

2-8-2016

# Soluble adenylyl cyclase mediates hydrogen peroxide-induced changes in epithelial barrier function

Pedro Ivonnet  
*University of Miami*

Hoshang Unwalla  
*Herbert Wertheim College of Medicine, Florida International University, hunwalla@fiu.edu*

Matthias Salathe  
*University of Miami*

Gregory E. Conner  
*University of Miami*

Follow this and additional works at: [https://digitalcommons.fiu.edu/all\\_faculty](https://digitalcommons.fiu.edu/all_faculty)

---

## Recommended Citation

Ivonnet, Pedro; Unwalla, Hoshang; Salathe, Matthias; and Conner, Gregory E., "Soluble adenylyl cyclase mediates hydrogen peroxide-induced changes in epithelial barrier function" (2016). *All Faculty*. 109.  
[https://digitalcommons.fiu.edu/all\\_faculty/109](https://digitalcommons.fiu.edu/all_faculty/109)

RESEARCH

Open Access



# Soluble adenylyl cyclase mediates hydrogen peroxide-induced changes in epithelial barrier function

Pedro Ivonne<sup>1</sup>, Hoshang Unwalla<sup>2</sup>, Matthias Salathe<sup>1</sup> and Gregory E. Conner<sup>3\*</sup>

## Abstract

**Background:** Elevated H<sub>2</sub>O<sub>2</sub> levels are associated with inflammatory diseases and H<sub>2</sub>O<sub>2</sub> exposure is known to disrupt epithelial barrier function, leading to increased permeability and decreased electrical resistance. In normal human bronchial epithelial (NHBE) cells, fully differentiated at the air liquid interface (ALI), H<sub>2</sub>O<sub>2</sub> activates an autocrine prostaglandin pathway that stimulates transmembrane adenylyl cyclase (tmAC) as well as soluble adenylyl cyclase (sAC), but the role of this autocrine pathway in H<sub>2</sub>O<sub>2</sub>-mediated barrier disruption is not entirely clear.

**Methods:** To further characterize the mechanism of H<sub>2</sub>O<sub>2</sub>-induced barrier disruption, NHBE cultures were treated with H<sub>2</sub>O<sub>2</sub> and evaluated for changes in transepithelial resistance and mannitol permeability using agonist and inhibitors to dissect the pathway.

**Results:** A short (<10 min) H<sub>2</sub>O<sub>2</sub> treatment was sufficient to induce resistance and permeability changes that occurred 40 min to 1 h later and the changes were partially sensitive to EP1 but not EP4 receptor antagonists. EP1 receptors were localized to the apical compartment of NHBE. Resistance and permeability changes were sensitive to inhibition of sAC but not tmAC and were partially blocked by PKA inhibition. Pretreatment with a PLC inhibitor or an IP3 receptor antagonist reduced changes in resistance and permeability suggesting activation of sAC occurred through increased intracellular calcium.

**Conclusion:** The data support an important role for prostaglandin activation of sAC and PKA in H<sub>2</sub>O<sub>2</sub>-induced barrier disruption.

**Keywords:** Soluble adenylyl cyclase, Hydrogen peroxide, Airway epithelium, EP1

## Background

Junctional complexes are composed of an assortment of proteins that anchor cells to each other and their basement membranes, thereby forming a stable tissue that serves to regulate passage of materials across the mucosa. Regulation of the apical junctional complex is key to epithelial barrier function. Numerous studies have shown changes in transepithelial permeability and electrical resistance can occur rapidly and reversibly and mirror changes in intercellular junction structure. Loss of barrier function is often associated with inflammation

[1]. A large number of studies have shown that H<sub>2</sub>O<sub>2</sub>, frequently elevated in inflammatory diseases, reversibly alters paracellular epithelial permeability and resistance (e.g., [2–4]). H<sub>2</sub>O<sub>2</sub> on epithelial surfaces can result from the respiratory burst of invading phagocytes or from epithelial cells themselves that produce H<sub>2</sub>O<sub>2</sub> through the enzymatic action of the NADPH oxidases Duox 1 & 2 [5–7].

The mechanism by which H<sub>2</sub>O<sub>2</sub> alters permeability and transepithelial resistance is multifactorial and differs between differentiated epithelia, endothelia and cell lines (e.g., [8]), but uniformly entails junctional protein redistribution (e.g., [2, 9–13]). Occludin, ZO1 and claudins are released from junctions after H<sub>2</sub>O<sub>2</sub> exposure. H<sub>2</sub>O<sub>2</sub> alteration of the epithelial barrier is known to rely on

\* Correspondence: gconner@miami.edu

<sup>3</sup>Department of Cell Biology, Miller School of Medicine, University of Miami, 1600 NW 10th Ave, Miami 33136 FL, USA

Full list of author information is available at the end of the article

increased protein tyrosine phosphorylation by inhibition of protein tyrosine phosphatase [8, 11], p38 MAP kinase activity [14] and dephosphorylation of occludin by PP2A in a Src kinase-dependent fashion [15]. Involvement of protein kinase C has been reported in some cases [16] but ruled out in others [3].

Studies in bovine tracheal epithelia [17], in human airway epithelial cell lines [18, 19] and more recently in fully differentiated normal human bronchial epithelial (NHBE) cells [20] show that acute exposure to  $H_2O_2$  stimulates an autocrine prostanoid signaling pathway that elicits an increase in CFTR-mediated anion secretion, which can be seen in Ussing chamber experiments as short circuit currents ( $I_{sc}$ ). The autocrine EP1 and EP4 pathways operate through G-proteins that indirectly stimulate sAC through increases in intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ), thereby amplifying the cAMP signal to increase CFTR conductance [21]. More prolonged exposures to  $H_2O_2$  induces a decrease in resistance with concomitant increase in permeability. These changes are believed to represent alteration of epithelial barrier function. Thus, to better understand the mechanism underlying the  $H_2O_2$ -induced junctional disruption, we explored the role of the  $H_2O_2$ -mediated decreases in resistance and increases in permeability using primary NHBE cell cultures re-differentiated at the air liquid interface. These experiments showed that the  $H_2O_2$ -induced effects on resistance and permeability depended not only on direct inhibition of tyrosine protein phosphatases by  $H_2O_2$ , but also on a G-protein coupled receptor (GPCR) transduction path that involves the  $Ca^{2+}$ -mediated stimulation of sAC activity and PKA.

## Methods

### Cell culture

Human airway epithelial cells were obtained from organ donors whose lungs were rejected for transplant. Consent was obtained through the Life Alliance Organ Recovery Agency of the University of Miami and the LifeCenter Northwest in WA according to IRB approved protocols. Epithelial cells from the lower trachea and bronchi were isolated as previously described [22, 23]. Air-liquid interface (ALI) cultures were allowed to differentiate for at least 2 weeks prior to experiments. All experiments were performed with date, passage and lung matched control cultures.

### Chemicals

DMEM, Ham's nutrient F-12 and Hank's balanced salt solution were purchased from Gibco, Life Technologies (Grand Island, NY). Gly-H 101 was from Calbiochem. Prostanoid receptor antagonists were from Cayman Chemicals. All other chemicals, unless otherwise stated, were purchased from Sigma Aldrich (St. Louis, MO).

### Ussing chambers

Snapwell filters containing differentiated NHBE cells were rinsed with Krebs-Henseleit (KH), and then mounted in Ussing chambers (EasyMount Chamber; Physiologic Instruments; San Diego, CA) with KH in apical and basolateral chambers. KH consisted of: 118 mM NaCl, 25 mM  $NaHCO_3$ , 4.7 mM KCl, 1.2 mM  $MgSO_4$ , 1.2 mM  $NaH_2PO_4$ , 1.2 mM  $CaCl_2$ , 5.5 mM glucose, pH 7.35 when gassed with 95 %  $O_2$ -5 %  $CO_2$ . Solutions were maintained at 37 °C by heated water jackets and continuously bubbled with a 95 %  $O_2$ -5 %  $CO_2$  mixture. To monitor  $I_{sc}$  and resistance, the transepithelial membrane potential was clamped at 0 mV with a 6-channel voltage clamp (model VCC MC6, Physiologic Instruments) using Ag/AgCl electrodes in agar bridges. Signals were digitized and recorded with DAQplot software (VVI Software, College Station, PA) via a LabJack A/D converter (LabJack Corp, Lakewood, CO). The input resistance of each filter was measured by application of 1 mV bipolar pulses of 2 s duration. After addition of amiloride,  $I_{sc}$  was allowed to stabilize and then  $H_2O_2$  was included in the apical perfusate. Time scales were initialized at the time of mounting in the chamber. To explore the mechanisms responsible for this change in  $I_{sc}$ , cultures were incubated apically and basolaterally with pharmacological agents for 20–50 min before and during apical exposure to  $H_2O_2$ .

### Permeability measurements

NHBE ALI cultures were preincubated for 10 min at 37 °C in KH-buffer (pH 7.35).  $^{14}C$  mannitol was added apically and initial apical to basolateral mannitol flux was determined [24]. Cells were washed apically and basolaterally three times to remove  $^{14}C$  mannitol, treated apically with  $H_2O_2$  or inhibitors/activators, fresh  $^{14}C$  mannitol added apically and final mannitol flux was determined. Apparent permeability  $P_{app}$  was calculated for initial and final mannitol flux using the following equation:  $P_{app} = dQ/dT/A \cdot C_0$  where  $dQ/dt$  is the flux determined from the amount transported ( $Q$ ) over time ( $T$ ) of the experiment,  $A$  is the surface area of the filter, and  $C_0$  is the initial concentration on the donor side. Results were expressed as mannitol  $P_{app}$  normalized to initial  $P_{app}$ .

### Intracellular $Ca^{2+}$ measurements

NHBE ALI cultures were loaded at room temperature for 2 h by gentle rocking of cultures in 10  $\mu$ M fura-2 AM (ThermoFisher Scientific, Waltham, MA) in Dulbecco's PBS containing 1 % glucose and 10 % FBS. Cultures were washed and mounted in a perfusion chamber in Krebs-Henseleit buffer at room temperature and ratiometric images captured and quantified as described previously [25].

### Immunocytochemistry

Fully differentiated NHBE cells in ALI cultures were fixed in 4 % paraformaldehyde in PBS, pH 7.4 for 15 min and permeabilized with 1 % Triton X-100 in PBS for 20 min at room temperature (RT). After permeabilization, cells were washed with PBS and then blocked with 3 % BSA in PBS for 1 h RT, followed by rabbit anti human EP1 receptor antibody (1  $\mu\text{g}/\text{ml}$ , Cayman Chemical #101740 I) and mouse anti human acetylated  $\alpha$ -tubulin (Sigma Cat. #T6793; 1:2000) in blocking solution and incubated overnight at 4 °C. Non-immune controls used normal rabbit IgG at 1  $\mu\text{g}/\text{ml}$ . EP1 and acetylated  $\alpha$ -tubulin were visualized with Alexa 488-labeled anti-rabbit antibody (4  $\mu\text{g}/\text{ml}$ , Invitrogen) and Alexa 647 labeled anti-mouse antibody respectively. Nuclei were labeled with 4,6-diamidine-2-phenylindole (DAPI, KPL). Samples on membranes were mounted on slides with Fluoro-Gel (EMS; Cat. #17985-10) and fluorescent images were acquired on a Leica DM6000 microscope with a SP5 confocal module at the University of Miami McKnight Analytical Imaging Core Facility.

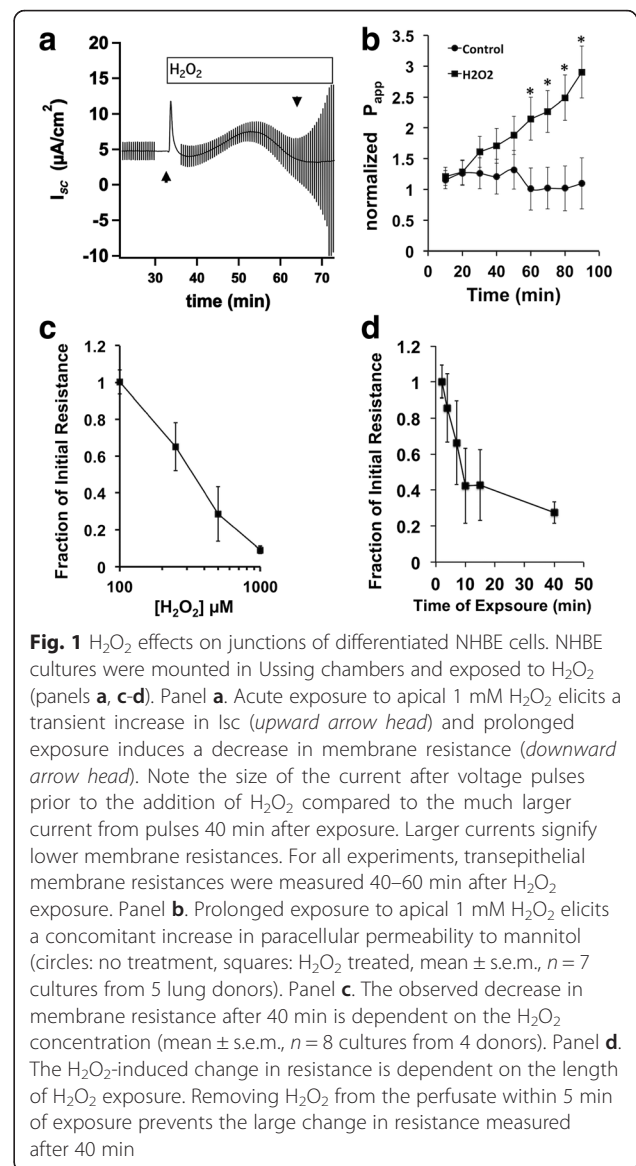
### Data analysis

The maximum change in  $I_{\text{SC}}$  following stimulation was normalized to baseline values obtained after addition of amiloride and other inhibitors. Replicate cultures from each lung donor and then all donors were averaged to give mean values. When multiple treatments were compared, changes were expressed as a fraction of date and lung matched controls. Mean values were compared by one-way ANOVA and if significant differences were obtained, by the Tukey Kramer HSD test. If means were not normal, the Wilcoxon or Kruskal-Wallis test was used (JMP software, SAS, Cary NC and Prism, Graphpad Software, La Jolla CA).

### Results

In Ussing chamber experiments, exogenously applied  $\text{H}_2\text{O}_2$  changes membrane ion conductance and resistance in NHBE cells. Acutely,  $\text{H}_2\text{O}_2$  leads to increases in CFTR-mediated conductance. Low  $\text{H}_2\text{O}_2$  concentrations elicit sustained increases in  $I_{\text{sc}}$  that plateau above baseline [20, 21]. High  $\text{H}_2\text{O}_2$  concentrations produce transient currents of larger amplitudes comprised of an increase to a peak followed by a decrease towards baseline (Fig. 1a, upward arrow head).

Prolonged apical exposures induced a large decrease in resistance (Fig. 1a, downward arrow head) that in some instances approached zero and a concomitant increase in paracellular permeability (Fig. 1b). Initial resistance values of differentiated NHBE cultures ranged from 200 to 1000  $\Omega/\text{cm}^2$ . Apical  $\text{H}_2\text{O}_2$  treatment decreased resistance as a function of starting resistance of the



**Fig. 1**  $\text{H}_2\text{O}_2$  effects on junctions of differentiated NHBE cells. NHBE cultures were mounted in Ussing chambers and exposed to  $\text{H}_2\text{O}_2$  (panels a, c-d). Panel a. Acute exposure to apical 1 mM  $\text{H}_2\text{O}_2$  elicits a transient increase in  $I_{\text{sc}}$  (upward arrow head) and prolonged exposure induces a decrease in membrane resistance (downward arrow head). Note the size of the current after voltage pulses prior to the addition of  $\text{H}_2\text{O}_2$  compared to the much larger current from pulses 40 min after exposure. Larger currents signify lower membrane resistances. For all experiments, transepithelial membrane resistances were measured 40–60 min after  $\text{H}_2\text{O}_2$  exposure. Panel b. Prolonged exposure to apical 1 mM  $\text{H}_2\text{O}_2$  elicits a concomitant increase in paracellular permeability to mannitol (circles: no treatment, squares:  $\text{H}_2\text{O}_2$  treated, mean  $\pm$  s.e.m.,  $n = 7$  cultures from 5 lung donors). Panel c. The observed decrease in membrane resistance after 40 min is dependent on the  $\text{H}_2\text{O}_2$  concentration (mean  $\pm$  s.e.m.,  $n = 8$  cultures from 4 donors). Panel d. The  $\text{H}_2\text{O}_2$ -induced change in resistance is dependent on the length of  $\text{H}_2\text{O}_2$  exposure. Removing  $\text{H}_2\text{O}_2$  from the perfusate within 5 min of exposure, however, prevented the change in resistance after 40 min

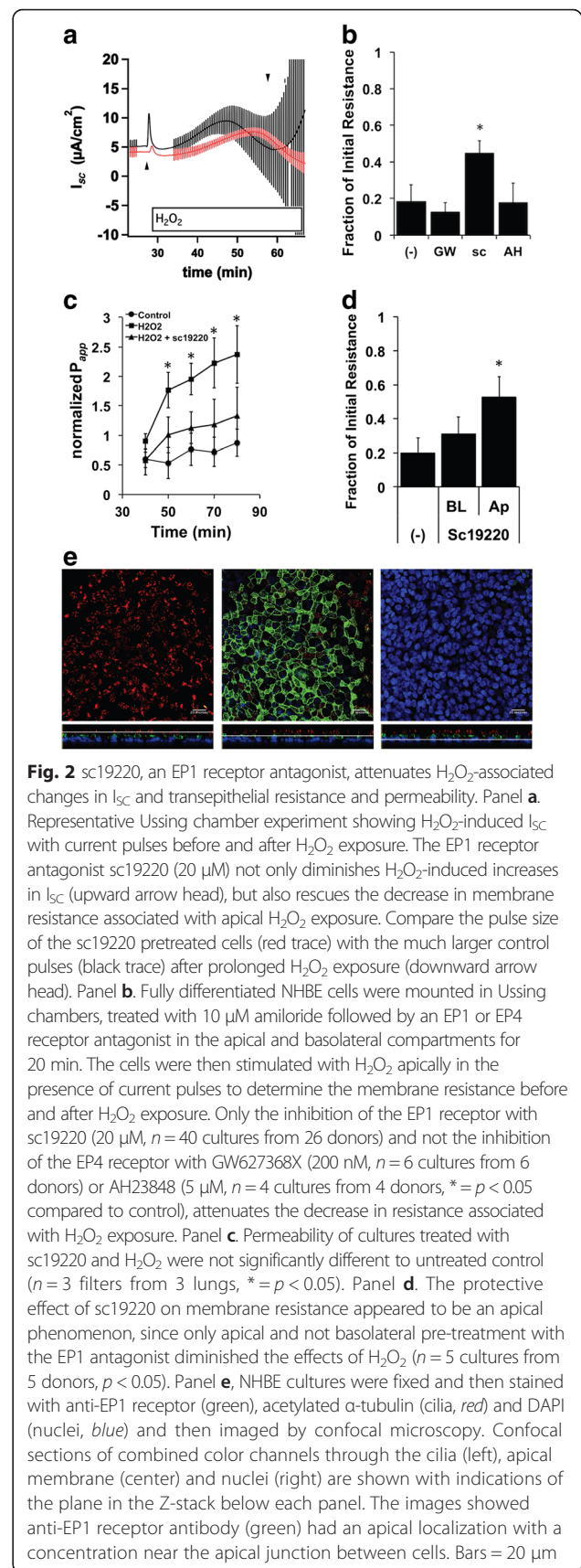
culture and not by a fixed absolute amount in each culture. Thus, resistance changes were expressed after normalization to the starting value for that culture at the beginning of the experiment. The decrease in membrane resistance depended on the  $\text{H}_2\text{O}_2$  concentration (Fig. 1c), started to occur approximately 15 min after  $\text{H}_2\text{O}_2$  exposure and lasted at least 1 h. The  $\text{H}_2\text{O}_2$ -induced change in resistance was observed even after exposure times as short as 10 min. Removal of  $\text{H}_2\text{O}_2$  within 5 min of exposure, however, prevented the change in resistance (Fig. 1d).

Pre-treating NHBE cells apically with 1 mM  $\text{H}_2\text{O}_2$  caused about a 50 % decrease in the forskolin-induced  $I_{\text{SC}}$  within 10 min of addition (control:  $20.7 \pm 3.6 \mu\text{A}$ ,  $n = 9$  lung donors vs  $\text{H}_2\text{O}_2$ -treated:  $9.2 \pm 2.1 \mu\text{A}$ ,  $n = 9$  lung donors, Additional file 1: Figure S1). Despite

H<sub>2</sub>O<sub>2</sub> having such a profound effect on resistance and CFTR activity, the effects were completely reversed within 24 h after treatment was stopped. Cultures exposed to apical 1 mM H<sub>2</sub>O<sub>2</sub> for 1 h recovered normal resistance in agreement with studies using cell lines [10] and also recovered CFTR activity as reflected in the forskolin-stimulated I<sub>SC</sub> returning to normal values when measured 24 h after H<sub>2</sub>O<sub>2</sub> exposure (Additional file 1: Figure S2). Additionally, cultures continuously exposed to apical 1 mM H<sub>2</sub>O<sub>2</sub> for 18 h also recovered normal resistance and forskolin-stimulated I<sub>SC</sub> within 8 h after H<sub>2</sub>O<sub>2</sub> removal.

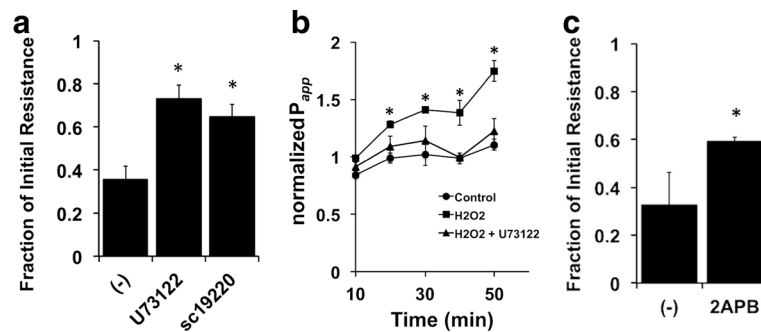
H<sub>2</sub>O<sub>2</sub> exposure leads to the production of prostaglandin compounds in NHBE cells and cell lines and results in stimulation of an EP1 and EP4-mediated, autocrine signaling pathway [19, 20]. In brain tissue [26] and intestinal epithelial cell lines [27, 28], EP1 receptor stimulation causes an increase in barrier permeability. Pretreating redifferentiated NHBE cells with 20 μM sc19220, an EP1 receptor antagonist, for 20 min attenuated the decrease in membrane resistance and increase in permeability seen after exposure to apical 1 mM H<sub>2</sub>O<sub>2</sub> (Fig. 2a–c). EP4 receptors were not involved in the H<sub>2</sub>O<sub>2</sub>-induced changes in resistance, since pretreatment for 20 min with EP4 receptor antagonists, GW627368X (200 nM) or AH23848 (5 μM), did not affect the H<sub>2</sub>O<sub>2</sub>-induced decrease in resistance (Fig. 2b). Also, application of sc19220 attenuated the decrease in membrane resistance when applied only to the apical compartment (Fig. 2d) and not to the basolateral compartment implicating EP1 receptors at the mucosal surface. Immunocytochemistry with anti-human EP1 antibody showed that EP1 receptors were localized to the apical portion of NHBE cells with the highest concentration at the apical cell border reminiscent of apical junctional complexes (Fig. 2e).

It is known that EP1 receptors are coupled to Gq and that this complex activates phospholipase C. The observation that EP1 receptor inhibition attenuates the changes associated with H<sub>2</sub>O<sub>2</sub> exposure suggested that the mechanistic pathway underlying the effect of H<sub>2</sub>O<sub>2</sub> on membrane resistance and permeability operated at least in part through increases in [Ca<sup>2+</sup>]<sub>i</sub>. NHBE cells pre-treated for 20 min with the phospholipase C inhibitor U73122 (20 μM) showed an attenuation of the H<sub>2</sub>O<sub>2</sub>-induced decrease in resistance similar to that seen with the EP1 antagonist sc19220 (Fig. 3a). Parallel mannitol flux experiments also showed a decrease in H<sub>2</sub>O<sub>2</sub>-induced permeability changes when NHBE cells were pre-treated with U73122 (Fig. 3b). Inhibition of IP<sub>3</sub> receptors with 2-aminoethoxydiphenyl borate (200 μM) also lead to a decrease in the H<sub>2</sub>O<sub>2</sub>-induced changes in resistance, further supporting a role of [Ca<sup>2+</sup>]<sub>i</sub> in this pathway (Fig. 3c).



**Fig. 2** sc19220, an EP1 receptor antagonist, attenuates H<sub>2</sub>O<sub>2</sub>-associated changes in I<sub>SC</sub> and transepithelial resistance and permeability. Panel **a**. Representative Ussing chamber experiment showing H<sub>2</sub>O<sub>2</sub>-induced I<sub>SC</sub> with current pulses before and after H<sub>2</sub>O<sub>2</sub> exposure. The EP1 receptor antagonist sc19220 (20 μM) not only diminishes H<sub>2</sub>O<sub>2</sub>-induced increases in I<sub>SC</sub> (upward arrow head), but also rescues the decrease in membrane resistance associated with apical H<sub>2</sub>O<sub>2</sub> exposure. Compare the pulse size of the sc19220 pretreated cells (red trace) with the much larger control pulses (black trace) after prolonged H<sub>2</sub>O<sub>2</sub> exposure (downward arrow head). Panel **b**. Fully differentiated NHBE cells were mounted in Ussing chambers, treated with 10 μM amiloride followed by an EP1 or EP4 receptor antagonist in the apical and basolateral compartments for 20 min. The cells were then stimulated with H<sub>2</sub>O<sub>2</sub> apically in the presence of current pulses to determine the membrane resistance before and after H<sub>2</sub>O<sub>2</sub> exposure. Only the inhibition of the EP1 receptor with sc19220 (20 μM, *n* = 40 cultures from 26 donors) and not the inhibition of the EP4 receptor with GW627368X (200 nM, *n* = 6 cultures from 6 donors) or AH23848 (5 μM, *n* = 4 cultures from 4 donors, \* = *p* < 0.05 compared to control), attenuates the decrease in resistance associated with H<sub>2</sub>O<sub>2</sub> exposure. Panel **c**. Permeability of cultures treated with sc19220 and H<sub>2</sub>O<sub>2</sub> were not significantly different to untreated control (*n* = 3 filters from 3 lungs, \* = *p* < 0.05). Panel **d**. The protective effect of sc19220 on membrane resistance appeared to be an apical phenomenon, since only apical and not basolateral pre-treatment with the EP1 antagonist diminished the effects of H<sub>2</sub>O<sub>2</sub> (*n* = 5 cultures from 5 donors, *p* < 0.05). Panel **e**. NHBE cultures were fixed and then stained with anti-EP1 receptor (green), acetylated α-tubulin (cilia, red) and DAPI (nuclei, blue) and then imaged by confocal microscopy. Confocal sections of combined color channels through the cilia (left), apical membrane (center) and nuclei (right) are shown with indications of the plane in the Z-stack below each panel. The images showed anti-EP1 receptor antibody (green) had an apical localization with a concentration near the apical junction between cells. Bars = 20 μm





**Fig. 3** Phospholipase C is an integral part of the  $H_2O_2$ -induced changes in resistance and permeability. Panel **a**. Fully differentiated NHBE cells were mounted in Ussing chambers, treated with  $10 \mu M$  amiloride and the phospholipase C inhibitor U73122 ( $20 \mu M$ ) in the apical and basolateral compartments for 20 min. Cells were then stimulated with apical  $1 mM H_2O_2$  in the presence of current pulses to determine the membrane resistance before and after  $H_2O_2$  exposure. Pre-treating NHBE cells with U73122 ( $n = 8$  cultures from 8 donors,  $* = p < 0.05$ ) attenuated the  $H_2O_2$ -induced decrease in membrane resistance in a similar fashion to that seen with the EP1 receptor antagonist sc19220 ( $n = 8$  cultures from 8 donors,  $* = p < 0.05$ ). Panel **b**. Parallel mannitol flux experiments also showed an attenuation of the concomitant increase in permeability associated with apical  $1 mM H_2O_2$  exposure ( $n = 4$  cultures from 2 lung donors,  $* = p < 0.05$  compared to control). Panel **c**. Inhibition of the  $IP_3$  receptor with 2-APB ( $200 \mu M$ ) also attenuates  $H_2O_2$ -induced decreases in resistance, further supporting a role for  $[Ca^{2+}]_i$  in the pathway ( $n = 3$  cultures from 3 donors,  $* = p < 0.05$ )

To confirm that  $H_2O_2$ -induced stimulation of phospholipase C via EP1 receptors led to an increase in  $[Ca^{2+}]_i$ , NHBE cells were loaded with fura-2 AM, mounted on the stage of a Nikon microscope and stimulated with  $400 \mu M H_2O_2$ .  $H_2O_2$  treatment resulted in an immediate increase in  $[Ca^{2+}]_i$  (Fig. 4a). Since calcium is an activator of both sAC and tmAC, we pre-treated NHBE cells with either the sAC inhibitor KH7 ( $25 \mu M$ ) or the tmAC inhibitor MDL-12,330A ( $25 \mu M$ ) to determine a possible role of these enzymes in the  $H_2O_2$ -induced change in resistance or permeability. Inhibition of sAC attenuated the  $H_2O_2$ -induced decrease in resistance or increase in permeability (Fig. 4b–c). On the other hand, inhibition of tmAC did not significantly alter the change in resistance associated with  $H_2O_2$  (Fig. 4b). Lower concentrations of KH7 ( $10 \mu M$ ) also attenuated the  $H_2O_2$ -induced decrease in resistance significantly (Fig. 4d). Inhibition of PKA with H-89 ( $15 \mu M$ ) led to a modest delay and attenuation of the  $H_2O_2$ -induced decrease in resistance. The involvement of PKA in the pathway became more evident when NHBE cells were washed free of  $H_2O_2$  after a 15 min exposure (Fig. 4e). Inhibitors of CFTR, COX, PKC and EPAC did not alter the  $H_2O_2$ -induced change in resistance (data not shown).

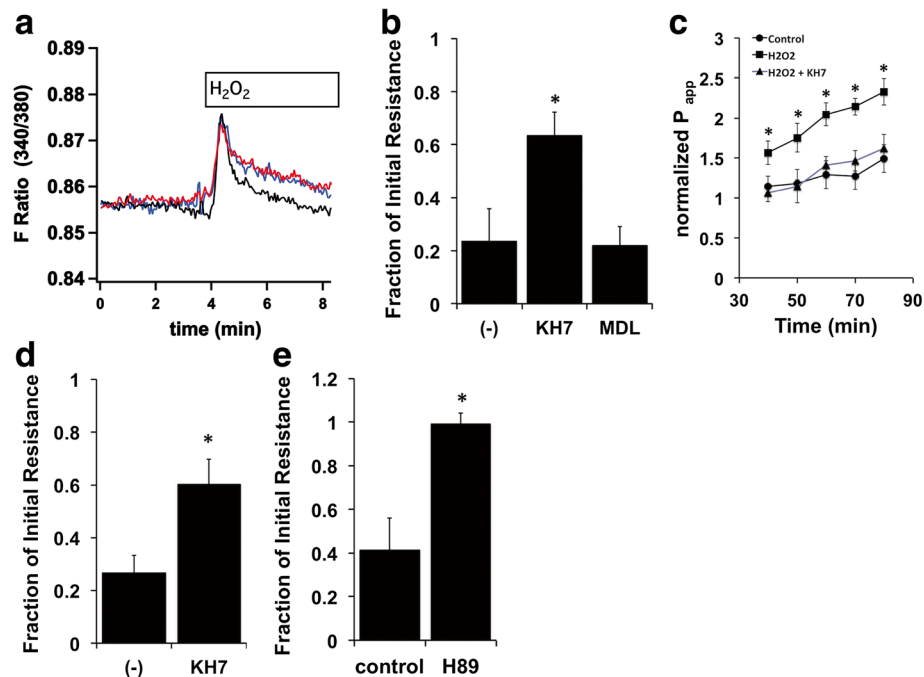
Maintenance of junctional integrity requires a balanced interplay between protein phosphorylation by kinases and dephosphorylation by phosphatases (e.g., [29]). This was evident when NHBE cells are treated with either a protein tyrosine kinase or phosphatase inhibitor. Pretreating NHBE cells with  $15 \mu M$  or  $30 \mu M$  AG82, a protein tyrosine kinase inhibitor, greatly inhibited the decrease in resistance and increase in permeability

after  $H_2O_2$  exposure (Fig. 5a–b). On the other hand, treating cells with phenylarsine oxide, a protein tyrosine phosphatase inhibitor, elicited a concentration-dependent decrease in resistance and increase in permeability similar to that seen with  $H_2O_2$  (Fig. 5c–d). Thus, it appeared that both PKA and a tyrosine kinase were involved in mediating  $H_2O_2$ -induced changes in barrier function.

## Discussion

Most studies of  $H_2O_2$  effects on junctions have used epithelial cell lines to demonstrate that  $H_2O_2$  compromises barrier function as measured by increased paracellular permeability and decreased transepithelial resistance. These studies on cell lines show that the pathway initiated by  $H_2O_2$  is multifactorial and cell type specific [8]. Thus, the mechanisms are not clear, especially not in primary cells or tissues. This study uses fully differentiated primary epithelia in vitro. The data suggested that the  $H_2O_2$  effect on bronchial epithelial junctions involves, in part, a specific PKA pathway, initiated by EP1 receptor stimulation, leading to  $Ca^{2+}$  activation of sAC and PKA, which in turn mediated changes in the function of apical junctions. In NHBE, the  $H_2O_2$ -activated PKA pathway was seen along with  $H_2O_2$  effects on activation of tyrosine kinases [30] and inactivation of tyrosine phosphatases [31] both known to interfere with junctions.

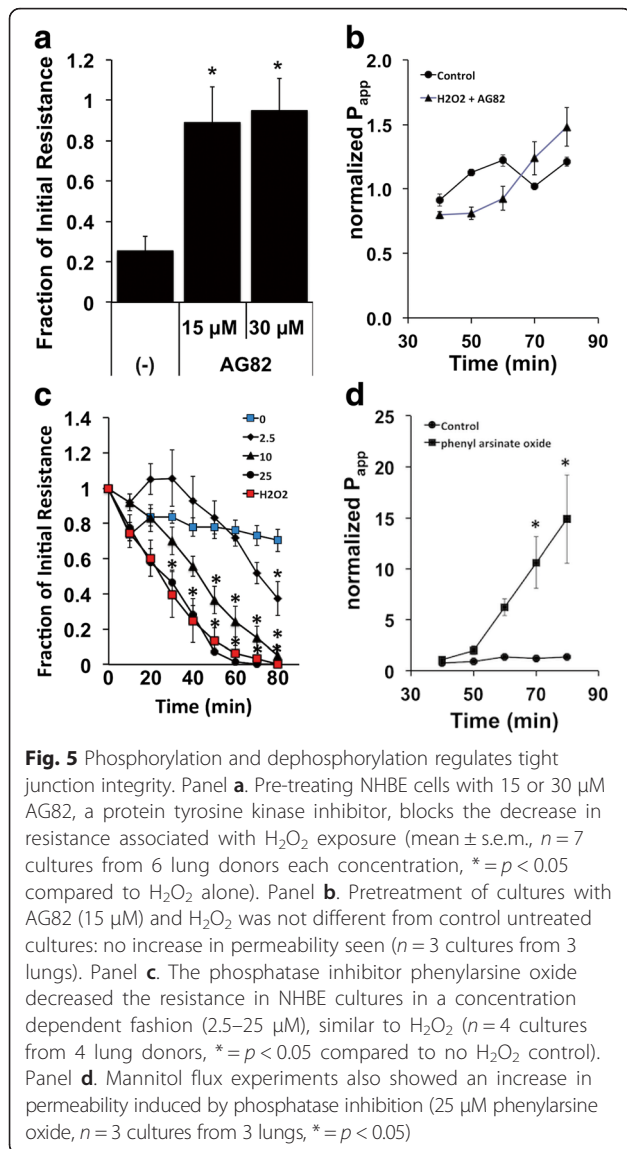
Previous studies have shown that  $H_2O_2$ -mediated decreases in resistance and increases in permeability occur through a variety of changes in protein phosphorylation, including inhibition of tyrosine phosphatases (e.g., [32]), increased tyrosine kinase activity (e.g., [29]) and increased serine/threonine dephosphorylation [15]. In NHBE cells,



**Fig. 4** H<sub>2</sub>O<sub>2</sub>-induced changes in permeability and resistance are mediated by sAC. Panel **a**. Treatment with 400 μM H<sub>2</sub>O<sub>2</sub> stimulated an increase in [Ca<sup>2+</sup>]<sub>i</sub> in NHBE cells loaded with fura-2 AM (traces from three regions of interest). Panel **b**. sAC, but not tmAC, is involved in the H<sub>2</sub>O<sub>2</sub>-induced change in resistance. Fully differentiated NHBE cells were mounted in Ussing chambers, treated with 10 μM amiloride and the sAC inhibitor KH7 (25 μM) or the tmAC inhibitor MDL-12,330A (25 μM) in the apical and basolateral compartments for 20 min. The cells were then stimulated with apical 1 mM H<sub>2</sub>O<sub>2</sub> in the presence of current pulses to determine the membrane resistance before and after H<sub>2</sub>O<sub>2</sub> exposure. Pre-treating NHBE cells with KH7 attenuated the H<sub>2</sub>O<sub>2</sub>-induced decrease in membrane resistance while the tmAC inhibitor did not have an effect (*n* = 6 cultures from 6 donors, \* = *p* < 0.05). Panel **c**. Inhibition of sAC attenuated the increase in permeability associated with prolonged H<sub>2</sub>O<sub>2</sub> exposure (*n* = 4 lungs). Panel **d**. Lower concentrations of KH7 (10 μM) also had a significant effect on the H<sub>2</sub>O<sub>2</sub>-induced change in resistance (*n* = 6 cultures from 6 donors, \* = *p* < 0.05). Panel **e**. NHBE cells were mounted in Ussing chambers and pretreated with 15 μM H89 for 20 min. The cells were then exposed to apical 1 mM H<sub>2</sub>O<sub>2</sub> for 15 min. Inhibition of PKA attenuates the H<sub>2</sub>O<sub>2</sub> induced decrease in resistance (*n* = 3 cultures from 3 donors, \* = *p* < 0.05)

direct treatment with the phosphatase inhibitor phenylarsine oxide induced a change in resistance similar to H<sub>2</sub>O<sub>2</sub>, and pre-treatment with the protein tyrosine kinase inhibitor AG82 caused the H<sub>2</sub>O<sub>2</sub>-induced decrease in resistance to be greatly attenuated. Treatment of NHBE cells with H<sub>2</sub>O<sub>2</sub> caused, in addition to changes in protein tyrosine phosphorylation, stimulation of PKA that also led to reduced resistance and higher permeability. Interestingly, H<sub>2</sub>O<sub>2</sub>-mediated changes in resistance could only be blocked by PKA inhibition if H<sub>2</sub>O<sub>2</sub> exposure was short (15 min), suggesting longer treatments activated a second parallel, PKA-independent pathway to alter intercellular junctions. Nevertheless, activation of sAC appeared absolutely necessary for H<sub>2</sub>O<sub>2</sub>-mediated changes in junctions since a sAC inhibitor blocked changes induced by both short and long exposures. These data suggest that sAC may be compartmentalized in a place allowing it to solely be responsible. sAC has been shown to be localized at the ciliated, apical membrane of NHBE and in cilia [33, 34].

Acute application of H<sub>2</sub>O<sub>2</sub> to normal human airway epithelial cells induces transient increases in CFTR conductance via a prostanoid signaling pathway involving increased release of prostaglandin E<sub>2</sub>, activation of prostaglandin E receptors, EP1 and EP4 [20, 21] that signal via G<sub>q</sub> and G<sub>s</sub>, respectively. The data here showed that EP1 but not EP4 signaling was a key aspect of H<sub>2</sub>O<sub>2</sub>-induced changes in transepithelial resistance and paracellular permeability. Inhibition of PLC and IP<sub>3</sub> receptors caused an attenuation of the H<sub>2</sub>O<sub>2</sub>-induced decrease in resistance and increase in permeability. However, blocking the EP1 signaling pathway was not sufficient to completely inhibit H<sub>2</sub>O<sub>2</sub> mediated junctional changes, supporting the idea that more than one pathway worked in parallel to induce changes in resistance and permeability. Since sAC is activated by increases in [Ca<sup>2+</sup>]<sub>i</sub>, the data were consistent with a pathway that involves H<sub>2</sub>O<sub>2</sub> induction of prostaglandin release [20, 21], activation of EP1 receptors to increase [Ca<sup>2+</sup>]<sub>i</sub> and activate sAC to produce cAMP for



PKA activity. Whether this pathway interacts with, or modulates tyrosine phosphorylation that also regulates junctions is not known.

Inflammatory airway diseases are often characterized by defective barrier function and coincidentally are associated with elevated  $\text{H}_2\text{O}_2$  presumably derived from NADPH oxidases of both infiltrating phagocytes as well as epithelial cells. The knowledge that  $\text{H}_2\text{O}_2$  contributes to barrier disruption suggests a possible mechanistic link between  $\text{H}_2\text{O}_2$  and tight junction changes in inflammation. The data presented here implicate EP1-mediated activation of sAC to increase epithelial cAMP and increase barrier disruption. In contrast, modulators that increase cAMP, relax airway smooth muscle and are used to treat asthma and COPD might also lead to counterproductive cAMP

increases in epithelial cells. Thus, the EP1 signaling pathway or sAC itself might be possible therapeutic targets to prevent deleterious changes in epithelia barrier function.

## Conclusion

$\text{H}_2\text{O}_2$ -induced changes in NHBE barrier function occur through an autocrine prostanoid signaling pathway that increased  $[\text{Ca}^{2+}]_i$ , activated sAC and altered epithelial resistance and permeability. Previous studies in brain and intestinal cells have shown prostaglandin-induced increases in permeability [26–28], suggesting a similar sAC-mediated mechanism of action of  $\text{H}_2\text{O}_2$  on epithelial barrier function may occur in a variety of tissues.

## Additional file

**Additional file 1: Figure S1.**  $\text{H}_2\text{O}_2$  inhibition of forskolin-stimulated  $I_{\text{SC}}$ . NHBE cultures were mounted in Ussing chambers and treated with either 10  $\mu\text{M}$  forskolin or 1 mM  $\text{H}_2\text{O}_2$  followed by 10  $\mu\text{M}$  forskolin. Panel a, representative traces of forskolin-induced CFTR activity with (red trace) and without (black trace)  $\text{H}_2\text{O}_2$  pre-treatment. Panel b,  $I_{\text{SC}}$  values with (treated) and without (control)  $\text{H}_2\text{O}_2$  pre-treatment (mean  $\pm$  s.e.m.,  $n = 9$  lung donors).  $\text{H}_2\text{O}_2$  treatment reduced the forskolin-stimulated  $I_{\text{SC}}$  by approximately 50 %. **Figure S2.** Recovery of resistance and forskolin-stimulated  $I_{\text{SC}}$  after  $\text{H}_2\text{O}_2$  treatment. NHBE cultures were exposed to apical 1 mM  $\text{H}_2\text{O}_2$  for 1h. After removal of  $\text{H}_2\text{O}_2$  the cultures were returned to the  $\text{CO}_2$  incubator for 23h and then placed in Ussing chambers for measurement of both resistance and forskolin-stimulated  $I_{\text{SC}}$  (time point 24h). Panel a,  $I_{\text{SC}}$  before and 23h after treatment are shown (mean  $\pm$  s.e.m.,  $n = 3$  lung donors). Forskolin-stimulated  $I_{\text{SC}}$  currents returned to normal within 23h after treatment. Panel b, Epithelial resistance before and 23h after treatment are shown (mean  $\pm$  s.e.m.,  $n = 3$  lung donors). Resistance returns to normal within 23h after treatment. (DOCX 633 kb)

## Abbreviations

$[\text{Ca}^{2+}]_i$ : intracellular calcium concentration; ALI: air liquid interface; GPCR: G-protein coupled receptor;  $I_{\text{SC}}$ : short circuit current; KH: Krebs-Henseleit buffer; NHBE: normal human bronchial epithelial; sAC: soluble adenylyl cyclase; tmAC: transmembrane adenylyl cyclase.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

PI conceived the project, collected the Ussing chamber and microscopy data and contributed to the design of experiments, analysis and interpretation of the data and to the drafting and revising of the document; UH collected the permeability data and contributed to analysis; MS contributed to interpretation of the data and drafting and revising of the document; GEC contributed to the design of experiments, analysis and interpretation of the data and to the drafting and revising of the document. All authors read and approved the final manuscript.

## Acknowledgements

The authors thank Nathalie Baumlin Schmid for providing cell cultures for these studies. This work was supported by FAMRI grants CIA 123060 to G.E.C. and CIA 103027 to M.S.

## Author details

<sup>1</sup>Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, Miller School of Medicine, University of Miami, 1600 NW 10th Ave, Miami 33136 FL, USA. <sup>2</sup>Department of Immunology, Herbert Wertheim College of Medicine,



Florida International University, Miami, FL 33199, USA. <sup>3</sup>Department of Cell Biology, Miller School of Medicine, University of Miami, 1600 NW 10th Ave, Miami 33136 FL, USA.

Received: 3 December 2015 Accepted: 26 January 2016

## References

- Al-Sadi R, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. *Front Biosci.* 2009;14:2765–78.
- Welsh MJ, Shasby DM, Husted RM. Oxidants increase paracellular permeability in a cultured epithelial cell line. *J Clin Invest.* 1985;76(3):1155–68. doi:10.1172/jci112071.
- Rochat T, Burkhard C, Finci-Cerkez V, Meda P. Oxidative stress causes a protein kinase C-independent increase of paracellular permeability in an in vitro epithelial model. *Am J Respir Cell Mol Biol.* 1993;9(5):496–504. doi:10.1165/ajrcmb.9.5.496.
- Seth A, Yan F, Polk DB, Rao RK. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol.* 2008;294(4):G1060–9. doi:10.1152/ajpgi.00202.2007.
- Forteza R, Salathe M, Miot F, Conner GE. Regulated hydrogen peroxide production by Duox in human airway epithelial cells. *Am J Respir Cell Mol Biol.* 2005;32(5):462–9.
- Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J.* 2003;17(11):1502–4.
- Harper RW, Xu C, Eiserich JP, Chen Y, Kao CY, Thai P, et al. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett.* 2005;579(21):4911–7.
- Jepson MA. Disruption of epithelial barrier function by H<sub>2</sub>O<sub>2</sub>: distinct responses of Caco-2 and Madin-Darby canine kidney (MDCK) strains. *Cell Mol Biol.* 2003;49(1):101–12.
- Waters CM, Savla U, Panos RJ. KGF prevents hydrogen peroxide-induced increases in airway epithelial cell permeability. *Am J Physiol.* 1997;272(4 Pt 1):L681–9.
- Chapman KE, Waters CM, Miller WM. Continuous exposure of airway epithelial cells to hydrogen peroxide: protection by KGF. *J Cell Physiol.* 2002;192(1):71–80.
- Rao RK, Baker RD, Baker SS, Gupta A, Holycross M. Oxidant-induced disruption of intestinal epithelial barrier function: role of protein tyrosine phosphorylation. *Am J Physiol.* 1997;273(4 Pt 1):G812–23.
- Rao RK, Li L, Baker RD, Baker SS, Gupta A. Glutathione oxidation and PTPase inhibition by hydrogen peroxide in Caco-2 cell monolayer. *Am J Physiol Gastrointest Liver Physiol.* 2000;279(2):G332–40.
- Kevill CG, Oshima T, Alexander JS. The role of p38 MAP kinase in hydrogen peroxide mediated endothelial solute permeability. *Endothelium.* 2001;8(2):107–16.
- Basuroy S, Seth A, Elias B, Naren AP, Rao R. MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. *Biochem J.* 2006;393(Pt 1):69–77. doi:10.1042/bj20050959.
- Sheth P, Samak G, Shull JA, Seth A, Rao R. Protein phosphatase 2A plays a role in hydrogen peroxide-induced disruption of tight junctions in Caco-2 cell monolayers. *Biochem J.* 2009;421(1):59–70.
- Yamaya M, Sekizawa K, Masuda T, Morikawa M, Sawai T, Sasaki H. Oxidants affect permeability and repair of the cultured human tracheal epithelium. *Am J Physiol.* 1995;268(2 Pt 1):L284–93.
- Seto V, Hirota C, Hirota S, Janssen LJ. E-Ring Isoprostanes Stimulate a Cl Conductance in Airway Epithelium via Prostaglandin E<sub>2</sub>-Selective Prostanoid Receptors. *Am J Respir Cell Mol Biol.* 2008;38(1):88–94. doi:10.1165/rcmb.2007-0117OC.
- Joy AP, Cowley EA. 8-iso-PGE<sub>2</sub> stimulates anion efflux from airway epithelial cells via the EP<sub>4</sub> prostanoid receptor. *Am J Respir Cell Mol Biol.* 2008;38(2):143–52. doi:10.1165/rcmb.2006-0295OC.
- Jones CL, Li T, Cowley EA. The prostaglandin E<sub>2</sub> type 4 receptor participates in the response to acute oxidant stress in airway epithelial cells. *J Pharmacol Exp Ther.* 2012;341(2):552–63. doi:10.1124/jpet.111.187138.
- Conner GE, Ivonne P, Gelin M, Whitney P, Salathe M. H<sub>2</sub>O<sub>2</sub> stimulates cystic fibrosis transmembrane conductance regulator through an autocrine prostaglandin pathway, using multidrug-resistant protein-4. *Am J Respir Cell Mol Biol.* 2013;49(4):672–9. doi:10.1165/rcmb.2013-0156OC.
- Ivonne P, Salathe M, Conner GE. Hydrogen peroxide stimulation of CFTR reveals an Epac-mediated, soluble AC-dependent cAMP amplification pathway common to GPCR signalling. *Br J Pharmacol.* 2015;172(1):173–84. doi:10.1111/bph.12934.
- Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med.* 2005;107:183–206.
- Nlend MC, Bookman RJ, Conner GE, Salathe M. Regulator of G-protein signaling protein 2 modulates purinergic calcium and ciliary beat frequency responses in airway epithelia. *Am J Respir Cell Mol Biol.* 2002;27(4):436–45.
- Unwalla HJ, Horvath G, Roth FD, Conner GE, Salathe M. Albuterol modulates its own transepithelial flux via changes in paracellular permeability. *Am J Respir Cell Mol Biol.* 2012;46(4):551–8. doi:10.1165/rcmb.2011-0220OC.
- Lieb T, Frei CW, Frohock JJ, Bookman RJ, Salathe M. Prolonged increase in ciliary beat frequency after short-term purinergic stimulation in human airway epithelial cells. *J Physiol.* 2002;538(Pt 2):633–46.
- Fukamoto K, Takagi N, Yamamoto R, Moriyama Y, Takeo S, Tanonaka K. Prostanoid EP<sub>1</sub> receptor antagonist reduces blood-brain barrier leakage after cerebral ischemia. *Eur J Pharmacol.* 2010;640(1-3):82–6. doi:10.1016/j.ejphar.2010.05.001.
- Tanaka MN, Diaz BL, de Souza W, Morgado-Diaz JA. Prostaglandin E<sub>2</sub>-EP<sub>1</sub> and EP<sub>2</sub> receptor signaling promotes apical junctional complex disassembly of Caco-2 human colorectal cancer cells. *BMC Cell Biol.* 2008;9:63. doi:10.1186/1471-2121-9-63.
- Rodriguez-Lagunas MJ, Martin-Venegas R, Moreno JJ, Ferrer R. PGE<sub>2</sub> promotes Ca<sup>2+</sup>-mediated epithelial barrier disruption through EP<sub>1</sub> and EP<sub>4</sub> receptors in Caco-2 cell monolayers. *Am J Physiol Cell Physiol.* 2010;299(2):C324–34. doi:10.1152/ajpcell.00397.2009.
- Kevill CG, Okayama N, Alexander JS. H<sub>2</sub>O<sub>2</sub>-mediated permeability II: importance of tyrosine phosphatase and kinase activity. *Am J Physiol Cell Physiol.* 2001;281(6):C1940–7.
- Rao R, Baker RD, Baker SS. Inhibition of oxidant-induced barrier disruption and protein tyrosine phosphorylation in Caco-2 cell monolayers by epidermal growth factor. *Biochem Pharmacol.* 1999;57(6):685–95.
- Hadari YR, Geiger B, Nativ O, Sabanay I, Roberts Jr CT, LeRoith D, et al. Hepatic tyrosine-phosphorylated proteins identified and localized following in vivo inhibition of protein tyrosine phosphatases: effects of H<sub>2</sub>O<sub>2</sub> and vanadate administration into rat livers. *Mol Cell Endocrinol.* 1993;97(1-2):9–17.
- Volberg T, Zick Y, Dror R, Sabanay I, Gilon C, Levitzki A, et al. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO J.* 1992;11(5):1733–42.
- Schmid A, Sutto Z, Nlend MC, Horvath G, Schmid N, Buck J, et al. Soluble adenylyl cyclase is localized to cilia and contributes to ciliary beat frequency regulation via production of cAMP. *J Gen Physiol.* 2007;130(1):99–109. doi:10.1085/jgp.200709784.
- Chen X, Baumlín N, Buck J, Levin LR, Fregien N, Salathe M. A soluble adenylyl cyclase form targets to axonemes and rescues beat regulation in soluble adenylyl cyclase knockout mice. *Am J Respir Cell Mol Biol.* 2014;51(6):750–60. doi:10.1165/rcmb.2013-0542OC.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
www.biomedcentral.com/submit

