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Local Effects of Two Intravenous Formulations of Pulmonary Vasodilators on Airway Epithelium

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BACKGROUND: Intravenous formulations of epoprostenol are frequently delivered via nebulizer to treat pulmonary hypertension in acutely ill patients. Although their efficacy as pulmonary vasodilators has been shown to be comparable to inhaled nitric oxide, the local effects of these formulations within the airways have not been determined. We hypothesized that the alkaline diluents of these compounds would lead to increased airway epithelial cell death and ciliary cessation. METHODS: Human bronchial epithelial cells were exposed to epoprostenol in glycine and arginine diluents or control fluid. Ciliary beat frequency, lactate dehydrogenase, and total RNA levels were measured before and after exposure. Results were compared between exposure and control groups. RESULTS: Ciliary beat frequency ceased immediately after exposure to epoprostenol with both diluents. Lactate dehydrogenase levels increased by 200% after exposure to epoprostenol and glycine diluent ($P = .002$). Total RNA levels were undetectable after exposure to epoprostenol and arginine, indicating complete cell death and lysis ($P = .015$). Ciliary beat frequency ceased after 30 s of exposure to epoprostenol and glycine ($P = .008$). There was no difference between cells exposed to epoprostenol and those exposed only to diluent. CONCLUSIONS: Exposure to intravenous formulations of epoprostenol in glycine and arginine caused increased cell death and ciliary cessation in bronchial epithelial cells. These findings suggest that undesired local effects may occur when these compounds are delivered as inhaled aerosols to patients. *Key words: airway pH; inhaled epoprostenol; inhaled prostacyclin; pulmonary vasodilator.* [Respir Care 0;0(0):1–●. © 0 Daedalus Enterprises]

Introduction

Inhaled nitric oxide (INO) and continuously nebulized inhaled epoprostenol have been used to treat pulmonary hypertension in neonatal, pediatric, and adult populations.[1,2] INO and epoprostenol exhibit similar effectiveness in increasing oxygenation, decreasing pulmonary artery pressure, and decreasing right-ventricular afterload in ARDS.[3-7] These effects have also been observed in patients who underwent cardiothoracic surgery.[8-10] In patients receiving heart or lung transplants, INO and epoprostenol are thought to decrease reperfusion injury while acting as a selective pulmonary vasodilator, improving hemodynamic indexes.[11]

Currently, INO is the most commonly used, FDA-approved inhaled pulmonary vasodilator, and it is approved for use in neonates (> 34 weeks gestation) with hypoxic

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respiratory failure and associated pulmonary hypertension.[12] Administration of INO outside of this indication is off-label but common across all age groups. Increasing health care costs coupled with declining reimbursements rates have led to efforts to decrease direct-delivery costs of INO. Continuously nebulized inhaled epoprostenol may be a cost-effective alternative to INO; the cost of INO is approximately $220.46/h compared to an hourly cost of $2.01–$10.05 for inhaled epoprostenol for a 70-kg adult patient.[2,13] These potential cost savings have led to interest in substituting inhaled epoprostenol in patients who would otherwise receive INO.[10]

Although there is evidence that epoprostenol offers clinical benefits similar to those of INO, safety data are very limited. Significant safety concerns remain regarding the continuous nebulization of epoprostenol, including ventilator malfunction, unrecognized nebulizer failure, and a lack of mandated back-up delivery systems.[1,2] Further, preparations of epoprostenol with either glycine or sucrose/L-arginine buffers have exceedingly high pH levels of 11–13, which may have negative effects on airway epithelia including increased inflammation, ciliary dysfunction, decreased transport of bronchodilators, and cell death.[1,14-19] An independent third-party report highlighted potential technical errors, which include infusion pump misconnections, over- or underdosing, a lack of alarms on nebulizers to identify stoppage of dose delivery, and ventilator component malfunction leading to auto-PEEP and pneumothorax.[20]

Given the alkaline properties of epoprostenol and its unknown effects on airway epithelium, we sought to evaluate the effects of glycine-buffered epoprostenol (EPO-GLY) and sucrose/L-arginine-buffered epoprostenol (EPO-ARG) on inflammatory biomarkers in differentiated human airway cells. Our group previously reported that human airway epithelial cells can be grown in an air-liquid interface that allows them to differentiate fully into functioning segments of airway epithelium.[21,22] This model allows for a controlled evaluation of airway epithelial function and response without systemic artifact. We hypothesized that exposure to EPO-GLY and EPO-ARG would increase levels of lactate dehydrogenase (LDH), a validated marker of cell stress and death, and that the effects seen in the epoprostenol groups will be caused by the alkaline GLY and ARG diluents as opposed to the epoprostenol.[23,24] We further hypothesized that exposure to these compounds would decrease airway epithelial ciliary beat frequency.

QUICK LOOK

Current knowledge

Intravenous formulations of epoprostenol are routinely delivered via nebulizer to acutely ill patients and are effective as pulmonary vasodilators. Concerns exist regarding their safe delivery and local effects within the airways.

What this paper contributes to our knowledge

Exposure of intravenous formulations of epoprostenol that are commonly delivered by nebulizer to the airway epithelium caused increased cell death and decreased ciliary beat frequency when tested in vitro. These effects were due to the high pH of the intravenous formulation rather than the epoprostenol itself.

Methods

Primary normal human bronchial epithelial cells used for all experiments described were from a healthy, adult, non-smoker donor (Lifeline Cell TechnologyConnecticut). We used the BronchiaLife Basal Media (Lifeline Cell Technology, Frederick, Maryland) during cell growth, and we used homemade differentiation media, as described previously, during cell differentiation.[21,22] Cells were differentiated on 6-mm Corning Transwells (Corning, New York) at 200,000 cells per well as previously described by our group and others.[21,22] Differentiation as pseudostratified, ciliated, columnar epithelial cells was verified with direct visualization of ciliary movement with a 20× microscope.

LDH protein levels were evaluated in culture media using commercially available Pierce LDH cytotoxicity colorimetric assay kits (Thermo Fisher Scientific, Waltham, Massachusetts). Assays were run according to the manufacturer's specifications in undiluted media. Total RNA levels were evaluated in airway exposure fluids using a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) as previously described by our group.[21]

Under 20× magnification with a microscope, ciliary motion was directly visualized uniformly across all of the cell culture. We also recorded ciliary motion on video using an iPhone SE (Apple, Cupertino, California) mounted on a Gosky Universal Adapter Mount (GOSKY Optics, Zhejiang, China) after locking focus and exposure.

Each video was transformed to grayscale (0 = black, 1 = white). Regions of interest were identified for each video by recording the coordinates of clusters of cells that demonstrated motion, and these regions were used for subsequent ciliary beat frequency analysis. Preference for each region of interest was given to cells where the cell wall was most clearly visible and not obscured by other cells. Next, individual regions were plotted (Fig. 1), and the number of peaks was calculated over the measurement epoch. The frequency was determined by adding the number of peaks and

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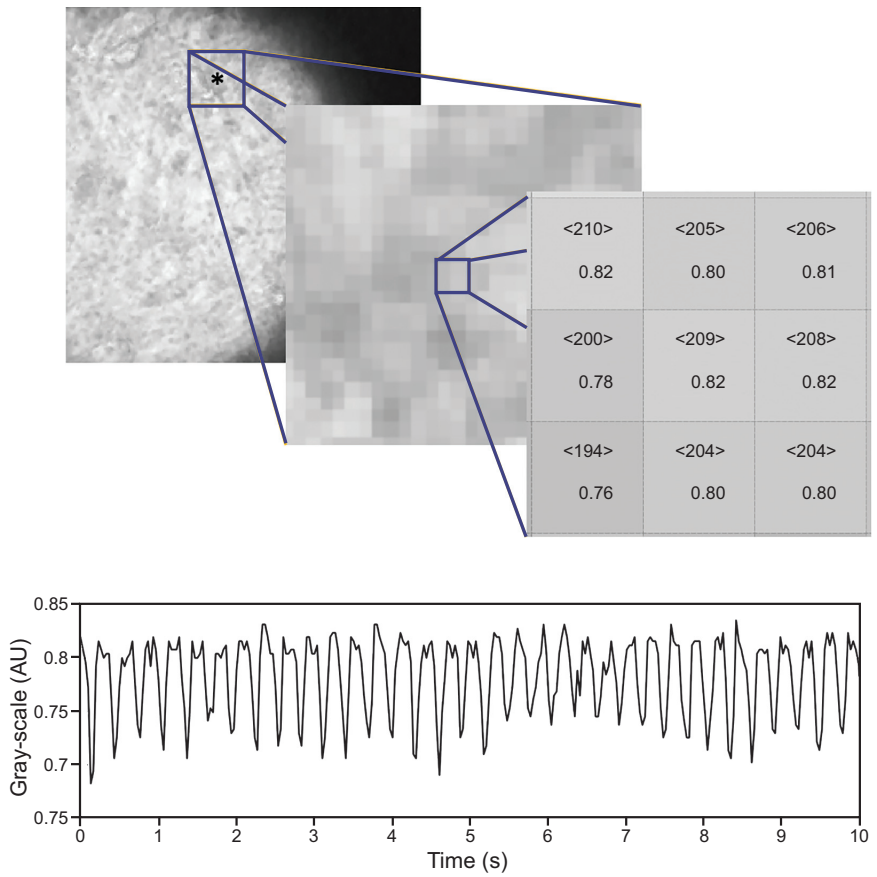

Fig. 1. Example of the calculation of ciliary beat frequency. First, a region of interest is manually recorded based on observed cell motion (indicated by ∗). The motion of the cells is analyzed by plotting the grayscale intensity over time. Ciliary beat frequency is calculated by adding the number of peaks detected and dividing by the total time of the recording.

dividing by the sampling time provided in the video (15 s for each video). In the cases where no cell motion could be detected by the software, this was noted in the study results.

After epithelial segments were fully differentiated and ciliary motion was confirmed, the Transwells were divided equally into control and exposure groups. The pH of all exposure fluids was measured with a standard Mettler Toledo pH probe immediately prior to exposure and were confirmed to be 7 for the phosphate-buffered saline (Wet Control), 12 for the EPO-GLY and GLY, and 12.9 for the EPO-ARG and ARG, as indicated on their package inserts. All groups other than the Dry Control group were exposed to 40 μL of sterile phosphate-buffered saline (ie, the minimum volume that uniformly coated the airway segments) to allow for uniform ciliary beat visualization. Video was recorded prior to exposure to establish a baseline. Following baseline establishment, 10 μL of Wet Control, GLY, ARG, EPO-GLY, or EPO-ARG was added to each group. Then epoprostenol (30,000 ng/mL concentration) was used in both epoprostenol groups because this concentration is commonly used in vivo. A Dry Control group was included in the protein analysis to determine any cell stress caused solely by exposure to fluid. Cells were visualized throughout the exposure with 20× magnification, and video was recorded. After 2 min of exposure, exposure fluids and cell culture media were removed and stored at –80°C until LDH and total RNA measurement occurred.

Statistical Analysis

Sample sizes of 4 per group were determined to be appropriate to achieve a level of significance of .05 and a power of 0.80 with an effect size of 0.9 (G*Power Software, Kiel University, Kiel, Germany). Statistical analysis was performed using SigmaStat (Systat Software, San Jose, California). The unpaired Student 2-tailed t test was used to compare LDH protein levels in control groups and stimulation groups. The Wilcoxon rank-sum test was used for non-Gaussian distributed data as determined with the Shapiro-Wilk test. The Wilcoxon matched-pairs signed rank test was used to assess differences in ciliary beat frequency between the control group and the treated group. A P value of $< .05$ was considered statistically significant. A Bonferroni correction was used to account for multiplicity.

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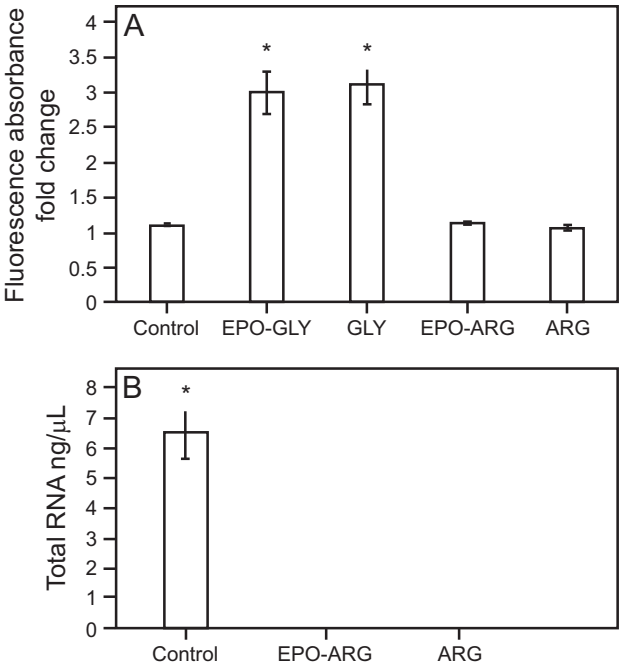

Fig. 2. A: Cells exposed to glycine-buffered epoprostenol (EPO-GLY) and glycine (GLY) released 200% more lactate dehydrogenase than the control group. No lactate dehydrogenase was measured from cells exposed to sucrose/L-arginine-buffered epoprostenol (EPO-ARG) or arginine (ARG). B: No RNA is detectable in the EPO-ARG and ARG cells after exposure, indicating complete death and lysis of cells.

Results

LDH levels in groups exposed to EPO-GLY and GLY were > 200% higher than the levels in the Wet Control group and the Dry Control group (Fig. 2A, $P = .002$). Exposure to EPO-ARG and ARG did not increase LDH levels compared to either control group, although ciliary cessation and cellular debris were noted within 30 s of exposure to these compounds. The exposure fluids of these groups were therefore analyzed for total RNA to determine if cell death occurred uniformly. Total RNA levels were undetectable in EPO-ARG and ARG exposure groups compared to control groups, indicating complete cell death and lysis (Fig. 2B, $P = .015$).

After 30 s of exposure to EPO-GLY, EPO-ARG, GLY, and ARG, all ciliary motion ceased by direct visualization. When evaluated using digital video analysis, the GLY exposure resulted in a reduction in mean ciliary beat frequency from 4.7 ± 1.3 Hz to 0 ± 0 Hz in control (Fig. 3, $P = .008$). Although ciliary motion was confirmed visually, the frequency analysis on the ARG exposure group could not be performed due to insufficient number of cilia detected by the software in these exposure videos.

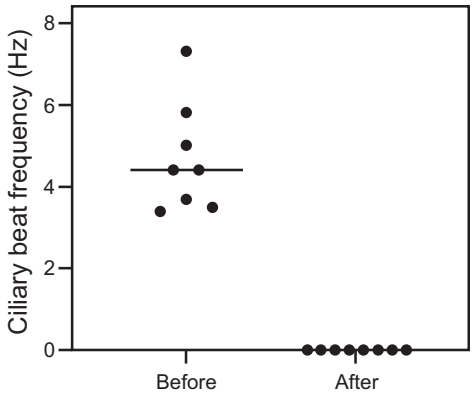

Fig. 3. Ciliary beat frequency before and after exposure to glycine-buffered epoprostenol (EPO-GLY). Ciliary beat frequency decreased to zero after exposure to EPO-GLY. $P = .008$.

Discussion

In this in vitro study, we describe the effects of 2 preparations of epoprostenol and their diluents on ciliary beat frequency and cell viability in healthy human bronchial epithelial cells. Apical exposure of pseudostratified, ciliated, columnar epithelium to epoprostenol simulated the conditions of airway exposure to these compounds from continuous aerosol delivery during invasive mechanical ventilation. Ciliary cell exposure to EPO-GLY, EPO-ARG, GLY, or ARG resulted in a statistically significant reduction in ciliary beat frequency and increased cell death compared to control cultures. There was no difference in outcomes between EPO-GLY and GLY or between EPO-ARG and ARG, suggesting that these results are caused by the ARG and GLY diluents rather than by the epoprostenol itself, although this cannot be completely ruled out. To our knowledge, this is the first study to demonstrate the direct effect of these compounds on airway cells.

Drug delivery can be inconsistent. The efficacy of any aerosolized medication is highly variable due to the efficiency of different nebulizers, physicochemical properties of different medications, and the various aerosol-generation technologies (ie, ultrasonic, vibrating mesh, and jet).[25,26] Nebulizers generate a range of particle sizes that have a direct effect on the effectiveness of the aerosolized medication.[27,28] Particles > 5 μm in diameter generated by the nebulizer are more likely to be deposited in the ventilator circuit and large airways.[29,30] Importantly, for nebulized medications that have a desired mechanism of action at the level of the alveolar-capillary membrane (eg, pulmonary vasodilators), deposition in large airways offers limited or no clinical benefit and carries a potential increased risk of adverse effects. Further, variability in nebulized particle size and the resultant changes in distribution of drug deposition lead to clinically important differences in physiologic response.[1] However, further work in this area is required.

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Previous studies have noted unwanted side effects such as a decrease in systolic blood pressure requiring clinical intervention in up to 30% of pediatric subjects receiving aerosolized epoprostenol.[31] However, the hemodynamic profile of adult subjects receiving aerosolized epoprostenol has been shown to be stable. This may be due to differences in volume distribution, ratio of surface area to body mass, and differences in absorption kinetics of the neonatal and pediatric populations compared to adults.[8,9,11,32]

The high pH of the diluents for epoprostenol may have undesirable effects on airway epithelium.[1,14-19] Large particles (ie, $\geq$ 9.5 μm in diameter) are reported to have an increased rate of deposition in the trachea.[29] This suggests that the proportion of aerosolized particles of large size would have an increased probability of collecting in the trachea and large conducting airways where ciliary function is essential. Poor nebulizer function and changes in aerosol output may increase heterogeneous deposition of medications in the respiratory tract and increase the proportion of these droplets. Of note, a single 2.5-mL nebulization of a glycine buffer formulated for inhalation and buffered to a pH of 9.8 is sufficient to alter airway pH.[33] Our findings suggest that continuous exposure to these aerosolized diluents may result in airway cell death and decreased mucociliary clearance, but clinical studies are needed to assess this possibility.

This is not the first report of therapeutic agents that have the potential to injure the airways when delivered as aerosols.[34] Unknown risks should be assumed when intravenous drug formulations are used for inhalational administration because the topical effects of these formulations on airway epithelium may not be known. We found a 200% increase in LDH levels in cells exposed to EPO-GLY and GLY and complete cell death according to RNA quantification in those exposed to EPO-ARG and ARG. These results suggest that the direct effect of high-pH solution on airway tissue results in cell death, although other mechanisms of cell death cannot be completely ruled out. We also observed complete cessation of ciliary beat frequency after exposure to these compounds. Mucociliary function is important in patients recovering from airway infection or respiratory failure. Decreasing mucociliary clearance and increasing cell death may worsen pulmonary system function; patients with ARDS or other critical pulmonary illness, who are often the recipients of inhaled vasodilators, may be even more susceptible to the effects we observed.[35]

In addition to the direct effects of epoprostenol and its diluents demonstrated in this study, patient safety concerns have been raised concerning the continuous nebulization of epoprostenol, such as technical sources of error, adverse effects, and physiologic deterioration.[31,36,37] Reported sources of technical error include the misconnection of infusion pumps, which can lead to over- or underdosing; nebulizers that lack alarms to alert the clinician of therapy interruption, which can result in physiologic deterioration; and ventilator malfunction from increased expiratory resistance, which limits exhalation and may result in pneumothorax.[2,20] Technical risk factors need to be further explored to develop processes to decrease the likelihood of adverse events.

This study had several limitations. As with all in vitro studies, the findings may be subclinical or not representative of clinical effect. However, these results suggest that consideration of unwanted effects of aerosolized epoprostenol with high-pH diluents is important. To control a uniform amount of drug exposure in each group, we elected to introduce the epoprostenol and diluents to the airway epithelia by volumetric pipette instead of via a nebulizer. This likely simulates the deposition, rainout, and collection of aerosolized medication in the larger airways more accurately than the entire delivery of aerosolized compounds to the targeted lower airways. In the future, it may be helpful to design an experiment that includes nebulization as a mechanism of introducing the agents. In addition, the method of ciliary motion measurement by digital video file analysis requires further optimization. Although ciliary motion was verified with direct visualization, the video files were unable to capture this movement in the ARG group. Higher-resolution cameras may make this technique more robust.

More work is needed to better understand the clinical effect that high-pH preparations have on airway tissue and relevant clinical outcomes. There is some debate in the literature as to the most effective aerosolized pulmonary vasodilator. It is recommended that nebulization of prostacyclin analogs approved and available in inhalational preparations should be considered for administration via the respiratory tract as a first-line aerosolized therapy for pulmonary hypertension or hypoxic respiratory failure.[1,2] Agents such as iloprost, which is pharmacologically similar to epoprostenol with lower viscosity, greater stability, a longer high-life, and more physiologic pH may be a more suitable consideration.[38]

Conclusions

To our knowledge, this is the first report of airway epithelium exposure to EPO-GLY and EPO-ARG. This exposure resulted in rapid cell death and cessation of ciliary movement in vitro. These results were observed even in the absence of epoprostenol, suggesting they are likely due to the high pH of the GLY and ARG diluents. Future clinical studies are indicated to assess the clinical importance of these findings.

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