Video Article

In vitro Cell Culture Model for Toxic Inhaled Chemical Testing

Shama Ahmad[1], Aftab Ahmad[1], Keith B. Neeves[2], Tara Hendry-Hofer[1], Joan E. Loader[1], Carl W. White[1], Livia Veress[1]

[1]Pediatric Airway Research Center, Department of Pediatrics, University of Colorado

[2]Department of Chemical and Biological Engineering, Colorado School of Mines

Correspondence to: Shama Ahmad at SHAMA.AHMAD@ucdenver.edu

URL: https://www.jove.com/video/51539
DOI: doi:10.3791/51539

Keywords: Bioengineering, Issue 87, air-liquid interface, chlorine exposure, toxic inhaled chemicals, Transepithelial Electrical Resistance, Immunocytochemistry

Date Published: 5/8/2014

Citation: Ahmad, S., Ahmad, A., Neeves, K.B., Hendry-Hofer, T., Loader, J.E., White, C.W., Veress, L. *In vitro* Cell Culture Model for Toxic Inhaled Chemical Testing. *J. Vis. Exp.* (87), e51539, doi:10.3791/51539 (2014).

Abstract

Cell cultures are indispensable to develop and study efficacy of therapeutic agents, prior to their use in animal models. We have the unique ability to model well differentiated human airway epithelium and heart muscle cells. This could be an invaluable tool to study the deleterious effects of toxic inhaled chemicals, such as chlorine, that can normally interact with the cell surfaces, and form various byproducts upon reacting with water, and limiting their effects in submerged cultures. Our model using well differentiated human airway epithelial cell cultures at air-liqiuid interface circumvents this limitation as well as provides an opportunity to evaluate critical mechanisms of toxicity of potential poisonous inhaled chemicals. We describe enhanced loss of membrane integrity, caspase release and death upon toxic inhaled chemical such as chlorine exposure. In this article, we propose methods to model chlorine exposure in mammalian heart and airway epithelial cells in culture and simple tests to evaluate its effect on these cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/51539/

Introduction

Exposure to toxic inhaled chemicals (TICs)/gases such as chlorine (Cl_2) remains an ongoing health concern in accidental exposures as well as in their potential use as a chemical threat agent. Although the lungs are the primary target, organs such as heart and brain are also affected[1-3]. *In vivo* models are generally used for testing toxicity from TICs, but *in vitro* assays for toxicity assessment are simpler, faster and more cost effective. *In vitro* models also allow for extensive investigation of agent-cell interactions that may be difficult to evaluate *in vivo*. Such *in vitro* exposure systems are rare and moreover, in some conventional models where toxic agents are added to the culture medium in which cells are submerged, the properties of the agents can change due to interactions and binding to components in the medium. In such scenarios cell culture systems such as air-liquid interface (ALI) cultures of primary human airway epithelial cells, proposed here, that can be directly exposed to gaseous agents could be promising.

Epithelial cells lining the airway are the first lines of defense against inhaled toxic chemicals. The human airway epithelium forms a physical barrier between the lumen and the underlying cells in the lung and participates in the response of the lung. It produces a number of cytokines and other pro- and anti-inflammatory agents as well as secretes mucus/airway surface liquid (ASL) covering the epithelium. One of the limitations in conventional submerged *in vitro* culture systems is also that the ASL and mucus that cover the epithelial surface is removed or diluted. This does not reflect the physiological condition of lung epithelial cells that are exposed to air. Thus, an ideal *in vitro* system for TIC toxicity testing should replicate this architecture. There is great interest in developing rapid screening methods that predict *in vivo* toxicity. Epithelial cells grown at the ALI differentiate and have well-differentiated structures and functions compared to cells grown submerged and serve a superior model of the airways.

In this study, we describe the use of air-liquid-interface culture of human airway (tracheobronchial) epithelial cells for testing poisonous inhaled gas toxicity and compare it with a submerged cell culture of cardiomyocyte, hence studying another important target of toxicity.

Protocol

1. Rat Cardiomyocyte Cultures

1. All experiments were performed under protocols approved by the institutional animal care and use committee, IACUC.
2. Obtain rat cardiomyocytes from the hearts (ventricles) of male rats (240-260 g) using methods described previously[4]. Briefly, anesthetize animals using an intraperitoneal injection of pentobarbital (100 mg/kg; confirm anesthesia by toe pinch method) and then remove hearts into 10.0 ml, 1 mM Ca^{2+} containing Krebs Ringer buffer, pH 7.4.

3. Rinse the hearts 5-6x to remove blood and then switch to a Ca^{2+} free Krebs Ringer buffer containing 0.02% protease and 0.06% collagenase A (5.0 ml/heart). Incubate the hearts in this solution for 10-15 min at 37 °C with occasional shaking.
4. After 10-15 min, wash out the enzymatic solution with Ca^{2+} free Krebs Ringer buffer for an additional 5 min. Release the cells from the flaccid tissue using a 25 ml pipette by pipetting the suspension up and down several times.
5. Separate the cells from tissue by filtering through a 70 µm nylon mesh and allow them to settle in Krebs Ringer buffer containing 0.1 mM Ca^{2+}. Suspend the cell pellet in 1.0 ml Krebs Ringer buffer containing 0.2 mM Ca^{2+}.
6. Carefully layer over 5.0 ml 60 µg/ml bovine serum albumin, BSA, in 15 ml centrifuge tubes, to separate cardiomyocytes from nonmyocytes and allow them to settle for 30 min. The cardiomyocytes are heavier and move to the bottom of the tube. Remove carefully the supernatant cells and media. Repeat this step once to further purify the cells.
7. Gradually transition by washing (in steps using 0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM Ca^{2+} containing buffer) the ventricular myocytes/ cardiomyocytes to 1.0 mM Ca^{2+} containing buffer and resuspended in ACCT medium consisting of Dulbecco's Modified Eagle Medium, DMEM containing 2 mg/ml BSA, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 IU/ml penicillin, and 100 µg/ml streptomycin.
8. Plate the cardiomyocytes in ACCT medium at a density of 100 to 150 cells/mm^2 on 100 mm or 35 mm laminin coated plastic culture dishes or 40 x 22 mm glass coverslips precoated with laminin (1 µg/cm^2). After 1 hr, wash the dishes with 2.0 ml ACCT to remove cells that are not attached. Add 10.0 ml of fresh media and incubate the cells.

2. Differentiated Air-liquid Interface (ALI) Culture of Human Airway Epithelial Basal Cells

1. Procure human tracheobronchial tissues from National Disease Research Interchange under Institutional Review Board approved protocols. Cell harvest and perform culture using a published procedure[5,6]. This procedure uses cell cultures as they are a precise model for human inhalation exposures to TICs, however, if needed one can isolate and grow at ALI mouse and/or rat airway epithelial cells[7,8].
2. Briefly, make small pieces (~¼ inch) of the tissue after removing all connective tissue and lymph nodes. Wash tissue several times in Lactated Ringer solution. Add tissues to a 50 ml conical tube and add protease solution (1% Protease/0.01% DNase in minimal essential media, MEM, tissue to fluid ratio (1:10)).
3. Rock the tissue at 4 °C for overnight. End the dissociation of the tissue by adding 10% fetal bovine serum. Scrape the epithelial surface with a surgical scalpel and collect the cells by centrifugation (2,000 rpm for 10 min).
4. Plate cells at passage one on collagen-coated snapwells in special ALI medium prepared as described in detail by Fulcher *et al*[9]. Culture for 5 days before changing to air-liquid interface (ALI) by removing apical media.
5. Feed the cells using ALI media every alternate day and allow the cells to differentiate for additional 2-3 weeks (observe numerous beating cilia and mucus secretion) before performing exposures. Wash the apical surface with warm PBS along with media changes.

3. Chlorine Exposure

1. Contain the chlorine exposure system (CES) inside a qualified chemical hood with an operational face velocity of 100 fpm that provides the necessary secondary containment to prevent exposure of personnel in the event of accidental chlorine leaks.
2. Operate the system under slight positive pressure (0.5 inches of water). Dry air is fed at 15 L/min and an appropriate level of chlorine is fed to attain the desired final chlorine concentration. The chambers have a locking lid with 4 mini BCU locks and a low durometer silicone gasket to provide the pressure seal.
3. Deliver the Cl_2 mixture through a Mass Flow Controller. The CES uses a compressed gas cylinder containing 1.0% Cl_2 in dry nitrogen.
4. Regulate the dilution airflow using the custom designed control panel and similarly regulate the Cl_2 concentration delivered to the exposure chambers. A low volume sampling pump pulls the exhaust from the chambers into a chlorine analyzer to monitor concentrations that are then recorded on a data logger connected to the analyzer.
5. Measure the flow rates within the chambers prior to exposure using a flow meter to assure equal delivery and exhaust rates.
6. Prepare the cell cultures for exposure by removing the supernatant media and adding fresh media (basolateral media in ALI cultures). Any pretreatments with agents could be performed at this time.
7. Expose the ALI cultures of airway epithelial cells to Cl_2 gas (50, 100, or 300 ppm for 30 min) in the two sealed polysulfone biocontainment chambers. The cardiomyocytes (submerged or confluent cultures on membranes) are exposed to 50 or 100 ppm Cl_2 for 15 min.
8. After exposure flush the chambers with air until the Cl_2 level falls below 1 ppm and can be safely opened to remove cells (within 5 min).

4. Transepithelial Electrical Resistance (TER) Measurement

1. Measure the TER of air-liquid-interface, ALI, cultures using an epithelial voltohmmeter with a pair of silver chloride "chopstick" electrodes.
2. Equilibrate the chopstick electrode in the ALI media 15 min before use.
3. Add warm media to the apical (1.0 ml) and basolateral (2.0 ml) surface and measure the TER using the chopstick electrodes of the voltohmmeter.
4. Dip the shorter arm of the electrode in apical media and the longer arm in the basolateral media. Click the 'measure' button on the voltohmmeter to evaluate the electrical resistance.
5. The voltohmmeter has the option to measure ohms or k ohms. Subtract the resistance across a cell-free culture support from the resistance measured across each cell layer to yield the transepithelial resistance (TER).

5. Caspase Measurement

1. Add fresh ALI media (2.0 ml) to the basolateral surface post exposure and incubate the cell at room temperature. Collect supernatant media at 4 and 24 hr.
2. Measure caspase 3/7 activity in the media supernatants by using a commercial caspase 3/7 assay kit.

6. Western Blot and Immunocytochemistry

1. Perform western blots using cell lysates as previously described[10]. Suspend the protein lysate (20 µg) in 5x reduced sample buffer and boil for 5 min. Subject the protein lysate to SDS-PAGE (4-15%) and transfer the separated proteins to a nitrocellulose membrane by electrophoretic blotting.
2. Block non-specific binding by incubating the membrane with 5% milk in wash buffer (PBS + 0.1% detergent) and probe the membranes with primary antibodies against SERCA2 or sarcomeric actin at 1:1,000 dilution, overnight at 4 °C. Next, wash and incubate membranes with the respective peroxidase-conjugated secondary antibodies and develop for detection as described before[10] using commercial peroxidase detection kit.
3. For immunocytochemistry treat live cells grown on inserts or glass coverslips in 6-well plates with 0.4% Triton-X-100 in 10 mM sodium citrate buffer for 20 min after rinsing with PBS.
4. Block nonspecific binding by treating the cells with 5% donkey serum for 20 min, and then incubate the cells with non-specific IgG or individual primary antibodies specific to sarcomeric actin or Ki-67.
5. Wash the cells with PBS and incubate with fluorescent-conjugated secondary antibodies to detect the primary antibody and a nuclear stain (1 µg/ml DAPI).
6. Wash with PBS and mount coverslips using commercial mounting media.
7. Visualize the staining using a fluorescent microscope.

Representative Results

Primary rod shaped cardiomyocytes attach on laminin matrices and spread and differentiate into confluent cultures (**Figure 1A** and its inset). These cells were further characterized on the basis of sarcomeric actin and SERCA2 expression (**Figures 1B** and **1C**). Rat cardiomyocytes are highly susceptible to chlorine toxicity as 15 min exposure to 100 ppm chlorine caused extensive cell rounding and death in submerged cultures and disruption of confluent layers on cells grown on laminin coated membranes (**Figure 1D**). There was also enhanced apoptotic cell death as indicated by caspase 3/7 release in cardiomyocytes grown on inserts (**Figure 1E**).

Exposure of differentiated human airway epithelium (**Figure 2**, inset to panel 1 showing a cross section of cell culture inserts with columnar, ciliated and goblet cells) to chlorine caused sloughing and lifting of cell membranes at both low (100 ppm) and high (300 ppm) concentrations (**Figure 2** panel A2 and A3). Damage by low chlorine concentrations was quickly reversed (**Figure 2** panel A5), however, cells exposed to higher chlorine concentrations had delayed or no repair ability (**Figure 2** panel A6) as shown by visual inspection as well as cell proliferation assessment by Ki-67 staining (**Figure 2** panel A9). Trans epithelial electrical resistance, TER measurements and caspase activity further confirmed these results and provide evidence for loss of membrane integrity and apoptotic cell death upon chlorine exposure (**Figure 2**, panel B and C). Thus our studies describe the development of an *in vitro* Cl_2 exposure system that causes loss of membrane integrity and death of airway epithelium and cardiomyocytes. This effect may not be due to non-physiological pH changes in the cardiomyocytes as the pH of the media after exposure to chlorine was maintained at ~7.4 as measured by using a pH meter in the collected media postexposure.

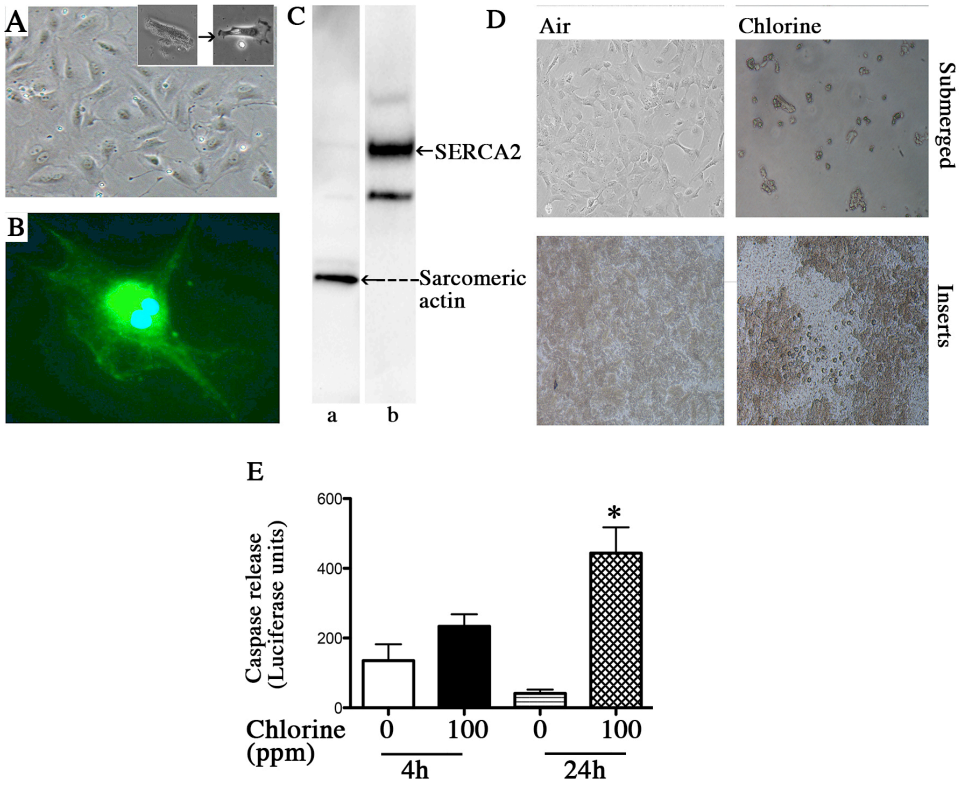

Figure 1. Rat cardiomyocyte isolation and exposure to chlorine. Rat cardiomyocytes were isolated as described in the protocol and plated on laminin-coated plastic dishes (panel A, a representative light microscopic image) or laminin coated inserts. Inset to panel A shows a rod shaped cardiomyocyte spreading and differentiating. The cardiomyocytes were also characterized based on the sarcomeric actin expression (panel B showing a representative image detected by immunofluorescence and lane a panel C showing a representative western blot scan) and abundant SERCA2 expression (panel C lane b). Chlorine exposure (100 ppm 15 min) caused extensive cell death in submerged cultures as well as disruption of the cell monolayer on the inserts (panel D showing the representative photomicrographs). Caspase 3/7 release (panel E) in the supernatant media of cardiomyocytes grown on inserts, at 4 hr and 24 hr post chlorine exposure was also measured as described in the text. Values shown are mean ± SEM and * indicates significant ($p < 0.05$) difference from 0 ppm control.

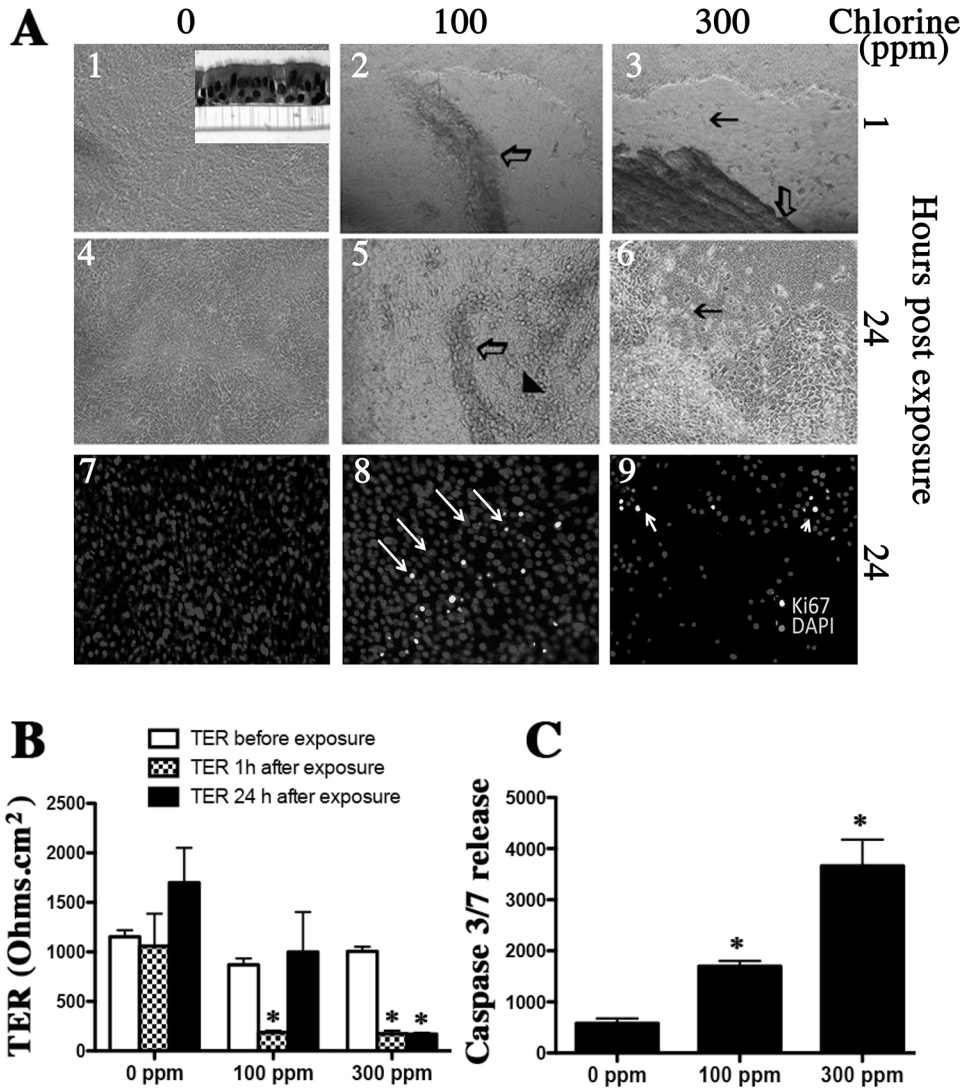

Figure 2. Effect of chlorine exposure on differentiated human airway epithelial air-liquid interface (ALI) cultures. Human airway epithelial basal cells were cultured on collagen coated snapwells. After day 5 the apical media was removed. Differentiated cultures (consisting of basal, ciliated, columnar, and goblet cells as shown in the inset in top left panel of panel A) were exposed to chlorine (100 or 300 ppm) for 30 min. The TER was measured and media was changed and cells incubated for 24 hr. At 24 hr TER was measured again and apical media was collected for caspase release measurement and the cell membranes were fixed for immunohistochemistry. The open arrow in panel A parts 2 and 3 shows sloughed off epithelial layer and black arrows show empty spaces on the insert. The arrowhead in panel A part 5 shows regenerated epithelium. Parts 7, 8, and 9 show cellular proliferation as assessed by Ki-67 immunostaining. Values shown are mean ± SEM and * indicates significant ($p < 0.05$) difference from 0 ppm control.

Discussion

The most common type of acute toxic exposures occurs when one breathes a poisonous chemical into the lungs. These chemicals may also be quickly taken up in the bloodstream and may impact other organs such as brain and heart. Inhalation toxicity of various agents using animal models are studied and reported widely, however the mechanisms are less well understood. This is a major hurdle in developing effective therapies. Absence of *in vitro* exposure systems is a primary reason behind the lack of mechanistic insights. Here we describe cell culture models of heart muscle and airways to study impact of toxic inhaled chemicals such as chlorine exposure. Chlorine reacts rapidly with aqueous surfaces to form hydrochloric and hypochlorous acid[3,11,12]. Chlorine can also combine with reactive oxygen species (ROS) to produce highly reactive compounds that may lead to oxidation of critical proteins and enzymes of the airway surfaces[13]. Studies using submerged cultures may only demonstrate effects of by products such as HOCl in case of chlorine rather than the gas itself. Exposure of differentiated ALI cultures of human airway epithelial cells described here would allow study of direct interactions of TICs such as chlorine with cell surfaces in absence of aqueous media similar to what occurs *in vivo*.

Using primary rat cardiomyocytes, we also describe that chlorine exposure causes rapid cell death in submerged cultures. These cells are unable to grow at ALI as they do not polarize and form tight junctions. Therefore, we also utilized confluent cultures of cardiomyocytes grown

on inserts with a thin layer of media. Exposure of these cell monolayers to chlorine demonstrated membrane disruption and enhanced caspase release suggesting apoptosis may play a role.

This study demonstrates that airways (the primary target of inhalation that can be replicated by ALI cultures) as well as organs such as heart that do not grow at ALI may be studied using *in vitro* exposure systems. These *in vitro* exposure models can easily be adapted to assess effects of other toxic inhaled chemicals (TICs). Using these models we anticipate to provide detailed mechanistic understanding of the toxicities and develop novel strategies to mitigate the toxic events associated with TIC/chlorine and then evaluate them further *in vivo*. Although, these exposures are short in duration the exposure system needs to better replicate cell culture conditions. Exposure to gases such as chlorine limits the use of humidity as it may corrode the device. The ALI cultures could be rocked to simulate respiration as previously utilized in our ozone exposure system[14,15]. We are currently working in these directions.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This research is supported by the CounterACT Program, National Institutes of Health (NIH), Office of the Director, and the National Institute of Environmental Health Sciences (NIEHS) Grant Number U54 ES015678 (CWW). SA is also supported by Children's hospital Colorado/Colorado School of Mines Collaboration Pilot Award #G0100394 and Children's Hospital Colorado Research Institue Pilot Award #G0100471.

References

1. Mohan, A., *et al.* Acute accidental exposure to chlorine gas: clinical presentation, pulmonary functions and outcomes. *Indian J Chest Dis Allied Sci.* **52**, 149-152 (2010).
2. Kose, A., *et al.* Myocardial infarction, acute ischemic stroke, and hyperglycemia triggered by acute chlorine gas inhalation. *Am J Emerg Med.* **27**, 1022 e1021-1024, doi:10.1016/j.ajem.2008.12.029 (2009).
3. Das, R., & Blanc, P. D. Chlorine gas exposure and the lung: a review. *Toxicol Ind Health.* **9**, 439-455 (1993).
4. Claycomb, W. C., & Palazzo, M. C. Culture of the terminally differentiated adult cardiac muscle cell: a light and scanning electron microscope study. *Dev Biol.* **80**, 466-482 (1980).
5. Ahmad, S. *et al.* Bcl-2 suppresses sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase expression in cystic fibrosis airways: role in oxidant-mediated cell death. *Am J Respir Crit Care Med.* **179**, 816-826, doi:10.1164/rccm.200807-1104OC (2009).
6. Ahmad, S. *et al.* Tissue factor signals airway epithelial basal cell survival via coagulation and protease-activated receptor isoforms 1 and 2. *Am J Respir Cell Mol Biol.* **48**, 94-104, doi:10.1165/rcmb.2012-0189OC (2013).
7. Lam, H. C., *et al.* Isolation of mouse respiratory epithelial cells and exposure to experimental cigarette smoke at air liquid interface. *J Vis Exp.* (48), e2513, doi:10.3791/2513 (2011).
8. Hosokawa, T. *et al.* Differentiation of tracheal basal cells to ciliated cells and tissue reconstruction on the synthesized basement membrane substratum in vitro. *Connect Tissue Res.* **48**, 9-18, doi:10.1080/03008200601017488 (2007).
9. Fulcher, M. L., *et al.* Well-differentiated human airway epithelial cell cultures. *Methods Mol Med.* **107**, 183-206 (2005).
10. Ahmad, S. *et al.* SERCA2 regulates non-CF and CF airway epithelial cell response to ozone. *PloS One.* **6**, e27451, doi:10.1371/journal.pone.0027451 (2011).
11. Martin, J. G. *et al.* Chlorine-induced injury to the airways in mice. *Am J Respir Crit Care Med.* **168**, 568-574, doi:10.1164/rccm.200201-021OC (2003).
12. Evans, R. B. Chlorine: state of the art. *Lung.* **183**, 151-167, doi:10.1007/s00408-004-2530-3 (2005).
13. Vliet, A., *et al.* Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem.* **272**, 7617-7625 (1997).
14. Ahmad, S. *et al.* Lung epithelial cells release ATP during ozone exposure: signaling for cell survival. *Free Radic Biol Med.* **39**, 213-226, doi:10.1016/j.freeradbiomed.2005.03.009 (2005).
15. Allen, C. B. An automated system for exposure of cultured cells and other materials to ozone. *Inhal Toxicol.* **15**, 1039-1052, doi:10.1080/08958370390226521 (2003).

