Induction of Cell Death, DNA Strand Breaks, and Cell Cycle Arrest in DU145 Human Prostate Carcinoma Cell Line by Benzo[a]pyrene and Benzo[a]pyrene-7,8-diol-9,10-epoxide

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Abstract: Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon, is a major environmental pollutant. In this study, the effects of this carcinogen/mutagen and one of its metabolites, benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), on human prostate carcinoma cell line DU145, were examined. Cell viability, DNA damage, and cell cycle progression were evaluated as toxic end-points. We have shown that B[a]P and BPDE inhibited cell viability following 48 hr of exposure. Furthermore, comet assay analyses revealed that both B[a]P and BPDE induced DNA strand breaks in a concentration-dependent fashion. Flow cytometric analyses showed that about 70% of DU145 cells were arrested by B[a]P at the G1 phase, while about 76% were arrested at G1 phase by BPDE. These data reveal that B[a]P and BPDE are cytotoxic and genotoxic to DU145 prostate cancer cells.

Keywords: Benzo[a]pyrene; benzo[a]pyrene-7,8-diol-9,10-epoxide; DU145; cell viability; DNA damage; cell cycle

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental and occupational pollutants formed from the combustion of organic compounds. Sources of PAHs include cigarette smoke, diesel exhaust, residential heating processes, and industrial coke production [1-2]. Benzo[a]pyrene B[a]P is the most studied PAH which serves as a model for the carcinogenic and mutagenic effects of PAHs [3-4]. Benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), is a highly reactive electrophilic metabolite of B[a]P that has been reported to cause mutations and cytotoxicity in both prokaryotic and eukaryotic cells [5]. BPDE forms covalent bonds with DNA, RNA, and proteins leading to mutations and cancer. BPDE is the proximate mediator of B[a]P-induced toxicity [6, 7].

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death among men in the United States [8]. DNA damage may be one of the first steps leading to various types of toxicities in human prostate cells. The present study will shed some insights into the cytotoxicity of B[a]P and BPDE on prostate cells. Currently no normal prostate cell lines are available. Therefore we used DU145 cells to help better understand the effects of B[a]P and its selected metabolite on normal prostate cells.

Our results demonstrated that varying concentrations of B[a]P and BPDE induced cell death, DNA strand breaks and cell cycle arrest in a dose-dependent manner.

Materials and Methods

Materials

Benzo[a]pyrene (B[a]P) and Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) were purchased from Midwest Research Institute (Kansas City, MO, USA). 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT dye) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The culture media and the calcium and magnesium-free phosphate buffered saline (PBS), Fetal bovine serum (FBS), RPMI 1640, penicillin–streptomycin (PS), N-[2-hydroxyethyl]piperazine-
N'2-et-hanesulfonic acid) (HEPES), sodium pyruvate, and glutamine were purchased from (GIBCO Laboratories, Grand Island, NY, USA).

**Cell Culture**

Human prostate cancer cells (DU145) was obtained from ATCC. All cells lines were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium with 0.25% glucose, 0.238% HEPES, 0.011% sodium pyruvate, 0.15% NaHCO3, 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml), and incubated at 37°C with 5% CO2 in an air atmosphere.

**Cell Viability**

The effects of B[a]P and BPDE on cell viability of DU145 was determined using the MTT assay. DU145 cells were plated in a 48-well plate at a density of 2×10^4 cells per well and cultured for 24 hours in routine culture medium. The cells were further cultured for 24 hours in assay medium composed of phenol red-free RPMI-1640 supplemented with 10% charcoal-stripped FBS. The cells were incubated with B[a]P at concentrations of 5.0, 20, and 50 µM and with BPDE at concentrations of 1.0, 5.0, and 10.0 µM, and DMSO (0.1%) for 48 hours. MTT (100 µl) was added to each well and allowed to incubate for 3 hours. The purple formazan product (produced by reduction of MTT by succinyl dehydrogenase in the mitochondria of living cells) was solubilized with 100µl of solution of isopropanol for about 10 minutes. The plate was read at a wavelength of 570 nm with background subtraction at 630-690nm using a plate reader (µ Quant, BioTek, Winooski, VT, USA). All reported experiments were performed three times in triplicates.

**DNA Strand Breaks**

The alkaline comet assay was used to evaluate DNA strand breaks [9-10]. Cells were cultured in 25cm^2 cell culture flasks. Confluent cell cultures were treated with DMSO (0.1%, vehicle control), B[a]P and BPDE for 48 hours. Cells were pelleted and centrifuged at 800 x G for 5 minutes. The pellets were resuspended in 2 mls of ice cold PBS. One hundred microliters of resuspended cells (~ 5,000 cells) from each treatment were added to 900µl low melting point agarose (0.75%). Frosted microscope slides were coated with normal melting point agarose followed by 100µl of low melting point agarase containing treated cells. After solidification on ice, the slides were placed in ice cold lysis buffer containing triton-X100 (pH~ 10) for 2 hours. After lysis, microscope slides were placed in alkaline electrophoresis buffer (pH>13.5) for 30 min to allow the DNA to unwind. After unwinding, the slides were electrophoresed for 30 min. at 280A and 25V in a cold room at 4°C and then neutralized with Tris buffer (pH 7.5) and placed in 100% ethanol for 5 min dehydration. DNA was stained with 100μl of propidium iodide (PI). The slides were analyzed using a fluorescent microscope and the Kinetic Imaging Comet assay software. A total of 150 cell images were analyzed per sample using a magnification of 400 X [9, 10]. The level of DNA damage was assessed based on the total length of the tail formed by the damaged DNA migrating from the nucleus towards the anode upon electrophoresis.

**Cell Cycle Analysis**

For cell cycle analysis, DU145 cells at density of 10^6 cells/ml were treated with DMSO (0.1%, vehicle control), B[a]P (5, 20, and 50 µM ) and BPDE (1, 5, and 10 µM) and incubated for 48 hours. Cells were prepared for flow cytometry analysis by fixing in 100% ice cold absolute alcohol. Cells were stained with 1ml of freshly prepared PI staining solution and RNase A for 1 hour in the dark at room temperature. For each sample, 20,000 events were acquired using FACSCalibur flow cytometer (Becton Dickerson, San Jose, CA, USA). CELLQuest software was used for the acquisition and analysis.

**Results and Discussions**

The cytotoxic effects of varying concentrations of B[a]P and BPDE were assessed in human prostate carcinoma cell line (DU145) after 48 hours and analyzed using the MTT assay. B[a]P at concentrations of 5, 20, and 50 µM caused a dose-dependent decrease in cell viability (Fig. 1).

![Figure 1: Cytotoxicity of B[a]P to DU145 cells as determined by the MTT Assay. Data presented are the means ± SEMs from three experiments in triplicate. The symbol (*) indicates statistical significance at the level of p<0.05 as compared with control. The symbol (**) indicates values that are statistically significant difference from 20µM B[a]P treatment group (p<0.05). The symbol (***) indicates statistically significant difference from 50 µM B[a]P treatment a significance of p<0.05.](image-url)

BPDE at concentrations of 1, 5, and 10 µM also caused a dose-dependent decrease in cell viability (Fig. 2). BPDE the active metabolite is more potent than B[a]P. A dose of 10 µM of BPDE reduced cell viability to 35% where as B[a]P at 50µM reduced cell viability to 50%.
Figure 2: Cytotoxicity of BPDE to DU145 cells as determined by the MTT Assay. Data presented are the means ± SEMs from three experiments in triplicate. The symbol (*) indicates values significantly different from the control. The symbol (**) indicates values significantly different the 1 µM BPDE treatment group. The symbol (***) indicates values that are statistically significant different from 5µM BPDE treatment group a significance of p<0.05.

DNA strand breaks analysis was performed on DU145 cells after exposure to varying concentrations of B[a]P and BPDE for 48 hours via the Comet assay (Fig. 3).

A: DMSO  B: 50 B[a]P  C: 10 BPDE

Figure 3: Induction of the extent of DNA strand breaks by DU145 cells treated with B[a]P and BPDE. A large comet tail is visible, indicating DNA strand breaks. Panel A represents 0.1% DMSO (vehicle control). In this cell there are no detectable DNA strand breaks as indicated by the absence of a comet tail. Panel B represents a cell treated with 50 µM B[a]P. In this cell the amount of DNA strand breaks was extensive as indicated by the large comet tail. Panel C represents a cell that was treated with 10µM BPDE. In this cell the amount of DNA strand breaks was extensive as indicated by the large comet tail. Original magnification 200 X, stained with PI. A significant change in comet shape can be observed compared to DMSO (vehicle control).

The Comet Assay is frequently used to detect DNA strand breaks in individual cells. The mean olive tail moment or simply tail moment is used because it incorporates the amount of DNA that has been damaged with the size of the strands. Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces; represented by the intensity of DNA in the tail [11]. DNA damage has been associated with mutation and cancer [12]. B[a]P at concentrations of 5, 20, and 50 µM produced mean olive tail moments of 3, 6, and 12 respectively (Fig. 4). Similar results were seen with BPDE at concentrations of 1, 5, and 10 µM with mean olive tail moments of 7, 11, and 18 respectively (Fig. 5). Therefore B[a]P and BPDE induced DNA strand breaks may play a role in the development of prostate cancer.

Figure 4: Comet assay analysis of DNA strand breaks in DU145 prostate carcinoma cell line after 48 hours treatment with B[a]P. The Comet assay analysis demonstrates that the degree of DNA damage by B[a]P was concentration dependent. At 50µM concentration the mean olive tail moment was 14. The symbol (*) indicates that the olive tail moment was statistically different from the control. The symbol (**) indicates that the olive tail moment was statistically different from at 5 µM of B[a]P. The symbol (***) indicates that the olive tail moment was statistically different from 20 µM concentration of B[a]P a significance of p<0.05.

Figure 5: Comet assay analysis of DNA strand breaks in DU145 prostate carcinoma cell line after 48 hours treatment with BPDE. The Comet assay analysis demonstrates that the degree of DNA damage by BPDE was concentration dependent. The symbol (*) indicates that the olive tail moment was statistically different from the control. The symbol (**) indicates that the olive tail moment was statistically different from the 5 µM BPDE treatment group. The symbol (***) indicates that the olive tail moment was statistically different from 10 µM BPDE treatment group at a significance of p<0.05.
Cell Cycle Analyses

To determine whether of B[a]P and BPDE results in the alteration of cell cycle progression, cells were treated with various concentrations of B[a]P and BPDE for 48 hours. Increasing concentrations of B[a]P resulted in increased percentage of cells arrested in G1. B[a]P at doses of 5, 20, and 50 μM resulted in cells arrested in G1 (Table 1). Increasing concentrations of BPDE showed increased percent of cells arrested in G1. Concentrations of BPDE of 1, 5, and 10 μM resulted in cells arrested in G1 (Table 2). The percent distributions at cell cycle phases demonstrated that majority of the cells were arrested at G1 phase (Fig. 6).

Table 1: The cell cycle distribution of DU145 after 48 h treatment with varying concentrations of B[a]P. Data presented are means ± SDs of three independent investigated experiments. Mean values of three replicates were analyzed for significant differences between treatments and control with one-way ANOVA.

| Cell line | B[a]P (μM) | % Distribution at cell cycle phase |
|-----------|------------|-----------------------------------|
|           |            | G1  | S  | G2/M |
| Control (0.1% DMSO) | 64.39 ± 0.03 | 14.18 ± 0.04 | 20.55 ± 0.08 |
| 5         | 68.54 ± 0.36* | 10.86 ± 0.29 | 20.07 ± 0.42 |
| 20        | 69.12 ± 0.15* | 10.04 ± 0.21 | 20.47 ± 0.48 |
| 50        | 70.39 ± 0.45* | 7.22 ± 0.56  | 22.41 ± 0.62 |

The symbol (*) indicates statistically significant compare to control (p<0.05).

Table 2: The cell cycle distribution of DU145 after 48 h treatment with varying concentrations of BPDE. Data presented are means ± SDs of three independent investigated experiments. Mean values of three replicates were analyzed for significant differences between treatments and control with one-way ANOVA.

| Cell line | BPDE (μM) | % Distribution at cell cycle phase |
|-----------|-----------|-----------------------------------|
|           |           | G1  | S  | G2/M |
| Control (0.1% DMSO) | 64.39 ± 0.03 | 14.18 ± 0.04 | 20.55 ± 0.08 |
| 1         | 69.26 ± 0.57* | 9.03 ± 0.65  | 20.89 ± 0.88 |
| DU145     | 20        | 69.12 ± 0.15* | 10.04 ± 0.21 | 20.47 ± 0.48 |
| 50        | 70.39 ± 0.45* | 7.22 ± 0.56  | 22.41 ± 0.62 |

The symbol (*) indicates statistically significant compare to control (p<0.05).

Figure 6: DNA content histograms representing the effect of B[a]P and BPDE on the cell cycle of DU145 prostate carcinoma cells. DU145 cells were incubated with B[a]P and BPDE for 48 hours. (A) Control (0.1% DMSO), (B) 50uM B[a]P treated cells, and (C) 10uM BPDE treated cells.
The observation that B[a]P and BPDE induced cell cycle arrest/delay in G1 phase allows one to propose several plausible mechanisms of action for this effect. One possibility is that B[a]P and BPDE induced this effect through a mechanism that could be mediated by p53. However, DU145 which is one of a prostate carcinoma cell lines widely used as a model for prostate cancer study expresses mutant p53 [13]. Another alternative mechanism for the B[a]P and BPDE-induced cell cycle arrest in G1 may involve the activation of the inhibitory cell cycle regulatory proteins p21/ or p27. This mechanism is currently being explored in our laboratory.

Our results demonstrate that B[a]P and BPDE induced cell death, DNA strand breaks, and cell cycle arrest in DU145. We propose that normal prostate cells will metabolize B[a]P in a similar fashion of DU145 cells. Therefore, the data obtained in these experiments indicate that DNA strand breaks and cell death may be a factor that leads to B[a]P toxicity in normal prostate cells.

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