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Microdoses Levels of Butadiene Diepoxide (BDO_2) Induced Toxicity in Prostate Cancer Cells

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ABSTRACT

Low-level exposure to environmental pollutants such as BDO_2 contributes directly and indirectly to an increase in PCa. The aim of this study was to define the cellular changes associated with micro-doses of Butadiene Diepoxide (BDO_2) in prostate cancer cells. We observed that micro-doses of BDO_2 resulted in dose- and time-dependent increases in cytotoxicity and increased expression of prostate tumor markers in LNCaP(AR^+) and DU145(AR^-) cells. There was an increased sensitivity of DU145(AR^-) cells to BDO_2 toxicity which was reversed by transient transfection of AR into theses cell. Exposure of prostate cells to BDO_2 increases cytotoxicity, and apoptosis, which correlates with increases in caspases and Bcl2 protein and mRNA levels. In cell DU145(AR^-) cell transient transfected with a functional AR, the levels of cytotoxicity and caspase activity were decreased in the presence of BDO_2, but BDO_2-induced apoptotic protein expression was unaltered. This study provides evidence that micro-doses of BDO_2 modulate prostate cell toxicity by promoting apoptosis and tumor gene expression.

Keywords: BDO_2; Cytotoxicity; Gene Expression; Prostate; Micro-Dose

1. Introduction

1,3-Butadiene is a gas used commercially in the production of styrene-butadiene rubber, plastics, and thermoplastic resins with the major environmental source been incomplete combustion of fuels from mobile sources (e.g., automobile exhaust). Tobacco smoke can be a significant source of 1,3-butadiene in indoor air. The reactive intermediates 1,2-epoxy-3-butene, 1,3,4-diepoxybutane, and 3,4-epoxy-1, 2-butanediol all play significant roles in the toxicity of 1,3-butadiene. These metabolites are capable of reacting with macromolecules such as DNA to induce a variety of genotoxic effects in mice and rats as well as in human cells *in vitro* [1-6]. The metabolism and genetic toxicity of 1,3-butadiene and its oxidative metabolites in humans and rodents is well established. Experimental animal studies support the theory that butadiene and its metabolites are human carcinogenic agents [3, 7-10]. Theses animal studies have suggest a specie difference in the carcinogenicity of 1,3-butadiene in mice and rats. Tumor induced by 1,3-butadiene occurs in the hematopoietic system—heart (hemangiosarcomas), lung, preputial gland, liver, mammary gland, ovary, and kidney and prostate [9-13]. Although the tumors induced by 1,3-butadiene in these tissues are thought to be due to genotoxic alteration, the exact genes that are mutated or altered in each type of tumor are unknown.

The mechanism of tumor induction by 1,3-butadiene in rodents and humans may be due to its metabolism to DNA-reactive intermediates, resulting in genetic alterations in protooncogenes and/or tumor suppressor genes. What is known, however, is that there is a quantitative relationship between exposure to 1,3-butadiene, its genotoxicity, and the induction of cancer in occupationally exposed male workers. To date, there have been no studies that examined the effects of 1,3-butadiene and its metabolites Butadiene Diepoxide (BDO_2) on male reproductive development or development of the prostate gland. BDO_2, the most active metabolite of 1,3-BD, a potential human carcinogen, is released into the environment as a result of petroleum byproducts, smoking or combustion of gasoline products. Once in ambient air, it readily enters the body by several routs such as inhalation or absorption through the skin where it is metabolized to a BDO_2 by cytochrome P450s [4,13]. In the body, BDO_2 may induce reproductive toxicity in target tissue

such as ovaries, testis, and prostate [1,8,14]. However, the biochemical mechanism of BDO_2 toxicity in prostate and BDO_2's effects on prostate cell function are undefined. Therefore, the objective of this study was to define some of the cellular changes that are associated with BDO_2 toxicity in prostate cells under androgen sensitive (LNCaP(AR^+)) and androgen insensitive DU-145 (DU145(AR^-)) conditions. We examined the effect of butadiene diepoxide in prostate by assessing its effect on the growth of LNCaP(AR^+) cells, production and secretion of prostate secretary protein, androgen receptor status, and induction of androgen dependent genes.

2. Material and Methods

2.1. Cell Culture

LNCaP(AR^+) and DU145(AR^-) cells were obtained from ATCC (Rockville, MD). Cells were maintained in RPMI 1640 (LNCaP(AR^+) or Kaighn's modification of Ham's F-12 (F12-K) medium supplemented with 10% FBS, 0.2 mM glutamine, 100 U/ml of penicillin, and 100 mg/ml streptomycin. Cells were kept in 5% CO_2 in a water-jacketed incubator and were passaged using a trypsin/EDTA solution (Sigma-Aldrich, Inc.) when they reached 80% - 90% confluence.

2.2. Cell Viability Analysis

For experiments involving cell growth and gene induction, LNCaP(AR^+) or DU145(AR^-) cells were grown for five days in RPMI 1640 medium containing 5% FBS that was stripped three times with dextran-coated charcoal. Cells were then grown for 24 hr in Cellgro® serum free-medium. Cells were plated in 96-well plates (8×10^5 cells/well) and allowed to attach overnight. BDO_2 in 0.1% DMSO was added in a series of concentrations (0, 10, or 100 nM) to a 96-well plate. As a control and a reference, 10^{-8} M DHT and 100 ng/ml TNF-α were added to separate wells of each plate. Each treatment and time point had eight replicates. In each treatment, the final concentration of vehicle solvent did not exceed 0.01% v/v in the medium. After 24 h exposure to the test compounds, the effect on cell viability and gene expression was determined. Cytotoxicity was determined by the CellTiter 96® AQ_{ueous} One Solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. After incubation with 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), absorbance at 490 nm was measured using a EL_X800UV universal microplate reader (Bio-Tek, Inc.). Cell viability was calculated as $\left[\left(A_{490(\text{control})} - A_{490(\text{treatment})}\right) / A_{490(\text{control})}\right] \times 100$.

For Tryphan blue staining, cells were plated in 12-well plates (10,000 cells/well) and induced with 100 nM BDO_2 for 24 hr. After induction, cells were harvested, stained with Tryphan blue, and the number of cells was determined using a hemocytometer.

2.3. RNA Extraction and Real Time RT-PCR Analysis

Total RNA was obtained from cells treated with 0, 10, or 100 nM BDO_2, 10 nM DHT or 5000 nM Resveratrol in the presence or absence of 50-fold flutamide for 24 hr by lysing with 1.0 ml of TRI reagent (*in vitrogen*). RNA was isolated and Taqman PCR was performed on the cDNA samples using an ABI PRISM 7500 Sequence Detection system (Applied Biosystems) as previously described [15,16]. Briefly, for each gene tested (see **Table 1**), PCR was carried out in a multiplex mode with each 25 µl reaction containing 5 µl of cDNA reaction (~100 ng), An increase in fluorescence was obtained at the annealing and extension step at 60˚C. The relative level of expression of each gene in the samples was determined using the relative $2^{\Delta\Delta Ct}$ expression method as described in detail in the ABI PRISM Sequence Detection system User Bulletin 2 [17].

2.4. Fluorescence Microscopy

For microscopy, 5×10^5 cells (LNCaP(AR^+) or DU145(AR^-) were grown on microscope slides and induced with 0, 10 and 100 nM of BDO_2 for 24 h. On each slide, cells were stained for 5 min with 5 µl of a 0.1 µg/µl solution of acridine orange and ethidium bromide. Two fluorescence parameters, green emission from acridine orange (525 nm) and red emission from ethidium bromide (620 nm) were examined under a fluorescence light microscope (Nikon Optiphot, Melville, NY, USA) for the nuclear changes that are typically associated with apoptosis. An index of apoptosis was calculated as the ratio of the number of cells per microscopic field with early and late apoptosis characteristics in treated samples relative to the total number of cells per microscopic field.

2.5. Analysis of Caspase-3/7 Activation

Caspase 3/7 activity was determined using an Apo-One® Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) as previously described [16]. Caspase-3/7-like activity was determined based on proteolytic cleavage of rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide, Z-DEVD-R110) using 100 µg of total protein.

2.6. Western Blot Analysis

Immunoblot of the LNCaP(AR^+) or DU145(AR^-) protein fraction was performed as previously described [18,19]. The membranes were immunostained with the following antibodies: anti-Bcl2 0.5 µg/ml and anti-Bax 0.5 µg/ml

Table 1. Regulation of androgen receptor target genes by butadiene diepoxide (BDO_2) in prostate cancer cells.

Genes	mRNA expression relative to control (−BDO_2)									GO Gene description
	LNCaP(AR^+) 10 nM BDO_2—24 h			DU145(AR^-) 10 nM BDO_2 −24 h			DU145(AR^+) pCMV-hAR 10 nM BDO_2 −24 h			
	Fold	**SEM**	**N**	**Fold**	**SEM**	**N**	**Fold**	**SEM**	**N**	
AR	0.74	0.10	9	0.88	0.24	4	3.35	0.42	5	Androgen Receptor; Transcription factor that mediates transcription of genes required for development of male reproductive tissues
BCL2	0.85	0.12	9	1.87	0.70	5	1.91	0.65	6	B-Cell Leukemia/Lymphoma-2; An anti-apoptotic protein known to regulate apoptotic pathway and protect against cell death
BAX	2.44	0.78	9	2.85	1.46	5	2.72	1.18	5	Bcl2-Associated X-protein; A pro-apoptotic protein that regulates apoptotic pathway and promotes cell death
PSA	2.16	0.42	6	1.08	0.43	3	2.15	0.14	3	Prostate-Specific Antigen; Prostate tumor marker
TSC22	1.09	0.06	3	1.18	0.04	3	2.44	0.54	5	Tuberous Sclerosis 22; A leucine zipper transcription factor whose expression is induced by TGF-b
NKX3-1	0.66	0.07	3	3.11	1.44	4	1.25	0.39	5	NK3 Homeobox; A novel human prostate-specific, androgen-regulated homeobox gene associated with prostate cancer progression
B2M	2.42	0.54	6	2.23	0.86	5	3.35	0.75	6	β-2-microglobulin (B2M) is a secreted protein expressed in human prostate cancer cell lines and tissues. Serum B2M levels are elevated in patients with metastatic, androgen-independent prostate cancer

Gene expression was measured using the two-step Taqman Real Time RT-PCR as described in *Materials and Methods*. The relative expression of each gene was calculated by the $2^{\Delta\Delta Ct}$ method. First, relative quantitation mRNA expression was performed by first normalizing the Ct values of the particular gene amplification against the Ct values of endogenous 18 S rRNA then the resulting Ct values were normalized using the Ct value of the vehicle control sample. The relative expression of the 0.0 mM BDO_2 control for each gene was set to zero.

(Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-AR 1.5 µg/ml, anti-PARP 0.5 µg/ml and anti-GDPH 0.5 µg/ml (Cell Signaling, Danvers, MA). The immunoblot signal was captured using an AlphaInnotech Fluorochem HD 9900 (Alpha Innotech, San Leandro, CA) equipped with a CDD camera. The images were analyzed with the AlphaEaseFC software (AlphaInnotech, San Leandro, CA) and curves and graphs were fitted with GraphPad Prism 5.0 software (GraphPad, San Diego, CA) [20].

2.7. Immunofluorescence Staining

DU-145 cells were transiently transfected with AR expression plasmid (0.79 µg/µl pCMVh-AR) and 72-hr post transfection cells were induced with 100 nM BDO_2. Twenty-four hours post BDO_2 induction, cells were fixed with 100% methanol (−20˚C for 10 min) and then cross linked with 4% paraformaldhye at room temp for 10 min. The slides were blocked with 1% rabbit serum solution at room temperature for one hour. Slides were probed with anti-AR at a 1:100 dilution for 1 hr at room temperature, washed and then incubated with fluorescence-labeled anti-rabbit IgG (1:5000 dilution) for an additional hour at room temperature. For dual antibody staining, slides were washed with TBS-T and blocked in 10% sheep serum for 1 hr and probed with anti-tubulin (1:200) for 1 hr. The tubulin signal was developed by addition of Cy3-labeled anti-mouse IgG for 1 hour. Slides were washed with TBS and stained with prolong gold anti-fade reagent containing DAPI (4,6-diamidino-2-phenylindole). Slides were visualized using a Nikon Optiphot fluorescent microscope with green fluorescent (525 nm) and red fluorescent (620 nm) filters.

2.8. Statistical Analysis

All numerical data were expressed as mean ± SEM. In each assay, three or four measurements were made. Means for the treatment groups were compared using analysis of variance and Duncan's multiple range test ($P < 0.05$). To analyze the absorbance density from western blot data, a two-tailed t test ($P < 0.05$) was used to compare the mean ($n = 3$) for each treatment group with the mean for the untreated control group. The GraphPad Prism 5.0 software program (GraphPad, San Diego, CA) was used for the statistical analyses [20].

3. Results

3.1. The Androgen Receptor Negative Prostate Cells Are More Sensitive to BDO_2 Exposure

In the body, BDO_2 may induce reproductive toxicity in target tissue such as ovaries, testis, and prostate (Anderson 1998; Boffetta *et al*. 2009; Schmiederer *et al*. 2005). However, the biochemical mechanism of BDO_2 toxicity in prostate and BDO_2's effects on prostate cell function are undefined. Therefore, the objective of this study was to define some of the cellular changes that are associated with BDO_2 toxicity in prostate cancer cells under androgen sensitive (LNCaP(AR^+)) and androgen insensitive DU-145 (DU145(AR^-)) conditions. The first objective was to establish an appropriate response of BDO_2 in

prostate cells by determining whether micro-doses of BDO_2 exhibit any cellular effect on prostate derived LNCaP(AR^+) and DU145(AR^-) cells. To answer this question, we assessed whether micro-doses of BDO_2 were toxic to both LNCaP(AR^+) and DU145(AR^-) prostate cells. Exposure of prostate cancer cells to 10 nM BDO_2 for 24 hr, a dose similar to 6 hr of exposure to the parent 1,3-BD compound, significantly decreased cell viability in both cells lines (**Table 2**). We observed that 10 nM BDO_2 induced a 25% - 30% decrease in cell viability in LNCaP(AR^+) and DU145(AR^-) cells. In control experiments, exposing both cell lines to 100 ng/ml TNF-α (as a positive control for cell death in these cell lines) resulted in a 56% ± 11% and 41% ± 5.3% decrease in the cell viability of LNCaP(AR^+) and DU145(AR^-) cells, respectively. Surprisingly, exposure of prostate cells to a high concentration (100 nM) of BDO_2, produced only a marginal decrease (~7.0%) in cell viability in LNCaP(AR^+) cells but a significant decrease (41%) in DU145(AR^-) cells after a 24-hr exposure. These data demonstrate a dose-dependent effect of BDO_2 on cell viability in DU145(AR^-) and suggest that these cells are more sensitive to BDO_2 than LNCaP(AR^+).

3.2. BDO_2 Induces Apoptosis in Prostate Cancer Cells at Low Concentration

To corroborate the viability assessment of BDO_2 in prostate cells and reconcile the apparent difference in sensitivity between the two-cell lines, we investigated whether the cytotoxicity of BDO_2 was related to the apoptosis status of these cells. LNCaP(AR^+) or DU145(AR^-) cells (5×10^5) were grown on microscope slides, induced with 100 nM BDO_2, then prepared for microscopic examination as described in Materials and Methods.

Slides were examined under a fluorescent light microscope for the nuclear changes that are typical of necrotic or apoptotic cells (**Figure 1**). Fluorescent staining with acridine orange and ethidium bromide revealed signs of nuclear condensation, nuclei fragmentation, and membrane budding, which are all hallmark features of apoptosis, and fewer cells showing signs of necrosis. Microscopic examination of apoptotic cells revealed that there was a dose-dependent increase in the number of apoptotic cells following 24 hr exposure to BDO_2 (**Figure 1**). Examination of cells treated with BDO_2 under phase-contrast microscopy showed a dramatic decrease in cell number and cellular shrinkage in DU145(AR^-) cells as compared to LNCaP(AR^+) cells **Figure 1(a)**. The apoptotic index was determined by quantifying the relationship between concentration of BDO_2 and the number of apoptotic cells **Figure 1(b)**. We calculated the apoptotic index by averaging the number of apoptotic cells per field (60 - 70 cells) then dividing by the total number of cells per field (120 - 140 cells). A concentration of 100 nM BDO_2 resulted in a 19% ± 3% increase in apoptosis in DU145(AR^-) cells as compared to an increase of 10% ± 2% in LNCaP(AR^+) cells **Figure 1(b)**. Thus, DU145 (AR^-) cells were twice as sensitive to BDO_2 than LNCaP(AR^+), suggesting that the absence of the AR rendered these cells more susceptible to BDO_2-induced cytotoxicity.

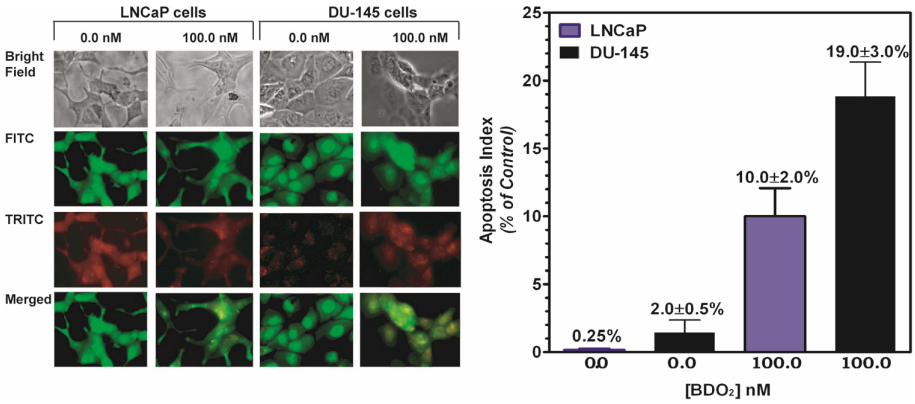
3.3. Androgen Receptor Protects Prostate Cells from BDO_2-Induced Toxicity

To demonstrate the importance of AR in the BDO_2-induced cellular effects in prostate cells, we transiently transfect the AR negative DU145(AR^-) cells with the full-length human wild-type AR cDNA pCMV expression plasmid. The transfected DU145(AR^+) cells were treated with and without BDO_2 for 24 hr and then cells were examined for morphological changes. In these experiments, un-transfected LNCaP(AR^+) and DU145(AR^-) cells were induced for 24 hr with and without BDO_2 and serve as positive and negative controls, respectively. (**Figure 2**, panels **(a)** and **(b)**). Immunocytochemistry analysis was performed on transfected and untransfected cells using anti-AR and anti-tubulin. Growth of untransfected DU145(AR^-) cells under serum starved condition (3X DCC media) or 3X DCC media plus BDO_2 for 24 hrs resulted in increased cell loss and disturbances of DU145(AR^-) cellular morphology (**Figure 2**, panels **(b)** and **(c)**). Transient transfection of these cells with a functional AR restored cell-cell contact, cell organization and increased cell numbers in the presence of BDO_2 (**Figure**

Table 2. Cytotoxicity of butadiene diepoxide (BDO_2) in prostate cells at micro-dose levels.

	BDO_2 (nM)			DHT (nM)	TNF-α (ng/ml)
Cell lines	0	10	100	10	100
LNCaP(AR^+)	100 ± 2.5 (24)	74.40 ± 6.11 (24)*	93.28 ± 4.67 (24) ns	123 ± 5.72 (10)*	56 ± 3.12 (12)*
DU145(AR^-)	100 ± 2.9 (23)	70.83 ± 2.69 (23)*	53.78 ± 2.62 (23)**	98.23 ± 4.62 (6) ns	41 ± 5.3 (6)*
DU145(AR^+)	100 ± 0.9 (10)	-	77.78 ± 2.62 (10)**	-	-

Cytotoxicity of BDO_2 in prostate cells was determined using MTT cell viability assay as described in Material and Methods. Note, results are reported as mean value ±SEM. (), number of sample. $^*p < 0.05$, control versus BDO_2 treatment in each cell line. Statistically significant differences were obtained using bonferroni's multiple test analysis. ns, not significant.



(a) (b)

Figure 1. BDO_2 induces apoptosis in prostate cancer cells at micro-dose levels. Cellular and nuclear morphology indicative of apoptosis induced by BDO_2 was examined as described in "Material and Methods". (a) Nikon Optiphot fluorescent microscope images taken with a 550 nm or a 620 nm filter. (First panel: Bright field, Second panel: Acridine Orange, Third panel: Ethidium Bromide, Fourth panel: a merger of second and third panels); (b) Apoptotic index. We calculated the apoptotic index by averaging the number of apoptotic cells per field (30 - 50 cells) then dividing by the total number of cells per field (120 - 140 cells). The results are shown as % change with respect to untreated cells. The values are the means ± SEM of three separate experiments performed in triplicate where five separate microscopic fields were examined.

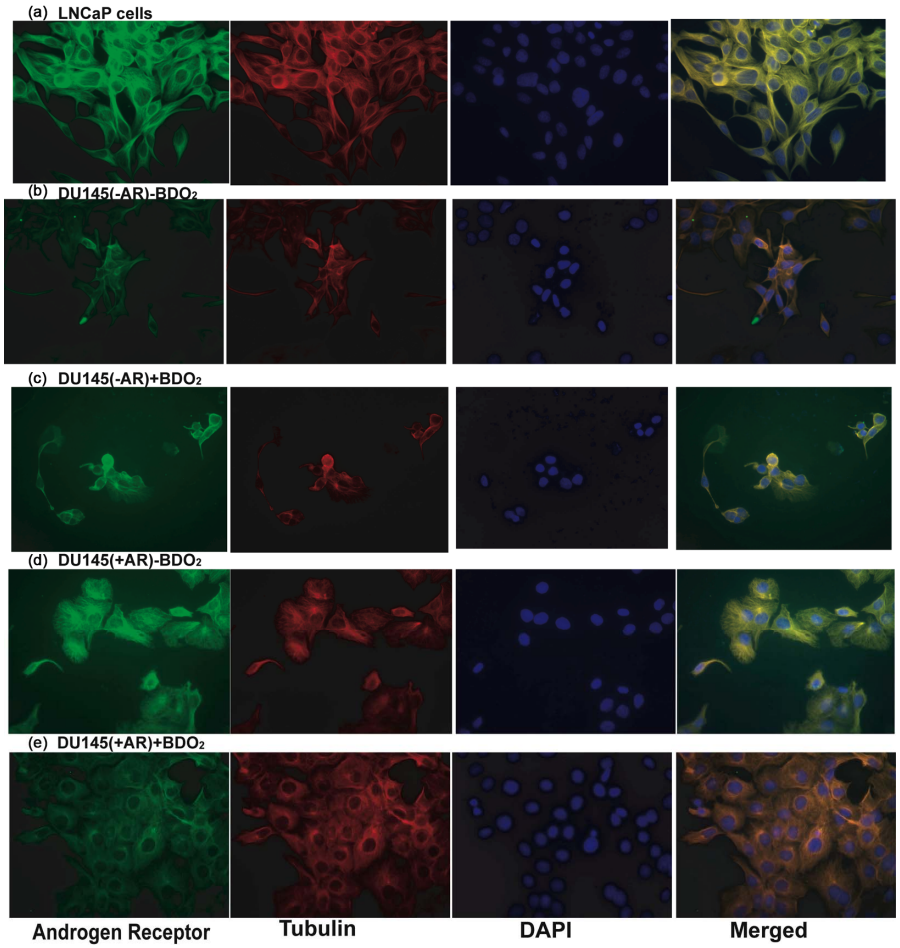


Figure. 2. Activation of androgen receptor function in cells transiently transfected with a CMV-hAR expression following BDO_2 exposure. AR transfected cells were induced with and without BDO_2, fixed with methanol and paraformaldehyde, stained with either anti-AR or FTCT-labeled anti-Tubulin antibody then the immunoflourescence detected using Cy3-labled secondary antibodies.

2, compare panels **(c)** and **(e)**). To corroborate our immunocytochemistry observations, we determined if transfection of a functional AR in DU145(AR^-) cells would render them less sensitive to BDO_2 exposure as observed in LNCaP cells. Therefore, DU145(AR^+) cell sensitivity to BDO_2 was assessed using cell viability and cell counting. Treatment of DU145(AR^+) cells with BDO_2 resulted in decreased toxicity (77.78% viable) as compared to untransfected DU145(AR^-) cells (54% viable) after 24 - 48 hr of exposure (**Table 2**).

3.4. BDO_2 Modulated the Expression of Down-Stream Apoptotic Executor Proteins

The process of apoptosis is well conserved in eukaryotic cells and involves both a receptor-mediated and a mitochondrial-mediated pathway. In prostate cancer cells, the presence of AR has been associated with increased cell survival [23]. Therefore, the levels of down-stream apoptotic executor caspase-3/7 and PARP cleavage were analyzed in both cell lines (**Figure 3**). The level of active caspase-3/7 was measured by proteolytic cleavage of rhodamine 110, from bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) in the Z-DEVD-R110 substrate and PARP cleavage was assayed by Western blot. Significant activation of caspase-3/7 was observed in both cell lines treated with BDO_2 (**Figure 3**). However, the DNA repair enzyme PARP was not activated following BDO_2 treatment as evidenced by the absence of the 89 kDa digest band (**Figure 4(b)**). We found that caspase activation paralleled the observed apoptotic effect of BDO_2 determined by morphological assessment (**Figure 1**). There was a 34% ± 7% and 60% ± 4% increase in caspase levels in LNCaP(AR^+) and DU145(AR^-) cells, respectively. Introduction of a function AR into DU145 (AR^-) cells resulted in a significant decrease in caspase expression and activity (**Figure 3**). There was a 10% to 12% decrease in caspase activity relative to control, which was similar to that observed with the RES, an know caspases inhibitor in prostate cells. These data strongly suggest that the presence of AR in DU145(AR^-) cells protects them from BDO_2-induced toxicity and/or improves cell morphology and integrity in the presence of BDO_2.

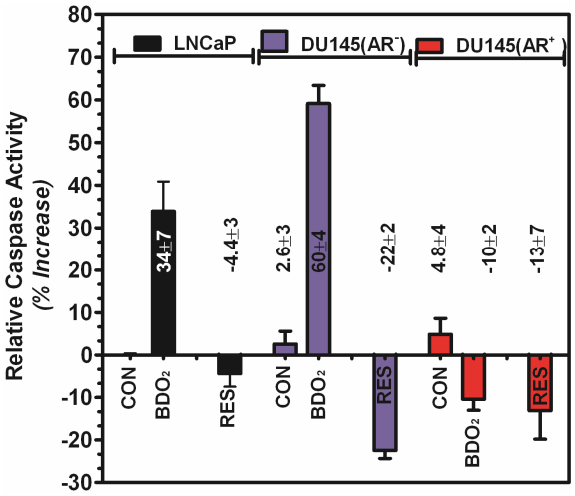


Figure 3. The presence of androgen receptor facilitates BDO_2-induced caspase activity in prostate cells. Cells (1E6) were grown in test medium, induced with 100 nM BDO_2 or 5 μM resveratrol (RES) for 24 hr and the resulting caspase-3/7 activity was determined using the Apo-One Homogeneous Caspase-3/7 assay as described in *Materials and Methods*. Caspase-3/7 activity was expressed as percent increase relative to untreated controls. The values are the means ± SEM of three separate experiments performed in quadruplets.

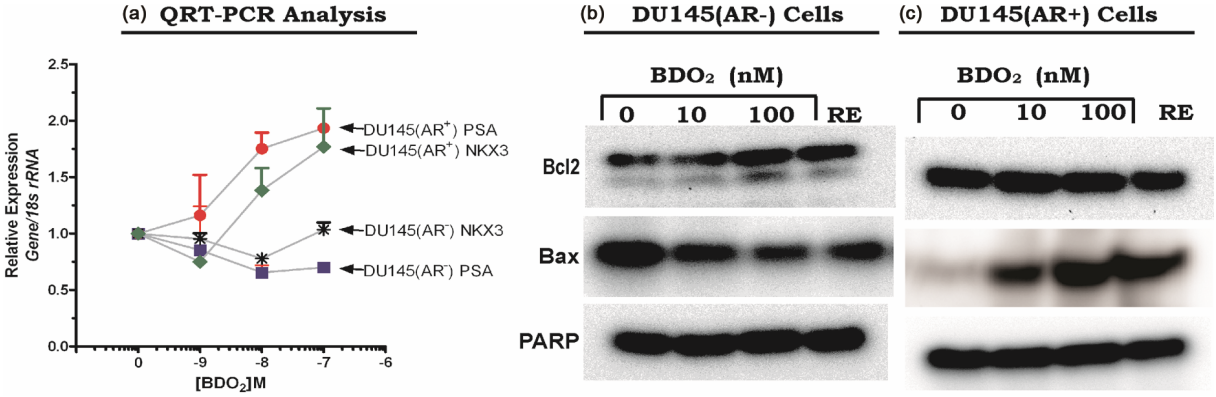


Figure 4. Activation of androgen receptor function in cells transiently transfected with a CMV-hAR expression following BDO_2 exposure. (a) Activation of androgen receptor related gene expression in DU145 cells following BDO_2 exposure. DU145(AR^-) cells or DU145(AR^{+}) cells were induced with 100 nM BDO_2 and DNase I-treated RNA, isolated from induced or un-induced cells, was subjected to two-step Taqman Real Time RT-PCR (see *Materials and Methods*). The relative expression of PSA mRNA expression was calculated by the $2^{\Delta\Delta Ct}$ method. DU145(AR^-) (b) and DU145(AR^+) (c) cells were grown in serum-free medium, treated with BDO_2 or resveratrol (RES) for 24 hr, and the levels of Bcl2, Bax, and PARP proteins were assessed by Western blot analysis. Details of the experiments are presented in *Materials and Methods*.

3.5. Androgen Receptor Restored Gene Expression but Not PSA Secretion in DU145(AR^+) Cells

To support the observation that AR mediates BDO_2 action and to validate the function of AR transfected into DU145(AR^-) cells, we examined two other AR-dependent processes, namely PSA secretion and AR-dependent gene expression (**Figure 4**). The parental DU145(AR^-) cells do not secret PSA due to the absence of functional AR protein in these cells. Therefore, we hypothesized that a gain of AR would restore PSA expression and secretion in DU145(AR^-) cells transfected with the wild type AR. To test this hypothesis, mRNA expression levels of two AR-responsive genes, PSA and NKX3-1, were measured by real-time quantitative RT-PCR in DU145 (AR^-) and DU145(AR^+) cells treated with BDO_2 for 24 hrs (**Figure 4**, and **Table 2**). There was reduction in the relative expression of PSA and NKX3-1 in the untransfected DU145(AR^-) cells following exposure to BDO_2 (**Figure 4**). Transfection of a functional AR into these cells resulted in a dose-dependent increase in the relative expression of PSA and NKX3-1 after 24 hr exposure to BDO_2 (**Figure 4(a)**). Transfection of AR into DU145(AR^-) cells increased the basal levels of both PSA and NKX3-1 by 1.5 to 2-fold above that observed in untransfected cells (**Figure 4(a)**). Analysis of the expression pattern of the pre- and pro-apoptotic proteins Bcl2 and Bax in DU145(AR^+) showed a doses-dependent increases in Bax protein levels in the presence of BDO_2. In DU145(AR^-) cells, we observed a high basal level of Bcl2, which increases by 50% - 60% after 100 nM BDO_2 treatment. The apparent increase in Bcl2 levels in DU145(AR^-) cells was transient and absence from DU145(AR^+) cell treated with BDO_2. In contrast, in DU145(AR^+) cells, we observed an decreases in Bcl2 levels with a corresponding increases in Bax protein expression (**Figures 4(b)** and **(c)**).

4. Discussion

Prostate cancer (PCa) development is influenced by factors such as increased age, ethnicity and exposure to environmental factors. Exposure of prostate cells to environmental compounds such as vinclozoline, PCB and cadmium has been characterized [30-32]. These compounds contribute to PCa through modulation of AR-dependent and estrogen-dependent pathways. We report for the first time a direct cellular effect of BDO_2 on prostate cancer cell function at micro-dose levels. We demonstrate that BDO_2 induces cellular toxicity by increasing apoptotic processes, while modulating the expression of genes involved in prostate cancer progression. We found that BDO_2 is able to modulate PCa cell activity via an apparent AR-pathway, although there are no known studies that indicate a direct binding of BDO_2 to AR. The AR-dependent activity of BDO_2 observed is best explained by BDO_2 covalently binding to AR through bifunctional cross linking, leading to ligand independent receptor activation. Such activation of AR would exemplify itself through increased gene expression of the AR cascade. We found that BDO_2 increased the expression of PCa tumor makers PSA, B2M and NK3. These data suggest that micro-doses of BDO_2 modulate prostate cell function at several levels and imply that BDO_2 is involved either in tumor induction in prostate cells or in tumor progression of (AR^+) prostate cancer cells.

One hypothesis for the induction of tumors by environmental compounds such as BDO_2 is that they function as tumor promoters by inhibiting the normal apoptotic processes in cells. The process of apoptosis is well conserved in eukaryotic cells and involves both a recaptor-mediated and a mitochondrial-mediated pathway. In prostate cancer cells, the presence of AR has been associated with increased cell survival because of enhanced Bcl2 protein expression [23]. To gain insight into the mechanism of BDO_2-induced cytotoxicity in LNCaP(AR^+) and DU145(AR^-) cells the dose-dependent expression levels of key apoptosis-related proteins were examined. Micro-dose concentrations of BDO_2 down-regulated anti-apoptotic Bcl2 expression and enhanced both the expression of the pro-apoptotic Bax protein and the activity of caspase-3/7. Interestingly, the activation of caspase-3/7 did not lead to PARP activation in either of the two prostate cell lines, despite an increase in apoptotic bodies in the presence of BDO_2. Failure to activate PARP under conditions where there is an increase in caspase-3/7 activity, a decrease in cell viability and an increase in apoptotic bodies suggests that some type of compensatory mechanism, which protects prostate cancer cells from the BDO_2-induced, mitochondrial-mediated apoptosis pathway, may be operating in these cells. We are currently testing the hypothesis that in BDO_2-treated cells, some type of autophagocytosis accompanies the activation of the mitochondrial apoptosis pathway or that BDO_2 is directly inducing autophagocytosis.

Tobacco smoke is an environmental source of BDO_2 which contributes directly to an increase in PCa [33]. Tobacco smoking produces constant low amounts of BDO_2 resulting in micro-dose exposure. Recent studies support the idea that smokers are more likely to develop aggressive PCa as compared to non-smokers [33]. Couple to this our current study, which provides evidence that exposure to micro-doses of BDO_2 may contribute to or modulate the development or progression of PCa. BDO_2 exposure increases cellular toxicity and alters apoptosis in prostate cells by increasing caspase activation without change in down stream targets such as PARP. Class I carcinogens such as BDO_2 have been

shown to induce apoptosis via death receptor pathways leading to inhibition of apoptosis. Thus, our work introduces the possibility that micro-doses of carcinogens such as BDO_2 may promote prostate tumors by modulation specific cellular activity.

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