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PCB153 reduces telomerase activity and telomere length in immortalized human skin keratinocytes (HaCaT) but not in human foreskin keratinocytes (NFK)

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ABSTRACT

Polychlorinated biphenyls (PCBs), ubiquitous environmental pollutants, are characterized by long term-persistence in the environment, bioaccumulation, and biomagnification in the food chain. Exposure to PCBs may cause various diseases, affecting many cellular processes. Deregulation of the telomerase and the telomere complex leads to several biological disorders. We investigated the hypothesis that PCB153 modulates telomerase activity, telomeres and reactive oxygen species resulting in the deregulation of cell growth. Exponentially growing immortal human skin keratinocytes (HaCaT) and normal human foreskin keratinocytes (NFK) were incubated with PCB153 for 48 and 24 days, respectively, and telomerase activity, telomere length, superoxide level, cell growth, and cell cycle distribution were determined. In HaCaT cells exposure to PCB153 significantly reduced telomerase activity, telomere length, cell growth and increased intracellular superoxide levels from day 6 to day 48, suggesting that superoxide may be one of the factors regulating telomerase activity, telomere length and cell growth compared to untreated control cells. Results with NFK cells showed no shortening of telomere length but reduced cell growth and increased superoxide levels in PCB153-treated cells compared to untreated controls. As expected, basal levels of telomerase activity were almost undetectable, which made a quantitative comparison of treated and control groups impossible. The significant down regulation of telomerase activity and reduction of telomere length by PCB153 in HaCaT cells suggest that any cell type with significant telomerase activity, like stem cells, may be at risk of premature telomere shortening with potential adverse health effects for the affected organism.

Introduction

Polychlorinated biphenyls (PCBs), first manufactured in 1929, are a group of 209 individual congeners with 1 to 10 chlorine atoms attached to the biphenyl ring (WHO, 1993). These man-made compounds are highly stable and have been used in numerous industrial products, such as diluents, hydraulic fluids, heat transfer fluids, dielectric fluids for capacitors and transformers and many more. Their lipophilic nature and resistance to degradation contribute to their ubiquitous environmental distribution and accumulation in the food chain (Bates et al., 1994); (Fürst et al., 1994). The general population thus is extensively exposed to PCBs through the consumption of contaminated foods, inhalation or skin absorption in work environments (Kogevinas et al., 1997). PCBs are neurotoxic, embryotoxic, immunosuppressant, and are considered to be a probable carcinogen (ATSDR, 2000; Danis et al., 2006; Silberhorn et al., 1990; Yoshizawa et al., 2009). In humans, skin lesions like chloracne and hyperkeratosis were the most common adverse health effects in patients exposed to PCBs/PCDFs through contaminated rice oil in the Yusho and Yusheng poisoning episodes in Japan and Taiwan, respectively (Kikuchi, 1984; Tsai et al., 2006). The toxicity of individual PCB congeners varies with their structure (Davis and Safe, 1990). Two major groups of PCBs can be distinguished, namely dioxin-like and non-dioxin-like PCBs. Dioxin-like congeners have few chlorines (1 or none) at the *ortho*-positions; therefore, they can adopt a more coplanar structure and bind to the aryl hydrocarbon receptor (AhR) (Bandiera et al., 1982). Non dioxin-like PCBs have at least two chlorine substitutions at the *ortho*-positions and are less coplanar due to steric hindrance. They exert their toxic effects via mechanisms independent of the AhR binding (Sipka et al., 2008).

Individual PCBs have different physical/chemical characteristics which influence their accumulation, uptake and metabolism in the environment and in organisms, giving rise to marked differences in PCB congener composition between the commercial PCB mixtures (Aroclor) and biological extracts. In most biological extracts PCB138 (2,2′,3,4,4′,5-hexachlorobiphenyl), PCB153 (2,2′,4,4′,5,5-hexachlorobiphenyl), and PCB180 (2,2′,3,4,4′,5,5-heptachlorobiphenyl) are the

Abbreviations: PCB153, 2,2′,4,4′,5,5′ hexachlorobiphenyl; HaCaT, Immortalized human skin keratinocytes; NFK, normal human foreskin keratinocytes.

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dominating components (Safe, 1994; Cogliano, 1998). Of the various congeners, PCB153 appears to be a predominant PCB congener present in human and fish tissues (Chao et al., 2004; Hayward et al., 2007). PCB153 is persistent in the environment and its half-life may exceed 100 years in marine sediments (Jönsson et al., 2002). PCB153 is a di-ortho-substituted PCB congener that does not activate the AhR and exhibits no dioxin-like activity. It was shown to cause several adverse biological effects, including DNA damage and promotion of tumorigenesis (Wei et al., 2009; Tharappel et al., 2002). One of the main mechanisms by which PCBs may exert their toxic effect is by increasing oxidative stress (Tharappel et al., 2002) and PCB153 has been shown to increase oxidative stress in human breast epithelial cells (Venkatesha et al., 2008).

Telomeres are specialized structures of tandem arrays of G-rich repeats (TTAGGG in humans) present at the end of chromosomes (Moyzis et al., 1988). Telomeres function to protect the double-stranded DNA ends from degradation, fusion, recombination (McClintock, 1941). Telomerase, an enzyme complex, contains a reverse transcriptase component (in humans it is called hTERT) and RNA component (in humans it is called hTR). It helps in adding TTAGGG to the telomeric ends to compensate the progressive loss of telomeric sequences during normal DNA replication (Greider and Blackburn, 1987). Both telomeres and telomerase play a vital role in a variety of biological activities in the body including but not limited to maintenance of cellular genome integrity and nuclear architecture, expression of growth-promoting genes, and cellular proliferation (Smith et al., 2003). Telomere DNA is highly sensitive to damage by oxidative stress (Henle et al., 1999; Oikawa and Kawanishi, 1999). The telomerase hTERT subunit is prevented from adding nucleotides to telomeric DNA via translocation of hTERT from the nucleus to mitochondria by oxidative stress (Haendeler et al., 2003). Dysfunctional telomeres and telomerase lead to various diseases like aging and cancer. Although telomeres and telomerase play a vital role in many diseases, very little research has been done so far concerning the effect of environmental contaminants on telomeres/telomerase and to our knowledge no research work has been done on the influence of the most prevalent PCB congener in our bodies, PCB153. Considering the ubiquitous exposure to PCBs through food and inhalation and skin exposure (Ludewig et al., 2008), this is an astonishing gap in our knowledge. Therefore this study is exploring the effect of PCB153 on telomerase activity, telomere length, and cell growth changes in immortalized human skin keratinocytes (HaCaT) and primary human foreskin keratinocytes (NFK).

Materials and methods

Materials. Dulbecco's Modified Eagle Medium (DMEM), Keratinocyte Serum Free Medium, Epidermal Growth Factor (EGF), Bovine Pituitary Extract (BPE), Fetal Bovine Serum (FBS), Penicillin–Streptomycin (P/S), 0.25% trypsin with EDTA, and Phosphate Buffered Saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), resazurin and propidium iodide were purchased from Sigma (St. Louis, MO, USA). Colcemide was bought from Alexis Biochemicals (San Diego, CA, USA). PCR reagents and kits were obtained from Qiagen (Valencia, CA, USA). PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) was synthesized and characterized as described previously (Schramm et al., 1985). The purity of PCB153 was greater than 99%, as assayed by gas-chromatography.

Cell culture and PCB exposure regiment. The human keratinocyte (HaCaT) cell line was obtained from Dr. C. Svenson, University of Iowa and maintained in DMEM with 10% FBS and 1% P/S in a humidified incubator at 37 °C with 5% CO_2. Human Normal Foreskin Keratinocytes (NFK) were obtained from Dr. Aloysius Klingelhutz, University of Iowa and maintained in Keratinocyte Serum Free Medium with 0.16 ng/ml EGF, 25 μg/ml BPE and 1% P/S in a humidified incubator at 37 °C with 5% CO_2. These cell types were chosen since skin is a major route of exposure and excretion for PCBs (ATSDR, 2000). HaCaT is a cell line obtained from spontaneously immortalized skin keratinocytes from normal skin (Boukamp et al., 1988). They have already been used for telomere length studies (Jacobus et al., 2008; Zhang et al., 2003) and are positive for telomerase activity (Harle-Bachor and Boukamp, 1996). NFK (Normal Foreskin Keratinocytes) were chosen to compare the effects observed in HaCaT cells with those in normal skin cells. NFK have only negligible telomerase activity.

The cytotoxicity of PCB153 was determined by seeding 30,000 HaCaT or NFK cells per well in 24-well tissue culture plates and exposing them to 2–20 μM PCB153 for six days with a change of medium containing fresh compound on the third day. Control cultures received solvent (DMSO, 0.05% final concentration) alone. After six days the medium was removed and cells were incubated with resazurin (5 μM, dissolved in fresh medium) for 2 h and the fluorescence intensity at 535 nm excitation and 590 nm emission wavelength in each well was measured using a microplate reader (TECAN, Seestrasse, Switzerland). The fluorescence intensity correlates with the number of living cells in the well, since cells metabolize non-fluorescent resazurin to the fluorescing resorufin. Based on these results a concentration of 5 μM (HaCat) and 2 μM (NFK) was selected for all following experiments.

Experiments with HaCaT cells were performed by seeding 100,000 cells in 10 cm diameter Petri dishes and exposing them continuously to 5 μM PCB153 for up to 48 days. Solvent controls received DMSO (0.05% final concentration) only. Media with test compound or solvent were changed every three days and cells were trypsinized, counted, and re-seeded at low density (100,000 per plate) in medium with test compound every sixth day. The remaining cells on days 6, 18, 30, 42 and 48 were used to determine telomerase activity, telomere length, and cell growth. In addition, cell viability and cell cycle distribution were measured on days 6, 30 and 48. Cell morphology was monitored throughout the exposure.

Similarly 100,000 NFK cells were seeded and exposed continuously to solvent only (0.05% DMSO) or 2 μM PCB153 for 24 days. Media with test compound or solvent were changed every other day. Cells were trypsinized, counted, and re-seeded at low density (100,000 per plate) in medium with fresh test compound every sixth day. Cells from day 6, 18 and 24 were used to determine telomerase activity, telomere length, and cell growth. Cell viability and cell cycle distribution were measured on days 6 and 24.

Exposure periods of up to 48 days were used to mimic the continuous and chronic human exposure to PCBs in daily life. The concentration of 5 μM PCB153 for HaCaT and 2 μM for NFK appears excessively high, but is justifiable considering that PCB levels in the blood of individuals living in Anniston, Alabama, vary from 0.003 to 6.5 μM (Hansen et al., 2003) and tissue levels of PCBs are usually by several magnitudes higher than blood levels.

Analysis of telomerase activity. Telomerase activity was measured following the method of Wege and coworkers (Senthilkumar et al., 2011; Wege et al., 2002). After trypsinization and centrifugation of cells, cell pellets were resuspended in cell lysis buffer at 1000 cells/μl and incubated on ice for 30 min. To compensate for the low telomerase activity in NFK cells a 10× higher number of cells was used in the NFK telomerase protocol than in the HaCaT protocol. Aliquots of cell lysate were centrifuged at 16,000 *g* for 20 min at 4 °C and the supernatant was used for qPCR. The SYBR Green based qPCR reaction was performed using a Telomerase Primer (TS) and Anchored Return Primer (ACX) (Kim and Wu, 1997), cell lysate supernatant, and Eppendorf MasterMix SYBR ROX (Fisher Scientific, PA, USA). SYBR green fluorescence was measured in an Eppendorf RealPlex Thermal Cycler (Eppendorf, Hamburg, Germany) and the Ct (threshold cycle value) was determined from the semi-log amplification plots and