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Evaluation of an in vitro screening model to assess phosgene inhalation injury

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ABSTRACT
Therapeutic development against exposure to toxic gases is hindered by the lack of appropriate models to evaluate candidate compounds prior to animal efficacy studies. In this study, an in vitro, air-liquid interface exposure model has been tested to examine its potential application for screening treatments for phosgene (carbonyl chloride)-induced pulmonary injury. Epithelial cultures on Transwell® inserts, combined with a Vitrocell® exposure apparatus, provided a physiologically relevant exposure environment. Differentiated human bronchial epithelial (16HBE) cultures were exposed for 8 min to phosgene ranging from 0 to 64 ppm and assessed for changes in transepithelial electrical resistance (TEER, epithelial barrier integrity), cellular viability (XTT) and post-exposure (PE) cellular metabolic energy status. Exposure to phosgene concentrations >8 ppm caused dose-dependent and significant decreases in TEER and XTT which did not recover within 24-h PE. In addition, at 64 ppm the rate of oxidative glutamine metabolism was significantly inhibited at 6 and 24 h after exposure. Glycolytic activities (glucose utilization and lactate production) were also inhibited, but to a lesser extent. Decreased glycolytic function can translate to insufficient energy sources to counteract barrier function failure. Consistent and sensitive markers of phosgene exposure were TEER, cell viability and decreased metabolism. As such, we have assessed an appropriate in vitro model of phosgene inhalation that produced quantifiable alterations in markers of lung cell metabolism and injury in human airway epithelial cells. Data indicate the suitability of this model for testing classes of anti-edemagenic compounds such as corticosteroids or phosphodiesterase inhibitors for evaluating phosgene therapeutics.

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RESEARCH ARTICLE

Introduction
Phosgene (carbonyl chloride COCl₂) is a heavier-than-air gas with the major route of exposure being inhalation. Toxic industrial chemicals (TICs), such as phosgene are commonplace in manufacturing. Since WWII phosgene has gained widespread use in industry as a chemical precursor in the production of pharmaceuticals, dyes, pesticides and polyurethane for foam rubber products. Although phosgene is nearly completely spent during industrial use, it can be released through failed processes. Moreover, the liberation of phosgene through the thermal decomposition of chlorinated hydrocarbons can pose a threat for welders, refrigeration mechanics and car repairmen (Borak & Diller, 2001). In addition, commonly used industrial degreasers contain chlorinated hydrocarbons, such as perchloroethylene, which can form phosgene when heated.

Phosgene manifests its toxicity mainly through acylation of proteins, but also through limited hydrolytic production of hydrochloric acid (Borak & Diller, 2001). Amino, hydroxyl and sulfhydryl groups appear to be the primary biomolecular targets for acylation (Babad & Zeiler, 1973), leading to marked inhibition of enzymes related to energy metabolism and to collapse of the air-blood barrier. Sub-lethal exposure to phosgene results in several distinct clinico-pathological phases consisting of pain in the eyes and throat, tightness in the chest, shortness of breath, wheezing and coughing (Zhu, 1985). Hypotension, bradycardia and, rarely, sinus arrhythmias can also occur. Ultimately, the latent effects of phosgene inhalation can cause non-cardiogenic pulmonary edema in humans resulting in death.

Although a variety of therapeutic strategies for phosgene-induced acute lung injury have been described in the literature (Borak & Diller, 2001; Diller, 1985; Diller & Zante, 1985; Grainge & Rice, 2010; Sciuto & Hurt, 2004), the mechanisms underlying phosgene-induced acute lung injury are imprecisely understood. Human in vitro lung models have proven useful for studying the mechanisms of lung injury (Hays et al., 2003), specifically using cultured 16HBE cell lines for sulfur mustard toxicity models (Pohl et al., 2009; Ray et al., 2008). Progress is being made toward in vitro-in vivo association for pulmonary absorption and in the use of cultured respiratory cells to evaluate toxicity and targeted therapeutic strategies (Aufderheide et al., 2003a,b). The work herein complements earlier in vitro research into the evaluation of the toxicity of phosgene applied directly to cultured cells.

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In human lung small airway epithelial cells, Cowan et al. (2004) showed that there was a low dose-dependent release of the inflammatory maker IL-8 at 24 h PE. More recently, Wijte et al. (2011) using human A549 epithelial cells, found that glutathione, alamarBlue© reduction and heme oxygenase 1 were decreased at 24 h following an exposure to 2 ppm phosgene.

We have tested a human lung/air-liquid interface exposure model to identify mechanistic targets such as the relationship between barrier function and metabolic pathways fundamentally involved in energy consumption. The model consists of human airway epithelial cell cultures grown on Transwell® inserts and exposed to various concentrations of phosgene gas using the Vitrocell© air/liquid exposure system (Aufderheide et al., 2003a). This system represents the most physiologically relevant in vitro exposure methodology available and has been used to test the toxicity on human lung cell lines following sulfur mustard exposure (Pohl et al., 2009). To test the model’s relevance, the exposed cultures were assayed for cellular viability (XTT) and for transepithelial electrical resistance (TEER), an indicator of epithelial barrier function, as well as the activities of glutamine and glycolysis metabolism.

Materials and methods

Physiological human in vitro lung model

16HBE14o-transformed human bronchial epithelial (16HBE) cell cultures were prepared on 12 mm polyester Transwell® inserts (Corning, Lowell, MA) and maintained in RPMI 1640 supplemented with 9% fetal bovine serum, L-glutamine and penicillin/streptomycin under standard culture conditions. All culture reagents were purchased from the American Type Culture Collection (ATCC, Manassas, VA). 16HBE cultures were a generous gift from Dr. Dieter Gruenert of the California Pacific Medical Center Research Institute and the University of California, San Francisco. Cells were maintained approximately one week until fully confluent (~6.5 ± 1.7 × 10^5/well) and TEER exceeded 500 Ω/cm^2.

Vitrocell® air/liquid exposure system

An air/liquid interface exposure apparatus was purchased from VitroCell Systems (Waldkirch, Germany) and teamed with a Brooks Instruments (Hatfield, PA) mass flow controller (MFC) and KNF Neuberger (Trenton, NJ) vacuum pump to create a compact exposure setup capable of being used entirely within a chemical safety hood. Phosgene (10%) in balance with nitrogen) was obtained from Matheson Tri-Gas (Montgomeryville, PA). Phosgene mixtures of varying concentrations were produced by mixing 10% phosgene with filtered dilution air from a compressor (Jun Air, Norresundby, Denmark) in calculated ratios. Gas ratios were controlled by additional Brooks mass flow control units, and gas concentrations were verified with a Foxboro Miran 1A gas analyzer (Inyesys Process Systems, Plano, TX). Gas mixture, exposure flow rates and system vacuum were controlled and monitored using a custom virtual instrument created in LabVIEW© (National Instruments, Austin, TX).

Lung culture exposure

Epithelial cultures on Transwell inserts were measured for TEER (described below), and the apical medium was aspirated to produce an air/liquid interface at the cell surface. Inserts were placed into the exposure apparatus, in basal contact with culture medium maintained at 37 °C. Three cultures at a time were simultaneously exposed to phosgene concentrations between 0 ppm, 1 ppm (4 mg/m^3), 2 ppm (8 mg/m^3), 4 ppm (16 mg/m^3), 8 ppm (32 mg/m^3), 16 ppm (64 mg/m^3), 32 ppm (128 mg/m^3) and 64 ppm (256 mg/m^3) for 8 min at a flow rate of 8.3 ml/insert/min. Exposure concentrations are expressed as the C×t (concentrations × time) product. For example, a C×t of 64 ppm-min (256 mg-min/m^3) represents an 8 ppm (32 mg-min/m^3) phosgene concentration delivered over an 8 min exposure. Immediately following exposure (t=0 h), inserts were returned to culture conditions.

Transepithelial electrical resistance (TEER)

The TEER of each insert culture was measured using the Millicell ERS© meter with AgCl “chopstick” style electrode (Millipore, Billerica, MA), used according to manufacturer’s instructions. Cultures were initially measured at the outset of the experiment, and a baseline value was taken (prior to incubation in fresh medium: Pre-EQ) (Figure 1). Cultures were switched to fresh medium and equilibrated at least 1 h and measured a second time (Post-EQ). Medium was aspirated immediately after this Post-EQ measurement, and the cultures were then exposed as described above. Immediately following exposure, cultures were returned to plates, fresh medium was applied to the apical compartment, and TEER measurements were subsequently taken hourly at 1 through 10 h and at 24 h. The first post-exposure (PE) measurement is indicated as “0”.

Cellular viability assay

The XTT assay (Biotium, Hayward, CA) assesses cellular function by the reduction of a diffusible, membrane permeable tetrazolium salt (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt). The XTT assay was used because it provides more sensitivity and a more dynamic range of responses than the MTT assay. The XTT assay (Biotium, Hayward, CA) assesses cellular function by the reduction of a diffusible, membrane permeable tetrazolium salt (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt). The XTT assay was used because it provides more sensitivity and a more dynamic range of responses than the MTT assay. The XTT assay was performed according to the manufacturer’s instructions. At 6 or 24 h PE, 500 μl of assay reagent/culture medium mixture was incubated in the apical compartment of each Transwell for 1 h at 37 °C. The orange product was quantified at 475 nm on a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Experiments were performed as independent biological replicates. Each experiment consisted of three wells run in parallel, so the sample size given for each resulting figure refers to the
number of independent experiments, each comprised of three replicates.

[^14]C-Glutamine oxidation assay

Glutamine metabolism was determined by measuring the rates of ^[^14]C^O_2 production from ^[^14]C-glutamine as follows. Within 10 min after exposure, each Transwell membrane was cut out and placed into the bottom of a 20 ml glass scintillation vial containing 6 mM ^[^14]C^-labeled glutamine (Amersham/GE Healthcare Life Sciences, Piscataway, NJ) in 2.0 ml culture medium at a final specific radioactivity of ^[^14]C^0.2 l Ci/l mole. The vial was sealed with a rubber stopper into which was fitted a plastic center well (Kontes, Vineland, NJ) containing a filter paper soaked with 0.1 mmol NaOH and then dried. The vials were incubated in a 37 °C shaking water bath. At 6 or 24 h after exposure 2 mmol of H_2SO_4 was injected to stop the reaction and release dissolved CO_2. The sealed vials were shaken overnight. The [^14]C^O_2 released was determined by liquid scintillation counting of the filter papers and corrected for background levels of [^14]C^O_2 evolved in the absence of cells. To calculate specific radioactivity, glutamine in the assay medium was determined using a YSI Model 2700 dual analyzer (Yellow Springs Instruments, Yellow Springs, OH) fitted with enzyme membranes selective for glutamine and glutamate. The instrument was calibrated with standard solutions obtained from YSI, and the data were corrected for glutamate cross-reaction with the glutamine sensor.

Glucose metabolism assays

The overall rates of glucose metabolism were measured as the rate of change of glucose and lactate in the medium as a function of time and phosgene concentration. Samples of media obtained from the vials used for CO_2 determinations were assayed for glucose and lactate using the YSI analyzer fitted with glucose- and lactate-selective membranes as described above.

Statistical analyses

Graphs were prepared and data analyzed using Prism (GraphPad Software, La Jolla, CA). Multiple groups were compared with one-way ANOVA with Tukey’s post-test. Results are displayed as mean ± SEM with statistically significant differences indicated as *p<.05; **p<.01; ***p<.001.

Results

Transepithelial electrical resistance (TEER)

Eight minutes of exposure to phosgene at concentrations ranging from 0 to 64 ppm decreased TEER in a dose-dependent relationship (Figure 1). As early as 1 h PE, 8 ppm phosgene or greater produced a significant loss in TEER compared to controls, which did not recover by 24 h. Exposure to 2 or 4 ppm phosgene caused a significant decrease in TEER by 6 h PE, which recovered by 7 h PE, although this was the
exception, as throughout most of the recorded PE period, those cultures exposed to less than 8 ppm did not significantly deviate from control-exposed. Relative to control exposure, phosgene at 1, 2, 4, 8, 16, 32 or 64 ppm caused decreases in TEER at 24 h PE of 1.5 ± 0.1%, 3 ± 0.2%, 10.7 ± 0.3%, 34.2 ± 2.6%, 46.8 ± 5.1%, 49.6 ± 6.1% and 62.1 ± 1.9%, respectively.

Cellular viability (XTT)

Eight minutes of exposure to phosgene at concentrations ranging from 0 to 64 ppm decreased XTT in a dose-dependent relationship. Exposure to phosgene at 16 ppm or greater (Ct ≥ 128 ppm·min, 512 mg·min/m³) produced significant loss of cell viability at 6 h PE, while exposure to phosgene at 8 ppm or less did not significantly impact viability at this time-point (Figure 2). Compared to controls at 6 h PE, 1, 2, 4, 8, 16, 32 or 64 ppm phosgene changed XTT by +5.7 ± 1.4%, +11.5 ± 1.7%, +5.9 ± 2.3%, −13.6 ± 2%, −19.0 ± 1.4%, −22.3 ± 1.7%, and −39.6 ± 3.4%, respectively.

At 24 h PE, exposure to 2 ppm phosgene or to ≥16 ppm phosgene produced significant loss of cell viability. Exposure to phosgene at 1, 4 or 8 ppm did not significantly impact viability at this time-point (Figure 3). Twenty-four hours PE, 1, 2, 4, 8, 16, 32 or 64 ppm phosgene decreased XTT by 16.7 ± 5.4%, 30.2 ± 6.6%, 24.6 ± 7.2%, 9.2 ± 6.5%, 38.1 ± 8.3%*, 46.8 ± 4.8%**, and 86.8 ± 6.0%***, respectively.

[^14]C]-Glutamine oxidation

Exposure to phosgene at 8 or 64 ppm decreased the rate of [^14]C]-glutamine oxidation in a dose-dependent fashion (Figure 4). Then 8 ppm phosgene did not significantly decrease [^14]C]-glutamine oxidation at 6 h PE (87 ± 10.1% of control rate) or 24 h PE (83 ± 22% of control rate). However, 64 ppm phosgene significantly decreased [^14]C]-glutamine oxidation to 34 ± 2.7%*** of control rate at 6 h PE and to 33 ± 8.0%** at 24 h PE.

Glucose utilization

Exposure to phosgene at 64 ppm resulted in modest decreases in the rates of glucose utilization, relative to control 0 ppm-exposed (Figure 5). This change was more pronounced at 6 h PE (65 ± 18% of control) than at 24 h PE (76 ± 5.2% of control), although the change was not statistically significant at either time-point. Exposure to phosgene at 8 ppm did not appear to change the rate of glucose utilization at either time-point (108 ± 18% of control at 6 h PE, 80 ± 9.1% of control at 24 h PE).

Lactate production

Exposure to phosgene at 8 ppm did not change the rate of lactate production at either 6 h PE (96 ± 7.1% of control) or 24 h PE (87 ± 7.0% of the control rate). However, exposure to 64 ppm phosgene significantly reduced the rates of lactate
production to $55 \pm 3.7\%$ of control rate at 6 h PE and to $66 \pm 3.5\%$ of control rate at 24 h PE (Figure 6).

**Discussion**

*In vitro* studies have clearly shown that phosgene exposure can have overwhelming effects on cellular homeostasis with regard to mediator release, activation of inflammatory pathways and compromised lung function (Cowan et al., 2004; Madden et al., 1991; Werreln et al., 1994; Wijte et al., 2011). *In vivo* phosgene studies support much of this evidence in a range of experimental exposure models (Frosolono & Pawlowski, 1977; Guo et al., 1990; Kennedy et al., 1989; Li et al., 2011; Madden et al., 1991; Pauluhn et al., 2007; Sciuto et al., 2005). The association between *in vitro* studies and *in vivo* studies to test medical countermeasures against phosgene toxicity is critical in terms of time and expense and identifying affected pathways.

We set out to test an *in vitro* exposure model with which to evaluate potential therapeutics for edemagenic gas inhalation exposure. Our baseline TEER measurements of $600-700 \Omega/cm^2$ in confluent 16HBE cells agree with that measured by Pohl et al. (2009) in their 31-day old mature cell model. In our model, exposure above 8 ppm (CT > 64 ppm·min; 256 mg·min/m³) resulted in TEER that did not recover by 24 h and was significantly different from control-exposed (*Figure 1*). Exposure below 8 ppm (CT < 64 ppm·min; 256 mg·min/m³), however, produced a temporary loss of TEER that recovered to near control values by 24 h, though XTT was still clearly affected at these lower exposure concentrations. We noted that the first post-exposure TEER measurement (T=0) for all groups including controls was always greatly reduced below baseline measurements and was highly consistent across all groups (*Figure 1*). We understand this to be an artifact of the mechanical stress associated with the exposure method, which requires medium removal from the apical surface, handling of the cultures, exposure at the newly created air-liquid interface and replacement of the medium back onto the apical surface; we believe this artifact is due to mild mechanical stress to the cultures during the process. We state that the ability of cultures to quickly restore and thereafter maintain barrier function is a valid indicator of proper tight junction integrity.

Exposure above 8 ppm (CT > 64 ppm·min; 256 mg·min/m³) produced significant loss of cell viability at 6 and 24 h PE. However, at 8 ppm phosgene had no significant PE effect on XTT (*Figures 2 and 3*), aerobic metabolism or anaerobic metabolism despite significantly reducing TEER. Thus, in our model, 8 ppm (64 ppm·min; 256 mg·min/m³) represents a threshold exposure level at which barrier function is compromised. Above 8 ppm cell damage appears to be irreparable and barrier function fails catastrophically. This observation is not at all surprising since maintaining cellular tight junctions, a key component in epithelial barrier function, is a homeostatic energetic process. Our data therefore suggest that the loss of viability and TEER induced by phosgene exposure may be attributable to or exacerbated by inhibition of cellular energy metabolism.

A limitation in this study is the disconnection between low cell viability shown in *Figure 3* and enhanced glucose utilization shown in *Figure 5*. We were surprised by this relationship and believe the following plausible explanations are required. The principal mechanism of phosgene toxicity involves the disruption of air-blood barrier function which results in pulmonary edema. Experiments have shown that the integrity of the air-blood barrier involving the “tightness” of epithelial junctions is the coordinated metabolic activity of adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), myosin light kinase and intracellular calcium levels in epithelial cells (de Lanerolle, 1989). The ATP cycle is the fundamental means of energy exchange in biological systems. Compromised tight junctions can be leaky and in lungs this is problematic with regard to enhanced fluid flux into the airspaces. Supporting evidence for this comes from several related studies. Postlethwait & Young (1980) found a 20% decrease in ATP concentration in edemagenic rats. In another study, Young et al. (1980) also showed a 100%
increase in glucose utilization in edemagenic rats. Currie et al. (1987) demonstrated that ATP is depleted early in rats following exposure to low levels of inhaled phosgene. Additionally, in isolated and perfused rabbit lungs, cAMP, a hydrolysis by-product of the substrate ATP, was shown to be significantly decreased after whole-body exposure to phosgene (Sciuto & Hurt, 2004). Considering the above, it appears that glycolysis is an important component of cellular function and is closely associated with the presence of ATP. The alignment of energy requirements in surviving epithelial cells, ATP and glucose uptake is demonstrated herein. The enhanced glucose utilization is likely a compensatory mechanism used by surviving epithelial cells to maintain the air-blood barrier. Another possibility is worth noting. The XTT assay used to evaluate XTT is actually a measure of reducing conditions within active mitochondria, which may explain the apparent increase in viability due to low-level phosgene exposure at 6 h PE or to 4 and 8 ppm phosgene at 24 h PE. It is likely that a mild toxic insult may increase observed viability using the XTT assay by increasing mitochondrial activity for the purpose of cell repair, whereas higher exposure levels do not leave sufficient mitochondrial function for this purpose.

Phosgene exposure at 8 ppm does not significantly affect metabolism, as measured by the rate of [14C]-O2 production from [14C]-glutamine (Figure 4). At 64 ppm however, phosgene causes metabolism to fail significantly as early as 6 h PE. Based on the statements above regarding phosgene-exposed decreases in ATP production, it not surprising that glutamine CO2 reduction rates are low as ATP is required for glutamine synthesis (Moran et al., 1994). Similarly, the rate of lactate production (a measure of glycolysis, Figure 6) was inhibited by 64-ppm phosgene, though not as severely as that of CO2 production, with no significant inhibition at 8 ppm. Mild, but not statistically significant, inhibition of glucose utilization was also observed at 64 ppm, but not 8 ppm. Our observation of phosgene-induced defects in energy metabolism is consistent with those of other investigators, in both in vitro (Wijte et al., 2011) and in vivo (Currie et al., 1985; Qin et al., 2008) exposure models.

It is also worth stating that our in vitro model may most accurately represent the earliest phase of phosgene inhalation injury, but because it lacks an immune system and other components of the whole animal, it cannot accurately mirror the inflammatory and free radical consequences of tissue damage observed in in vivo models. However, the exposure model did demonstrate repeatability, and phosgene exposure decreased TEER and XTT in dose-dependent relationships, indicating an appropriate in vitro model for therapeutic development efforts.

Herein we have outlined an effective method for performing phosgene gas exposures with an in vitro model of the human airway. In this system, we have replicated an in vivo inhalation exposure by utilizing cultured human airway epithelium and performing exposures to phosgene gas dilutions at an air-liquid interface, thus closely simulating conditions within the living lung. We have demonstrated that our model responds consistently to phosgene exposure, which we have shown affects both airway barrier function and the utilization of cellular energy. Based on this assessment, we believe that our in vitro model system is an appropriate platform for screening potential therapeutics for inhalation injury from phosgene or other toxic industrial compounds. Therapeutic screening would involve testing classes of compounds that possess anti-edemagenic effects such as corticosteroid treatment. Because smooth muscle contraction, a critical regulatory component of barrier function, is partly controlled by ATP and cAMP levels, phosphodiesterase inhibitors may also be important. The significance of this in vitro model is that these compounds can be added directly to the media for testing. This cell model provides a rapid, inexpensive screening tool to down-select phosgene therapeutics prior to efficacy testing in an appropriate animal's model.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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