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Hypothiocyanite produced by human and rat respiratory epithelial cells inactivates extracellular H1N2 influenza A virus

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Abstract

Objective and design.—Our aim was to study if an extracellular, oxidative antimicrobial mechanism inherent to tracheal epithelial cells is capable of inactivating influenza virus.

Material or subjects.—Epithelial cells were isolated from tracheas of male Sprague-Dawley rats. Both human and rat tracheobronchial epithelial cells were differentiated in air-liquid interface cultures.

Treatment.—A/swine/Illinois/02860/09 (swH1N2) influenza A virions were added to the apical side of the airway cells for 1 hour in the presence or absence of lactoperoxidase or thiocyanate.

Methods.—Characterization of rat epithelial cells (morphology, Duox expression) occurred via western blotting, PCR, hydrogen peroxide production measurement and histology. The number of viable virions was determined by plaque assays. Statistical difference of the results was analyzed by ANOVA and Tukey's test.

Results.—Our data show that rat tracheobronchial epithelial cells develop a differentiated, polarized monolayer with high transepithelial electrical resistance, mucin production and expression of dual oxidases. Influenza A virions are significantly inactivated by human and rat epithelial cells via a dual oxidase-, lactoperoxidase- and thiocyanate-dependent mechanism.

Conclusions.—Differentiated air-liquid interface cultures of rat tracheal epithelial cells provide a novel model to study Duox-influenza interactions. The dual oxidase/lactoperoxidase/thiocyanate extracellular oxidative system producing hypothiocyanite is a fast and potent anti-influenza mechanism inactivating H1N2 viruses prior to infection.

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Keywords

Dual oxidase; hypothiocyanite; influenza; airway epithelium; hydrogen peroxide

1. Introduction

Tracheobronchial epithelial cells (TBEC) in the airways provide the first line of defense against inhaled pathogenic infectious agents [1]. The respiratory epithelium alerts the innate immune system to initiate inflammation [1]. TBECs themselves are capable of fighting pathogens by producing reactive oxygen species, mucins and antimicrobial peptides [1–4]. TBECs orchestrate an oxidative extracellular antiviral system present in the airway surface liquid consisting of the protein lactoperoxidase (LPO), the thiocyanate anion (SCN^-) and hydrogen peroxide (H_2O_2) [5–7]. LPO and SCN^- are both present in large quantities in the airway surface liquid [6, 7]. LPO oxidizes SCN^- using H_2O_2 into hypothiocyanite ($OSCN^-$), which has demonstrated antimicrobial effects [8–11]. This antimicrobial system was originally described in milk and saliva [8, 9, 11], and only recently in the airways [5, 6].

Cellular H_2O_2 is derived from two NADPH oxidases highly expressed in TBECs: dual oxidase 1 and 2 (Duox1, Duox2) [5, 12, 13]. Duox1 and Duox2 are the most likely candidates to provide H_2O_2 for the H_2O_2/LPO/SCN^- antimicrobial system since these enzymes are the dominant NADPH oxidases expressed in TBECs [5, 14, 15], and are ideally localized to the apical membrane to produce extracellular H_2O_2 into the airway surface liquid [5, 16]. The H_2O_2/LPO/SCN^- antimicrobial system is effective against several microbes including viruses [17, 18]. Its virucidal effects have been described for HIV and RSV but not for other viruses including influenza [17, 18].

Influenza A virus (IAV) causes yearly epidemics with high morbidity and deaths in humans [19, 20]. IAVs have several subtypes that are classified based on their hemagglutinin (HA) and neuraminidase (NA) surface proteins [21]. The IAV subtypes most commonly infecting humans are H1N1, H1N2, and H3N2 [21]. A recent study showed that enzyme-free $OSCN^-$ has antiviral activity against a pandemic influenza strain (A/H1N1/2009) [22]. Different IAV subtypes (H1N1, H3N2) were shown to elicit discrete responses (Duox up-regulation) in TBECs [23]. These data suggest that the Duox/H_2O_2/LPO/SCN^- system in TBECs is a potent anti-influenza mechanism of the respiratory innate immune system [22, 23]. The potential role of the H_2O_2/LPO/SCN^- antimicrobial system in inactivation of the IAV subtype H1N2 has yet to be determined. H1N2 viruses represent a serious public health problem in humans and pigs [24]. The H1N2 IAV subtype resulted from reassortment between H1N1 and H3N2 subtypes [25] and are endemic in the United States [26].

The purpose of this study was to determine if TBECs are capable of inactivating extracellular IAV virions of the H1N2 subtype by the Duox/H_2O_2/LPO/SCN^- system. Air-liquid interface (ALI) cultures of polarized, differentiated TBECs provide the best *in vitro* model of the respiratory epithelium. These cultures develop transepithelial electric resistance (TEER); contain ciliated, goblet and basal cells; produce mucins, and release cytokines upon microbial stimulus [14, 15, 27–31]. We and others have also shown that TBECs express Duox at their later, differentiated stage in ALI cultures [14, 15, 23, 32]. Human TBECs

are difficult to obtain, costly and exhibit large donor-to-donor variation. For this reason, we established a complementary model: ALI cultures of primary rat TBECs. These cells are easier to isolate, cost-effective, less prone to variations and are suitable for larger scale studies; therefore rat TBECs provide an excellent alternative to human TBECs *in vitro* [33].

In this article, we provide detailed characterization of ALI cultures of primary rat TBECs. Rat cells express both, Duox1 and Duox2, and produce extracellular H_2O_2 in a calcium-dependent manner. We also show that rat TBECs inactivate H1N2 IAV to a remarkable extent by the H_2O_2/LPO/SCN^- system. These data were further confirmed using ALI cultures of normal human bronchial epithelium (NHBE). In summary, our data establishes rat TBECs as a model to study influenza-Duox interactions and show for the first time that H1N2 subtype of IAV is efficiently inactivated by the Duox/H_2O_2/LPO/SCN^- antiviral system.

2. Materials and Methods

2.1. Animals.

Male Sprague-Dawley rats were purchased from Harlan Laboratories (South Easton, MA). The animals were between 15–20 weeks old at the time of euthanasia via CO_2. All animal-related procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia (Rada, IACUC protocol numbers: A2012 11-004-Y2-A1, A2015 03-030-Y1-A0.)

2.2. Culture of primary human and rat airway epithelial cells.

Primary normal human bronchial epithelial cells (NHBE) were purchased from Lonza (Walkersville, MD) and cultured as previously described [15]. Briefly, cells were seeded onto 24-well polyester (0.4 micron pore) membrane transwells (Costar), precoated with 0.3% rat tail collagen I (Sigma), at a density of 2.0×10^4 cells/ well. B-ALI™ growth medium was used in the apical and basal chambers until the cells reached confluence. The upper chamber medium was aspirated and the lower medium was replaced with B-ALI™ Differentiation medium (Lonza, Walkersville, MD). Cells were maintained on the air-liquid interface (ALI) for 4–5 weeks by feeding every other day with ALI differentiation medium (the surface of ALI cultures was washed with sterile HBSS every other day). Antibiotics (penicillin and streptomycin, Life Technologies, Grand Island, NY) were supplemented in the media up to four days before experiments.

Rat tracheas were removed under sterile conditions from male Sprague-Dawley rats. The tracheas were incubated overnight at 4°C in Dulbecco's modified Eagle's medium (DMEM; Sigma) and Ham's nutrient F-12 medium (F-12; Sigma) (1:1) with 5% protease (Sigma). Fetal bovine serum 10% (FBS; Hyclone) was then added to the incubation medium (DMEM/F-12) and TBECs were flushed out. The cells were collected by centrifugation (450g, 4°C, 10 min) and were washed twice with DMEM/F-12 containing 10% FBS. Growth medium for RTE cells consisted of DMEM/F-12 supplemented with 1% L-Glutamine, 1% Pen/Strep, 10 μg/mL insulin, 0.1 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 5 μg/mL transferrin, 5ug/mL Transferrin, 25 ng/mL epidermal growth factor

(all reagents from Sigma), 1% bovine pituitary extract (Life technologies), 3 mg/mL bovine serum albumin, 50 nM retinoic acid. Polyester permeable membranes on culture inserts (6.5-mm-diameter, 0.4-μm-pore-size; Costar) were precoated with 100 μL 0.3% rat tail collagen I (Sigma). Rat TBEcs were plated onto the apical surface of the inserts with 0.2 mL of growth medium in the upper (apical) compartments of the culture plates (6.0×10^4 cells/membrane). Cultures were grown in 95% air and 5% CO2 at 37°C. After 72 hours, media in the apical chamber was changed. The medium was changed every other day using 0.5 and 0.2 mL growth medium in the basal and apical compartments, respectively. After 7 days the cells were confluent (determined visually and by measuring transepithelial electrical resistance (TEER)). The medium was removed from the apical chamber taking the cells to ALI. The basal chamber was changed every other day. Cells were cultured between 3–4 weeks on ALI when used for experiments. 4 days prior to experiments cells were given antibiotic-free medium. To characterize their morphology, rat TBEC cultures were fixed with 4% paraformaldehyde (GE Healthcare Life Sciences, Pittsburgh, PA) and subjected to H&E and Mayer's mucicarmine staining (detecting mucins) (UGA, Histology Laboratory, Athens, GA) (Figure 1.).

2.3. Influenza A virus.

Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (HyClone) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C with 5% CO_2. A/swine/Illinois/02860/09 (swH1N2) IAV viral stocks were cultured in MDCK cells using infection medium (DMEM containing high glucose supplemented with 1 mM L-glutamine with 1-μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK]-treated trypsin).

2.4. Western blotting

NHBE cells were lysed by Nonidet P-40 lysis buffer (Boston Bioproducts, Ashland, MA) containing 150 μM PMSF (Fluka Biochemika) and protease inhibitor cocktail (Sigma-Aldrich) [15]. Protein levels were determined using the bicinchoninic acid assay (Pierce, Grand Island, NY). Lysates were electrophoresed on SDS-polyacrylamide gels (8%; Tris-glycine gel, Invitrogen). Gels were blotted on nitrocellulose membrane (Invitrogen) using the iBlot dry blotting system (Life Technologies, Carlsbad, CA). Blots were blocked overnight in TTBS (TBS buffer containing 5% milk powder and 0.05% Tween 20). Blots were incubated with primary anti-Duox antibody (rabbit, polyclonal; 1/2000) [34] followed by incubation with secondary HRP-linked anti-rabbit IgG antibody GE Healthcare; 1/1000). Blots were developed by chemiluminescence using the Lumigen DS detection kit (GE Healthcare).

2.5. RNA isolation and RT-PCR

RNA was isolated from rat TBECs by RNAzol (Sigma-Aldrich, St. Louis, MO)/chloroform extraction followed by isopropanol precipitation as described [35]. RNA Concentrations and purities were determined using a Nanodrop spectrophotometer. cDNA synthesis was carried out with the Thermoscript cDNA synthesis kit (Life Technologies) using 1 μg total RNA, oligo dT primers and RNaseH treatment. To detect *duox1*, *duox2* and *actin* gene expressions, the following gene-specific primers were used in the PCR

reaction (PCR Thermocycler, Eppendorf): rat *duox1* (F: CTGGAGCTCT CCGGGTTT, R: GGCACTGAGG AGGCTGACTA, product: 767 bp); rat *duox2* (F: GGTGGAGATC AGTGTGGTGA, R: GCTAGGAAGC CCCTCTGC, product: 665 bp); rat *actin* (F: GGAAATGCAC TCCCTTGTGT, R: TGTTAGCTTT GGGGTTCAGG, product: 453 bp). PCR program: 94 C (0.5 min), 62 C (0.5 min), 72 C (1.5 min), 37 cycles.

2.6. Measurement of H_2O_2 production.

H_2O_2 production was measured through horseradish peroxidase (HRP)-mediated oxidation of homovanillic acid (HVA) as previously described [14]. Flourescence was measured using Varioskan Flash fluorescence microplate reader (ThermoScientific, excitation wavelength 320nm, emission wavelength 405 nm) for one hour taking readings every minute. H_2O_2 production was then calculated and expressed as nmol H_2O_2/hr/10^6 cells.

2.7. Viral inactivation assay.

After human or rat TBECs had been on ALI for the respective time, 4 days prior to use cells were switched to antibiotic-free medium and the apical chamber was washed with HBSS (Mediatech, Manassas, VA)once. Each component of the system was tested at the following concentrations 100 μM ATP, 6.5 μg/ml LPO, 400 μM SCN^- (in HBSS as assay medium). The reaction volume was set to 40 μL with the appropriate concentration of each component. Virus was diluted to an MOI of 0.1 (NHBE cells ~5000 viral particles, rat TBECs ~8000 viral particles). Catalase (15,000 U/mL, Sigma-Aldrich, St. Louis, MO) was also used in one of the reactions to inhibit the system. Once the components were assembled, the 40 μL was pipetted to the apical chamber of the transwell and placed in a 37 °C 5% CO_2 incubator for one hour. After one hour supernatants from each respective well were collected and stored at −80^c. Plaque assays were performed to determine viral concentrations as previously described [24].

2.9. Statistics.

Data for the viral inactivation assay were log-transformed and significance was calculated using a one-way ANOVA and a Tukey post-hoc test performed using Minitab17. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3. Results

3.1. Characterization of ALI cultures of differentiated rat TBECs.

Recently published results suggest that the Duox/H_2O_2/LPO/SCN^- system responds to H1N1 and H3N2 subtypes of IAV [22, 23]. Whether the third IAV subtype commonly infecting humans, H1N2, can be inactivated by TBECs is unknown. To study this, we used ALI cultures of primary, differentiated rat TBECs as an *in vitro* model to study epithelial-influenza interactions. Rat TBECs were cultured on ALI (Fig. 1. scheme) in 24-well transwells for 3 weeks and subjected to different assays to characterize them. Hematoxylin and eosin staining revealed that TBECs formed the characteristic monolayer containing polarized epithelial cells with apical cilia (Fig. 1A). Basal cells near the transwell membrane support can be visualized (Fig. 1A). Mayer's mucicarmine staining detecting mucins as large glycoproteins identified positively stained (purple), mucin-producing Goblet

cells (Fig. 1B). Unstained polarized cells are ciliated epithelial cells (Fig. 1B). Differentiated TBECs develop high transepithelial electric resistance (TEER) suggesting formation of tight junctions [36]. Figure 1C shows the time-dependent development of high TEER values in rat TBECs. These data confirm that rat TBECs form polarized, mucus-producing, ciliated monolayers in our hands providing an excellent *in vitro* model of the respiratory epithelium.

3.2. Rat TBECs express functional Duox1 and Duox2.

In humans, dual oxidases are expressed in TBECs *in vivo* [5] or *in vitro* in ALI cultures [14, 15, 32, 37]. Duox proteins were also detected in *in vitro* cultures of rat and cow TBECs although detailed description of their culture conditions were not provided [32]. Here we provide detailed characterization of Duox expression and function in rat TBECs. As shown in Figure 2A, polarized rat TBECs express Duox protein (~180 kDa molecular weight) to similar extent in all three animals studied. Although the antibody favors detection of Duox1, it is not isoform-specific [38]. Therefore we detected gene expression levels of each isoform, Duox1 and Duox2, by reverse transcriptase PCR. Figure 2B shows that both isoforms are expressed in rat TBECs. Reverse transciptase was omitted in control samples to show specificity for amplification of DUOX1 and DUOX2 and lack of contaminating host DNA. (Fig. 2B). To show that rat TBECs contain functional Duox enzymes, we measured extracellular H_2O_2 release by horse radish peroxidase (HRP)-mediated oxidation of homovanillic acid (HVA) – as previously described [14, 16]. Rat TBECs spontaneously produced extracellular H_2O_2 (referred to as basal level) that could be still enhanced by known activators of Duox: ATP and ionomycin (Fig. 2C) [15, 28, 39]. In the current study we used ATP to stimulate Duox activity (see later Figs. 4. and 5.). Duox requires an increase in cytosolic calcium to be activated [37, 40]. Scavenging extracellular calcium by EGTA largely reduces basal H_2O_2 output of rat TBECs suggesting that Duox enzymes are the main H_2O_2 producers in rat TBECs, and that extracellular calcium is required for their activation (Fig. 2D). In summary, rat TBECs express both Duox isoforms that are the suggested source of H_2O_2 production.

3.3. Rat TBECs inactivate H1N2 influenza A virus in a H_2O_2/LPO/SCN^--dependent manner.

Although enzyme-free $OSCN^-$ has virucidal effect against the pandemic A/H1N1/2009 influenza virus [22], and NHBE cells upregulate Duox in response to H1N1 and H3N2 IAV strains [23], there are no data to show direct IAV inactivation by the Duox/H_2O_2/LPO/SCN^- system and response of TBECs to the H1N2 subtype. To address this knowledge gap, we exposed TBEC cultures to the H1N2 IAV strain A/Swine/Illinois/02860/09 (swH1N2) at a multiplicity of infection (MOI) of 0.1 for 1.5 hour (see explanatory scheme in Figure 3). swH1N2 is an endemic swine strain originally isolated in the state of Illinois in 2009 [24, 41]. Supernatants were collected from infected TBEC cultures to prepare 10-fold serial dilutions (Fig. 3.). Diluted viral suspensions were added to Madin-Darby Canine Kidney Epithelial Cells (MDCK) (Fig. 3.). Plaques were counted after three days of incubation, and changes in viable virion concentrations were calculated (Fig. 3.). Although Duox enzymes show high spontaneous activity in rat TBECs, ATP was added to enhance their H_2O_2 output (Fig. 2.). LPO and SCN^- were added as indicated at levels found in human airways (SCN^-: 400 μM, LPO 6.5 μg/ml) (Fig. 4.) [32, 37]. Figure 4 shows that a significant 2–3 log reduction in the number of viable H1N2 IAV virions occurred when the complete

H_2O_2/LPO/SCN^- system was reconstituted on top of rat TBEC cultures. Omitting LPO, SCN^- or both resulted in complete loss of virion inactivation (Fig. 4.), indicating that the full system needs to be present and $OSCN^-$ is responsible for viral inactivation.

3.4. H1N2 IAV inactivation by the H_2O_2/LPO/SCN^- system of NHBE.

To determine if primary cultures of human bronchial epithelial cells behave similarly to rat TBECs, we exposed human NHBE cells to H1N2 IAV under the same conditions as the rat cells (Fig. 4) and followed influenza virion inactivation as described. Similarly to the results shown in Figure 4., NHBE caused H1N2 inactivation when the full H_2O_2/LPO/SCN^- system was assembled. If LPO, SCN^- or both were left out, influenza inactivation was entirely inhibited (Fig. 5.). This highlights again the crucial antiviral role of the final product, $OSCN^-$ against influenza, and proves that NHBE cells can produce sufficient H_2O_2 by Duox to fuel this antiviral system. In addition, we also show that the H_2O_2 scavenger catalase entirely blocks the virion-inactivating effect of the H_2O_2/LPO/SCN^- system (Fig. 5, see “CAT”). This further suggests that Duox-derived H_2O_2 is required and sufficient for influenza inactivation. Overall, the Duox/H_2O_2/LPO/SCN^- system of both rat and human cultures of primary airway epithelial cells have strong antiviral activity against the IAV H1N2 strain. This further confirms that rat TBECs provide an excellent model to study IAV-Duox interactions in the respiratory epithelium.

Discussion

TBECs are the earliest responders to influenza challenge in the airways [1, 42]. Their reaction to the first invading virions is essential in determining the later fate of the inflammatory response. One of the inflammatory mechanisms by which TBECs fight pathogens is the production of reactive oxygen species [1, 5, 23]. TBECs release hydrogen peroxide into the airway surface liquid that is used by LPO to oxidize its most abundant substrate, SCN^- to produce antimicrobial $OSCN^-$ [6, 7, 43, 44]. LPO is produced primarily in serous acini of submucosal glands in the airways, not in the epithelium [5, 45]. Its main substrate, SCN^- is present extracellularly in submillimolar concentrations in human airways [43]. SCN^- is transported from the blood trough the epithelium via several suggested transport proteins [46–48]. Both, LPO and SCN^- are abundantly present in airway secretion and H_2O_2 production represents the rate-limiting factor in the activity of the system. In intact airways, H_2O_2 is primarily provided by two NADPH oxidases, Duox1 and Duox2 [5]. Both Duox enzymes and their maturation factors (Duox activators) localize to the apical plasma membrane of bronchial epithelial cells ideally suited to produce extracellular H_2O_2 [5, 16]. *In vitro* ALI cultures of differentiated and polarized human respiratory epithelium express high amounts of Duox, produce apical H_2O_2 and have been shown to kill several microorganisms in an H_2O_2-dependent manner [15, 23, 32, 43]. However, working with cultures of primary human cells has significant limitations: 4–5 weeks of culturing time, cost and most importantly large donor-to-donor variations. We used ALI cultures of rat TBECs to complement our data obtained with human cells. Obtaining primary rat cells is cost effective, the cells are faster to culture, show little variation among donors and allow larger scale studies. Our rat TBECs reconstitute all the features of human cultures and express both dual oxidases (Figs. 1–2.). This is in accordance with previous studies that also used rat

TBEC ALI cultures [32, 49–51]. Our data showing that rat TBECs inactivate IAV similarly to human cultures indicates that rat TBECs provide an excellent, alternative model to study influenza-human epithelial cell interactions (Figs. 4–5.).

TBECs are capable of producing sufficient H_2O_2 to supply the anti-influenza effect of LPO (Figs. 4–5.). All three components (Duox/H_2O_2, LPO, SCN^-) are necessary to inactivate IAV. Omitting one, two or all three components results in no significant inactivation of IAV. Thus, not only enzyme-free [22] but also enzyme-derived $OSCN^-$ efficiently inactivates IAV. Detailed responses of human epithelial cells to H1N1 and H3N2 subtypes of IAV have been shown but their direct extracellular inactivation by the Duox-based system has not been documented [23]. Our studies are the first to show that extracellular influenza viruses can be inactivated efficiently by epithelial-derived $OSCN^-$. This adds influenza to the growing list of pathogenic infectious agents that the H_2O_2/LPO/SCN^- system is capable of inactivating/killing [15, 18, 32, 43]. We also show that Duox and LPO are efficient against H1N2 subtype of IAV.

There are two isoforms of Duox, both of which are highly expressed in TBECs [2, 5, 15, 23, 52]. Which isoform is more important to produce H_2O_2 in TBECs is unclear at this point. It is well-accepted that in human TBECs Duox1 is the main Duox isoform expressed [5, 14]. Basal Duox2 expression is lower but Duox2 can be induced to a larger extent by microbial stimuli [53]. While the *in vivo* role of Duox1 is still unknown, the main physiological function of Duox2 is clearly to provide H_2O_2 for thyroid hormone biosynthesis. This is evident since both Duox2-deficient mice and human patients with Duox2 mutations develop hypothyroidism [54, 55]. Our data indicate that rat TBECs express both Duox1 and Duox2 at similar levels. Although Duox2 has been proposed to be the main isoform in rat TBECs [32], this conclusion was drawn from an siRNA transfection experiment which showed minimal effect on apical H_2O_2 output without showing isoform specificity of the siRNAs. The situation is likely more complicated and both isoforms could be activated by different stimuli or could overtake each other's function to provide redundancy. Therefore, further data are needed to clearly identify the main Duox isoform responsible for the antimicrobial and inflammatory effect of TBECs.

It is important to emphasize the very fast action of the Duox/H_2O_2/LPO/SCN^- system to inactivate IAV. Under our experimental conditions, IAV was only added for 1 hour on the top of TBECs. During this time there is a significant decrease in the concentration of viable extracellular virions if the whole system is assembled (Figs. 4–5). We assume that this rapid and robust inactivation of IAV by the Duox-based system largely reduces their potential to infect TBECs and to cause inflammation. To our current knowledge very few, if any, airway immune responses act so quickly [1, 56]. Antimicrobial peptides are already present in the airway surface liquid but we estimate that the $OSCN^-$-generating mechanism has a larger virucidal capacity and is more manipulative (it can be turned on or off fast) [57, 58]. These features make the Duox/H_2O_2/LPO/SCN^- antiviral system ideal for pharmaceutical intervention. By enhancing its anti-influenza effect, we could eliminate extracellular virions still on the airway surface before establishing infections in TBECs and triggering inflammation. All the downstream effects of IAV infection could be theoretically prevented at the earliest intervention time point possible.

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Abbreviations

ALI	air-liquid interface
Duox	dual oxidase
HA	hemagglutinin
LPO	lactoperoxidase
MDCK	Madin-Darby canine kidney cells
MOI	multiplicity of infection
NA	neuraminidase
NHBE	normal human bronchial epithelium
$OSCN^-$	hypothiocyanite
SCN^-	thiocyanate
TBEC	tracheobronchial epithelial cell
TEER	transepithelial electrical resistance

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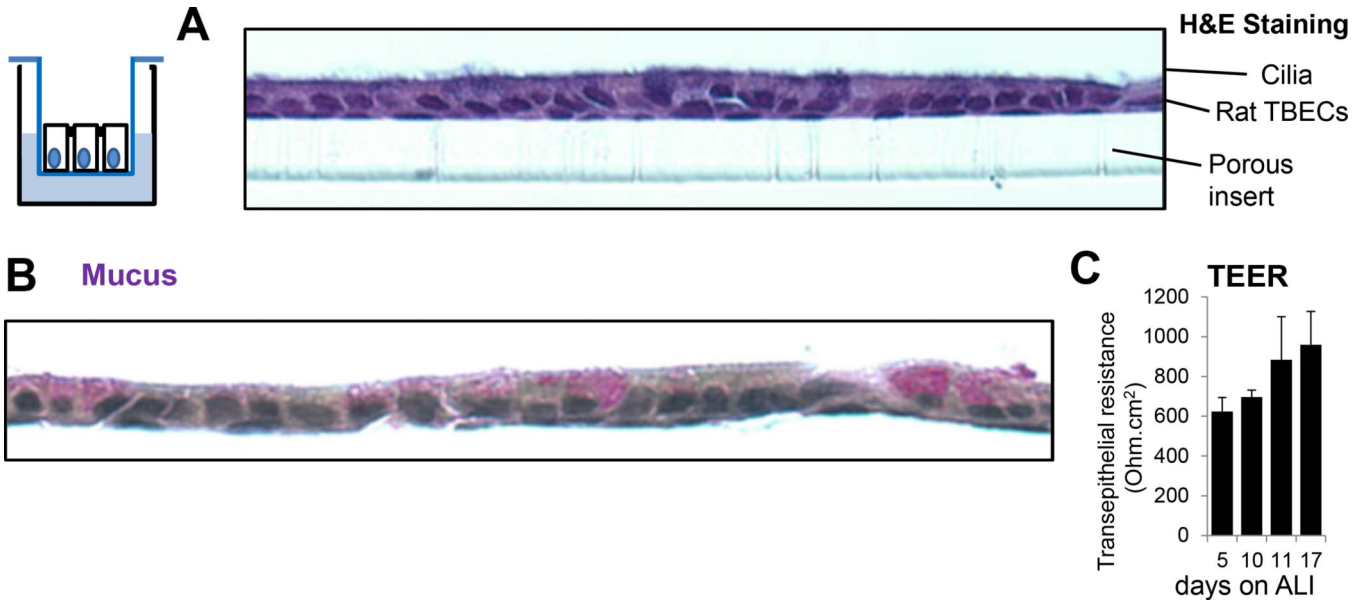

Figure 1. Air-liquid interface cultures of polarized, differentiated rat tracheal epithelial cells provide an excellent *in vitro* model of the bronchial epithelium.

(A) H&E staining shows formation of a ciliated monolayer of polarized cells after 17 days of culture on ALI. One representative results, n=3. (B) Mayer's mucicarmine staining detects mucins. These large glycoproteins were identified by positive staining (purple) originating from mucin-producing Goblet cells. One representative result, n=3. (C) Transepithelial resistance (Ohm·cm^2) was measured on days 5, 10, 11, 17 post ALI using a voltohmmeter. Mean+/−S.E.M., n=7. TEER, transepithelial electrical resistance; TBEC, tracheobronchial epithelial cell; ALI, air-liquid interface.

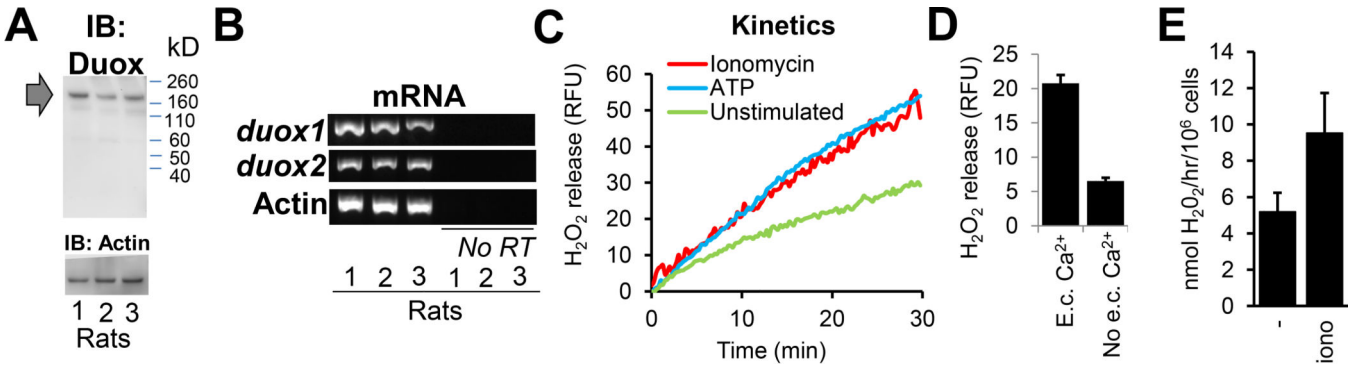

Figure 2. Rat tracheal epithelial cells express Duox and produce extracellular H_2O_2 in a calcium-dependent manner.

Air-liquid interface cultures of primary rat tracheal cells were cultured for 17 days and subjected to the following analyses: (A) Duox protein was detected in cell lysates by western blotting. Samples of three animals shown. (B) Gene expression levels of rat duox1, duox2 and actin genes were detected by reverse transcriptase PCR. Samples of three animals shown. (C) Kinetics (left) and endpoint (right) measurements of extracellular hydrogen peroxide production detected by homovanillic acid oxidation assay. Kinetics: one representative result, n=3. ATP (100 μM), ionomycin (1 μM). Endpoint: mean+/−S.E.M., n=3. Extracellular calcium was chelated by addition of 1mM EGTA. IB, immunoblot; RT, reverse transcriptase; RFU, relative fluorescence unit.

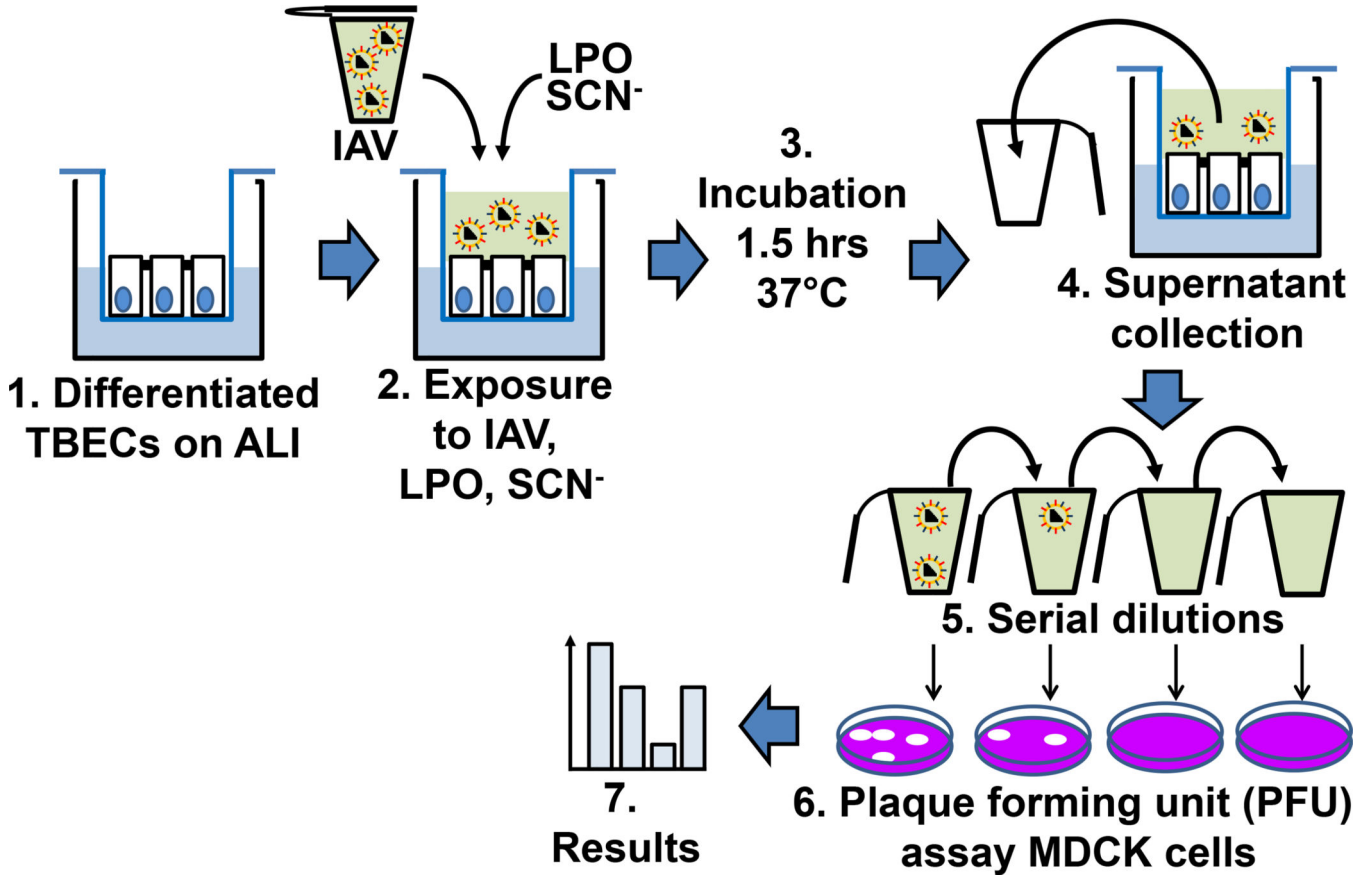

Figure 3. Steps of measuring inactivation of extracellular influenza virions on tracheobronchial epithelial cells.

ALI cultures of differentiated TBECs **(1)** are exposed to IAV in combination with exogenous LPO and SCN^- **(2)** for 1 hour at 37°C **(3)**. Supernatants were then collected **(4)**, 10-fold serial dilutions were performed **(5)** and plaque forming unit assays on MDCK cells **(6)** were used to determine the PFU/mL of virus remaining **(7)**. MDCK, Madin-Darby canine kidney cells; PFU. Plaque forming unit; LPO, lactoperoxidase; SCN-, thiocyanate; IAV, influenza A virus; ALI, air-liquid interface; TBEC, tracheobronchial epithelial cell.

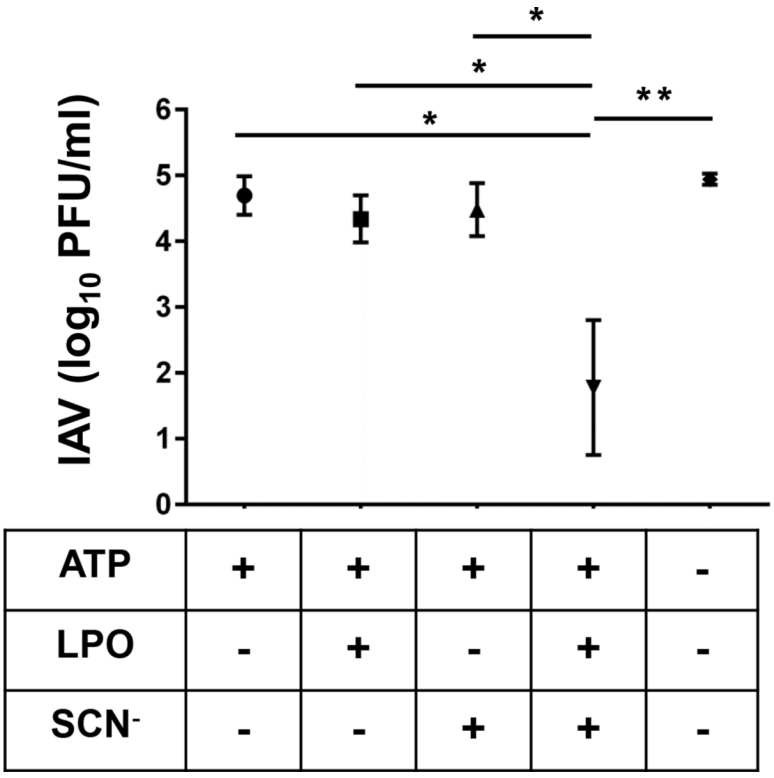

Figure 4. Rat TBECs inactivate influenza virions in a Duox/H_2O_2/LPO/SCN^--dependent manner.

ALI cultures of differentiated primary Sprague-Dawley rat TBECs were exposed to 8000 PFU of H1N2 IAV (swH1N2) in presence or absence of ATP (Duox activator, 100 μM), LPO (6.5 μg/ml) or SCN^- (400 μM) in the indicated combinations. After 1 hr incubation supernatants were collected and concentration of viable virus particles was determined by PFU assay using MDCK cells. Mean+/−S.E.M., n=4. ANOVA, Brown-Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$. IAV, influenza A virus; PFU, plaque forming unit.

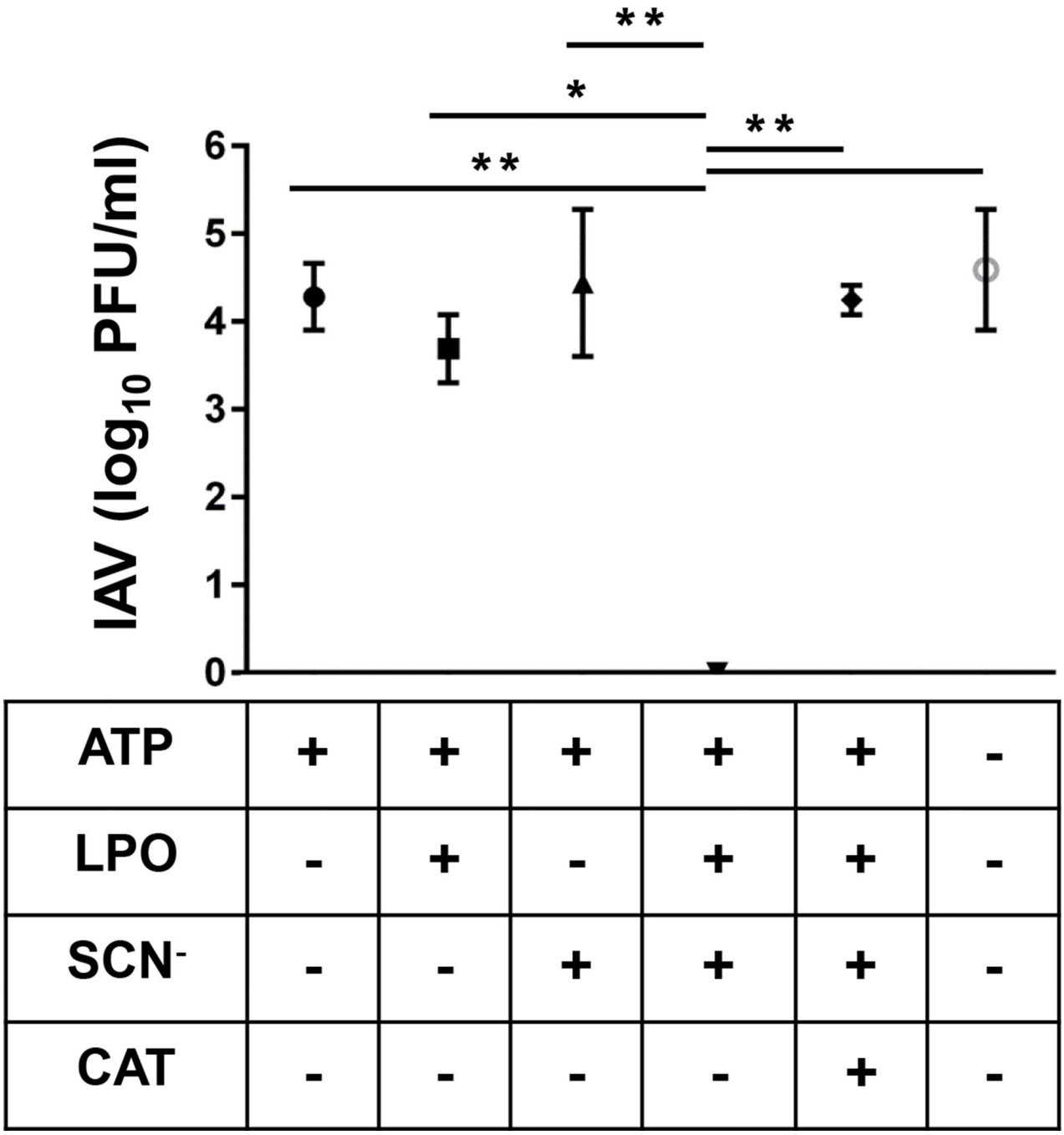

Figure 5. Human bronchial epithelial cells inactivate influenza virions in a Duox/ H_2O_2/LPO/SCN^- -dependent manner.

ALI cultures of differentiated human NHBE cells were exposed to 5000 PFU of H1N2 IAV (swH1N2) in presence or absence of ATP (Duox activator, 100 μM), LPO (1 μM), SCN^- (400 μM) or catalase (CAT, 15.000 U/ml) in the indicated combinations. After 1 hr incubation supernatants were collected and concentration of viable virus particles was determined by PFU assay using MDCK cells. Mean+/−S.E.M., n=2. ANOVA, Brown-

Forsythe test, Tukey's multiple comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. IAV, influenza A virus; PFU, plaque forming unit.