Alveolar epithelial microfilament assembly disrupted by 1,4-benzoquinone, a byproduct of diesel combustion

Anai Perez and Adriana J. LaGier

Grand View University, College of Social and Natural Sciences, Department of Biology, Des Moines, IA 50131

Introduction

Elevated hospital admissions and mortality in patients with preexisting pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, are increased by exposure to air pollutants, mainly those that are waste byproducts of gasoline and diesel motor vehicles (1). These pulmonary diseases are constantly causing traumatic injury to the epithelial cells lining the lungs. This causes the epithelial cells to migrate across the provisional matrix, a scaffold containing fibronectin and fibrin that directs the cells into the injury, to restore the epithelial integrity (2). It has been shown that diesel exhaust, a well-known air pollutant, delays the directed cell movement of lung epithelial cells (3). In this study, we sought to determine if a chemical component of diesel exhaust particles disrupted directed cell movement by inhibiting cytoskeletal functions. BQ, a product of the combustion of diesel as it passes through an engine, has been identified to be present in the exhaust of diesel and was utilized for this study to determine if it influences cell migration (3). We hypothesized that BQ would delay epithelial cell migration to seal the wound.

Materials & Methods

Cell Culture - A549 cells are human alveolar basal epithelial cells that were chosen because they have previously been utilized in similar air pollution studies (4). A549 cells were developed in 1972 by D. J. Giard (5) from cancerous lung tissue obtained from a 58 year old Caucasian male. The A549 cells (ATCC) were grown to confluent monolayer in a C60 tissue culture dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 µg/ml), and amphotericin B (0.5 µg/ml) in 5% CO_2 at 37°C. All chemicals were obtained from Fisher Scientific unless otherwise stated.

1,4-benzoquinone Stock Preparation - The 25 µM concentration of BQ shown previously to disrupt cytoskeleton (4) was bracketed by a factor of 5. To begin, we made a 20 mM stock (0.034g of BQ, at a molecular weight of 108.10, added up to 14.5 mL of water in a 50 ml tube. From previously stated concentration of stock, we made a working stock of 250 µM, 50 µM, and 10 µM utilizing DMEM.

Wound Scratch Model - We used a previously described *in vitro* scratch assay as an inexpensive method for analysis of cell migration (6). Once A549 cells were confluent, they were washed twice with Hank's Balanced Salt Solution (HBSS) and a small linear wound was lightly made with a 200 µl plastic pipette tip. All wounds were viewed immediately after creation by phase-contrast microscopy. Cells were subsequently washed with HBSS and placed in either 500 µl DMEM sans FBS [FBS(-) plate] or DMEM plus 1.0% FBS [FBS(+) plate]. In the first three wells per plate, 500 µl DMEM sans FBS was added to the culture media and was the no BQ control (Mock). The other sets of triplicate wells containing 500 µl media were treated with 500 µl of the 10 µM, 50 µM, and 250 µM working stocks in the same manner as the control [BQ5, BQ25, BQ125, respectively]. Measurements were then made using Image J software (7).The mean area represented the average percent of the initial wound. Values were normalized to time 0 values.

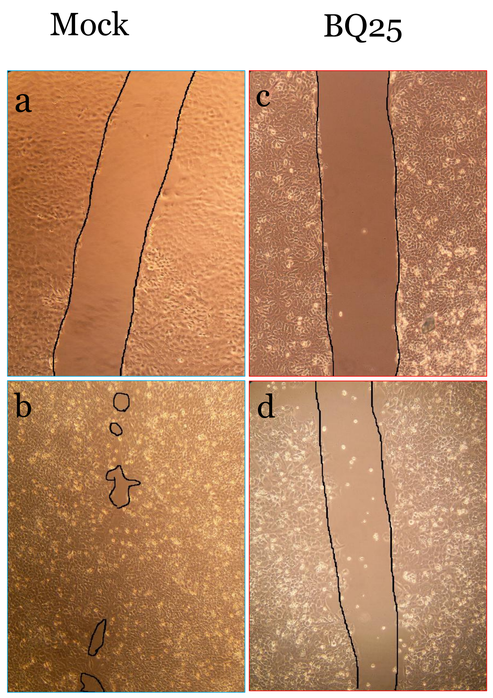
Whole Cell Lysates and Protein Quantification- After 30 minute incubation at 37°C, cells were washed & 1 mL of RIPA buffer (RIPA base+ inhibitors) was added to each well. C6-well plate was incubated on ice for 30 minutes and scraped with rubber policeman every 10 min. Whole cell lysate + RIPA buffer was centrifuged for 5 min. and supernatant was transferred to another tube. Standard curve for protein quantification was created using 1000 µL of working biuret reagent and protein standard (2 mg/mL) to create concentrations of 0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 (mg/mL). 500 µL whole cell lysates+1000 µL of biuret reagent were done to obtain concentrations. Spectrophotometer was used to obtain absorbance's at 540 nm after 20 minute incubation at 37°C .BQ(-) 30 min. had a concentration of .9408 µg/µL and BQ(+) had a concentration of 1.98 µg/µL.

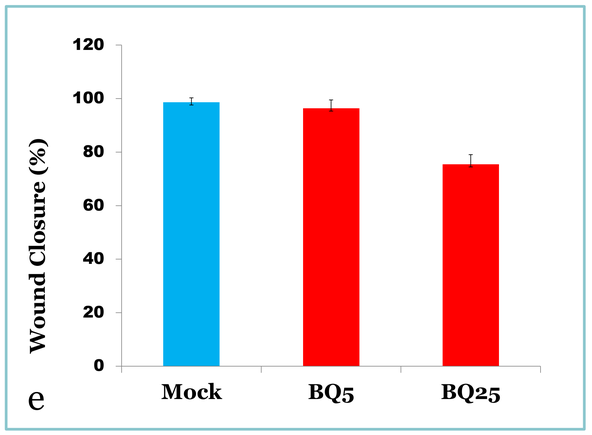
SDS-PAGE Gel Electrophoresis- 17 µg of each sample, water and loading buffer were loaded into the gel. It was a 1:5 ratio of loading buffer. Pre-cast 12% denaturing gel was used. 1X electrophoresis buffer was made from 10X electrophoresis buffer. Gel was run for 45 min and then was stained with SimplyBlue Safe stain. Analysis was was done with Image J.

Statistical Data- All data presented in graphs represent mean ± SD for triplicates in one independent experiment. Cell migration data represents one of three experiments with similar results. Data were analyzed by analysis of variance (ANOVA) and by Bonferroni's corrections for multiple comparisons, using Prism 4 (GraphPad Software Inc., San Diego, CA). Statistical significance shown when $p<0.05$.

Results

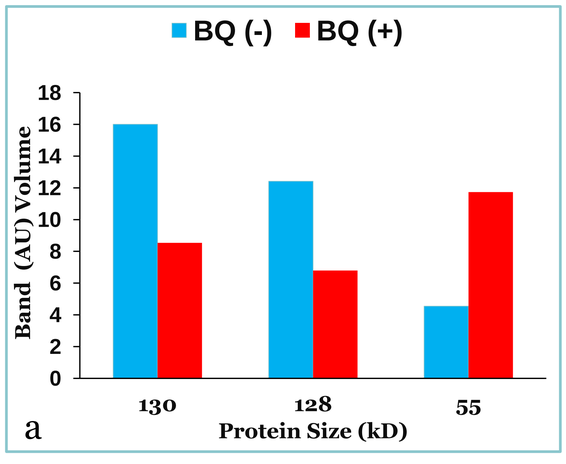
Figure 1: BQ alteration in Directed Cell Migration





Phase contrast microscopy provides visualization of BQ induced cell migration alterations.
Figures 1a and 1c : representative of wounded A549 cells on day 0 of treatment.
Figures 1b and 1d: Shows the wound after 4 days of migration.
Figure 1e: Statistical values of wound closure displaying decrease in cell migration by about 21 %.

Figure 2: Increased Volume of 55 kD Protein when Treated with BQ



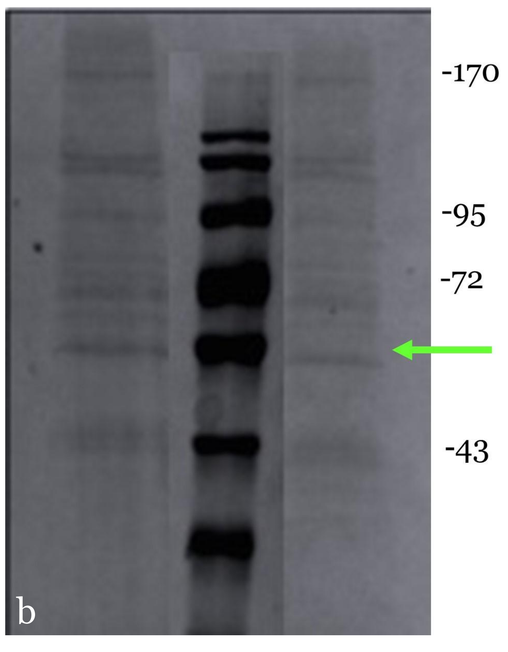


Figure 2a: Thirty minute incubation with (blue bar) or without (red bar) BQ treatment of wounded A549 cells. Protein size (kD) determined with SDS-PAGE shows BQ treatment an increased volume of a 55 kD protein but decreased others.
Figure 2b: Representative picture of a SDS-PAGE gel displaying BQ(-) and BQ(+) treated A549 cells at 30 minute incubation at 37°C . Arrows indicates band at about 55 kD with comparison to ladder (L).

Conclusions

The scratch-wound model showed that 1,4-Benzoquinone (a product made from using automobiles), at a concentration of 25 µM, altered directed migration of A549 lung cells, by affecting wound closure. In this study a new observation was made that wounded A549 cells incubated with BQ for 30 min. had an increase in a 55 kD protein. Corresponding decreases in proteins at 130 kD and 128 kD indicate that changes in protein levels were not driven by loading different amounts of total protein.

Future Directions

Cell migration of A549 lung cells can be altered by certain pollutants such as BQ because it affects diseases such as COPD, pulmonary diseases, and lung cancer(1). Further investigation of how cytoskeleton and membrane proteins of epithelial cells function, is important for creation of drug therapies.
Literature mining to characterize the unknown 55 kD band led to a protein called Src kinase whose relative size is 59 kD and is involved in cell migration (8)
Previous studies indicate ezrin (protein known to function in cell migration) is a substrate of Src kinase. A study done with A549 cells using inhibitors of Src kinase reduced metastasis, hallmark of cancer that requires cell migration(9).
We therefore hypothesize that an interaction between ezrin and Src kinase plays a role in BQ induced changes in cell migration.

Acknowledgments

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