. doi:10.1016/j.envint.2010.01.001.

2-(4-chlorophenyl)benzo-1,4-quinone induced ROS-signaling inhibits proliferation in human non-malignant prostate epithelial cells

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Abstract

Polychlorinated biphenyls (PCBs) and their metabolites are environmental chemical contaminants which can produce reactive oxygen species (ROS) by auto-oxidation of dihydroxy PCBs as well as the reduction of quinones and redox-cycling. We investigate the hypothesis that 2-(4-chlorophenyl) benzo-1,4-quinone (4-Cl-BQ), a metabolite of 4-chlorobiphenyl (PCB3), induced ROS-signaling inhibits cellular proliferation. Monolayer cultures of exponentially growing asynchronous human non-malignant prostate epithelial cells (RWPE-1) were incubated with 0–6 micromolar of 4-Cl-BQ and harvested at the end of 72 h of incubation to assess antioxidant enzyme expression, cellular ROS levels, cell growth, and cell cycle phase distributions. 4-Cl-BQ decreased manganese superoxide dismutase (MnSOD) activity, protein, and mRNA levels. 4-Cl-BQ treatment increased dihydroethidium (DHE) fluorescence, which was suppressed in cells pre-treated with polyethylene glycol conjugated superoxide dismutase (PEG-SOD). The increase in ROS levels was associated with a decrease in cell growth, and an increase in the percentage of S-phase cells. These effects were suppressed in cells pretreated with PEG-SOD. 4-Cl-BQ treatment did not change the protein levels of phosphorylated H2AX at the end of 72 h of incubation, suggesting that the inhibition in cell growth and accumulation of cells in S-phase at the end of the treatments were probably not due to 4-Cl-BQ induced DNA double strand break. These results demonstrate that MnSOD activity and ROS-signaling perturb proliferation in 4-Cl-BQ treated *in vitro* cultures of human prostate cells.

Keywords

Polychlorinated biphenyls; PCB3; reactive oxygen species; MnSOD; superoxide; prostate epithelial cells

1. Introduction

Polychlorinated biphenyls are a group of environmental contaminants that were widely used as coolants and lubricants in transformers and as a dielectric in capacitors (Safe 1993).

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Although the production of PCBs was discontinued in the USA in 1977, the persistent bioaccumulation and ubiquitous distribution of PCBs raised concerns about the long term health effects (Safe 1993). PCBs are strongly lipophilic and very highly stable compounds. Serum levels of PCBs in Americans average about 10 ppb (~30 nM); occupationally exposed individuals may have PCB blood levels in the hundreds of ppb (Warshaw et al., 1979). The blood levels of PCBs in individuals living in Anniston, Alabama vary widely (0.003 – 6.5 μ M (Hansen et al., 2003). PCB levels in adipose tissue are much higher than those in blood, with levels in low ppm range (Johnson-Restrepo et al., 2005). Once ingested and absorbed, PCBs may remain in the body for extended time periods.

The biological effects of individual PCBs are determined by the number and position of chlorines in the biphenyl rings, which also determine their chemical and physical properties. PCBs in general and lower halogenated PCBs in particular are metabolized by microsomal enzymes to mono- and di-hydroxy metabolites. Metabolism of the di-hydroxy metabolites, catechols and hydroquinones, may result in semiquinones and quinones that are highly reactive electrophiles (Srinivasan et al., 2001). There are at least two mechanisms by which PCB metabolites can produce ROS: (a) autoxidation of di-hydroxy PCBs, and (b) reduction of quinones *via* Michael addition of GSH with autoxidation. There is considerable evidence that semiquinone radical can be a primary source for formation of hydrogen peroxide from the hydroquinone/quinone redox system (Eyer 1991; Guo et al., 2002; Hall et al., 1994; Song et al., 2008). Consistent with these earlier reports, we have previously shown that 4-Cl-BQ undergoes redox-cycling resulting in increased production of superoxide and hydrogen peroxide (Venkatesha et al., 2008). An increased flux of superoxide would result in an increased flux of hydrogen peroxide. We used electron paramagnetic spectroscopy to demonstrate that a semiquinone radical was formed in 4-Cl-BQ-treated MCF-10A human non-malignant breast epithelial cells, suggesting that this species could be the source of the apparent higher flux of ROS levels (Venkatesha et al., 2008).

The steady-state level of ROS is a balance between production of ROS and their removal by antioxidants and antioxidant enzymes. ROS (superoxide and hydrogen peroxide) are produced by two metabolic sources: the mitochondrial electron transport chain and enzymatic reactions. Superoxide is converted to hydrogen peroxide by superoxide dismutase, and catalase neutralizes hydrogen peroxide to water. Manganese superoxide dismutase (MnSOD) is a nuclear encoded and mitochondrial matrix localized protein, which is essential and biologically significant to aerobic cells (McCord and Fridovich 1969; Oberley et al., 1989; Oberley et al., 1981). Our previously published results showed that MnSOD activity regulates a “ROS-Switch” facilitating a superoxide-signal regulating proliferation and a hydrogen peroxide-signal supporting quiescence in human normal skin fibroblasts (Sarsour et al., 2008). We hypothesize that 4-Cl-BQ induced changes in MnSOD activity and ROS-signaling regulate cellular proliferation.

Epidemiological studies indicate that exposure to PCBs might be causally linked to an increased incidence of prostate cancer (Ritchie et al., 2003; Ritchie et al., 2005). Metabolites of PCB3 are known to possess tumor initiating activities in rat liver (Espandiar et al., 2003; Espandiar et al., 2004). PCB3 and its dihydroxylated metabolites have been shown to upregulate prostaglandin H synthase (PGHS) in ‘hormonally sensitive’ tissues like prostate, breast, and ovary. PGHS has both cyclooxygenase and peroxidase activity (Wangpradit et al., 2009). Increased levels of cyclooxygenase-2 and peroxidase activity have been correlated with higher incidence of prostate cancer (Yoshimura et al., 2000). Epidemiological studies also show men exposed to PCBs have a higher risk of developing prostate cancer (Prince et al., 2006; Ritchie et al., 2003). The present study investigates the hypothesis that 4-Cl-BQ-induced ROS-signaling perturbs cellular proliferation in human non-malignant prostate epithelial cells.

2. Materials and methods

2.1. Chemicals

2-(4-Chlorophenyl)benzo-1, 4-quinone (4-Cl-BQ) was provided by Dr. Hans-Joachim Lehmler from the Occupational & Environmental Health, University of Iowa. 4-Cl-BQ was synthesized and characterized as described previously (Amaro et al., 1996; Schramm et al., 1985). The purity of 4-Cl-BQ was determined by gas chromatography and found to be >98%. 4-Cl-BQ stock solutions were prepared using dimethyl sulfoxide (DMSO); the final concentration of DMSO in culture medium was kept below 0.5%. Control cultures were adjusted to the same concentrations of DMSO as the 4-Cl-BQ-treated cells. Polyethylene glycol conjugated (PEG) superoxide dismutase was obtained from Sigma Chemical Co.

2.2. Cell culture

RWPE-1 human non-malignant prostate epithelial cells were obtained from the American Tissue Culture Collection (ATCC). RWPE-1 cells are spontaneously immortalized and these cells possess characteristics of normal epithelial cells. Monolayer cultures were grown in keratinocyte growth medium (Gibco) supplemented with 10% FBS, growth factors and antibiotics. Cells were grown at 37°C, 5% CO₂ and 95% humidity. Exponentially growing asynchronous cultures were treated with 0–6 µM 4-Cl-BQ for 24–72 hours. 4-Cl-BQ dose selection was based on a recent study where it was reported that the blood levels of PCBs in individuals living in Anniston, Alabama varied from 0 – 6.5 µM (Hansen et al., 2003).

Cell growth was measured by counting cell number using a Beckman Z1 particle counter (Beckman Coulter). Cell population doubling time (Td) was calculated from the exponential portion of the growth curve using the following equation:

$$Td = 0.693t / \ln(Nt/N0)$$
 where t is time, and Nt and N0 represent cell numbers at time t and initial time, respectively.

2.3. Flow cytometry assays

Monolayer cultures were trypsinized and fixed in 70% ethanol. Ethanol-fixed cells were washed once with PBS and treated with RNase A (1 mg/ml) for 30 min, followed by staining with propidium iodide (PI; 35 µg/mL). PI-fluorescence was measured using a FACScan flow cytometer. Data from 10,000 events were collected in list mode and the percentage of cells in each phase of the cell cycle was calculated using MODFIT software.

Cellular steady-state levels of superoxide were measured using the fluorescent dye, dihydroethidium (DHE, Molecular Probes). Control and 4-Cl-BQ treated cells were trypsinized and washed with PBS buffer containing 5 mM pyruvate, followed by incubation with 10 µM DHE for 40 minutes. DHE-fluorescence was analyzed by flow cytometry using an excitation wavelength 488 nm, and emission 585 nm band-pass filter. The mean fluorescence intensity of 10,000 cells was analyzed in each sample and corrected for autofluorescence. The mean fluorescence intensity (MFI) was calculated relative to untreated control. Pretreatment of cells with PEG-SOD was used to determine the specificity of DHE-fluorescence for measurements of cellular steady-state levels of superoxide.

2.4. Antioxidant enzyme assays

MnSOD enzymatic activity was measured using an indirect competition assay between SOD and nitroblue tetrazolium (NBT) following the method of Spitz and Oberley (Spitz and Oberley, 1989). Specific activity was reported as Units per mg protein. The protein levels in each sample were quantitated using the Lowry assay (Lowry et al., 1951).

2.5. Immunoblotting

Total cellular proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane (BIORAD Labs.). Blots were incubated with antibodies to MnSOD (Upstate Biotech), catalase (Athens Research and Technology) and γ -H2AX (Upstate Biotech). Immunoreactive polypeptide was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced-hemiluminescence detection reagents (GE Healthcare) following manufacturer-supplied protocols. Blots were reprobed with antibodies to actin (Santa Cruz Biotechnology). Results were quantitated using AlphaImager 2000 software (Alpha Innotech) and calculated relative to actin levels in individual samples. Fold-change was calculated relative to untreated control.

2.6. Reverse transcription and quantitative polymerase chain reaction (RT-Q-PCR)

One microgram of total cellular RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems) following manufacturer-supplied protocol. One-tenth of the cDNA was subjected to Real-Time PCR amplification using primer-pairs specific to MnSOD, and 18S (MnSOD forward primer: 5'-GGCCTACGTGAACAACCTGAA-3', reverse primer: 5'-CTGTAAACATCTCCCTTGGCCA-3'; 18S forward primer: 5'-CCTTGGATGTGGTAGCCGTTT-3', reverse primer: 5'-AACTTTCGATGGTAGTCGCGG-3'). The ABI PRISM 7000 sequence detection system (Applied Biosystems) was used with the following cycle parameters: Cycle 1 (95°C for 10 min); Cycle 2 (95°C for 15 sec, 60°C for 1 min) $\times$ 30 cycles. The relative MnSOD mRNA levels were calculated as follows: ΔC_T (sample) = C_T (MnSOD) - C_T (18S); $\Delta\Delta C_T = \Delta C_T$ (post-treatment time point) - ΔC_T (control); Relative expression = $2^{-\Delta\Delta C_T}$.

2.7. Statistics

Statistical significance was determined by one and two-way ANOVA with Tukey's post hoc test and Student t-tests using GraphPad Prism, version 4 and SPSS. Results are presented as mean $\pm$ standard deviation. Homogeneity of variance was assumed with 95% confidence interval level. Results from at least $n \geq 3$ with $p < 0.05$ are considered significant.

3. Results

3.1. 4-Cl-BQ treatment of human prostate epithelial cells decreased MnSOD activity, protein, and mRNA levels

We have shown previously that PCBs cause oxidative stress in MCF-10A human non-malignant mammary epithelial cells (Venkatesha et al., 2008). To determine if PCB-induced oxidative stress could be due to changes in antioxidant enzyme expression, exponentially growing asynchronous cultures of human non-malignant prostate epithelial cells (RWPE-1) were incubated with 0–6 μ M of 4-Cl-BQ for 48 h and harvested for analysis of MnSOD activity, protein and mRNA levels. Results showed 0.3 μ M of 4-Cl-BQ treatment decreased MnSOD protein levels approximately 50%, which remained low (40% of control) in 3 and 6 μ M treated cells (Fig. 1A, left panel). Interestingly, the same treatment did not show any change in catalase protein levels (Fig. 1A, right panel). To further investigate if the decrease in MnSOD protein levels was also associated with changes in MnSOD activity, cells from replicate dishes were harvested and MnSOD activity was measured by the NBT-superoxide dismutase biochemical activity assay (Spitz and Oberley 1989). MnSOD activity was approximately 50 U/mg in exponentially growing asynchronous RWPE-1 cultures. 4-Cl-BQ treatment decreased MnSOD activity to 40 U/mg in 0.3 μ M and 30 U/mg in 3 and 6 μ M treated cells (Fig. 1B). Results from the quantitative RT-PCR assay showed approximately 30% decrease in MnSOD mRNA levels in 0.3, 3, and 6 μ M 4-Cl-BQ treated vs. untreated control cells (Fig. 1C).

3.2. 4-Cl-BQ treatment increased cellular ROS levels

MnSOD converts superoxide produced by the univalent reduction of oxygen in the mitochondria to hydrogen peroxide; MnSOD activity is known to regulate cellular ROS levels. The steady state level of cellular ROS is maintained by a balance between the production of ROS and their removal by antioxidants. To determine whether changes in MnSOD activity (Fig. 1) perturb cellular superoxide levels, RWPE-1 cells were incubated with 0–6 μ M 4-Cl-BQ for 48 h and harvested for flow cytometry analysis of DHE-fluorescence. The mean fluorescence intensity (MFI) increased 3–4 folds in 3 and 6 μ M 4-Cl-BQ treated cells compared to control (Fig. 2B). PEG-SOD was used to determine the specificity of the DHE-fluorescence for the measurements of superoxide. Control and 4-Cl-BQ treated cells were incubated with PEG-SOD followed by flow cytometry measurements of DHE-fluorescence. This treatment decreased DHE-fluorescence in control as well as 4-Cl-BQ treated cells (Fig. 2B). These results suggest that the increase in DHE-fluorescence in cells incubated with 4-Cl-BQ was due to an increase in the steady state levels of cellular superoxide.

3.3. MnSOD activity regulates proliferation in cells treated with 4-Cl-BQ

To determine whether 4-Cl-BQ-induced decrease in MnSOD activity and increase in cellular superoxide levels could perturb proliferation, a cell growth assay was performed. RWPE-1 cultures were incubated with 3 μ M 4-Cl-BQ for 0–72 h, and cell numbers counted at indicated times (Fig. 3A). Results showed 4-Cl-BQ significantly delayed cell growth at 48 and 72 h of 4-Cl-BQ treatment. Cell population doubling time was calculated to be 22 h in control compared to 33 h in 4-Cl-BQ treated cells. Interestingly, this delay in proliferation was reversed in cells that were pre-treated with PEG-SOD followed by incubation with 4-Cl-BQ (Fig. 3B).

3.4. 4-Cl-BQ induced growth-delay was associated with an increase in the percentage of S-phase

Cellular growth is a net-result of proliferation and cell death. To determine if the 4-Cl-BQ-induced growth delay was associated with cell death and/or inhibition in cell cycle phase transit, a flow cytometry assay measuring DNA content was performed. Exponentially growing asynchronous RWPE-1 cultures were treated with 3 μ M 4-Cl-BQ for 0–72 h and fixed in ethanol. DNA content of ethanol-fixed cells was analyzed by flow cytometry. Representative PI-histograms of DNA content in control and 4-Cl-BQ treated cells are shown in Figure 4, and the percentages of cell cycle phase distributions are presented in Table I. The absence of a cell population with sub-G₁ DNA content (Fig. 4) indicates that the 3 μ M 4-Cl-BQ treatments did not result in any cell death at the end of 24, 48, and 72 h treatment. However, the same treatment resulted in the redistribution of cells in different cell cycle phases (Table I). The percentage of cell cycle phases at the time of addition of 4-Cl-BQ were as follows: G₁, 46%; S, 42%; G₂+M, 12% (Table I). The percentage of G₁ in control cells increased to 54 and 57% at 48 and 72 h without any significant change in percent S-phase, suggesting that these cells are probably progressing towards quiescent growth. In contrast, the percentage of S-phase in 4-Cl-BQ treatment did show an increase from 42% at 0 h to 49% at 48 h and 52% at 72 h (Table I). 4-Cl-BQ-induced increase in the percentage of S-phase and decrease in cell number (Fig. 3) indicate that the 4-Cl-BQ treatment could interfere with DNA synthesis delaying transit through S-phase.

To determine if the accumulation of cells in S-phase could be due to DNA damage, an immunoblot assay was performed to measure the levels of phosphorylated H2AX (γ -H2AX) in control and 4-Cl-BQ treated cells at the end of a 72 h incubation. An increase in the levels of γ -H2AX is indicative of DNA double strand break. Results (Fig. 5) showed no significant difference in the levels of γ -H2AX in control compared to 4-Cl-BQ treated cells, suggesting that the inhibition in cell growth and accumulation of cells in S-phase at the end of the treatments were probably not due to 4-Cl-BQ induced DNA double strand break.

3.5. Pretreatment with PEG-SOD inhibits S-phase accumulation in cells incubated with 4-Cl-BQ

To further determine if MnSOD activity regulates proliferation in cells incubated with 4-Cl-BQ, RWPE-1 cells were pretreated with 150 U of PEG-SOD followed by incubation with 3 μ M 4-Cl-BQ for 72 h. The percentage of cell cycle phases was determined by flow cytometry measurements of DNA content in ethanol-fixed cells. The percentage of S-phase in 4-Cl-BQ treated cells increased to 51% at 72 h compared to 40% in untreated control cells (Fig. 6). Interestingly, pretreatment of cells with PEG-SOD suppressed 4-Cl-BQ induced accumulation of cells in S-phase. These results support the hypothesis that MnSOD activity and ROS-signaling regulate proliferation in 4-Cl-BQ treated human prostate cells.

4. Discussion

Epidemiological studies indicate increased mortality of capacitor and electric utility workers from various cancers (Bertazzi et al., 1987; Brown 1987; Gustavsson et al., 1986; Loomis et al., 1997; Sinks et al., 1992). Recent evidences suggest that exposure to PCBs might be causally linked to an increased incidence of breast and prostate cancer (Demers et al., 2002; Laden et al., 2002; Moysich et al., 2002; Ritchie et al., 2005). PCBs that are constitutive androstane receptor (CAR)-agonists (e.g. PCB153) have been suggested to increase the risk of prostate cancer presumably *via* the CYP450 mediated metabolism (Ritchie et al., 2005). Although PCB3 or its metabolite 4-Cl-BQ is not a known CAR-agonist, we hypothesize that 4-Cl-BQ induced perturbations in cellular ROS levels could regulate many of the cellular processes including cell proliferation.

4-Cl-BQ is a metabolite of 4-chlorobiphenyl (PCB3), a common PCB-congener found in air. The concentrations of 4-Cl-BQ (0.03–6 μ M) used in this study were selected based on the findings that the levels of PCBs in the blood of individuals living in Anniston, Alabama vary widely (0.003 – 6.5 μ M) (Hansen et al., 2003). Our results showed a decrease in MnSOD activity in RWPE-1 human non-malignant prostate epithelial cells that were incubated with 0.3–6 μ M 4-Cl-BQ for 48 h (Fig. 1). The decrease in MnSOD activity was associated with a corresponding decrease in its protein and mRNA levels. Since the cellular localization of 4-Cl-BQ is unknown, it is unclear how 4-Cl-BQ treatment could down regulate MnSOD expression. It is possible that the 4-Cl-BQ treatment can negatively impact upon the transcription/post-transcription and/or translation/post-translational regulation of MnSOD expression. Alternatively, the 4-Cl-BQ-induced decrease in MnSOD activity could result from a redistribution of cells in S-phase (Fig. 4, Table I). This hypothesis is consistent with our previously published results demonstrating an inverse correlation between the percentage of S-phase and MnSOD activity (Menon et al., 2007; Sarsour et al., 2008).

MnSOD is a nuclear encoded and mitochondrial matrix localized antioxidant enzyme known to convert mitochondria-generated superoxide to hydrogen peroxide. A decrease in MnSOD activity can result in an increase in cellular ROS levels. RWPE-1 cells incubated with 4-Cl-BQ showed a dose-dependent increase in DHE-fluorescence (Fig. 2). 4-Cl-BQ-induced increase in DHE-fluorescence was suppressed in cells pretreated with PEG-SOD, suggesting that the increase in DHE-fluorescence could result from an increase in the steady-state levels of superoxide. Our results are comparable to a previous study in the literature reporting approximately 50–200 fold increase in 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CDCFH₂) fluorescence in HL-60 cells that were incubated with 2.5 μ M of 4-Cl-BQ, indicating that the 4-Cl-BQ treatment increased cellular ROS levels (Srinivasan et al., 2001). The difference in fold change, 3–4 fold increase in DHE fluorescence in the present study compared to 50–200 fold increase in CDCFH₂ fluorescence in the earlier study, could be due to the differences in the assays (DHE vs. CDCFH₂) and cell lines (HL-60 vs. RWPE-1) used in the two studies.

The decrease in MnSOD activity (Fig. 1) and increase in DHE-fluorescence (Fig. 2) suggest that 4-Cl-BQ can induce mitochondria-generated ROS, presumably superoxide. Alternatively, it is possible that 4-Cl-BQ having a quinone “like” structure can undergo redox-cycling leading to an increase in cellular ROS levels. Furthermore, there is considerable evidence that semiquinone radicals generated from the hydroquinone/quinone redox system can be a primary source for formation of ROS (Eyer 1991;Guo et al., 2002;Hall et al., 1994;Song et al., 2008). Consistent with this hypothesis, we have previously shown that semiquinone radicals are indeed formed in 4-Cl-BQ treated MCF-10A human non-malignant mammary epithelial cells (Venkatesha et al., 2008). Therefore, the increase in cellular ROS levels in 4-Cl-BQ treated RWPE-1 cells could originate from the formation of a semiquinone radical.

4-Cl-BQ resulted in a significant decrease in cell number at 48 and 72 h post-treatment (Fig. 3A). Cell population doubling time in control cells was 22 h, which increased to 33 h in 4-Cl-BQ treated cells (Fig. 3A). The increase in doubling time was associated with an increase in the percentage of cells in S-phase (Fig. 4, Table I). Although the percentage of cells in G₂+M phase was comparable in control and 4-Cl-BQ treated cells, the percentage of cells in G₁ phase was lower in 4-Cl-BQ treated cells compared to control. These results suggest that the treatment of cells with 4-Cl-BQ could recruit more G₁ cells into S-phase. This hypothesis is consistent with our previously published results demonstrating a prooxidant event facilitating the entry of G₁ cells into S-phase (Menon et al., 2003). Alternatively, 4-Cl-BQ treatment could delay progression through S-phase, which could then result in an increase in the percentage of cells in S-phase. A delay in transit through S-phase could contribute to the increase in cell population doubling time in cells incubated with 4-Cl-BQ (Fig. 3). 4-Cl-BQ induced perturbation in cell cycle phase distributions was also observed in V79 hamster fibroblasts (Zettner et al., 2007). The authors in this previous study showed 4-Cl-BQ treatment decreased the percentage of G₁ cells, while the same treatment resulted in an increase in the percentage of S-phase cells. Interestingly, 4-Cl-BQ induced changes in cell cycle phase redistributions did not affect V79 cell viability and proliferation compared to control. Our results show a correlation between 4-Cl-BQ induced accumulation of cells in S-phase and inhibition in RWPE-1 cell proliferation. Cell population doubling increased from 22 h in control to 33 h in 4-Cl-BQ treated cells (Fig. 3).

The decrease in cell number (Fig. 3A) could also be due to cell death. However, the percentage of cells with sub-G₁ DNA content in 4-Cl-BQ treated cells was not significantly different than control cells (Fig. 4). Likewise, there was no difference in the protein levels of γ H2AX in control compared to cells treated with 4-Cl-BQ (Fig. 5). These results suggest that the accumulation of cells in S-phase and an increase in cell population doubling time in cells incubated with 4-Cl-BQ were probably not due to DNA double strand break. Instead, 4-Cl-BQ induced decrease in MnSOD activity and an increase in cellular superoxide levels could regulate cell population doubling time and accumulation of cells in S-phase. This notion is further evident from the results presented in Figure 3B and 6. Cells pretreated with PEG-SOD followed by incubation with 4-Cl-BQ suppressed the accumulation of cells in S-phase and subsequently inhibited the decrease in cell number.

In summary, our results show that short-term (24–72 h) incubation with 4-Cl-BQ decreased MnSOD activity and increased cellular superoxide levels. The increase in cellular superoxide levels was associated with an increase in the percentage of cells in S-phase and cell population doubling time. Cells pretreated with PEG-SOD reversed the effects of 4-Cl-BQ. Because MnSOD is a mitochondrial matrix localized enzyme, these results support the hypothesis that mitochondrial generated ROS-signaling regulates cellular responses to 4-Cl-BQ exposure. This hypothesis is further supported by our recent microscopic observation of co-localization of MitoSOX red oxidation with MitoTracker green in 4-Cl-BQ treated human mammary (MCF-10A) and prostate (RWPE-1) epithelial cells (Zhu et al., 2009).

Abbreviations

CAR	Constitutive androgen receptor
4-Cl-BQ	2-(4-chlorophenyl)benzo-1, 4-quinone
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
MFI	Mean fluorescence intensity
MnSOD	Manganese superoxide dismutase
NBT	Nitroblue tetrazolium
PBS	Phosphate-buffered saline
PCB	polychlorinated biphenyls
PEG-SOD	polyethylene glycol conjugated superoxide dismutase
PGHS	prostaglandin H synthase
PI	Propidium iodide
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction.

Acknowledgments

We thank the staff of the Flow Cytometry and Radiation & Free Radical Research Core Facilities, and Ms. Amanda L. Kalen for critical reading of the manuscript. 4-Cl-BQ was provided by Dr. Hans-Joachim Lehmler from the Occupational & Environmental Health, University of Iowa. Funding from NIEHS (P42 ES 013661) and NIH (CA 111365) supported this work.

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Figure 1. 4-Cl-BQ treatment decreased MnSOD activity, protein, and mRNA levels in human prostate epithelial cells

Exponentially growing asynchronous cultures of RWPE-1 human non-malignant prostate epithelial cells were treated with 0–6 μM 4-Cl-BQ for 48 h and harvested for measurements of MnSOD protein (A), activity (B), and mRNA (C) levels. Immunoblotting of catalase and actin were included for comparison of results. Fold change was calculated relative to control. Asterisk indicates significant difference compared to control ($n = 3$, $p < 0.05$).

Figure 2. 4-Cl-BQ treatment increased cellular steady-state levels of ROS

Exponentially growing asynchronous cultures of RWPE-1 cells were treated with 0–6 μ M 4-Cl-BQ for 48 h and incubated with a prooxidant-sensitive dye, dihydroethidium (DHE). DHE-fluorescence was analyzed by flow cytometry, and the mean fluorescence intensity (MFI) was calculated using CellQuest software. Representative histograms are shown in (A): (–) DHE, autofluorescence of control cells; (+) DHE, control cells incubated with DHE; (+) 4-Cl-BQ, cells treated with 3 μ M 4-Cl-BQ followed by DHE staining; (+) 4-Cl-BQ + PEG-SOD, cells treated simultaneously with 3 μ M 4-Cl-BQ and 100 units of PEG-SOD followed by DHE-staining. (B) Fold-change in MFI calculated relative to 0 h untreated control. *, # indicates significant difference compared to untreated control and PEG-SOD treated control respectively ($n = 3$, $p < 0.05$).

Figure 3. MnSOD activity regulates proliferation of human prostate cells incubated with 4-Cl-BQ RWPE-1 cells were treated with 3 μ M 4-Cl-BQ and cell numbers counted at the indicated time (A). (B) In a separate set of experiments, cells were pretreated with 150 U of PEG-SOD followed by incubation with 3 μ M 4-Cl-BQ. Cell numbers were counted at the end of 72 h incubation. Asterisk indicates significant difference compared to control; Ψ indicates significance compared to 4-Cl-BQ and PEG-SOD treated cells ($n = 3$, $p < 0.05$).

Figure 4. Increased percentage of S-phase in cells incubated with 4-Cl-BQ

Exponentially growing asynchronous cultures of RWPE-1 were treated with 3 μM 4-Cl-BQ for the indicated times and fixed in ethanol. Ethanol fixed cells were treated with RNase A and stained with propidium iodide (PI). Cell cycle phase distributions were analyzed by flow cytometry measurements of DNA content of PI-stained cells. Representative DNA histograms of control and 4-Cl-BQ treated cells are shown and the areas representing cells with DNA content less than G₁ (sub-G₁), G₁, S, G₂ are marked. The percentage of cells in each cell cycle phase is calculated using MODFIT and results are shown in Table I.

Figure 5. 4-Cl-BQ induced delay in cell growth was not associated with DNA double strand break
Total cellular protein extracts were isolated from control and cells incubated with 3 μ M 4-Cl-BQ for 72 h. Proteins were separated by SDS-PAGE and immunoblotted for the protein levels of phosphorylated H2AX (γ -H2AX).

Figure 6. Prior treatment with PEG-SOD inhibits 4-Cl-BQ induced accumulation of cells in S-phase
RWPE-1 cells were treated with 150 U of PEG-SOD followed by incubation with 3 μ M 4-Cl-BQ for 72 h. Ethanol fixed cells were analyzed for DNA content and the percentage of cell cycle phase calculated using MODFIT software. *, # indicates significant difference compared to untreated control and 4-Cl-BQ + PEG-SOD treatment respectively (n = 3, p<0.05).

Table IPercent cell cycle phase in 3 μ M 4-Cl-BQ treated RWPE-1 cells

Treatment Hours		G ₁	S	G ₂
Control	0	46 $\pm$ 1.2	40 $\pm$ 1.1	11 $\pm$ 0.6
	8	50 $\pm$ 1.5	36 $\pm$ 0.8	14 $\pm$ 1.8
	24	53 $\pm$ 1.4	39 $\pm$ 1.2	8 $\pm$ 0.4
	48	54 $\pm$ 1.7	37 $\pm$ 0.9	9 $\pm$ 0.4
	72	57 $\pm$ 1.6	38 $\pm$ 0.5	3 $\pm$ 2.8
4-Cl-BQ	0	46 $\pm$ 0.5	42 $\pm$ 0.6	12 $\pm$ 0.2
	8	49 $\pm$ 1.1	42 $\pm$ 1.3	9 $\pm$ 1.5
	24	49 $\pm$ 0.9	41 $\pm$ 1.4	10 $\pm$ 1.3
	48	42 $\pm$ 0.7	*49 $\pm$ 0.5	9 $\pm$ 0.7
	72	45 $\pm$ 1.7	*52 $\pm$ 1.4	3 $\pm$ 0.3

Average $\pm$ SD; n = 3, p < 0.05