Environment International 36 (2010) 924–930

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

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

Leena Chaudhuri, Ehab H. Sarsour, Prabhat C. Goswami *

Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa, Iowa City, Iowa, United States

ARTICLE INFO

Available online 16 February 2010

Keywords:
Polychlorinated biphenyls
PCB3
Reactive oxygen species
MnSOD
Superoxide
Prostate epithelial cells

ABSTRACT

Polychlorinated biphenyls (PCBs) and their metabolites are environmental chemical contaminants which can produce reactive oxygen species (ROS) by auto-oxidation of di-hydroxy 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 μM 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 pretreated 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.

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). 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 μM) (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,

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.

* Corresponding author. B180 Medical Laboratories, Free Radical and Radiation Biology Program, Department of Radiation Oncology, University of Iowa, Iowa City, IA 52242-1181, United States. Tel.: +1 319 335 8019; fax: +1 319 335 8039.
E-mail address: prabhat-goswami@uiowa.edu (P.C. Goswami).

0160-4120/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envint.2010.01.001

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., 1981, 1989). 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, 2005). Metabolites of PCB3 are known to possess tumor initiating activities in rat liver (Espandiari et al., 2003, 2004). PCB3 and its di-hydroxylated metabolites have been shown to upregulate prostaglandin H synthase (PGHS) in 'hormonally sensitive' tissues like prostate, breast, and ovary. PGHS has both cycloxygenase and peroxidase activity (Wangpradit et al., 2009). Increased levels of cycloxygenase-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 and 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% CO_2 and 95% humidity. Exponentially growing asynchronous cultures were treated with 0–6 μM 4-Cl-BQ for 24–72 h. 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 to 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 min. 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-chemiluminescence 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′-CTGTAACATCTCCCTTGGCCA-3′; 18S forward primer: 5′-CCTTGGATGTGGTAGCCGTTT-3′, reverse primer: 5′-AACTTTC-GATGGTAGTCGCCG-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 s, 60 °C for 1 min) × 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 = $2^{-\Delta\Delta CT}$.