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Induction of DNA damage in human urothelial cells by the brominated flame retardant 2,2-bis(bromomethyl)-1,3-propanediol: Role of oxidative stress

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ABSTRACT

2,2-bis(bromomethyl)-1,3-propanediol (BMP) is an extensively used brominated flame retardant found in urethane foams and polyester resins. In a 2-year dietary study conducted by the National Toxicology Program, BMP caused neoplastic lesions at multiple sites including the urinary bladder in both rats and mice. The mechanism of its carcinogenic effect is unknown. In the present study, using SV-40 immortalized human urothelial cells (UROtsa), endpoints associated with BMP induced DNA damage and oxidative stress were investigated. The effects of time (1–24 h) and concentration (5–100 μM) on BMP induced DNA strand breaks were assessed via the alkaline comet assay. The results revealed evidence of DNA strand breaks at 1 and 3 h following incubation of cells with non-cytotoxic concentrations of BMP. Strand breaks were not present after 6 h of incubation. Evidences for BMP associated oxidative stress include: an elevation of intracellular ROS formation as well as induction of Nrf2 and HSP70 protein levels. In addition, DNA strand breaks were attenuated when cells were pre-treated with *N*-acetyl-L-cysteine (NAC) and oxidative base modifications were revealed when a lesion specific endonuclease, human 8-hydroxyguanine DNA glycosylase 1 (hOGG1) was introduced into the comet assay. In conclusion, these results demonstrate that BMP induces DNA strand breaks and oxidative base damage in UROtsa cells. Oxidative stress is a significant, determinant factor in mediating these DNA lesions. These early genotoxic events may, in part, contribute to BMP-induced carcinogenesis observed in rodents.

1. Introduction

2,2-bis(bromomethyl)-1,3-propanediol (BMP), or as it is known commercially dibromoneopentyl-glycol, is a brominated flame retardant used in unsaturated polyester resins, molded products, rigid polyurethane foam, and as an additive in the manufacture of plastic polymers(Larsen, 1973). BMP is also used as a chemical intermediate in the production of other flame retardants. Between 1986 and 2002, the estimated annual production of BMP in the United States was as high as 10 million pounds (EPA, 2002). This production has resulted in the environmental contamination, as BMP has been identified in dust particles and wastewater (EPA, 1983). Once in the environment, BMP is slowly degraded. Its half life is estimated to be over 100 years in groundwater (Ezra et al., 2006, 2010). Therefore, humans may be exposed to BMP by multiple routes (NTP, 1996). The widespread production and use of BMP, as well as evidence that it is a contaminate present in dust and water, heighten the importance of understanding the potential risk of mammalian exposure to BMP.

When administered orally, the acute toxicity of BMP (LD_{50}: 3458 mg/kg) in rats is low (Keyes et al., 1979). In a thirteen-week sub-chronic toxicity study, results obtained in rats and mice that received BMP daily either in feed or by oral gavage demonstrated transitional cell hyperplasia in their kidneys and urinary bladders (Elwell et al., 1989). Results of a 2-year dietary bioassay conducted by the National Toxicology Program (NTP) reported that BMP is a multisite carcinogen in both sexes of rats and mice (Dunnick et al., 1997; NTP, 1996). Increased incidences of neoplasms were observed in a variety of tissues including kidney, urinary bladder, skin, mammary gland, oral cavity, esophagus, forestomach, small and large intestine, mesothelium, lung, thyroid gland, hematopoietic system, seminal vesicle and pancreas after BMP exposure. Such a wide spread distribution of tissues that developed neoplasms suggests that BMP might be a direct acting carcinogen. Based on these animal studies, BMP is reasonably anticipated to be a human

Abbreviations: AP-sites, apurinic/apyrimidinic sites; BMP, 2,2-bis(bromomethyl)-1,3-propanediol; CCK-8, cell counting kit-8; carboxy-DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; EPA, U.S. Environmental Protection Agency; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; hOGG1, human 8-hydroxyguanine DNA glycosylase 1; NAC, *N*-acetyl-L-cysteine; NTP, National Toxicology Program; 8-OHgua, 7,8-dihydro-8-oxo-guanine.

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carcinogen (NTP, 2011). Although BMP is carcinogenic, results of microbial mutagenicity assays are uniformly negative, except for a positive response in one study (with 30% Aroclor 1254-induced hamster liver S9) (NTP, 1996; Zeiger et al., 1992).

Induction of DNA lesions is believed to be one of the initial steps in chemical induced carcinogenesis. DNA can be damaged by such mechanisms as a direct attack of the chemical or its metabolites or via events associated with oxidative stress. Oxidation products formed in DNA include strand breaks, abasic sites and oxidized bases. Within the last group, attention has been focused on 7,8-dihydro-8-oxo-guanine (8-OHgua) as a major maker for oxidative DNA damage with a clear mutagenic potential (Hwang and Kim, 2007; Van Loon et al., 2010). The single cell gel electrophoresis assay (alkaline version), which measures DNA strand breaks and alkali-labile sites i.e. AP-sites or abasic sites, can be modified to measure oxidative DNA base modifications. For example, human 8-hydroxyguanine DNA glycosylase 1 (hOGG1) is an endonuclease that specifically removes 8-OHgua and is used for the detection of oxidative DNA lesions (Mihaljevic et al., 2011; Smith et al., 2006).

Due to the lack of significant data on the mechanism of BMP induced carcinogenesis and since the urinary bladder was one of the target tissues in rat and mice, the present study investigated if BMP caused DNA damage and oxidative stress in an immortalized, non-malignant, p53 deficient human urothelial cell line (UROtsa cells). This cell line has been used as an effective model for studies on human bladder transitional epithelium and for investigation of both acute and chronic arsenic-induced cellular insults (Eblin et al., 2008; Rossi et al., 2001; Sens et al., 2004). In the studies reported here the ability of BMP to cause DNA strand breaks and oxidative base lesions in these cells was characterized via both the standard and the hOGG1 modified comet assay. Oxidative stress and its involvement in BMP mediated genotoxicity were evaluated by intracellular ROS generation, expression of NF-E2 related factor 2 (Nrf2) and Heat shock protein 70 (HSP70), as well as the protective capability of *N*-acetyl-L-cysteine (NAC), a well-established antioxidant on BMP induced DNA strand breaks.

2. Material and methods

2.1. Chemicals

BMP (Lot No. 04119MD) with a purity of 98% was obtained from Sigma–Aldrich (St. Louis, MO). Hydrogen peroxide was purchased from JT baker (Phillipsburg, NJ) and was diluted with sterilized distilled water (dH_2O) before use. Dulbecco's Modified Eagle Medium (DMEM), Penicillin/Streptomycin, trypsin-EDTA and trypan blue were acquired from Gibco Invitrogen Corporation (Carlsbad, CA) and Fetal Bovine Serum (FBS) from Atlanta Biologicals (Lawrenceville, GA). Nrf2 (rabbit polyclonal) and HSP70 (mouse monoclonal) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and EMD Bioscience, Inc. (Calbiochem®, San Diego, CA) respectively. 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA) were obtained from Molecular Probes, Invitrogen Detection Technology (Eugene, OR). Other generally used chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless stated otherwise, and used without further purification.

2.2. Cell culture and BMP exposure

The UROtsa cells were generously provided by Drs. Donald and Maryann Sens (University of North Dakota). Cell culture conditions were as previously described (Wnek et al., 2010). Particularly, stock cultures were maintained in a growth medium of DMEM containing 5% (v/v) FBS, 50 unit/ml penicillin and 50 μg/ml streptomycin. Growth medium was changed every 3 days. Cultured cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Confluent cells were removed from plates with trypsin-EDTA (0.25%) and sub-cultured at approximately 5×10^5 cells/flask in 75-cm^2 culture flasks. Results of the periodical screening test for mycoplasma contamination indicated all UROtsa cells used in this study were negative.

BMP was dissolved in and serially diluted with 100% Ethanol (EtOH). Incubations were carried out from 0 to 24 h in growth medium. All control and treated cultures received the same final concentration of vehicle (0.5% EtOH).

2.3. Cytotoxicity assay

Cell viability was determined by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD) which measures the conversion of the highly water-soluble tetrazolium salt to formazan. The amount of formazan is proportional to the number of living cells. Briefly, UROtsa cells were seeded in 96-well plates (1×10^4 cells/well) and incubated in fresh DMEM medium at 37 °C for 24 h. Then the cells were treated with vehicle or various concentration of BMP (10–1500 μM). After incubation for 1, 6 and 24 h, 10 μl of CCK-8 solution were added to each well and the plates were further incubated for 2 h at 37 °C. The absorbance at 450 nm was measured with a microplate reader (BioTek, Winooski, VT). Cell viability following BMP and vehicle exposure compared to untreated (naïve) control. The results were expressed as the percentage of viability with respect to naïve control.

2.4. Standard and hOGG1 modified comet assay

Standard and modified comet assays were done using the CometAssay® and hOGG1 FLARE® Assay kit (Trevigen, Gaithersburg, MD). Cells were seeded in 12-well plate at 5×10^4 cells/well and maintained for 48 h until chemical exposure. In some cases, cells were pretreated with NAC (2 mM) for 1 h before incubation with vehicle or BMP. Agents known to produce effects in the comet assay (H_2O_2 or $KBrO_3$) were used as positive controls. Following the different treatments, cells were collected and quantified. 30 μl of cell suspensions were added to 200 μl low melting agarose and the cell/agarose mixture (50 μl) was then added to the comet slide. The slides were placed in the dark at 4 °C for 30 min after which they were immersed in the lysis solution for 35 min. Next, the slides were placed in fresh alkali solution (300 mM NaOH and 1 mM Na_2EDTA in dH_2O, PH > 13) for 35 min at room temperature. Electrophoresis was conducted in the same buffer at 4 °C for 35 min at 25 V (0.8 V/cm), 300 mA. Following electrophoresis, the slides were washed using dH_2O and then immersed in 70% EtOH for 5 min. The slides were air dried and stained with 100 μl diluted SYBR® green I for 1 h. In the hOGG1 modified assay, following the incubation with the lysis buffer, the slides were immersed in three changes of FLARE buffer® for 10 min, each time at room temperature. Then 75 μl of hOGG1 enzyme solution (0.08 U/gel) was added to the gel area and at the same time, a buffer-only control was also included. Gels were covered with Parafilm® and incubated in a humidified chamber for 1 h at 37 °C. The slides were then placed in the alkaline solution and processed as in the standard comet assay.

Comet slides were analyzed at 40× magnifications under an Olympus ImT-2 epifluorescence microscope (Center Valley, PA) equipped with an excitation filter of 460–500 nm, a 100 W mercury lamp, a long pass filter at 515 nm and a Hamamatsu Orca 100 digital camera (Bridgewater, NJ). The images were collected using Hamamatsu SimplePCI digital imaging software (Sewickey, PA) and the analysis of DNA damage was performed by Comet Score program (V1.5, Tritek Corp). Computer generated % tail DNA ($T_{\%\,DNA}$) was used as the parameter to assess DNA damage. In the hOGG1 modified comet assay, the difference in the extent of DNA strand breaks (Net $T_{\%\,DNA}$) between hOGG1-treated and buffer-treated control slides was determined to give a quantitative measurement of hOGG1-sensitive sites. For each exposure group a total of 100 cells were analyzed using systemic random sampling (McArt et al., 2009). All experiments were repeated at least three times independently.

2.5. Intracellular ROS measurement

Intracellular ROS were determined by using H_2DCFDA and carboxy-DCFDA as the sensitive non-fluorescent precursor dyes according to Tome et al. (2006). Both dyes are taken into the cells and enzymatically hydrolyzed by intracellular esterases. Carboxy-DCFDA will fluoresce once the acetate groups are removed, but H_2DCFDA fluoresces only in the presence of ROS (H_2O_2, $HO^•$). When used in cellular systems, H_2DCFDA is generally considered as a marker of oxidative stress rather than an indicator of specific reactive species (Gomes et al., 2005). Cells were seeded in 96-well plates at 5×10^3 cells/well and incubated for 1 h at 37 °C in serum free DMEM medium containing 20 μM H_2DCFDA or 1 μM carboxy-DCFDA. Afterwards, cells were washed twice with PBS and exposed to vehicle, BMP (10–100 μM) or H_2O_2 (100 μM) in PBS for 1 h. ROS were measured periodically using a fluorescent plate reader (BioTek, Winooski, VT) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. Fluorescence due to H_2DCFDA was corrected for the relative carboxy-DCFDA fluorescence to account for differences in dye uptake. ROS level was expressed as percentage of the fluorescence in vehicle control.

2.6. Western immunoblotting analysis

Cells were rinsed with ice cold Tris Buffer Saline (TBS) twice and then lysed in lysis buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 20 mM $Na_4P_2O_7$, 1 mM PMSF, 200 μM NaF, 230 nM Aprotinin, 21 μM Leupeptin and 5 mM Na orthovanadate). Protein assays (Bio-Rad, Hercules, CA) were performed on cell lysates to correct for loading. Equal amounts of protein (30 μg) were resolved by SDS-PAGE, transferred onto a PVDF membrane (Bio-Rad, Hercules, CA) and immunoblotted for Nrf2, HSP70 and β-actin. Proteins were visualized by Visiglo HRP chemilum substrate kit (Amresco, Solon, OH) on genemate