

Sustained Protein Kinase D Activation Mediates Respiratory Syncytial Virus-Induced Airway Barrier Disruption

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Abbreviation used:

AJ: Adherens junction

AJC: Apical junctional complex

- 26 dsRNA: Double-stranded RNA
- 27 LDH: Lactate dehydrogenase
- 28 pAb: Polyclonal antibody
- 29 PKC: Protein kinase C
- 30 PKD: Protein kinase D
- 31 polyI:C: Polyinosinic:polycytidylic acid
- 32 TEER: Transepithelial electrical resistance
- 33 TJ: Tight junction
- 34 TLR: Toll-like receptor
- 35 ZO-1: Zonula occludens protein 1

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ABSTRACT:

Understanding the regulation of airway epithelial barrier function is a new frontier in asthma and respiratory viral infections. Despite recent progress, little is known about how Respiratory Syncytial Virus (RSV) acts at mucosal sites, and very little is known about its ability to influence airway epithelial barrier function. Here we studied the effect of RSV infection on airway epithelial barrier using model epithelia. 16HBE14o- bronchial epithelial cells were grown on Transwell® inserts and infected with RSV strain A2. We analyzed: (i) epithelial apical junctional complex (AJC) function, measuring transepithelial electrical resistance (TEER) and permeability to FITC-conjugated dextran, and (ii) AJC structure using immunofluorescent staining. Cells were pre-treated with and without protein kinase D (PKD) inhibitors. UV irradiated RSV served as negative control. RSV infection led to a significant reduction in TEER, and increase in permeability. Additionally it caused disruption of the AJC, and remodeling of the apical actin cytoskeleton. Pre-treatment with two structurally unrelated PKD inhibitors markedly attenuated RSV-induced effects. RSV induced phosphorylation of the actin-binding protein cortactin in a PKD-dependent manner. UV inactivated RSV had no effect on AJC function or structure. Our results suggest that RSV-induced airway epithelial barrier disruption involves PKD-dependent actin cytoskeletal remodeling, possibly dependent on cortactin activation. Defining the mechanisms by which RSV disrupts epithelial structure and function should enhance our understanding of the association between respiratory viral infections, airway inflammation, and allergen sensitization. Impaired barrier function may open a potentially new therapeutic target for RSV mediated lung diseases.

INTRODUCTION

Respiratory Syncytial Virus (RSV) is the most common respiratory pathogen in infants and young children¹ and an important cause of death in childhood². RSV has been identified as a source of morbidity and mortality in elderly and high-risk adults³. RSV infects airway epithelial cells and is thought to cause tissue pathology by inducing the expression of pro-inflammatory mediators leading to airway inflammation and ultimately an anti-viral immune response⁴. RSV also induces the expression of anti-apoptotic genes and promotes epithelial cell survival, which is probably a strategy to ensure viral replication in infected cells⁵.

Emerging evidence points to a role for airway barrier dysfunction during respiratory viral infections⁶ as well as in stable asthmatics⁷. The airway barrier is made up of the surface mucus layer as well as apical junctional complexes (AJC) that regulate paracellular permeability⁸. Previously we demonstrated that polyinosinic:polycytidylic acid (polyI:C), a synthetic double-stranded RNA and viral mimetic, induces potent breakdown of the airway epithelial AJC in a protein kinase D (PKD) dependent manner⁹. Protein kinase D (PKD), formerly known as PKC μ , is a serine/threonine protein kinase family consisting of three isoforms (PKD1-3)¹⁰. The PKD family is involved in a number of important cell functions including survival, migration, differentiation, proliferation, and membrane trafficking¹¹. Interestingly, PKD was recently shown to be an upstream regulator of cortactin, an actin binding protein involved in actin polymerization and regulation of junctional structures in other cell types^{12,13}. Although activation of epithelial PKC plays a role in the early stages of RSV infection^{14,15}, we have limited understanding of the expression and function of PKD in epithelial cells in the context of naturally occurring viral infections. Furthermore, whether cortactin-dependent actin polymerization is involved in AJC disassembly in the airway is not known.

In this current study we sought to address these gaps in our knowledge by studying the effect of RSV infection on airway epithelial AJC structure and function. We tested the hypothesis that RSV mediates AJC disassembly and remodeling of the perijunctional F-actin cytoskeleton in a PKD-dependent manner. We show that RSV induces potent breakdown of AJC structure and function in the absence of cell death, and we propose a model in which RSV replication leads to sustained PKD activation, phosphorylation of cortactin, actin remodeling and AJC disassembly. These findings provide new knowledge about RSV effects on the airway barrier and identify new pharmacologic targets to explore in the treatment of RSV-induced lung infections.

METHODS:

Antibodies

The following primary monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) were used to detect junctional and signaling proteins by immunofluorescence labeling and immunoblotting: anti-occludin, anti-ZO-1 mAbs, anti-E-cadherin mAb (Invitrogen, Camarillo, CA); anti- β -catenin mAb (BD Bioscience); anti-TLR-3 (Abcam, Cambridge, MA); phospho-PKD/PKC (ser 744/748) and PKD/PKCmu, cleaved caspase 3 pAb (Cell Signaling, Danvers, Mass), Ph-cortactin pAb (anti-cortactin [pS405] phosphospecific pAb) (Protea Bioscience, San Jose, CA), and anti-cortactin (p80/85) clone 4F11 mAb (Millipore, Billerica, MA). Fluorescently labeled phalloidin 594 and 488 (Invitrogen, Camarillo, CA) were used to visualize actin filaments. Anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa-488 or Alexa-568 were obtained from Invitrogen (Camarillo, CA). Mouse and Rabbit secondary HRP were purchased from GE healthcare (Fairfield, Conn).

Chemical and reagents

PKC inhibitors Gö6976, Gö6983 were obtained from EMD Biosciences (Billerica, MA). PKC inhibitor

GF109203X was obtained from Calbiochem. PKD inhibitor, CID755673, was a kind gift from Dr. Elizabeth R. Sharlow, (University of Virginia, this is also commercially available at Tocris # 3327). Fluorescein–conjugated 3-Kd dextran was obtained from Invitrogen (Camarillo, CA). Synagis® (palivizumab) was obtained from MedImmune.

Airway epithelial cell culture

16HBE14o- human bronchial epithelial cells (a gift from Dr. Dieter C. Gruenert, University of California San Francisco) were cultured in collagen-coated transwells as previously described⁹. Cells were cultured on 0.4 µm or 5.0 µm pore size filters (Transwell, Costar, Cambridge, Mass) for immunofluorescent staining and permeability studies, respectively. For biochemical experiments cells were cultured in 24-well plastic plates.

Respiratory Syncytial Virus

Polarized human airway epithelial cells were infected apically with RSV derived from RSV A2 strain (a kind gift Dr. Edward E. Walsh, University of Rochester, NY). In some experiments we used rgRSV (RSV derived from the RSV-A₂ expressing the green fluorescent protein gene), a kind gift from Dr. Mark Peeples (Nationwide Children's Hospital Research Institute, Columbus, OH, and Dr. Peter Collins, National Institutes of Health, Bethesda, MD) as described^{16 17}. UV Inactivated RSV (UV-RSV) was used as negative control. UV inactivation was performed by exposure of RSV to UVB irradiation for 20 minutes. Inactivation of RSV replication was confirmed by plaque forming assay.

Transepithelial electrical resistance (TEER)

TEER was measured with an EVOMX voltohmmeter (World Precision Instruments, Sarasota, FL) as previously described⁹. Data are presented as percent changes compared to time zero. Experiments

were performed when TEER reached $>500 \Omega \times \text{cm}^2$.

Paracellular flux of fluorescent markers

Permeability assay was performed by measuring flux of apically added FITC-conjugated dextran 3 kDa (Invitrogen, Camarillo, CA) from the apical to basal chamber across epithelial monolayers ⁹. Results were expressed as the fold changes from baseline.

Cell cytotoxicity and apoptosis assays

All cultures were monitored daily for cytopathic effect by light microscopy. Following the indicated time points, supernatant was collected for cytotoxicity assay by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) extracellularly (LDH detection kit, Clontech lab. CA) ⁹. To analyze cell death by apoptosis protein was extracted and western blot was performed with a cleaved caspase 3 pAb (Cell Signaling, Danvers, Mass).

Immunofluorescence staining of junctional proteins

As in previous studies ⁹, after indicated treatments, cell monolayers were fixed, and analyzed by immunofluorescence microscopy. Fixed cells were incubated with specific primary antibodies directed against ZO-1, occludin, E-cadherin, and β -catenin, followed by incubation with AlexaFluor labeled secondary antibodies, and mounting with Prolong Gold antifade mounting medium (Invitrogen, Camarillo, CA). Immunofluorescently labeled cell monolayers were examined with a scanning confocal microscope. Single plane confocal images were taken with an Olympus FV1000 laser scanning confocal microscope at the University of Rochester Medical Center Confocal and Conventional Microscopy Core. A 100X U Plan S Apo 1.4 NA (oil) objective was used and images were taken at 512x512 resolution with a Kalman setting of 14. All images were optimized so that fluorescence intensity remained in the linear range. Images were processed with Adobe Photoshop software.

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172 **Analysis of TJ proteins by means of immunoblotting**

173 After the indicated treatments, cell monolayers grown in cell-culture plates were washed with cold
 174 PBS and lysed in RIPA lysis buffer (Sigma). Protein concentration was quantified by icinchoninic acid
 175 protein assay (Pierce, Cheshire, United Kingdom), followed by resolving on SDS-PAGE and
 176 transferring to PVDF (polyvinylidene difluoride) membranes (Bio-Rad). Membranes were incubated
 177 overnight at 4°C with indicated primary antibodies, then for one hour at room temperature with
 178 horseradish peroxidase–conjugated secondary antibodies. The blots were exposed to ECL (RPN
 179 2106; GE Healthcare, Fairfield, Conn) and subjected to autoradiography. GAPDH (Abcam,
 180 Cambridge, MA) was used as a lane loading control. Densitometric quantification was performed
 181 using NIH ImageJ software.

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183 **Extraction of RNA and quantitative Real-Time Polymerase Chain Reaction Analysis**

184 Total RNA was extracted from epithelial cells using E.Z.N.A total RNA kit (OMEGA bio-tek, Norcross,
 185 GA), cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and was
 186 amplified by real time PCR with a iQ5 multicolor Real-Time PCR Detection System (Bio-Rad,
 187 Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and using primers targeting
 188 the sequence 87 bp of the RSV strain A2 genome which encodes for the viral fusion (F) protein (5'
 189 CACCCTGTTGGAAAC 3'and 5' CTCTGTCAGTTCTTG 3'-from sigma Aldrich).

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191 **Statistical analysis**

192 Data was analyzed using Prism software (GraphPad, San Diego, CA), and Microsoft Excel. Data are
 193 representative of three or more experiments and are means $\pm$ SEMs. The data were evaluated
 194 statistically with ANOVA and the Student t test, with Bonferonni correction for multiple comparisons.
 195 Significance was considered at a *P* value of less than 0.05.

RESULTS:

RSV infection decreased resistance and increased permeability of polarized airway epithelial cells

RSV is known to infect human and mouse airway epithelial cells leading to cytokine/chemokine production and airway inflammation ⁴. We previously showed that polyI:C, a mimic of viral infection, induced paracellular permeability of airway epithelial cells ⁹. Here we hypothesized that RSV can disrupt apical junctional complexes (AJC's). To test this possibility, we used polarized 16HBE140–human bronchial epithelial cells, which maintain tight and adherent junctions similar to primary human bronchial epithelial cells ^{6, 9}. We began by growing 16HBE cells on semi-permeable inserts (see Methods) and infected them apically with RSV strain A2 (courtesy Dr. Edward Walsh, University of Rochester, NY) at a multiplicity of infection (MOI) ranging from 0.05 to 1.0. RSV induced a marked decrease in transepithelial electrical resistance (TEER), consistent with barrier disruption (Fig. 1A). The effect of RSV was both dose- and time-dependent, resulting in sustained decreases in TEER for up to 72 hrs (Fig. 1A). RSV infection also led to significant increases in paracellular flux of fluorescein–conjugated 3 kDa dextran (Fig. 1B), indicating increased permeability to macromolecules. In subsequent studies, we used RSV at MOI of 0.5.

RSV infection did not induce cell cytotoxicity or apoptosis

To investigate whether RSV infection induced cytotoxicity or apoptosis in epithelial cells, we infected cells with RSV at MOI of 0.5 and collected cell supernatants at different time points for a cytotoxicity assay (see Methods). As a positive control, we treated cells with 2% Triton, whereas cell culture medium was used as negative control. Importantly, similar to other reports ^{5, 18, 19}, we found no evidence of RSV-induced cell cytotoxicity (Fig. 2A). In an independent experiment confluent polarized epithelial cells were infected with RSV (MOI of 0.5) and whole cell lysates analyzed by Western blot

for cleaved caspase 3 as a marker of apoptosis. We found no evidence of apoptosis in RSV infected epithelia (Fig. 2B).

RSV infection induces AJC disassembly in airway epithelial cells which requires live replicating virus

We next examined the effect of RSV or UV-inactivated RSV on AJC structure and function. Polarized 16HBE140- cell monolayers were infected apically with live RSV (MOI of 0.5) or equivalent amounts of UV-irradiated RSV, and AJC structure was determined using immunofluorescence labeling and confocal microscopy for different TJ (occludin, ZO-1) or AJ (E-cadherin, β -catenin) proteins. In comparison with control uninfected cells, which showed a normal “chicken wire” appearance of AJ and TJ structure, RSV infected cells exhibited marked disruption of AJC with evidence of gaps and breaks in junctional strands (arrowheads, Fig. 3A). In spite of the breakdown of AJC epithelial nuclei remained intact (data not shown).

Interestingly, UV irradiated RSV did not disrupt apical junction structure, and in contrast to non-irradiated intact virus, UV-RSV also did not induce junctional dysfunction as determined by measuring TEER (Fig. 3B), and paracellular flux of fluorescein-conjugated 3 kDa dextran (data not shown).

RSV infection induced PKD phosphorylation

In other cell types, apical junctional complex dysfunction can occur via suppression of expression of junctional components or by expression-independent mechanisms. Western blot of whole cell lysates revealed that total expression of key junctional components was largely unaltered following RSV infection (Fig. 3C and D). This contrasted with marked disruption of AJC formation at the apical membrane as detected using confocal microscopy (Fig. 3A). Expression-independent AJC disruption often involves junctional internalization driven by actin cytoskeletal remodeling²⁰. When we analyzed actin structure in RSV-infected epithelial cells, we found marked redistribution of cortical actin fibers

(Fig. 4A).

Formerly known as PKC μ , PKD is now known to play an important role in cell motility and cytoskeletal rearrangements^{11, 12}, but very little is known about this signaling molecule in the airway. We examined activation of PKD in RSV-infected epithelial cells by monitoring PKD phosphorylation (Ser^{744/748}) by Western blot. Interestingly, RSV infection resulted in biphasic PKD phosphorylation with both early (2-3 hours) and late (48 hours) kinetics (Fig. 4B and C). Only the early wave of PKD phosphorylation occurred using UV inactivated RSV (Fig. 4D and E), suggesting that this early phosphorylation depended on viral entry whereas the later phase required viral replication (see below).

RSV-induced epithelial permeability is mediated by PKD

Previous studies have shown the role of PKC signaling in epithelial cell tight junction integrity²⁰, and activation of different PKC family members plays a role in the early stages of RSV infection^{14, 15}. In order to determine whether these protein kinases contributed to RSV-induced AJC dysfunction, we used a panel of inhibitors targeting both classical and atypical PKC family members (Gö6983, and GF109203X) as well as PKD (Gö6976). Interestingly, only Gö6976 (Fig. 5A), but not other inhibitors (data not shown), attenuated the effect of RSV infection on epithelial permeability and AJC structure. To confirm the role of PKD signaling in RSV induced disrupted barrier structure, we next used a novel benzoxoloazepinolone PKD inhibitor (e.g. CID755673, provided courtesy Dr. E. Sharlow, UVA), which is selective, cell permeable and structurally unrelated to Gö6976^{21, 22}. This compound also strikingly prevented RSV-induced barrier disruption confirming a role for PKD in RSV induced AJC disassembly (Fig. 5A) and increased permeability (Fig. 5B). Similarly RSV, but not UV-RSV, upregulated expression of the key viral sensor TLR3 (Fig. 6A and B). This indicates that live replicating virus is required to induce epithelial junctional dysfunction. Interestingly the PKD antagonist also blocked the upregulation of TLR3 (Fig. 6A)

RSV-induced phosphorylation of cortactin is dependent on PKD signaling

To further dissect the mechanism of RSV-induced AJC disruption we sought to examine the molecular events involved in AJC disruption induced by RSV and dependent on PKD. PKD was recently shown to be an upstream regulator of cortactin, an actin binding protein involved in actin polymerization^{12, 13}. We next assessed the expression of total and phospho-cortactin (Ser⁴⁰⁵) at different time points following RSV infection. Similar to the case of PKD, RSV infection resulted in a biphasic pattern of cortactin phosphorylation (Fig. 7A and B). Importantly, both phases of cortactin-phosphorylation were dependent upon PKD, but only the early phase occurred using UV-RSV (Fig. 7C and D). Confocal microscopy revealed increased co-localization of actin and cortactin in response to RSV infection (yellow staining, Fig. 7E). Taken together, these data support a model in which RSV infection and replication induce sustained PKD phosphorylation leading to cortactin-dependent remodeling of the actin cytoskeleton, destabilizing membrane apical junction complexes.

PKD inhibitor did not inhibit early RSV infection or RSV replication.

One possible explanation for the observed effects of PKD inhibitors is that they prevent RSV uptake or replication. To test this, epithelial cells were infected with RSV at a multiplicity of infection of 0.05-0.5 in the presence or absence of PKD inhibition. Palivizumab (Synagis), a known monoclonal antibody against RSV fusion protein, was used as a positive control for suppression of viral infection. Importantly, we observed no significant differences in RSV infectivity in the presence of PKD inhibition (Fig. 8A and B). In addition, PKD inhibition did not affect viral replication, as similar viral loads were recovered from RSV-infected cells in the presence or absence of the inhibitor (Fig. 8C).

DISCUSSION:

Apical junctional complexes are an integral part of the airway epithelial barrier. Although epithelial

barrier dysfunction is increasingly associated with disease states including asthma ⁷, the molecular mechanisms involved are not well understood. Tight junctions and adherens junctions are dynamic structures that are responsive to diverse environmental stimuli ²³. Here we show that RSV, a common respiratory virus associated with substantial morbidity, induces epithelial barrier dysfunction in a PKD-dependent manner. RSV infection causes barrier dysfunction without altering expression of key junctional components, but instead appears to induce disassembly of epithelial junctional complexes by destabilizing the cortical actin cytoskeletal in a cortactin-dependent manner. Our results highlight a novel molecular pathway of airway barrier dysfunction that likely contributes to RSV-induced airway inflammation.

Respiratory viruses including rhinovirus (RV) and RSV have been previously shown to cause airway epithelial barrier dysfunction via different mechanisms. Hershenson and colleagues have shown that RV causes epithelial junction dysfunction in a NOX1-dependent manner ^{6, 24}, whereas Singh et al. showed that RSV infection reduced transepithelial electrical resistance across airway epithelial monolayers ²⁵. Our results clearly show that RSV infection leads to marked disassembly of AJC resulting in increased paracellular permeability (Fig.1A and B). Inducible junction dysfunction may promote viral replication by allowing paracellular escape of basolateral viral particles ²⁶. We speculate that another consequence of virus-induced epithelial permeability might be increased “outside/in” translocation of inhaled particles that deposit in the airway. In the case of inhaled aeroallergens, increased paracellular translocation will facilitate uptake by intra-epithelial dendritic cells and may help explain the association between respiratory viral infection and allergen sensitization in asthma.

The observation that RSV also paradoxically promotes epithelial cell survival ^{5, 18, 19, 27} raises the intriguing possibility that a “leaky airway” may persist after acute infection. At present there are no assays of outside/in airway epithelial barrier function in human subjects in routine clinical use, but

development of such assays may prove useful in the future to determine the clinical significance of the mechanisms we report here.

Originally known as PKC μ , this molecule was renamed PKD since it has different structure and substrate specificity than other PKC family members^{10, 28}. In other cell types, PKD regulates cell shape and motility in part by controlling actin dynamics, but it has not been well-studied in the lung^{12, 29-32}. A recent study uncovered a key role for PKD in a mouse model of hypersensitivity pneumonitis³³, and the *C. elegans* PKD homolog DKF-2 was found to regulate intestinal innate immunity³⁴. Thus PKD may play a broader role in regulating airway mucosal immune responses, and further study of the expression and function of this signaling molecule in asthma and other airway diseases seems warranted. The recent development of selective benzoxoloazepinolone PKD inhibitor (see Fig. 5, and 7) should accelerate research in this area^{22, 35-37}. This compound is a potent and cell-permeable inhibitor for PKD. Although any chemical inhibitor might have potential off-target effects, we used two structurally unrelated antagonists which prevented RSV induced junctional disassembly as well as PKD phosphorylation, thus fulfilling two key criteria of inhibitor specificity³⁸.

We found that RSV, similar to the synthetic double stranded RNA polyI:C⁹, both induced phosphorylation of PKD on Ser^{744/748}, a key step in PKD activation³⁹. Both RSV infection and exposure disrupted apical junctional complexes in a PKD-dependent manner, and both also led to PKD-dependent cortactin phosphorylation (Fig. 7 and data not shown). Thus even though they engage different pattern recognition receptors, RSV and polyI:C converge on PKD and cortactin to cause epithelial barrier dysfunction.

Our kinetic analysis uncovered temporally distinct patterns of activation of signaling molecules in response to RSV and UV-inactivated RSV, which allow us to construct the following model. UV-RSV

induced only the early phase of PKD and cortactin phosphorylation (Fig. 4D, and 7C), without affecting AJC structure or function (Fig. 3A and B). In contrast, non-irradiated RSV induced both early and sustained PKD activation and cortactin phosphorylation (Fig. 4B, and 7A). This suggests that live replicating virus is required to cause sustained PKD activation and sufficient cortactin-dependent actin remodeling to cause AJC disassembly. This model is consistent with earlier work by Monick et al. showing that RSV induced biphasic PKC and MAPK activation in A549 cells ¹⁴. Since RSV (and not UV-RSV) also resulted in upregulation of TLR3 (Fig. 6A and B), which we previously found was responsible in part for polyI:C-induced AJC disassembly ⁹, we speculate that sustained viral replication generates a positive feed-back loop amplifying signaling modules that lead to barrier dysfunction.

RSV was discovered more than 50 years ago and continues to be a chief cause of hospitalization worldwide not only in infants, but also in older and high risk adults ³. RSV infection in childhood has been linked to chronic inflammation and asthma ⁴⁰⁻⁴², and although we have a very detailed understanding of RSV immunopathogenesis ⁴³⁻⁴⁷, current treatment strategies for RSV are largely supportive. Palivizumab, which is approved for RSV prophylaxis in high-risk infants, has only moderately decreased hospital admission due to RSV infection ^{48, 49}. Here we report a new pathway of RSV induced junctional complex disassembly that likely contributes to airway epithelial barrier dysfunction and inflammation in vivo. Therapeutic strategies that interfere with virus induced barrier dysfunction and restore epithelial barrier integrity may hold promise in the future.

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FIGURE LEGENDS:

FIG 1 RSV infection disrupts barrier function of airway epithelial cells. Confluent epithelial cell monolayers were infected with RSV at MOI of 0.05-1.0 for 72 hours. (A) TEER was measured by a voltohmmeter at different time points after infection. RSV causes a dose- and time-dependent decrease in TEER, where data are expressed relative to TEER measured at time zero (values approximately $500 \Omega \times \text{cm}^2$, which was set as 100%). (B) Permeability assay was performed with 3-Kd fluorescein-conjugated dextran at 48 and 72 hours post RSV infection. Data show that RSV causes a time and dose dependent increase in permeability. Data are representative of 2-3 independent experiments. Differences between control and RSV infected cells were analyzed by ANOVA. *** $p < 0.001$.

FIG 2 RSV infection does not induce cell cytotoxicity or apoptosis. (A) 16HBE140- cell monolayers were infected with RSV MOI of 0.5, or control medium for 0-48 hours. Results (A) show cytotoxicity index of infected cells compared with untreated cells. Medium alone was used as negative control, and lysed cells by Triton were served as positive control. (B) Confluent polarized epithelial cells were infected with RSV MOI of 0.5, or medium control. Cell lysates were immunoblotted and probed with antibodies against cleaved caspase 3. Cell lysate from cytochrome-c treated and untreated jurkat

cells (Cell Signaling) were used as positive and negative controls. A representative blot of 3 independent experiments has been shown.

FIG 3 RSV-induced AJC disassembly requires live replicating virus. Polarized 16HBE140-monolayers were infected with RSV at MOI of 0.5, control medium, or equal amount of UV-irradiated RSV for 48 hours. (A) Apical junctional complexes were visualized by immunofluorescent staining and confocal microscopy. Arrows indicate normal chicken wire appearance of membrane AJC. Arrowheads indicate gaps in AJC integrity, and ZO-1 localized to intracellular vacuoles. (B) TEER was measured using a voltohmmeter at indicated time points. UV inactivated RSV did not cause epithelial junctional disassembly or changes in TEER. Images are representative of at least 3 independent experiments. Differences between control, UV-irradiated RSV, and RSV infected cells were analyzed by ANOVA. *** $p < 0.001$. (C) RSV infection did not change AJC protein expression. Total cell lysates of control and RSV infected epithelial cells were analyzed by Western blot with antibodies directed against different TJ and AJ components. Immunoblots representative of different time points post infection are shown. (D) Shows densitometric quantification of at least 3 independent experiments.

FIG 4 RSV infection induces redistribution of cortical actin fibers and PKD phosphorylation. Confluent polarized epithelial cells were infected with RSV MOI of 0.5, or medium control for the indicated time points. (A) Actin cytoskeleton was visualized by fluorescently labeled phalloidin and confocal microscopy. (B) Cell lysates were analyzed by Western blot with antibodies against phospho-PKD/PKC (ser^{744/748}), total PKD, and GAPDH. RSV infection induced PKD phosphorylation beginning at 3 hours, with another peak at 24 hours. The expression of total PKD also slightly increased at later time points (≥ 24 hrs). Images are representative of 3 independent experiments. (C) Lower panel shows densitometric quantification of at least 3 independent experiments. Polarized epithelial cells

were infected with UV irradiated RSV. Like live virus, UV irradiated virus (D) led to increased expression of phospho-PKD in early time points. However the second peak was not observed in UV inactivated RSV confirming that late effect requires live replicating virus. (E) Lower panel shows densitometric quantification of at least 3 independent experiments.

FIG 5 RSV-induced epithelial junctional disassembly is mediated by PKD. (A) Confluent 16HBE140-cell monolayers were infected with RSV at MOI of 0.5 for 48 hours in the presence or absence of two structurally unrelated PKD inhibitors (Gö6976, 10 μ M, Calbiochem, and CID755673, 10 μ M, Tocris). TJ and AJ were visualized by immunofluorescent staining and confocal microscopy. Note intact AJ and TJ staining in control non-infected cells (arrows), which was disrupted in RSV infected cells (arrowheads). AJ and TJ surface expression were restored in RSV-infected cells pre-treated with two structurally unrelated PKD inhibitors. (B) Cells were infected with RSV at MOI of 0.5 for 48 hours in the presence or absence of PKD inhibitors, and permeability assay with 3Kd fluorescein-conjugated dextran was performed at 48 hours post treatment. Both inhibitors significantly attenuated the barrier disrupting effects of RSV. Images are representative of at least 3 independent experiments. Data are presented as mean $\pm$ SEM values of 3 experiments *** $p < 0.001$.

FIG 6 RSV induces up-regulation of TLR3, which requires live replicating infection and is inhibited by PKD inhibitor. (A) Confluent polarized epithelial cells were infected with RSV MOI of 0.5, or medium control at indicated time points. Cell lysates were immunoblotted and probed with antibodies against TLR3. (B) UV irradiation of RSV did not change virus induced TLR expression. Polarized epithelial cells were infected with equal amount of UV irradiated RSV and protein was subjected to western blot analysis. No change in TLR3 expression was noted with UV irradiation, which again indicates the requirement of live replication virus in this process. Images are representative of at least 3 independent experiments.

587

588 **FIG 7** RSV infection induced phosphorylation of cortactin, which was inhibited by a PKD inhibitor.
 589 Confluent polarized epithelial cells were infected with RSV MOI of 0.5, or medium control for indicated
 590 time points. Cell lysates were analyzed by Western blot with antibodies directed against phospho-
 591 cortactin (pS405), total cortactin (p80/85, clone 4F11), and GAPDH. (A) RSV infection caused
 592 cortactin phosphorylation, which was blocked by PKD inhibition. (B) Densitometric quantification of at
 593 least 3 independent experiments. (C) UV-inactivation of RSV changed the pattern of cortactin
 594 phosphorylation. Polarized epithelial cells were infected with UV irradiated RSV. Like live virus,
 595 irradiated virus led to expression of phospho-cortactin in early time points. However the second peak
 596 was not observed in UV inactivated RSV confirming that late effect requires live replicating virus. (D)
 597 Densitometric quantification of at least 3 independent experiments. (E) Confocal images of epithelial
 598 cells show marked redistribution of cortical actin fibers, redistribution of cortactin in RSV infected cells
 599 into intracellular vesicles. Lower panel shows increased co-localization of actin and cortactin in
 600 response to RSV infection (yellow staining). Data are representative of 3 independent experiments.

601

602 **FIG 8** PKD inhibitor did not inhibit early RSV infection or viral replication in epithelial cells. Polarized
 603 epithelial cells were pre-incubated with PKD inhibitor for 2 hours followed by infection with rgRSV at
 604 MOI 0.05-0.5, rinsed twice with PBS after 2 hours, followed by incubation at 37°C for 16 hours.
 605 Palivizumab, a monoclonal antibody against RSV fusion protein (F-protein), was used as a positive
 606 control, which was also added 2 hours before RSV infection. 16 hours post RSV infection, live cells
 607 were subjected to fluorescent microscopy. (A) GFP+ cells are readily apparent after rgRSV infection,
 608 which was completely inhibited using Palivizumab. In contrast, the PKD inhibitor did not affect the
 609 number of GFP+ cells (representative example in A, lower right, average of 3 experiments in panel
 610 (B)). (C) In parallel, inhibition of PKD did not affect RSV viral replication, as determined in cells
 611 infected with RSV A2 and RT-PCR with primers that amplify the RSV A2 F protein. Images are

512 representative of 3 independent experiments. Data are representative of three experiments, with n =
513 3 per experiment.

514

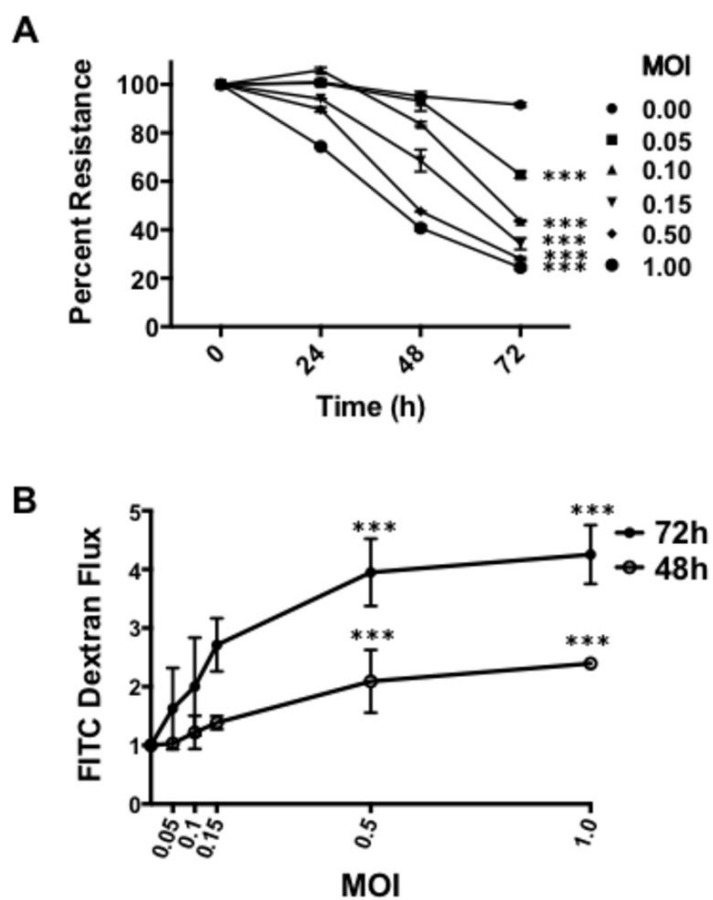




Fig 3

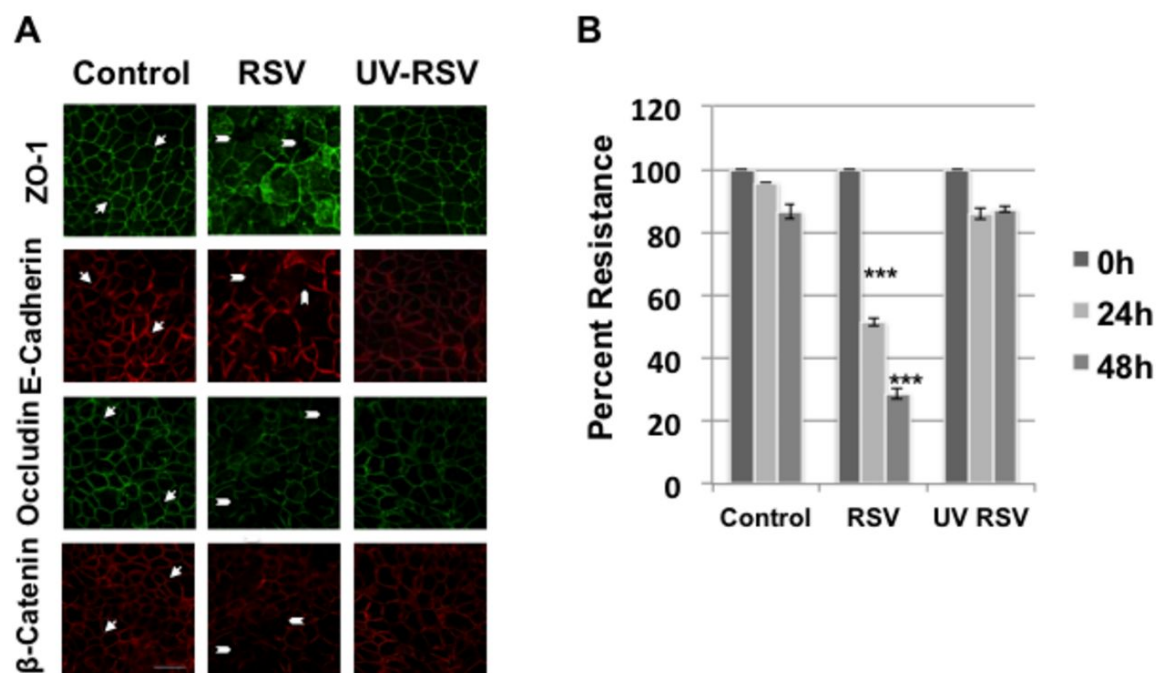


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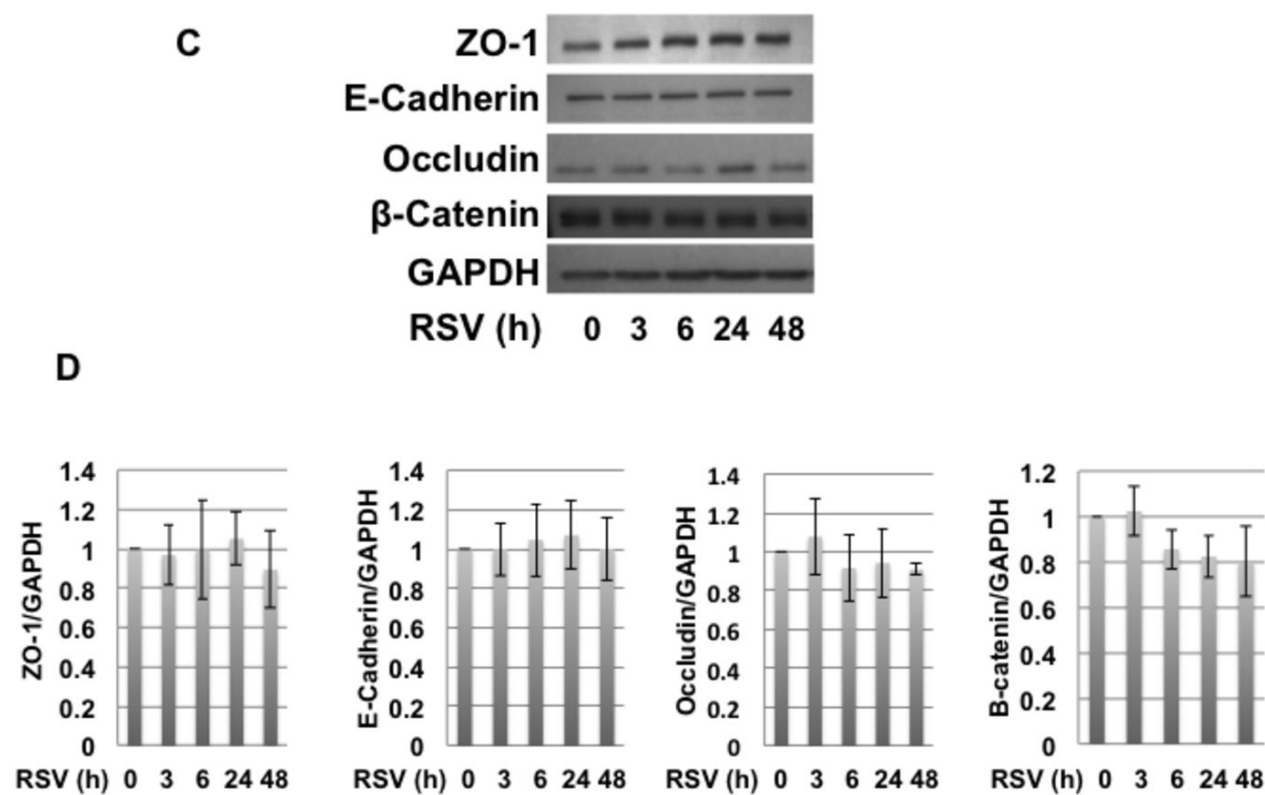


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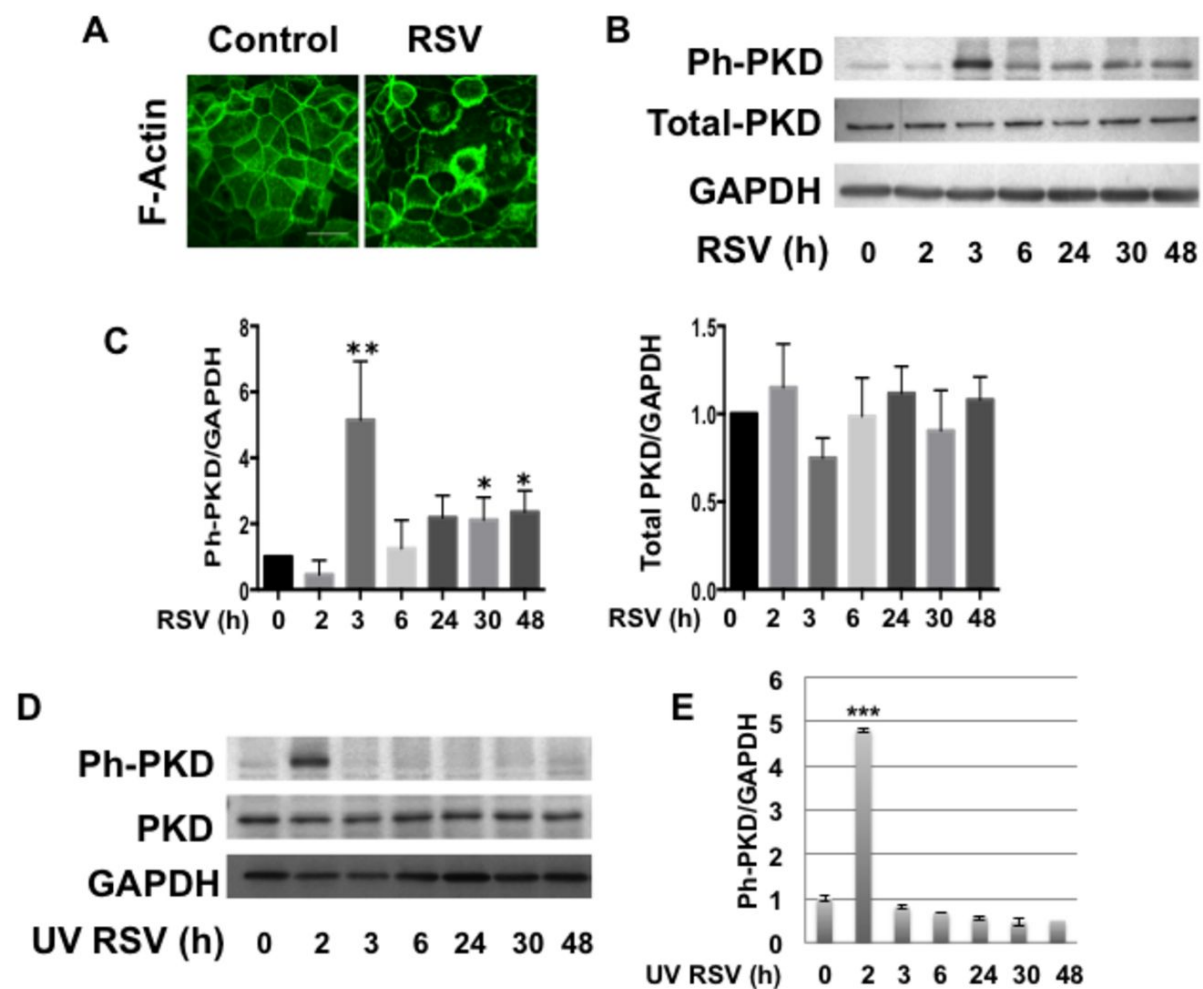


Fig 5

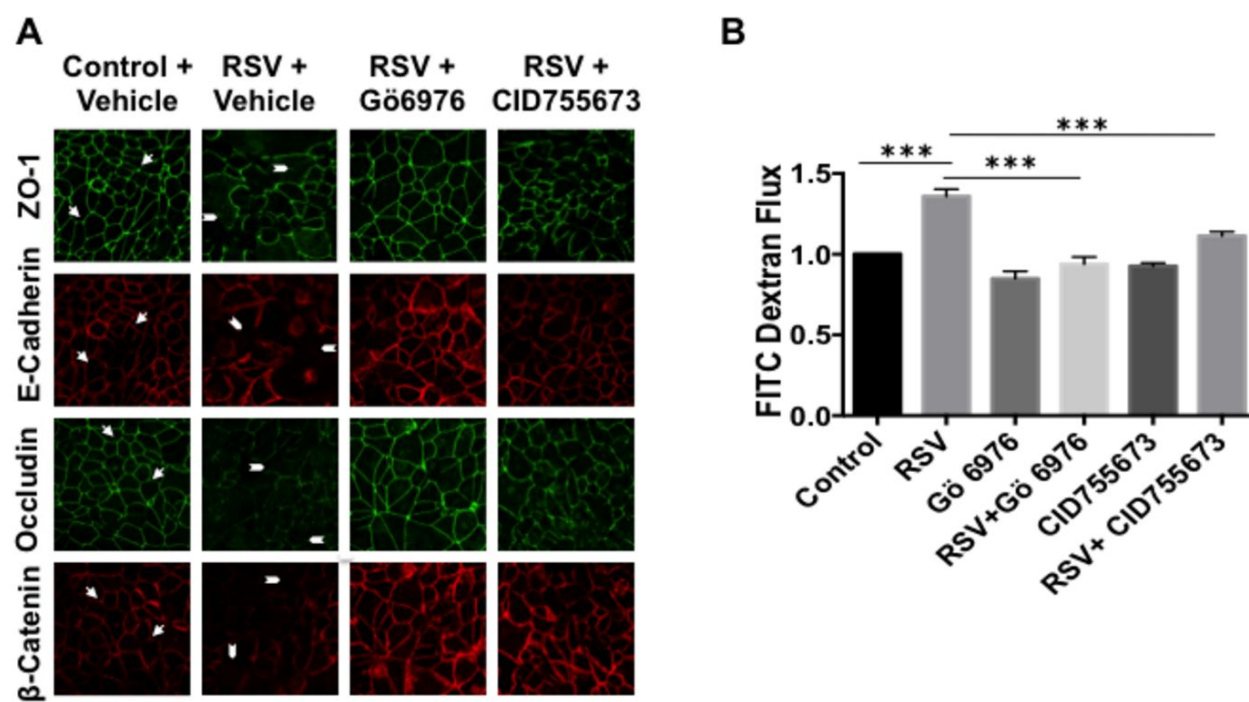


Fig 6

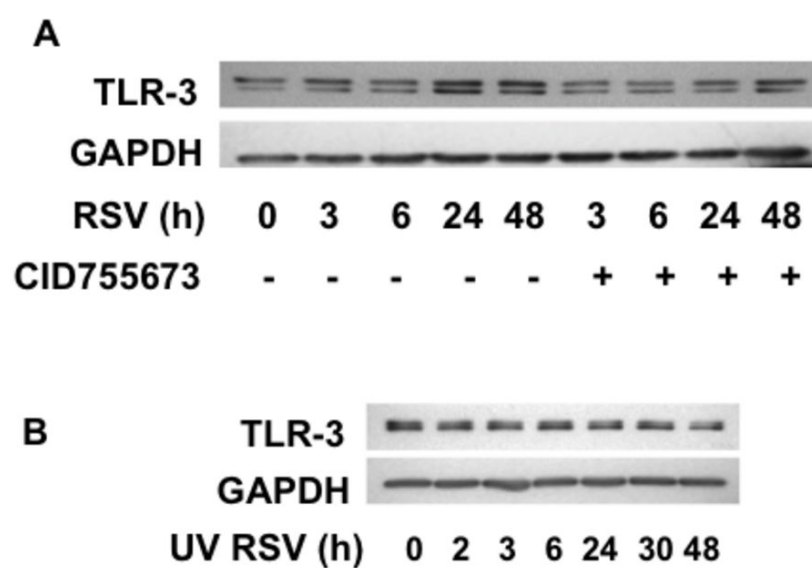


Fig 7

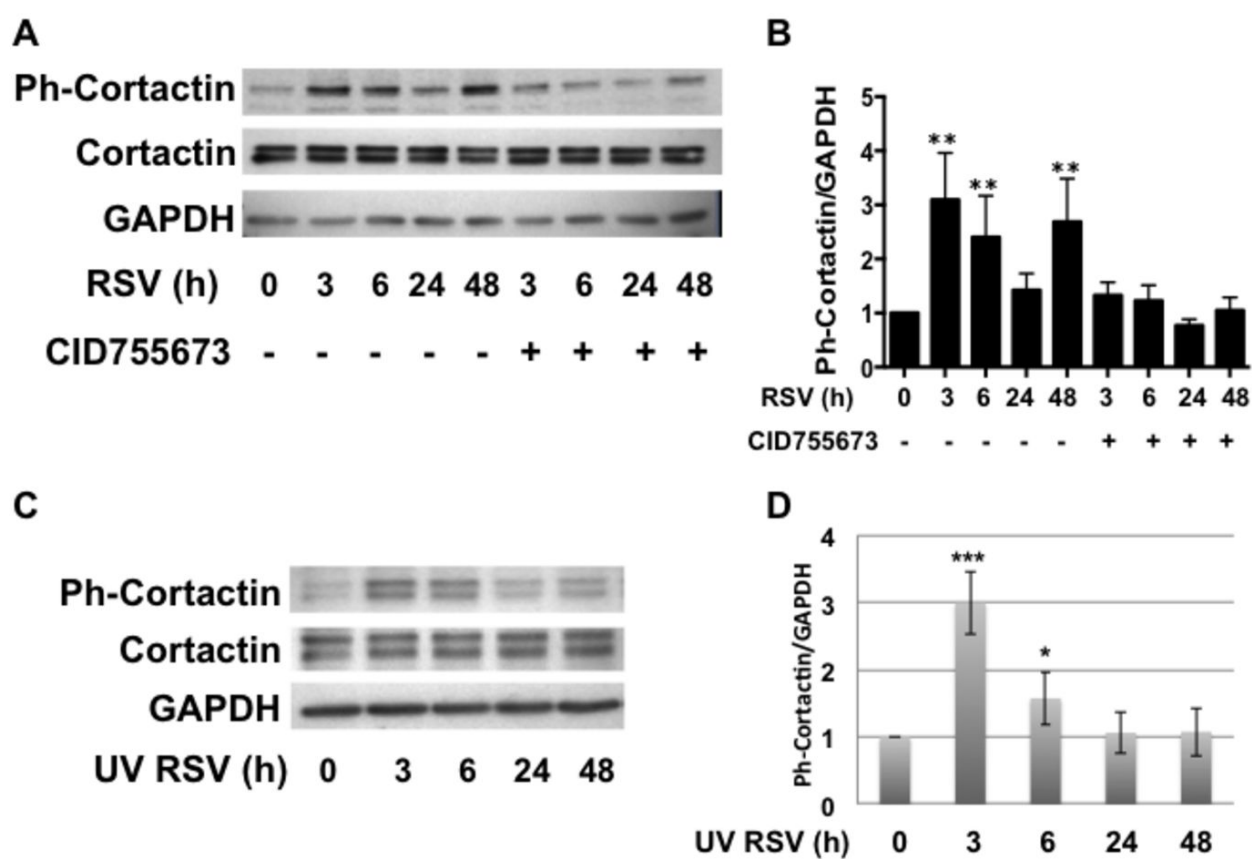


Fig 7

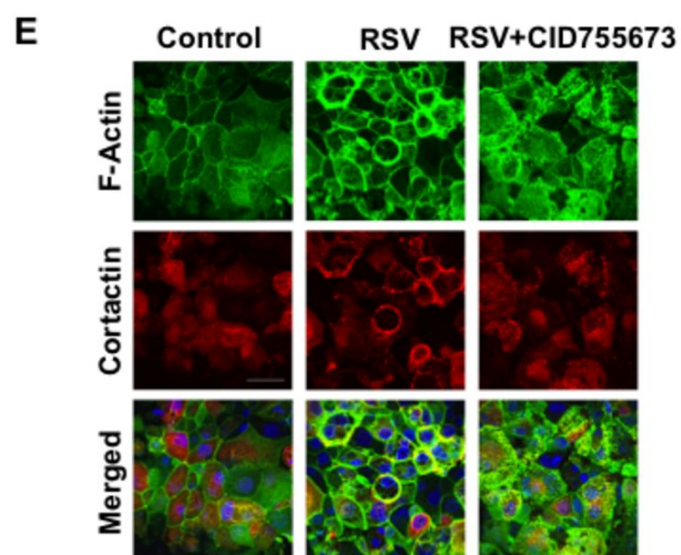


Fig 8

