Investigating the role of hydroquinone, benzoquinone and the AhR in benzene-initiated toxicities

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Introduction

Benzene is a leukemogen found in agents such as cigarette smoke, industrial solvents, and gasoline. In the mid 1900's chronic occupational exposure to benzene was correlated with aplasticaneamia and acute myelogenous leukemia[1]. Since then, occupational exposure to benzene has been drastically reduced and the required threshold exposure limit in the United States is currently 1 ppm per 8 hour day in a 40 hour week. However, a recent study has demonstrated that workers exposed to less than 1 ppm benzene, which is considered safe under U.S. occupational guidelines, had fewer white blood cells than unexposed workers[2]. In order for benzene toxicity to occur, it is thought that it must first be metabolized into a number of hydroxylated metabolites[3]. Evidence from several studies has suggested that hydroquinone (HQ) and benzoquinone (BQ) are the most toxic metabolites involved in the development of benzene toxicity[3]. Activation of the aryl hydrocarbon receptor (AhR) by environmental toxicants has been associated with carcinogenesis[4]. Activation of the AhR causes this receptor to translocate to the nucleus and bind regions of DNA known as dioxin response elements (DREs)[4,5]. AhR binding to DREs leads to enhanced transcription and upregulation of a number of genes including cytochrome P450 1A1 (CYP1A1)[4,5]. Interestingly, benzene-initiated hematotoxicity is absent in mice lacking both alleles for the AhR suggesting an imperative role for this receptor in benzene toxicities[5]. Using mouse hepatoma1c1c7 cells we investigated the role of this receptor in benzene initiated-toxicity utilizing the dual luciferase assay, immunofluorescence and western blot techniques. With these techniques we were able to evaluate the ability of benzene, HQ or BQ to cause AhR translocation to the nucleus, activate DRE regions within the mouse CYP1A1 promoter, and induce CYP1A1 expression.

Materials and Methods

Cell culture and treatment:

Wild type mouse hepatoma hepa1c1c7 cells (obtained from Dr. Julio Herrara, University of Minnesota) were propagated in a-MEM (with L-glutamine and without ribonucleosides, deoxyribonucleosides and sodium bicarbonate) supplemented with 10% FBS, 100 units/ml penicillin/streptomycin solution and 2.2% weight/volume sodium bicarbonate. Cells were treated with concentrations of benzene, HQ or BQ ranging from 0 µM to 1 mM over periods of time ranging from 1 to 24 hours. Cells were also treated with 120 µM B(a)P or 10 nM TCDD as positive controls.

Dual luciferase assay:

Dual luciferase assays were used to determine if benzene, HQ and/or BQ activate the DRE region of the CYP1A1 promoter. The reporter plasmid used to measure DRE activation was pGudLuc1.1 (kindly provided by Dr. Denison, University of California). This vector contains the 500 bp dioxin response domain from the 5'-flanking region of the mouse cytochrome CYP1A1 gene, which contains four DREs upstream of the mouse mammary tumor virus (MMTV) long terminal repeat promoter. The MMTV promoter drives transcription of the luciferase gene in the pGL2-basic vector (from Promega). A second reporter plasmid, phRLnull, was used to control for transfection efficiency. Hepa1c1c7 cells (2.5 X 10^4 cells/well) were transiently transfected with the pGudLuc1.1 and phRLnull vectors using FuGENE. Transfected cells were allowed to grow for 16 hours before media was replaced and B(a)P, benzene, HQ or BQ were added for an additional 24 hours. Cells were then lysed using passive lysis buffer and frozen at -20°C. Samples were analyzed using the Dual Luciferase Assay from Promega.

Immunofluorescence microscopy:

Hepa1c1c7 cells were washed twice with PBS and fixed for 20 minutes with 4% paraformaldehyde in PBS. Fixed cells were permeabilized with 0.05% TritonX 100 in PBS at 4°C for 10 minutes followed by a 1 hour incubation in blocking solution (1% BSA in PBS). Cells were then incubated for 1 hour at room temperature with rabbit polyclonal, affinity purified antibodies made to amino acids 61-419 of the murineAhR (kindly provided by Dr. R. Pollenz, University of South Florida). After being washed the samples were incubated with AlexaFluor 568 goat anti-rabbit IgG secondary antibody, DAPI and AlexaFluor 488 phalloidin for 1 hour (Molecular Probes).

Western blot analysis:

Total cell lysates were prepared, fractionated by SDS-PAGE and transferred to nitrocellulose membranes according to established procedures[6]. Membranes were blocked with 10% non-fat dry milk in Tris-buffered saline Tween (TBST) for 1 hour. Blots were then incubated with rabbit polyclonal IgG anti-CYP1A1 primary antibody (Santa Cruz) at 1:1000 dilution in TBST containing 5% milk. The blots were then washed (twice with TBST for 5 minutes then twice for 15 minutes) followed by incubation for 1 hour with anti-rabbit horseradish peroxidase-conjugated secondary antibody at a 1:1000 dilution. The blots were then washed and bands were visualized using an enhanced chemiluminescence detection system.

Results

Dual luciferase assay results:

Using dual luciferase assays with the pGudLuc1.1 plasmid in hepa1c1c7 cells our results demonstrate significant DRE activation after exposure to 300 mM HQ or BQ for 24 hours. DRE activation was not observed in cells exposed to benzene (Figure 1).

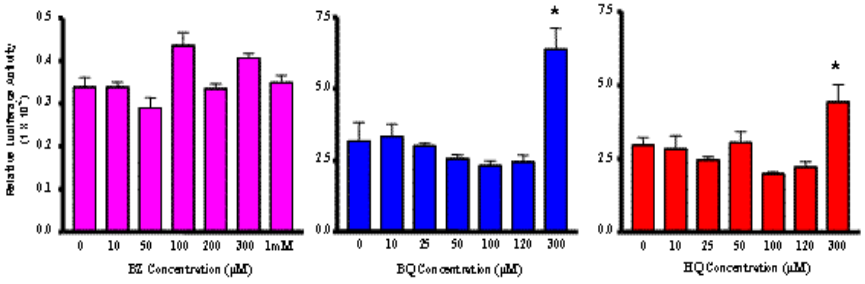

Figure 1. DRE activation in Hepa1c1c7 cells exposed to benzene (BZ; 0 mM to 1 mM), HQ (0 mM to 300 mM) or BQ (0 mM to 300 mM) for 24 hours. Significance indicated by *, $p<0.05$.

Immunofluorescence results:

Immunofluorescent staining of hepa1c1c7 cells demonstrated that under normal conditions, as expected, the AhR resides predominantly in the cytoplasm (Figure 2A). Our results show that after exposure to HQ (50 mM) for 90 minutes the AhR resides predominantly in the nucleus (Figure 2B).

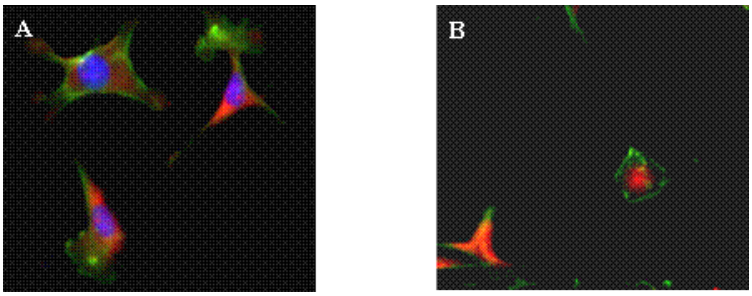

Figure 2. Immunofluorescence microscopy results. Hepa1c1c7 cells were stained for the AhR (red) after 90 min of

drug treatment. To verify morphology, cells were stained with phalloidin (green) and DAPI (blue). (A) Control; (B) 50 µM HQ with DAPI blue colour removed from picture in order to visualize the AhR.

Western blot results:

Western Blot analysis of cells exposed to BZ (10 mM), HQ (120 mM) or BQ (120 mM) for either 24 or 4 hours did not show induction of CYP1A1 (Figure 3).

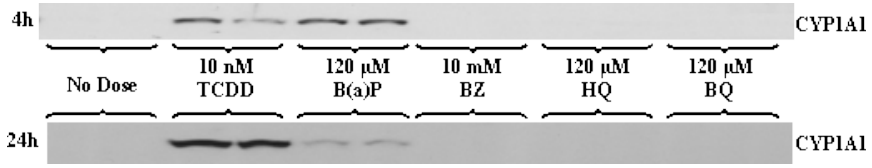

Figure 3. Western blot analysis of CYP1A1 protein expression in hepa1c1c7 cells. TCDD (10 nM) and B(a)P (120 mM) were used as positive controls.

Discussion

As a constituent of tobacco smoke, gasoline and automobile emissions there is a high potential for benzene exposure to the general population. Concerns over high benzene levels in the air from the oil industry and motor vehicle emissions have lead to many geographical and epidemiological studies, some of which suggesting a higher risk of developing leukemia in populations living in high traffic areas or near large petrol refineries[7]. The mechanism in which benzene causes hematotoxicities and leukemias is unknown. Studies utilizing AhR knock out mice have shown that the AhR is imperative for benzene to initiate toxicity[5]. In the present study we demonstrate that the highly reactive benzene metabolites HQ (300 mM) and BQ (300 mM) each significantly increase DRE activity in hepa1c1c7 cells at the mouse CYP1A1 promoter after 24 hours of exposure (Figure 1). In addition, our preliminary results have shown translocation of the AhR to the nucleus in hepa1c1c7 cells exposed to 50 µM HQ for 90 minutes (Figure 2). This evidence supports the hypothesis that HQ and BQ may be AhR agonists, which may be a possible mechanism underlying the development of benzene toxicity. However, through western blot analysis, benzene (10 mM), HQ (120 mM) or BQ (120 mM) did not elicit an induction of CYP1A1 protein expression after exposure for either 4 or 24 hours (Figure 3). A lack of CYP1A1 induction by HQ and BQ is interesting considering the positive results we demonstrated for these compounds in regards to AhR activation through the dual luciferase assay and indirectly through immunofluorescence. While studies evaluating CYP1A1 induction after exposure to a broader range of time points and concentrations of benzene, HQ or BQ are ongoing, these preliminary findings suggest a possibility of selective gene transcription through AhR activation by different compounds. Results from these studies will further our understanding of the molecular mechanism by which benzene and its metabolites cause toxicity and will help elucidate possible mechanisms behind leukemogenesis. In addition, broadening the awareness that benzene is a harmful and carcinogenic compound may help set higher safety standards for occupational exposure to benzene, ultimately making work environments more safe.

Acknowledgements

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