Articles in PresS. Am J Physiol Cell Physiol (April 26, 2017). doi:10.1152/ajpcell.00043.2017

1

2

3 # Airway epithelial anion secretion and barrier function following exposure to fungal
4 aeroallergens: Role of oxidative stress.

5

6 Nathan A. Zaidman*[+], Kelly E. O'Grady^[+], Nandadevi Patil[†], Francesca Milavetz[†], Peter
7 J. Maniak[†], Hirohito Kita[♦] and Scott M. O'Grady*[†]

8

9 [†]Department of Animal Science, University of Minnesota, St. Paul, MN, 55108;
10 [*]Department of Integrative Biology and Physiology and ^Department of Chemical Engineering
11 and Materials Science, University of Minnesota, Minneapolis, MN, 55455;
12 [♦]Department of Immunology, Mayo Clinic, Rochester MN, 55905
13

14

15 [+]Nathan Zaidman and Kelly O'Grady contributed equally to this study.

16

17 Address correspondence to:

18 Scott M. O'Grady, Ph.D.
19 Department of Animal Science
20 University of Minnesota
21 480 Haecker Hall, 1364 Eckles Ave.
22 St. Paul, MN, 55110
23
24

25 Running title: Anion secretion, barrier function and oxidative stress

26

27 Key words: Chloride secretion, tight junctions, ATP release, intracellular calcium

28

29

30

31

Copyright © 2017 by the American Physiological Society.

32 **Abstract**

33 Aeroallergens produced by *Alternaria alternata* can elicit life-threatening exacerbations
34 of asthma in patients sensitized to this fungus. In this study, the effect of *Alternaria* on ion
35 transport mechanisms underlying mucociliary clearance and airway epithelial barrier function
36 was investigated in human airway epithelial cells. Apical exposure to *Alternaria* induced an
37 increase in anion secretion that was inhibited by blockers of CFTR and Ca^{2+}-activated Cl^-
38 channels. Stimulation of anion secretion was dependent on Ca^{2+} uptake from the apical
39 solution. *Alternaria* exposure also produced an increase in reactive oxygen species (ROS) that
40 was blocked by pretreatment with the oxidant scavenger glutathione (GSH). GSH and the
41 NADPH oxidase inhibitor/complex 1 electron transport inhibitor diphenylene iodonium chloride
42 (DPI) blocked ATP release and the increase in intracellular [Ca^{2+}] evoked by *Alternaria*.
43 *Alternaria* also decreased transepithelial resistance and a portion of this affect was dependent
44 on the increase in ROS. However, the *Alternaria*-induced increase in unidirectional dextran (mw
45 = 4000 Da) flux across the epithelium could not be accounted for by increased oxidative stress.
46 These results support the conclusion that oxidative stress induced by *Alternaria* was responsible
47 for regulating Ca^{2+}-dependent anion secretion and tight junction electrical resistance that would
48 be expected to affect mucociliary clearance.

49

50

51

52

53

54

55

56 **Introduction**

57 *Alternaria alternata* is a saprophytic fungus with worldwide distribution (15;47). Although
58 considered primarily as an outdoor fungus, it is also found in damp, poorly ventilated buildings
59 (33;40). *Alternaria* spore counts are generally highest during warm, dry and windy weather
60 conditions in late summer and early autumn (17;41). These spores contain some of the most
61 potent airborne allergens capable of inducing IgE mediated allergic airway inflammation
62 (16;18;29;42). Moreover, asthma severity appears to be more strongly associated with
63 *Alternaria* exposure than with sensitization to other aeroallergens, including house dust mite and
64 pollens (18;29). *Alternaria* spores are known to induce respiratory distress and airway hyper-
65 responsiveness in asthma patients and individuals sensitized to *Alternaria* are more likely to
66 develop asthma than non-sensitized individuals (25;45). In some populations, sensitization to
67 *Alternaria* is particularly high, especially in farm workers where greater occupational exposure to
68 *Alternaria* spores frequently occurs (1;27;35;40;49). Furthermore, a study investigating the
69 effects of atmospheric CO_2 on *Alternaria alternata* revealed a three-fold increase in sporulation
70 and more than twice the level of antigenic protein content/spore in response to elevated CO_2
71 concentrations above 600 μmol mol^{-1} (52). These results suggest that continuing increases in
72 atmospheric CO_2 associated with global climate change will increase both the level of *Alternaria*
73 exposure and antigenicity of spores that come in contact with the airways.

74 Earlier studies of human airway epithelial cells have shown that exposure to *Alternaria*
75 extracts induces the synthesis and secretion of IL-33, a cytokine that is known to induce Th2-
76 type inflammation in airways (9;19;20;26;45). IL-33 secretion by the epithelium occurs as a
77 consequence of ATP release following exposure to *Alternaria* aeroallergens, resulting in
78 activation of $P2X_7$ receptors and sustained elevations in intracellular $[Ca^{2+}]_i$ (26;39). This
79 prolonged increase in $[Ca^{2+}]_i$ activates processing enzymes that cleave IL-33 into its 19 KDa
80 secreted form and stimulates translocation of the processed cytokine from its storage site within

81 the nucleus into the cytoplasm where it is ultimately secreted into the extracellular fluid. Once
82 released, IL-33 can bind to ST2 receptors expressed by multiple immune cell types. ST2
83 receptor activation induces the production and secretion of Th2 cytokines necessary for eliciting
84 allergic inflammation within the airway mucosa (9).

85 Additionally, more recent experiments have demonstrated that *Alternaria* exposure
86 induces oxidative stress in human airway epithelial cells and that this effect is important in the
87 initiation of IL-33 processing and release (19;21;48). This finding is reminiscent of an earlier
88 study where exposure to pollen proteins induces allergic airway inflammation by increasing the
89 production of reactive oxygen species (ROS) by lung epithelial cells (2;4). The oxidative stress
90 was produced by an endogenous NADPH oxidase(s) associated with pollen grains since
91 removal of pollen NADPH oxidase activity inhibited the inflammatory response. However,
92 exposure of airway epithelial cells to *Alternaria* extract was shown to activate NADPH dual
93 oxidase 1 (DUOX1) located within the apical membrane of the epithelium following $P2Y_2$
94 receptor activation. DUOX1-dependent H_2O_2 production in turn, stimulated Src and EGFR
95 signaling that ultimately resulted in calpain-2 activation and subsequent processing and release
96 of IL-33 (19). Interestingly, the mycotoxin altertoxin (ATX) II, a perylene quinone-type
97 compound produced by *Alternaria alternata* was found to interact with the nuclear factor
98 erythroid-derived 2-like antioxidant response element (Nrf2/ARE) pathway in HT29 intestinal
99 epithelial cells to induce a concentration-dependent depletion of glutathione (GSH) within the
100 cytoplasm, suggesting that ATX II may constitute at least one component of *Alternaria* extract
101 that can produce oxidative stress (23).

102 Exposure of airway epithelial cells to *Alternaria* allergens has also been shown to
103 significantly reduce barrier function (31). Measurements of transepithelial resistance (TER) and
104 FITC-labeled dextran (mw = 4000) showed that exposure to *Alternaria* extracts produced
105 concentration-dependent decreases in TER within 1 hour and increased apical-to-basolateral

106 flux of FITC-dextran after 24 hours of *Alternaria* exposure. Heat treatment of the extract was
107 effective at inhibiting the actions of *Alternaria* on epithelial barrier function, suggesting that the
108 activity of an enzyme within the extract was responsible for barrier disruption. Moreover,
109 experiments with various protease inhibitors including 4-(2-Aminoethyl) benzenesulfonyl fluoride
110 hydrochloride (AEBSF) suggested that serine proteases were the predominant class of
111 proteolytic enzymes responsible for the barrier disrupting effects of *Alternaria* allergens.

112 In the present study the effects of *Alternaria* aeroallergen exposure on ion transport
113 function of human airway epithelium was investigated using an immortalized airway epithelial
114 cell line with well characterized ion transport properties. Our objective was to define the
115 transport mechanisms that are stimulated by these allergens and to identify the underlying
116 signaling pathways responsible for their actions on the epithelium. Our experiments revealed
117 that *Alternaria* stimulates both Ca^{2+}-dependent and CFTR-dependent anion secretion and that
118 treatment with oxidant scavengers such as GSH and N-acetyl cysteine (NAC) inhibit this
119 response by reducing oxidative stress. In addition, GSH partially blocked the decrease in
120 transepithelial resistance produced by *Alternaria* exposure. These findings demonstrate that
121 *Alternaria* allergens are capable of modulating the transport properties of the airway epithelium.
122 However prolonged exposure leads to disruption of epithelial barrier function that would
123 ultimately reduce mucociliary clearance.

124

125 **Materials and Methods**

126 *Materials*

127 16HBE14o$^-$ cells were obtained from Dr. Dieter Gruenert, University of California, San
128 Francisco (5). Eagle's minimum essential medium with Earl's salts (MEM), fetal bovine serum,
129 nonessential amino acids, penicillin-streptomycin, CellROX orange and phosphate-buffered
130 saline (PBS) and Hank's balanced salt solution (HBSS) were obtained from ThermoFisher

131 Scientific (Pittsburgh, PA). The acetoxymethyl ester form of Fura-2, (Fura-2-AM) and 1,2-Bis(2-
132 aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) were procured from
133 Invitrogen/Life Technologies (Carlsbad, CA, USA). The following compounds, ethylene glycol-
134 bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4,4'-diisothiocyano-2,2'-
135 stilbenedisulfonic acid (DIDS), 5-[(4-Carboxyphenyl)methylene]-2-thioxo-3-[(3-
136 trifluoromethyl)phenyl-4-thiazolidinone ($CFTR_{inh}$-172), ionomycin, diphenyleneiodonium chloride
137 (DPI), glutathione (GSH), N-acetyl cysteine (NAC), hydrogen peroxide (H_2O_2),
138 phenylmethylsulfonyl fluoride (PMSF), trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane,
139 L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine, N-(trans-Epoxysuccinyl)-L-leucine 4-
140 guanidinobutylamide (E64), 2-[[(4-phenoxyphenyl)sulfonyl]methyl]-Thiirane (SB-3CT), 4-(2-
141 Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 6-tert-butyl-2-(furan-2-
142 carboxamido)-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid ($CaCC_{inh}$-Ano1) were from
143 Sigma-Aldrich Chemical Co., St. Louis, MO). Trypsin was obtained from Lonza (Allendale NJ)
144 and GM6001 from EMD Millipore (Billerica, MA). Antibodies for E-cadherin (ab76055), ZO-1
145 (ab59720) and β-catenin (ab32572) were purchased from Abcam (Cambridge, MA). Antibody
146 validations in human cells for E-cadherin (ab76055), ZO-1 (ab59720) and β-catenin (ab32572)
147 have been previously published (7;24;51).

148 *Alternaria alternata extract*

149 *Alternaria* extract was purchased from Greer Laboratories (Lenoir, NC). The precise
150 composition of the extract is unknown but earlier studies with human eosinophils indicated that
151 proteinases within the extract play an important role in mediating *Alternaria* activity (33).

152 *Cell Cullture*

153 Immortalized human bronchial epithelial cells (16HBE14o$^-$ cells) were grown in MEM
154 with 10% fetal bovine serum, 1% nonessential amino acids and 1% penicillin-streptomycin at 37
155 ^{o}C in a humidified CO_2 (5%) incubator. Monolayers used in voltage clamp experiments were

156 grown on Snapwell® membrane filters (Fisher Scientific (Pittsburgh, PA) until transepithelial
157 resistance (TER) exceeded 500 $\Omega \cdot cm^2$. Cells used for Ca^{2+} imaging experiments were grown on
158 2-well chamber slides (Lab-Tek®, VWR International, Chicago, IL).

159 *Electrophysiology*

160 Transepithelial resistances were measured using an EVOM epithelial voltohmeter
161 connected to Ag/AgCl electrodes (World Precision Instruments, New Haven, CT). Monolayers
162 were mounted in Ussing chambers and bathed on the apical and basolateral sides of the
163 epithelium with symmetric physiologic saline solution (in mM): 130 NaCl, 6 KCl, 1.5 $CaCl_2$, 1
164 $MgCl_2$, 20 $NaHCO_3$, 0.3 NaH_2PO_4, and 1.3 Na_2HPO_4 (pH 7.4), maintained at 37°C and gassed
165 with 95% O_2-5% CO_2. Cells were apically stimulated with *Alternaria* extract at concentrations
166 indicated in the figure legends and all three anion channel blockers were added to the apical
167 solution. Voltage clamp experiments were performed using DVC1000 epithelial voltage/current
168 clamps (World Precision Instruments, New Haven, CT) and short circuit current (Isc) data was
169 acquired using Axoscope 8.1 software (Molecular Devices, Sunnyvale CA).

170 *Calcium Imaging*

171 Ca^{2+} imaging experiments were performed following a previously published protocol
172 (35). Briefly, 16HBE14o$^-$ cells were gown on chamber slides for 48-72 hours. Prior to loading
173 cells with Fura-2, the media was replaced with Ca^{2+}/Mg^{2+} free HBSS solution containing 10 mM
174 HEPES buffer. Cells were loaded with Fura 2-AM for 1 hour, then washed with Ca^{2+}/Mg^{2+}
175 containing HBSS before mounting onto the stage of a Nikon Diaphot inverted fluorescence
176 microscope. Fluorescence was visualized with a Nikon UV 20X objective at excitation
177 wavelengths of 340 and 380 nm and a single emission wavelength of 510 nm. Image acquisition
178 and data analysis were performed using Image-1 MetaMorph software. Changes in intracellular
179 $[Ca^{2+}]$ ($[Ca^{2+}]_i$) were determined from the fluorescence ratio (F_{340}/F_{380}) following calibration using
180 the Fura-2-AM calcium imaging calibration kit from ThermoFisher Scientific (Pittsburgh, PA).

181 *Oxidative stress measurements*

182 Cells were grown for 48-72 hours on 2 well chamber slides in standard MEM/fetal calf serum
183 containing media. Oxidative stress induced by *Alternaria* exposure was measured following
184 incubation with CellROX orange reagent according to the manufacturer's instructions
185 (ThermoFisher Scientific, Pittsburgh, PA). After loading for 30 minutes, cells were exposed to
186 *Alternaria* (100 μg/ml) or 5 mM glutathione (GSH) + *Alternaria* (100 μg/ml) or not exposed to
187 either GSH or *Alternaria* (basal conditions) for 20 minutes. Images were captured with a Nikon
188 Diaphot fluorescence microscope equipped with a Diagnostics Instruments Model 14.1
189 monochrome camera and SPOT Advanced imaging software. Relative fluorescence intensity
190 measurements were made using Image J software.

191 *ATP release measurements*

192 ATP measurements in the extracellular media were performed in real time as previously
193 described using a luciferase/luciferin bioluminescence ATP determination kit (ThermoFisher
194 Scientific, Pittsburgh, PA) (39). ATP evoked photon emission was measured using a Glomax
195 20/20 luminometer (Promega, Madison WI). Initially, standard curves were generated over a
196 range of 1-1000 nM ATP in standard reaction solution (SRS). The ATP concentrations were
197 averaged after subtraction of the back-ground luminescence. Cultured epithelial monolayers
198 grown on 35 mm dishes were then loaded into the luminometer and the apical surface covered
199 with SRS containing luciferin/luciferase. Monolayers were then stimulated with *Alternaria* or
200 pretreated with DPI (100 μM) or GSH (5 mM) for 30 minutes prior to stimulation with *Alternaria*.
201 Luminescence was measured over a period of 20 minutes after the addition of *Alternaria* and
202 the signal converted to [ATP] using the previously generated calibration curve.

203 *Dextran flux measurements*

204 Monolayers were initially grown on 12 mm Snapwell® filters until they reached
205 resistances between ~600-1000 $\Omega \cdot cm^2$. Media was removed and the cells washed twice with

206 serum-free MEM. Fluorescein isothiocyanate (FITC) labeled dextran (mw 4000 Da, final
207 concentration; 2.0 mg/ml) was added to the apical solution 1 hour before treatment with
208 *Alternaria* or H_2O_2 (0.5 mM). Samples of the apical and basolateral solutions were taken at
209 several time points (-1, 0, 1 and 4 hours). Fluorescence intensity was measured using a Biotek
210 Synergy 2 fluorescence plate reader equipped with filters for detecting FITC (excitation/emission
211 wavelengths = 485 nm/530 nm). To determine dextran concentration, a standard curve was
212 generated using varying FITC-dextran concentrations (μM: 0.0625, 0.125, 0.25, 0.5, 1, 2) and
213 used to calculate the unidirectional fluxes at time 0, 1 and 4 hours following *Alternaria* or H_2O_2
214 exposure.

215 *Immunocytochemistry*

216 Monolayers used for characterizing the effects of *Alternaria* and H_2O_2 on TER were fixed
217 with 4% paraformaldehyde for 30 minutes and incubated with 0.1% TritocX-100 for 20 minutes
218 before being washed and blocked with 3% bovine serum albumin (BSA) for 2 hours. Monolayers
219 were then incubated with primary E-cadherin, ZO-1 or β-catenin antibodies overnight in 3%
220 BSA. The cells were then incubated in secondary Alexa Fluor antibodies diluted in phosphate
221 buffered saline solution (PBS) for 45 minutes then washed five times with PBS. Next,
222 monolayers were treated with 4′,6-Diaminido-2-phenylindole (DAPI) diluted in PBS for 10 min
223 and then washed again with PBS. Filters were subsequently mounted on slides and antibody
224 labeling was examined by confocal microscopy using an Olympus FV1000 confocal microscope
225 with a 20X fluorescence objective.

226 *Statistics*

227 Data are presented as the mean ± SEM. Statistical significance was determined by
228 using an unpaired two-tailed *t*-test (for single comparisons) or an ANOVA followed by Dunnett's
229 post-test for multiple comparisons with a common control or an ANOVA followed by a Tukey's
230 post-test for multiple comparisons. A value of $p < 0.05$ was considered significant.

231 Concentration-response relationships were analyzed using PRISM 6 software (GraphPad
232 Software Inc., La Jolla, CA).

233

234 **Results**

235 Apical addition of *Alternaria* extract to monolayers of immortalized human bronchial
236 epithelial cells produced an increase in short circuit current (Isc) consistent with anion secretion.
237 Figure 1A and B shows the kinetics of *Alternaria*-evoked increases in Isc at concentrations of 50
238 and 100 μg/ml and a concentration-response relationship for *Alternaria* ranging from 50-200
239 μg/ml. The rate and magnitude of the Isc increase was found to be dependent on concentration.
240 However, stable measurements of Isc could not be obtained at *Alternaria* concentrations greater
241 than 200 μg/ml. Treatment of monolayers with the disulfonic stilbene derivative DIDS (250 μM),
242 an inhibitor of calcium-activated Cl^- channels (CaCCs) as well as certain anion exchange
243 transporters (27;42) produced an initial rapid inhibition of Isc, followed by a slower, time-
244 dependent decrease in current to baseline levels (Figure 1C). Similarly, pretreatment with 250
245 μM DIDS effectively abolished the increase in Isc typically observed following apical addition of
246 50 μg/ml *Alternaria*. A concentration-response relationship for DIDS is shown in Figure 1D. The
247 IC_{50} value was calculated to be 100 μM based on a non-linear least squares fit using a three
248 parameter logistic function (Prism 6.0). The effect of an inhibitor of the A01 (TMEM16A) calcium
249 activated Cl^- channel ($CaCC_{inh}$-A01) was also tested and its effect on Isc is shown in Figure 1E.
250 $CaCC_{inh}$-A01 inhibited approximately 50% of the *Alternaria*-stimulated Isc and blocked the
251 further decrease in current produced by treatment with 100 μM DIDS, indicating that DIDS and
252 $CaCC_{inh}$-A01 target a common apical transport pathway involved in anion secretion (Figure 1E).
253 *Alternaria* also stimulated CFTR-dependent anion secretion as indicated by inhibition of the
254 *Alternaria* stimulated Isc with $CFTR_{inh}$-172 shown in Figure 1F.

255 Apical addition of *Alternaria* (50 μg/ml) also produced a large and sustained increase in
256 $[Ca^{2+}]_i$ (Figure 2A and B). Moreover, adding 1 mM EGTA to the apical solution with the purpose
257 of lowering extracellular $[Ca^{2+}]$ prior to treatment with *Alternaria*, abolished the increase in $[Ca^{2+}]_i$
258 as shown in panel 4 of figure 2A and in the red trace of figure 2B. This result indicated that the
259 *Alternaria*-evoked increase in $[Ca^{2+}]_i$ was a consequence of Ca^{2+} uptake from the extracellular
260 solution. Pretreating cells with a cell permeable Ca^{2+} chelating agent (BAPTA-AM) at 50 and
261 100 μM also reduced the increase in $[Ca^{2+}]_i$ associated with apical *Alternaria* exposure (Figure
262 2C and D). Furthermore, pretreatment with BAPTA-AM also significantly blocked the Isc
263 increase induced by *Alternraria* (Figure 2D), suggesting a direct role for $[Ca^{2+}]_i$ in regulating
264 *Alternaria*-evoked anion secretion. In an earlier experiment (Figure 1C), DIDS was shown to
265 have a biphasic effect on the *Alternaria* Isc response such that pretreatment with 250 μM DIDS
266 completely blocked the increase in Isc. The slow, time-dependent component of the DIDS
267 response suggested that the compound may be blocking anion secretion by a mechanism
268 independent of inhibiting apical membrane anion channels. To test this hypothesis we
269 examined the effect of DIDS on the *Alternaria*-induced increase in $[Ca^{2+}]_i$ and found that
270 pretreatment with 250 μM DIDS completely blocked the response, but had no effect on the
271 increase in $[Ca^{2+}]_i$ produced by apical addition of the Ca^{2+} ionophore, ionomycin (4 μM) (Figure
272 2E and F). This result suggested that 250 μM DIDS is capable of blocking the Ca^{2+} uptake
273 mechanism that is activated by *Alternaria* in addition to its effect on CaCCs.

274 Figure 3 shows the results of experiments designed to determine if *Alternaria* exposure
275 produces ROS accumulation leading to oxidative stress. CellROX orange is a cell permeable
276 fluorescent compound that responds to oxidative stress by increasing fluorescence (18;26;49).
277 CellROX orange loaded cells exhibited a low level of fluorescence under basal, unstimulated
278 conditions (Figure 3A), however, exposure to 100 μg/ml *Alternaria* for 15 minutes produced a
279 significant increase in fluorescence consistent with ROS generation and oxidative stress (Figure

280 3B). In contrast, cells that were initially pretreated with 5 mM glutathione (GSH) prior to
281 *Alternaria* exposure showed significantly reduced levels of fluorescence compared to *Alternaria*
282 treatment alone (Figure 3C and D). This result demonstrated that oxidant scavenging by GSH is
283 sufficient to block *Alternaria*-evoked ROS generation.

284 Earlier studies using primary normal human bronchial epithelial (NHBE) cells showed
285 that *Alternaria* exposure induces ATP release (19;26;39). We tested whether a similar response
286 could be detected in 16HBE14o$^-$ cells by measuring the release of ATP by photon counting in
287 real time using monolayers where the apical solution contained luciferin and luciferase. Results
288 presented in Figure 4A show that exposure to *Alternaria* (50 μg/ml) induces ATP secretion into
289 the apical solution starting at ~5 min and continues to increase up to 20 minutes in the presence
290 of *Alternaria* allergens. This increase in ATP release was dramatically blocked by pretreatment
291 with 5 mM GSH, and to a lesser extent following pretreatment with DPI, an inhibitor of NADPH
292 oxidase activity and of complex 1 of the electron transport chain (30) (Figure 4B). Subsequent
293 Ca^{2+} imaging experiments revealed that 5 mM GSH and 100 μM DPI were highly effective at
294 inhibiting *Alternaria*-evoked increases in $[Ca^{2+}]_i$ (Figure 4C and D) The effects of GSH and DPI
295 on the steady-state levels of $[Ca^{2+}]_i$ following *Alternaria* exposure are shown in figure 4E. The
296 dose-response relationship of N-acetyl cysteine (NAC) a clinically used oxidant scavenger on
297 the steady-state increase in $[Ca^{2+}]_i$ induced by *Alternaria* is reported in figure 4F. These data
298 indicate that *Alternaria*-evoked oxidative stress underlies the increase in both ATP release and
299 Ca^{2+} uptake that occurs following apical treatment with *Alternaria*.

300 The effects of *Alternaria* (100 μg/ml) and exogenously added hydrogen peroxide (0.5
301 mM) on epithelial barrier function was investigated by measuring both transepithelial resistance
302 (TER) and unidirectional (apical-to-basolateral) dextran (mw = 4000 Da) fluxes (Figure 5).
303 Exposure to *Alternaria* produced a decrease in TER from 760 ± 35 to 151 ± 4 $\Omega \cdot cm^2$ (n = 6)
304 over a period of 4 hours. This change in resistance reflects both the increase in transepithelial

305 Cl^- conductance and an increase in tight junction ion permeability. Addition of 0.5 mM H_2O_2 to
306 the apical solution also reduced the TER (674 ± 60 to 331 ± 18 $\Omega \cdot cm^2$; n = 6) but not to the
307 same extent as *Alternaria* after 4 hours (Figure 5A). Moreover, the time course of the H_2O_2
308 response was appreciably slower compared to that of *Alternaria*, which caused nearly a
309 complete loss of resistance after 1 hour of exposure (note: the resistance of the filters alone =
310 ~100 $\Omega \cdot cm^2$). When monolayers were initially pretreated with 5 mM GSH and then exposed to
311 either H_2O_2 or *Alternaria*, the effect of H_2O_2 was completely blocked whereas the response to
312 *Alternaria* was only partially inhibited (Figure 5B). However, pretreatment of monolayers with
313 various proteinase inhibitors including PMSF (a pan-specific serine proteinase inhibitor), E64 (a
314 cysteine protease inhibitor) GM6001 (a pan-specific matrix metalloproteinase (MMP) inhibitor),
315 SB-3CT (an MMP2 and MMP9 inhibitor) and AEBSF (a pan-specific serine proteinase inhibitor)
316 each failed to inhibit the decrease in TER evoked by *Alternaria* (Table 1). As a control, we
317 tested the effects of a low concentration of trypsin (7 μg/ml) on TER and discovered that it
318 significantly increased resistance by >2 fold and sustained this increase for 4 hours in the
319 continuous presence of the enzyme. Pretreatment with a serine proteinase inhibitor (PMSF)
320 effectively blocked the rise in TER whereas E64, a cysteine proteinase inhibitor did not (Figure
321 5C). To determine if the effect of *Alternaria* on TER was due to activation of PAR2 receptors,
322 we tested the effects of the PAR2 activating peptide SLIGKV-NH_2 (20 μM) and found that it had
323 no effect on resistance after 4 hours of treatment (data not shown). The results of trypsin
324 experiments indicated that low concentrations of the enzyme do not reduce TER and in fact
325 cause an increase that can be blocked by a serine proteinase inhibitor, but is poorly blocked by
326 a cysteine proteinase inhibitor. However, these inhibitors at the same concentrations were
327 ineffective at blocking the *Alternaria* response. Figure 5D shows that *Alternaria* increases the
328 permeability of epithelial monolayers to macromolecules as indicated by an increase in the
329 unidirectional flux of dextran (mw = 4000) after 1 and 4 hours of allergen exposure. In contrast

330 pretreatment with AEBSF was ineffective at blocking the stimulated dextran flux after 4 hours,
331 although a significantly lower flux was observed after a 1 hour exposure to *Alternaria* (Figure
332 5C). H_2O_2 had no significant effect on the dextran flux after 1 hour, however a significant
333 increase was detected after 4 hours. To determine if *Alternaria* or H_2O_2 exposure for 4 hours
334 produced visible signs of monolayer destruction, we performed confocal imaging using
335 antibodies that labeled tight junction proteins including β-catenin, E-cadherin and ZO-1 (Figure
336 6). Our results indicated that epithelial integrity remained intact, with no observable disruption
337 of tight junctions or loss of epithelial cells, although some decrease in the intensity of β-catenin
338 and ZO-1 staining was observed in monolayers treated with H_2O_2. These findings support the
339 conclusion that *Alternaria* increases both ionic and macromolecular permeability of the
340 epithelium, but that these changes do not involve observable destruction of monolayer integrity
341 or junctional complexes between the epithelial cells.

342

343 **Discussion**

344 16HBE14o$^-$ cells were originally isolated from a 1 year-old male heart-lung transplant
345 patient and immortalized following transfection with the SV40 T-antigen (5;12). These cells
346 have been shown to retain differentiated epithelial morphology including tight junctions, cilia and
347 a transepithelial resistance comparable to primary bronchial epithelial cells. Monolayers of
348 16HBE14o$^-$ cells exhibit β-adrenergic receptor and cAMP-dependent regulation of anion
349 secretion, mediated through activation of apical CFTR channels. Moreover, exposure to
350 bradykinin or the Ca^{2+} ionophore, ionomycin produced increases in intracellular [Ca^{2+}] and
351 stimulated Ca^{2+}-dependent Cl^- secretion (12). Using this cell line as an experimental model, we
352 investigated the effects of *Alternaria* aeroallergens on transepithelial anion transport, a process
353 that underlies mucociliary clearance. Our experiments demonstrated that *Alternaria* exposure
354 induced a sustained, concentration-dependent increase in Isc, consistent with stimulation of

355 anion secretion. Furthermore, treatment with inhibitors of CFTR and the Ca^{2+}-activated Cl^-
356 channel Ano1 significantly inhibited the Isc, suggesting that both channels are activated
357 following apical treatment with *Alternaria*. However, it is worth noting that $CFTR_{inh}$-172 has been
358 previously shown to inhibit the ClC-2 Cl^- channel as well as CFTR. Earlier whole cell patch
359 clamp experiments with recombinant ClC-2 and CFTR channels expressed in HEK293 cells
360 revealed that $CFTR_{inh}$-172 inhibited both forskolin/IBMX stimulated CFTR currents and
361 lubiprosone-stimulated ClC-2 currents with equal potency (13). Therefore, care must be taken
362 not to conclude that inhibition of anion transport by $CFTR_{inh}$-172 results from CFTR inhibition
363 alone, especially in cells that are known to express both ClC-2 and CFTR. In a prior study, a
364 molecular approach involving the use of ClC-2 and ClC-3 deficient mice was used to rule out the
365 possible involvement of these ClC channels as contributors to the Cl^- conductance present in
366 the basolateral membrane of airway epithelia (22). In the present study ClC-2/CFTR knockdown
367 experiments were not performed, therefore we are unable to exclude the possibility that ClC-2
368 channels may be contributing to *Alternaria*-evoked anion secretion. The disulfonic stilbene
369 compound DIDS, a known Ca^{2+}-dependent Cl^- channel inhibitor, produced a biphasic effect on
370 Isc resulting in complete inhibition of the *Alternaria*-evoked Isc response. The initial decrease in
371 Isc was consistent with rapid inhibition of anion channel activity, whereas the slow, time-
372 dependent decrease in current appeared to involve a separate mechanism necessary for
373 sustained anion secretion. We speculate that the slow component of the DIDS response is
374 related to inhibition of Ca^{2+} uptake and an associated decrease in $[Ca^{2+}]_i$.

375 *Alternaria* exposure also produced a sustained increase in intracellular $[Ca^{2+}]$ with
376 characteristics similar to results from earlier studies using primary human bronchial epithelial
377 (NHBE) cells (25;38) and 16HBE14o$^-$ cells (3). Chelation of extracellular Ca^{2+} with EGTA prior
378 to addition of *Alternaria* abolished the $[Ca^{2+}]_i$ increase indicating that *Alternaria* exposure had
379 activated a Ca^{2+} uptake mechanism located in the apical membrane. Similarly, pretreatment

380 with the cell permeable Ca^{2+} chelating agent BAPTA-AM also blocked the *Alternaria*-induced
381 increase in $[Ca^{2+}]_i$ and significantly reduced the Isc response, demonstrating that stimulation of
382 anion secretion was dependent on the increase in $[Ca^{2+}]_i$. Comparison of the kinetics of the
383 *Alternaria* effect on both Isc and $[Ca^{2+}]_i$ shows a greater delay in the onset of ATP release and
384 the Ca^{2+} response. This apparent discrepancy in the temporal cause and effect relationship
385 between the initiation of ATP secretion, Ca^{2+} uptake and stimulation of Cl^- secretion is most
386 likely due to the fact that the ATP and Ca^{2+} measurements were performed at room temperature
387 (~22 ^oC), whereas the Isc measurements were conducted at 37 ^oC. This 15 degree difference
388 in temperature would be expected to slow the effect of *Alternaria* on signaling mechanisms
389 involved in regulation of Cl^- secretion.

390 Pretreatment of cells with DIDS also inhibited the *Alternaria*-evoked increase in $[Ca^{2+}]_i$,
391 however the ionomycin response was not affected. This result established that the
392 concentration of DIDS used to completely block the *Alternaria*-induced Isc response inhibited
393 Ca^{2+} uptake across the plasma membrane, which accounts for the slow, time-dependent
394 decrease in Isc that occurs following inhibition of anion channel activity. Previous experiments
395 with NHBE cells showed that $P2X_7$ receptors were responsible for the increase in $[Ca^{2+}]_i$
396 produced by *Alternaria* (23) and activation of $P2X_7$ receptors by 2,3-O-(4-benzoylbenzoyl) ATP
397 (BzATP) in pancreatic duct epithelial cells was shown to be blocked by high concentrations
398 (>100 μM) of DIDS (8). P2X receptor activation in 16HBE14o$^-$ cells is likely to occur since the
399 present study showed that *Alternaria* stimulated a robust and sustained increase in ATP release
400 from the epithelium. Moreover, previous studies have shown that $P2X_7$ receptor activation with
401 BzATP increases $[Ca^{2+}]_i$ in submandibular gland acinar and duct epithelial cells and stimulates
402 Cl^- secretion by activation of CFTR and Ca^{2+}-activated Cl^- channels (32;37;38).

403 Recently, *Alternaria* exposure was shown to induce oxidative stress in NHBE cells and
404 this response could be blocked by pretreatment with oxidant scavengers such as GSH or by

405 Nrf2 modulators such as CDDO-Me (bardoxolone) or resveratrol (19;21;48). Oxidative stress
406 following treatment with *Alternaria* was directly observed in the current study by an increase in
407 fluorescence associated with a cell permeable ROS indicator. Addition of exogenous GSH
408 completely blocked this change in fluorescence, consistent with ROS scavenging.
409 Pretreatment with ROS scavengers and DPI, an NADPH oxidase/complex 1 electron transport
410 inhibitor (30), also inhibited ATP release and the increase in $[Ca^{2+}]_i$ induced by *Alternaria*,
411 signifying that oxidative stress was critical for triggering signaling pathways that activate ATP
412 secretion and Ca^{2+}-uptake across the apical membrane, ultimately stimulating Cl^- secretion. In
413 an earlier study using Calu-3 cells, a human submucosal gland serous cell line, ROS exposure
414 was shown to stimulate CFTR-dependent anion secretion (11). Although measurements of
415 $[Ca^{2+}]_i$ were not performed, pretreatment with the adenylyl cyclase inhibitor SQ22536,
416 completely blocked CFTR activation, indicating that ROS exposure produced an increase in
417 cAMP which is known to regulate both apical CFTR channels and basolateral KCNQ1/KCNE3 K
418 channels. ROS exposure is also known to stimulate the production of prostaglandin-like
419 compounds known as isoprostanes (10). Exposure of Calu-3 cells to the isoprostane 8-iso-
420 prostaglandin E2 was shown to stimulate CFTR-dependent anion secretion in part by activation
421 of the thromboxane A2 (TPα) receptor. Previous studies have shown that ligand-dependent
422 activation of TPα receptors increases adenylyl cyclase activity, leading to an increase in [cAMP]
423 and activation of ion channels involved in anion secretion. A similar mechanism could
424 potentially contribute to the *Alternaria*-induced CFTR activation observed in the present study.
425 Oxidative stress was also shown to induce increases in $[Ca^{2+}]_i$ and Cl^- secretion in human nasal
426 epithelial cells and 16HBE14o$^-$ cells following treatment with exogenous H_2O_2 (14). The
427 increase in $[Ca^{2+}]_I$ was abolished when EDTA was added to the extracellular media indicating
428 that H_2O_2 exposure stimulates a Ca^{2+} uptake mechanism in the plasma membrane. In addition,
429 oxidative stress also produced inhibition of Na-K ATPase activity which resulted in a time-

430 dependent increase in intracellular [Na^+]. The results of this study are consistent with the
431 conclusion that oxidative stress induced by *Alternaria* is responsible for increasing both $[Ca^{2+}]_i$
432 and anion secretion in airway epithelial cells.

433 Earlier studies of the house dust mite allergen Der p 1, a protein with cysteine protease
434 activity, on epithelial barrier function showed time dependent disruption of tight junctions and
435 loss of ZO-1 content in 16HBE14o$^-$ cell monolayers (50). Morphologic changes in tight junction
436 structure were associated with increased junctional permeability as determined by
437 measurements of ^{14}C-mannitol fluxes. Tight junction disruption was linked to cleavage of ZO-1
438 and occludin, suggesting that sensitization and allergic inflammatory responses to house dust
439 mite allergens may depend in part on loss of airway epithelial tight junction integrity. Similarly,
440 alkaline serine proteinase activity associated with allergens Pen ch 13 and Asp f 13 derived
441 from two species of fungi, *Pencillium* and *Aspergillus* have been shown to induce the release of
442 PGE_2, IL-8 and TGF-β_1 from airway epithelial cells and to cleave occludin within the tight
443 junctions of 16HBE14o$^-$ cells (44;46). Although measurements of tight junction permeability
444 were not performed, the authors speculated that occuldin degradation could facilitate allergen
445 transport across the epithelial barrier to promote an inflammatory response. More recently, the
446 effects of Pen ch 13 were investigated in a human lung adenocarcinoma cell line (NCI-H441
447 cells) where it was shown to induce degradation of occludin, ZO-1 and E-cadherin after 2 hours
448 of exposure and to reduce TER (6). The results of the present study showed that *Alternaria*
449 induces a significant decrease in TER within one hour after exposure. In contrast, treatment with
450 0.5 mM H_2O_2 was less complete after four hours of exposure. Interestingly, pretreatment with
451 several proteinase inhibitors including PMSF, E64, AEBSF and two MMP inhibitors failed to
452 block the effects of *Alternaria* on TER. Moreover, in control experiments using a low
453 concentration of trypsin added to the apical surface, TER increased by two fold and this effect
454 was blocked by pretreatment with the serine proteinase inhibitor PMSF, but only slightly

455 reduced by the cysteine proteinase inhibitor E64, a result confirming the serine proteinase
456 selectivity of PMSF. In contrast to the lack of effect of proteinase inhibitors on the decrease in
457 TER evoked by *Alternaria*, pretreatment with GSH produced a partial inhibition of the response,
458 suggesting that oxidative stress contributed in part to the decrease in electrical resistance of the
459 monolayer. Furthermore, fluorescent dextran flux experiments demonstrated that *Alternaria*
460 produced a significant increase in tight junction macromolecule permeability within 1 hour of
461 exposure, whereas oxidative stress induced by H_2O_2 required 4 hours of treatment to produce a
462 significant increase in permeability that was significantly less than observed after treatment with
463 *Alternaria*. Pretreatment with the serine proteinase inhibitor AEBSF affected the magnitude of
464 the unidirectional dextran flux after 1 hour, but at 4 hours the flux was not significantly different
465 than *Alternaria* treatment alone. These findings indicated that acute oxidative stress
466 predominately affects the electrical resistance of the epithelium, which would increase
467 paracellular ion movement and reduce the shunt resistance of the monolayer. This would
468 ultimately diminish net transepithelial electrolyte transport and reduce overall mucociliary
469 clearance. However, the increase in macromolecule permeability induced by *Alternaria* appears
470 to be less affected by oxidative stress, but the exact mechanism is not clear from the results of
471 the present study. It is possible that a proteinase(s) that are insensitive to the inhibitors used in
472 this study may be involved, but additional experiments will be required to reveal their identity.

473 A model that summarizes the key findings of this study is presented in Figure 7. The
474 results suggest that apical exposure to *Alternaria* allergens induces oxidative stress, possibly by
475 activation of NADPH oxidase activity or by uncoupling mitochondrial respiration. Consequently,
476 ATP release from the epithelium occurs which activates Ca^{2+} uptake through a DIDS-sensitive
477 conductive pathway in the apical membrane. The increase in $[Ca^{2+}]_i$ stimulates apical Cl^- efflux
478 and transepithelial Cl^- secretion which is completely blocked by $CaCC_{inh}$-Ano1, a known inhibitor
479 of Ca^{2+}-activated Cl^- channels and $CFTR_{inh}$-172, an inhibitor of CFTR as well as ClC-2. The

480 increase in oxidative stress also contributes to a time-dependent decrease in transepithelial
481 resistance, however the increase in dextran permeability appears to be independent of the
482 increase in oxidative stress and may involve proteinase activation.

483

484 **Acknowledgements**

485 This work was supported by grants from the National Institute of Biomedical Imaging and
486 Bioengineering Grant (F31 EB-018707 to N. A. Zaidman) and the National Heart, Lung, and
487 Blood Institute (R01 HL-110539 to H. Kita and S. M. O'Grady).

488

489 **Table 1.** Effect of proteinase inhibitors on the *Alternaria*-induced decrease in TER.

490

	untx Control		*Alt* (100 mg/ml)		PMSF (0.5 mM)		E64 (10 μM)		GM6001 (10 μM)		SB-3CT (1 μM)		AEBSF (200 μM)	
Time:	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr	0 hr	4 hr
($\Omega \cdot cm^2$)	654 ± 34	521 ± 26	718 ± 33	151 ± 4*	664 ± 64	158 ± 3*	662 ± 46	166 ± 2*	1026 ± 27	150 ± 3*	1042 ± 14	154 ± 2*	847 ± 104	151 ± 7*

491

492 **Figure Legends**

493 **Figure 1.** *Alternaria* exposure stimulates transepithelial anion secretion. **A**. Kinetics of the Isc
494 response following apical exposure to 50 and 100 μg/ml *Alternaria* extract. **B**. Concentration-
495 response relationship for *Alternaria*-evoked increases in steady-state Isc (n = 4). **C**. Kinetics of
496 apical DIDS (250 μM) post-treatment and pre-treatment on the *Alternaria*-evoked (50 μg/ml) Isc
497 response (n = 6). **D**. Concentration-response relationship showing the inhibitory effects of apical
498 DIDS on the *Alternaria*-induced (50 μg/ml) increase in Isc (n = 5). The IC_{50} = 1.23x10^{-4} M. **E.**
499 Kinetics of apical $CaCC_{inh}$ (40 μM) and DIDS post-treatment on the *Alternaria*-evoked Isc
500 response (n = 6). **F**. Summary bar graph showing the effects of $CFTR_{inh}$-172 (20 μM), DIDS
501 (250 or 100 μM) and $CaCC_{inh}$ (40 μM) on steady-state Isc increase evoked by *Alternaria* (50
502 μg/ml). Asterisk (*) indicates a significant difference between the basal and *Alternaria*-stimulated

503 Isc. (†) indicates significant differences between the *Alternaria*-stimulated and inhibitor ($CFTR_{inh}$-

504 172, $CaCC_{inh}$-Ano1 or DIDS)-treated Isc values.

505

506 **Figure 2.** *Alternaria*-induced changes in intracellular [Ca^{2+}]. **A**. Images of 16HBE14o$^-$ cell

507 monolayers labeled with Fura 2-AM before and after 700 seconds of stimulation with 50 µg/ml

508 *Alternaria* extract and after pretreatment with 1 mM EGTA in the extracellular solution. Scale bar

509 = 20 µm. **B**. Kinetics of the *Alternaria* effect on $[Ca^{2+}]_i$ in the absence and presence of 1 mM

510 EGTA in the extracellular solution (n = 25 cells for each condition). **C**. Effects of BAPTA-AM

511 pretreatment (50 and 100 µM) on *Alternaria*-evoked increases in $[Ca^{2+}]_I$ (n = 25 cells for each

512 condition). **D.** BAPTA pretreatment significantly inhibits the effect of *Alternaria* on $[Ca^{2+}]_i$ (n = 25

513 cells) and Isc (n = 8 monolayers) (*) indicates significantly different from the basal Isc. (†)

514 indicates significant differences between basal and *Alternaria*-treated conditions. **E**.

515 Pretreatment with 250 µM DIDS blocks the *Alternaria*-evoked increase in $[Ca^{2+}]_i$ but does not

516 inhibit ionomycin-induced increases in $[Ca^{2+}]_i$ (n = 25 cells for each condition). **F**. Summary of

517 the effects of DIDS on *Alternaria* and ionomycin-induced increases in $[Ca^{2+}]_i$ (n = 25 for each

518 condition).

519

520 **Figure 3**. *Alternaria* induces oxidative stress in 16HBE14o$^-$ cells. **A**. Basal fluorescence of cells

521 loaded with CellROX orange. **B**. *Alternaria*-evoked (50 µg/ml) increase in fluorescence after 15

522 minutes of exposure to the extract. **C**. Pretreatment with 5 mM GSH blocks the increase in

523 fluorescence produced by *Alternaria* exposure. **D**. Summary of the changes in relative

524 fluorescence intensity obtained from images of basal, *Alternaria* stimulated and 5 mM GSH +

525 *Alternaria* stimulated conditions (n = 25 cells for each condition). (*) indicates significantly

526 different from the basal condition. Scale bar = 20 µm.

527

528 **Figure 4**. ROS scavengers and DPI inhibit the effects of *Alternaria* on ATP release and $[Ca^{2+}]_i$.
529 **A**. Kinetics of ATP release evoked by *Alternaria* (100 μg/ml). **B**. Effects of DPI (100 μM) and
530 GSH (5 mM) pretreatment (5 minutes before *Alternaria* addition) on ATP release evoked by 50
531 μg/ml *Alternaria*. (*) indicates significantly different from the *Alternaria*-treated condition. **C**. GSH
532 inhibits *Alternaria*-evoked increase in $[Ca^{2+}]_i$ (n = 25 cells for each condition). **D**. DPI inhibits
533 *Alternaria*-evoked increase in $[Ca^{2+}]_i$ (n = 25 cells for each condition). **E**. Summary of the effects
534 of GSH and DPI on $[Ca^{2+}]_i$ (n = 25 cells for each condition). (*) indicates significantly different
535 from the *Alternaria*-treated condition. **F**. Concentration-response effects of N-acetyl cysteine
536 (NAC) on $[Ca^{2+}]_i$ (n = 25 cells for each condition).

537

538 **Figure 5**. *Alternaria* and H_2O_2 exposure reduces transepithelial resistance (TER) and increases
539 macromolecule permeability of 16HBE14o$^-$ cell monolayers. **A**. Time dependent decreases in
540 TER following exposure to apical *Alternaria* (♦; 100 μg/ml) or H_2O_2 (■; 500 μM) compared to
541 untreated controls (•) (n = 6 for each condition). **B.** Pretreatment with 5 mM GSH completely
542 blocks the inhibitory effect of H_2O_2 on TER and partially inhibits the effects of *Alternaria* on TER
543 (n = 6). **C**. Effects of serine protease exposure (trypsin, 7 μg/ml) or *Alternaria* (100 μl/ml) on
544 TER before and after pretreatment with the serine protease inhibitor PMSF (0.5 mM) or the
545 cysteine protease inhibitor E64 (10 μM). (n = 3 for each condition). **D**. Effects of *Alternaria* and
546 H_2O_2 on apical-to-basolateral unidirectional FITC-Dextran (mw = 4000 Da) fluxes at time zero, 1
547 hour after treatment and 4 hours after treatment. (*) indicates significantly different from time
548 zero (0). (n = 6 for each condition).

549

550 **Figure 6**. Effects of *Alternaria* and H_2O_2 on tight junction complexes in monolayers of
551 16HBE14o$^-$ cells. **A**. Immunocytochemistry showing tight junction expression of E-cadherin, β-
552 catenin and ZO1 under untreated control conditions. **B**. Exposure to *Alternaria* for 4 hours
553 produced no measurable change in the expression pattern for E-cadherin, β-catenin or ZO1. **C**.
554 Treatment with 0.5 mM H_2O_2 for 4 hours diminishes the intensity of β-catenin and ZO1 labeling
555 along the lateral membranes. Scale bar = 20 μm.

556

557 **Figure 7.** A model summarizing the key findings of this study. See Discussion for details.

558
559
560
561
562
563 **References**
564

565 1. Adhikari, A., T. Reponen, S. A. Lee, and S. A. Grinshpun. Assessment of human exposure
566 to airborne fungi in agricultural confinements: personal inhalable sampling versus
567 stationary sampling. *Ann Agric Environ Med* 11: 269-77, 2004.

568 2. Bacsi, A., N. Dharajiya, B. K. Choudhury, S. Sur, and I. Boldogh. Effect of pollen-mediated
569 oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in
570 allergic conjunctivitis. *J Allergy Clin Immunol* 116: 836-43, 2005.

571 3. Boitano, S., A. N. Flynn, C. L. Sherwood, S. M. Schulz, J. Hoffman, I. Gruzinova, and M.
572 O. Daines. Alternaria alternata serine proteases induce lung inflammation and airway
573 epithelial cell activation via PAR2. *Am J Physiol Lung Cell Mol Physiol* 300: L605-14,
574 2011.

575 4. Boldogh, I., A. Bacsi, B. K. Choudhury, N. Dharajiya, R. Alam, T. K. Hazra, S. Mitra, R. M.
576 Goldblum, and S. Sur. ROS generated by pollen NADPH oxidase provide a signal that
577 augments antigen-induced allergic airway inflammation. *J Clin Invest* 115: 2169-79, 2005.

578 5. Boncoeur E., V. Saint Criq, E. Bonvin, T. Roque, A. Henrion-Caude, D.C. Gruenert, J.
579 Jacquot and O. Tabary. Oxidative stress induces extracellular signal-regulated kinase 1/2
580 mitogen-activated protein kinase in cystic fibrosis lung epithelial cells: Potential
581 mechanism for excessive IL-8 expression. *Int. J. Biochem Cell Biol.* 40: 432-446, 2008.

582 6. Chen, J. C., J. G. Chuang, Y. Y. Su, B. L. Chiang, Y. S. Lin, and L. P. Chow. The protease
583 allergen Pen c 13 induces allergic airway inflammation and changes in epithelial barrier
584 integrity and function in a murine model. *J Biol Chem* 286: 26667-79, 2011.

585 7. Chen, Y.C., S. Statt, R. Wu, H. Chang, J.W. Liao, C.N. Wang, W.C. Shyu and C.C. Lee.
586 High mobility group box 1-induced epithelial mesenchymal transition in human airway
587 epithelial cells. *Sci Rep* 6: 18815-28, 2016.

588 8. Christoffersen, B. C., M. J. Hug, and I. Novak. Different purinergic receptors lead to
589 intracellular calcium increases in pancreatic ducts. *Pflugers Arch* 436: 33-9, 1998.

590 9. Cohen, E. S., I. C. Scott, J. B. Majithiya, L. Rapley, B. P. Kemp, E. England, D. G. Rees,
591 C. L. Overed-Sayer, J. Woods, N. J. Bond, C. S. Veyssier, K. J. Embrey, D. A. Sims, M. R.
592 Snaith, K. A. Vousden, M. D. Strain, D. T. Chan, S. Carmen, C. E. Huntington, L. Flavell,
593 J. Xu, B. Popovic, C. E. Brightling, T. J. Vaughan, R. Butler, D. C. Lowe, D. R. Higazi, D. J.
594 Corkill, R. D. May, M. A. Sleeman, and T. Mustelin. Oxidation of the alarmin IL-33
595 regulates ST2-dependent inflammation. *Nat Commun* 6: 8327, 2015.

596 10. Cowley, E. A. Isoprostane-mediated secretion from human airway epithelial cells. *Mol*
597 *Pharmacol* 64: 298-307, 2003.

598 11. Cowley, E. A. and P. Linsdell. Oxidant stress stimulates anion secretion from the human
599 airway epithelial cell line Calu-3: implications for cystic fibrosis lung disease. *J Physiol* 543:
600 201-9, 2002.

601 12. Cozens, A. L., M. J. Yezzi, K. Kunzelmann, T. Ohrui, L. Chin, K. Eng, W. E. Finkbeiner, J.
602 H. Widdicombe, and D. C. Gruenert. CFTR expression and chloride secretion in polarized
603 immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 10: 38-47, 1994.

604 13. Cuppoletti, J., J. Chakrabarti, K.P. Tewari and D.H. Malinowska. Differentiation between
605 human ClC-2 and CFTR Cl− channels with pharmacological agents. *Am J Physiol [Cell*
606 *Physiol]* 307:C479-C492, 2014.

607 14. Dazy, A. C., F. Auger, D. Bailbe, S. Blouquit, A. Lombet, and F. Marano. The toxicity of
608 H_2O_2 on the ionic homeostasis of airway epithelial cells in vitro. *Toxicol In Vitro* 17: 575-80,
609 2003.

610 15. De Lucca, A. J. Harmful fungi in both agriculture and medicine. *Rev Iberoam Micol* 24: 3-
611 13, 2007.

612 16. Denning, D. W., B. R. O'Driscoll, C. M. Hogaboam, P. Bowyer, and R. M. Niven. The link
613 between fungi and severe asthma: a summary of the evidence. *Eur Respir J* 27: 615-26,
614 2006.

615 17. Filali Ben Sidel, F., H. Bouziane, M. Del Mar Trigo, F. El Haskouri, F. Bardei, A.
616 Redouane, M. Kadiri, H. Riadi, and M. Kazzaz. Airborne fungal spores of Alternaria,
617 meteorological parameters and predicting variables. *Int J Biometeorol* 59: 339-46, 2015.

618 18. Gabriel, M. F., I. Postigo, C. T. Tomaz, and J. Martinez. Alternaria alternata allergens:
619 Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy. *Environ Int*
620 89-90: 71-80, 2016.

621 19. Hristova, M., A. Habibovic, C. Veith, Y. M. Janssen-Heininger, A. E. Dixon, M. Geiszt, and
622 A. van der Vliet. Airway epithelial dual oxidase 1 mediates allergen-induced IL-33
623 secretion and activation of type 2 immune responses. *J Allergy Clin Immunol* 137: 1545-
624 1556.e11, 2016.

625 20. Iijima, K., T. Kobayashi, K. Hara, G. M. Kephart, S. F. Ziegler, A. N. McKenzie, and H.
626 Kita. IL-33 and thymic stromal lymphopoietin mediate immune pathology in response to
627 chronic airborne allergen exposure. *J Immunol* 193: 1549-59, 2014.

628 21. Iijima K., T. Kobayashi, M. Uchida, E.L. Anderson, D. Squillace, G.M. Kephart, S.M.
629 O'Grady, H. Kita: Oxidative Stress Responses to the Fungal Allergen Alternaria Mediate
630 IL-33 Secretion By Airway Epithelial Cells and Type 2 Immunity in the Airways. *J. Allergy*
631 *Clin Immnol* 137:AB199, 2016.

632 22. Itani O.A,, F.S. Lamb, J.E. Melvin and M.J. Welsh. Basolateral chloride current in human
633 airway epithelia. *Am J Physiol [Lung Cell Mol Physiol]* 293(4): L991-L999, 2007.

634 23. Jarolim, K., G. Del Favero, G. Pahlke, V. Dostal, K. Zimmermann, E. Heiss, D. Ellmer, T.

635 D. Stark, T. Hofmann, and D. Marko. Activation of the Nrf2-ARE pathway by the Alternaria
636 alternata mycotoxins altertoxin I and II. *Arch Toxicol* 2016.

637 24. Jones W.M., A.T. Chao, M. Zavortink, R. Saint and A. Bejsovec. Cytokinesis proteins Tum
638 and Pav have a nuclear role in Wnt regulation. *J Cell Sci* 123:2179-89, 2010.

639 25. Kauffman, H. F. and S. van der Heide. Exposure, sensitization, and mechanisms of
640 fungus-induced asthma. *Curr Allergy Asthma Rep* 3: 430-7, 2003.

641 26. Kouzaki, H., K. Iijima, T. Kobayashi, S. M. O'Grady, and H. Kita. The danger signal,
642 extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and
643 innate Th2-type responses. *J Immunol* 186: 4375-87, 2011.

644 27. Krysinska-Traczyk, E. [Microflora of the farming work environment as an occupational risk
645 factor]. *Med Pr* 51: 351-5, 2000.

646 28. Kunzelmann, K., Y. Tian, J. R. Martins, D. Faria, P. Kongsuphol, J. Ousingsawat, F.
647 Thevenod, E. Roussa, J. Rock, and R. Schreiber. Anoctamins. *Pflugers Arch* 462: 195-
648 208, 2011.

649 29. Kustrzeba-Wojcicka, I., E. Siwak, G. Terlecki, A. Wolanczyk-Medrala, and W. Medrala.
650 Alternaria alternata and its allergens: a comprehensive review. *Clin Rev Allergy Immunol*
651 47: 354-65, 2014.

652 30. Lambert, A. J., J. A. Buckingham, H. M. Boysen, and M. D. Brand. Diphenyleneiodonium
653 acutely inhibits reactive oxygen species production by mitochondrial complex I during

654 reverse, but not forward electron transport. *Biochim Biophys Acta* 1777: 397-403, 2008.

655 31. Leino, M. S., M. Loxham, C. Blume, E. J. Swindle, N. P. Jayasekera, P. W. Dennison, B.
656 W. Shamji, M. J. Edwards, S. T. Holgate, P. H. Howarth, and D. E. Davies. Barrier
657 disrupting effects of alternaria alternata extract on bronchial epithelium from asthmatic
658 donors. *PLoS One* 8: e71278, 2013.

659 32. Li, Q., X. Luo, W. Zeng, and S. Muallem. Cell-specific behavior of P2X7 receptors in
660 mouse parotid acinar and duct cells. *J Biol Chem* 278: 47554-61, 2003.

661 33. Matsuwaki, Y., K. Wada, T.A. White, L.M. Benson, M.C. Charlesworth, J.L. Checkel, Y.
662 Inoue, K. Hotta, J.U. Ponikau, C.B. Lawrence and H. Kita. Recognition of fungal protease
663 activities induces cellular activation and eosinophil-derived neurotoxin release in human
664 eosinophils. *J Immunol* 183: 6798-6816 2009.

665 34. McGrath, J. J., W. C. Wong, J. D. Cooley, and D. C. Straus. Continually measured fungal
666 profiles in sick building syndrome. *Curr Microbiol* 38: 33-6, 1999.

667 35. Monso, E. Occupational asthma in greenhouse workers. *Curr Opin Pulm Med* 10: 147-50,
668 2004.

669 36. Noble, J. A., S. A. Crow, D. G. Ahearn, and F. A. Kuhn. Allergic fungal sinusitis in the
670 southeastern USA: involvement of a new agent Epicoccum nigrum Ehrenb. ex Schlecht.
671 1824. *J Med Vet Mycol* 35: 405-9, 1997.

672 37. Novak, I. Purinergic signalling in epithelial ion transport: regulation of secretion and

673 absorption. *Acta Physiol (Oxf)* 202: 501-22, 2011.

674 38. Novak, I., I. M. Jans, and L. Wohlfahrt. Effect of P2X(7) receptor knockout on exocrine
675 secretion of pancreas, salivary glands and lacrimal glands. *J Physiol* 588: 3615-27, 2010.

676 39. O'Grady, S. M., N. Patil, T. Melkamu, P. J. Maniak, C. Lancto, and H. Kita. ATP release
677 and Ca2+ signalling by human bronchial epithelial cells following Alternaria aeroallergen
678 exposure. *J Physiol* 591: 4595-609, 2013.

679 40. Prester, L. and J. Macan. Determination of Alt a 1 (Alternaria alternata) in poultry farms
680 and a sawmill using ELISA. *Med Mycol* 48: 298-302, 2010.

681 41. Rodriguez-Rajo, F. J., I. Iglesias, and V. Jato. Variation assessment of airborne Alternaria
682 and Cladosporium spores at different bioclimatical conditions. *Mycol Res* 109: 497-507,
683 2005.

684 42. Sanchez, H. and R. K. Bush. A review of Alternaria alternata sensitivity. *Rev Iberoam*
685 *Micol* 18: 56-9, 2001.

686 43. Scudieri, P., E. Sondo, L. Ferrera, and L. J. Galietta. The anoctamin family: TMEM16A
687 and TMEM16B as calcium-activated chloride channels. *Exp Physiol* 97: 177-83, 2012.

688 44. Shen H.D., M.F. Tam, R.B. Tang and H. Chou. Aspergillus and Penicillium allergens:
689 focus on proteases. *Curr Allergy Asthma Rep* 7(5):351-356, 2007.

690 45. Snelgrove, R. J., L. G. Gregory, T. Peiro, S. Akthar, G. A. Campbell, S. A. Walker, and C.

691 M. Lloyd. Alternaria-derived serine protease activity drives IL-33-mediated asthma
692 exacerbations. *J Allergy Clin Immunol* 134: 583-592.e6, 2014.

693 46. Tai, H. Y., M. F. Tam, H. Chou, H. J. Peng, S. N. Su, D. W. Perng, and H. D. Shen. Pen ch
694 13 allergen induces secretion of mediators and degradation of occludin protein of human
695 lung epithelial cells. *Allergy* 61: 382-8, 2006.

696 47. Thomma, B. P. Alternaria spp.: from general saprophyte to specific parasite. *Mol Plant*
697 *Pathol* 4: 225-36, 2003.

698 48. Uchida, M, E.L. Anderson, D.L. Squillace, N. Patil, P.J. Maniak, K. Iijima, H. Kita and S.M.
699 O'Grady. Oxidative stress serves as a key checkpoint for IL-33 release by airway
700 epithelium . *Allergy* In Press, DOI: 10.1111/all.13158, 2017.

701 49. Viegas, C., E. Carolino, J. Malta-Vacas, R. Sabino, S. Viegas, and C. Verissimo. Fungal
702 contamination of poultry litter: a public health problem. *J Toxicol Environ Health A* 75:
703 1341-50, 2012.

704 50. Wan, H., H. L. Winton, C. Soeller, D. C. Gruenert, P. J. Thompson, M. B. Cannell, G. A.
705 Stewart, D. R. Garrod, and C. Robinson. Quantitative structural and biochemical analyses
706 of tight junction dynamics following exposure of epithelial cells to house dust mite allergen
707 Der p 1. *Clin Exp Allergy* 30: 685-98, 2000.

708 51. Weyemi, U., C.E. Redon, R. Choudhuri, T. Aziz, D. Maeda, M. Boufraqech, P.R. Parekh,
709 T.K. Sethi, M Kasoji, N Abrams, A Merchant, V.N. Rajapakse and W.M. Bonner. The
710 histone variant H2A.X is a regulator of the epithelial-mesenchymal transition. *Nat*

711 *Commun.* 15;7: 10711-23, 2016.

712 52. Wolf, J., N. R. O'Neill, C. A. Rogers, M. L. Muilenberg, and L. H. Ziska. Elevated
713 atmospheric carbon dioxide concentrations amplify Alternaria alternata sporulation and
714 total antigen production. *Environ Health Perspect* 118: 1223-8, 2010.

715
716

Figure 1

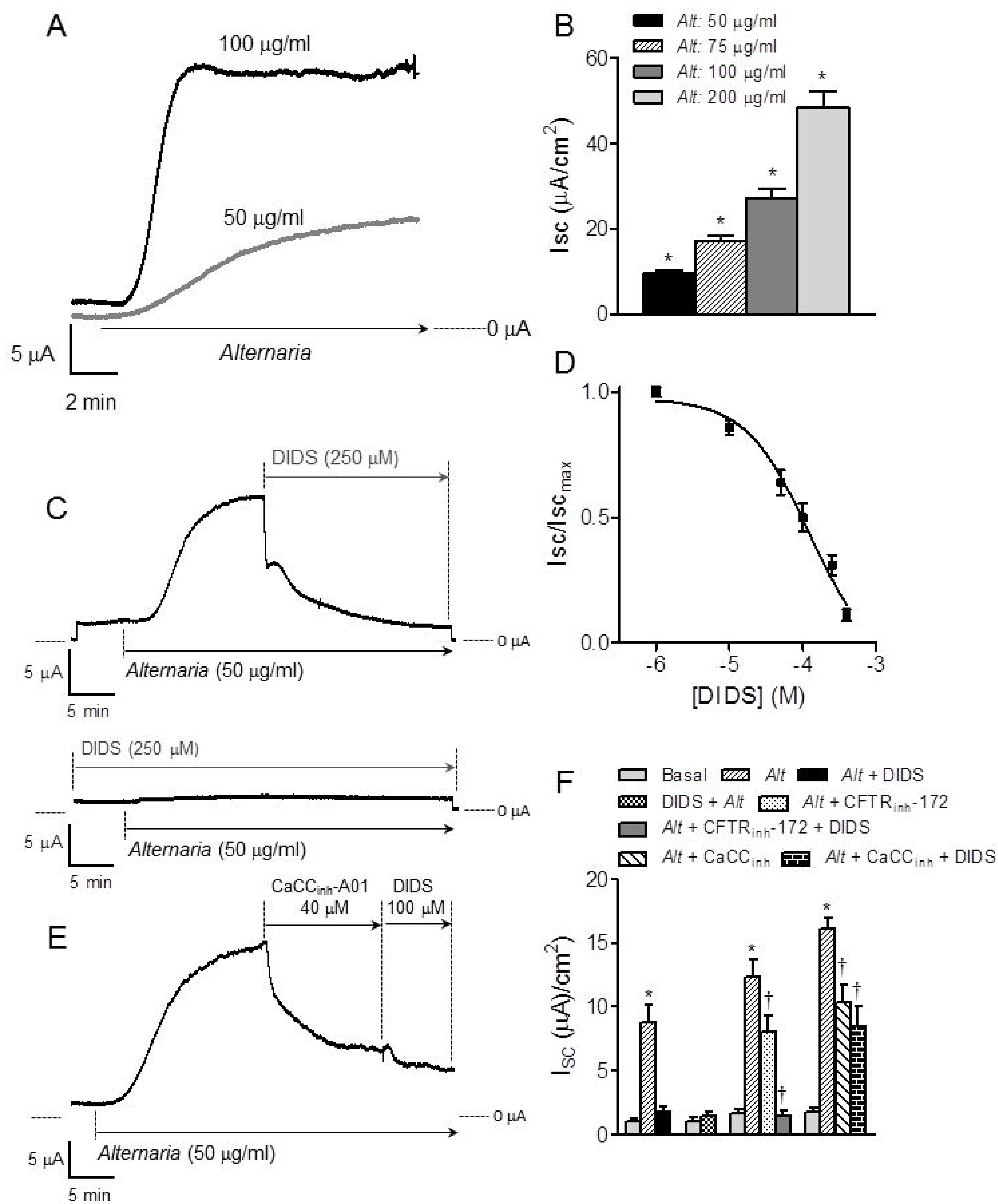

Figure 2

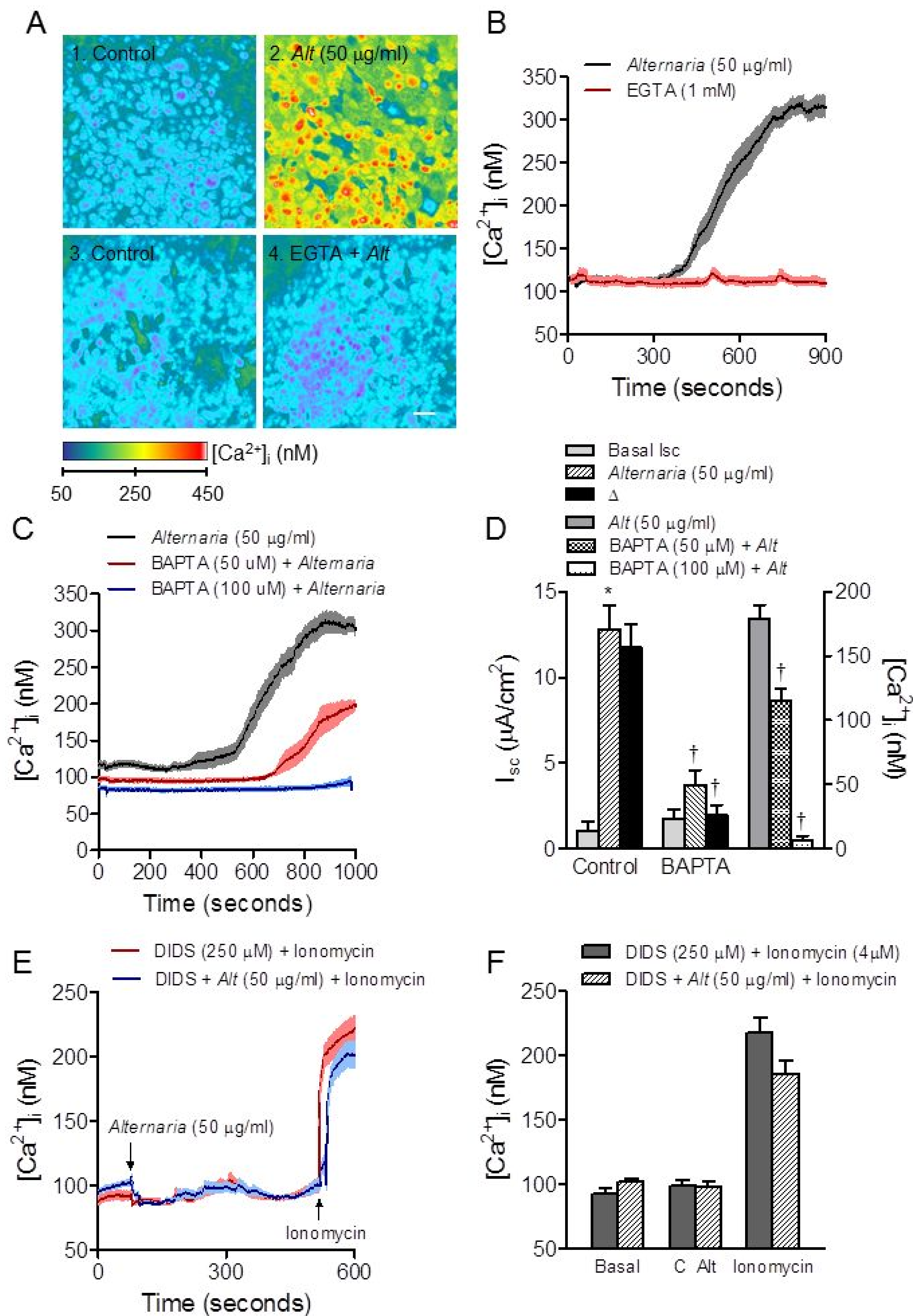

Figure 3

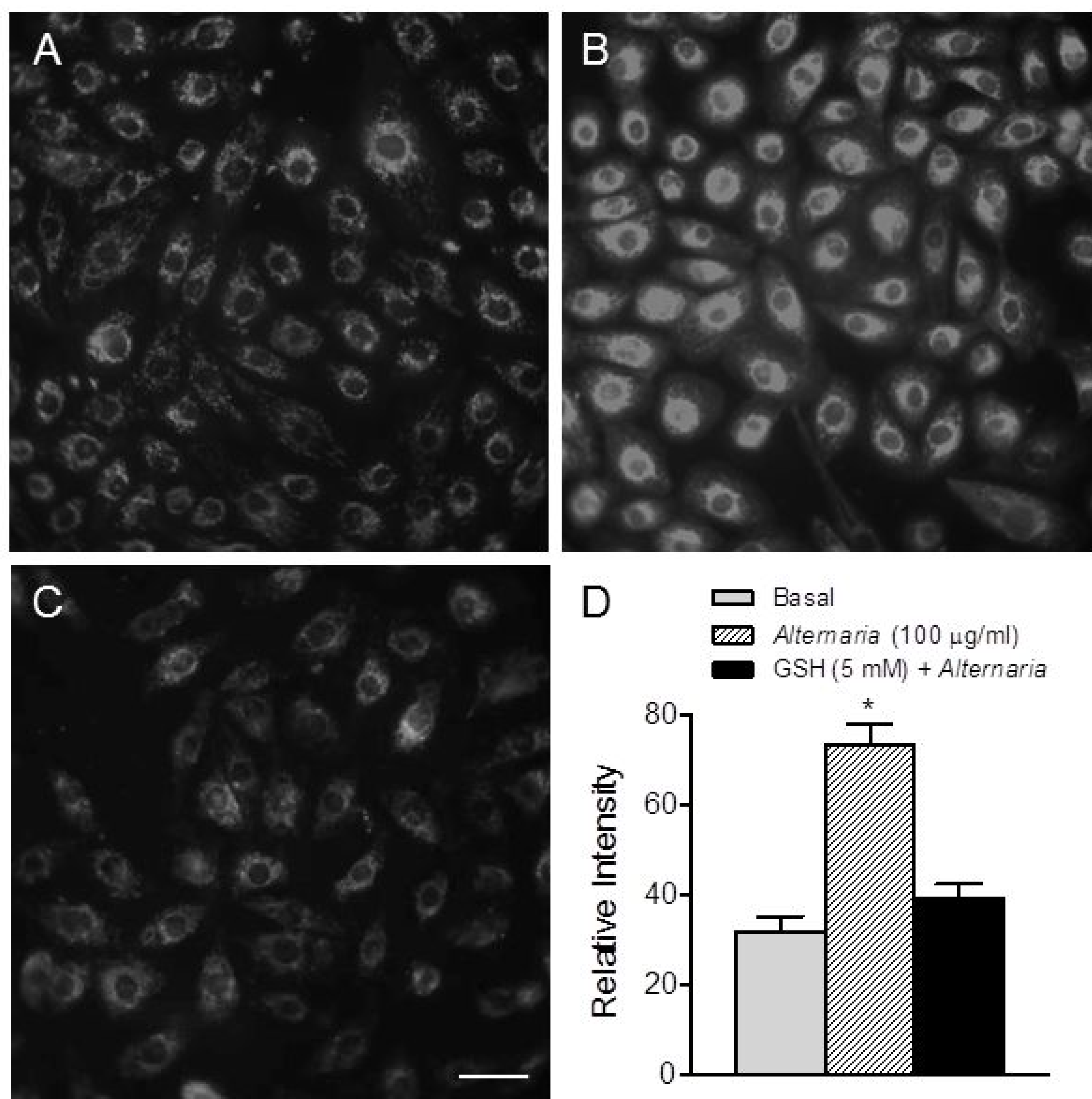

Figure 4

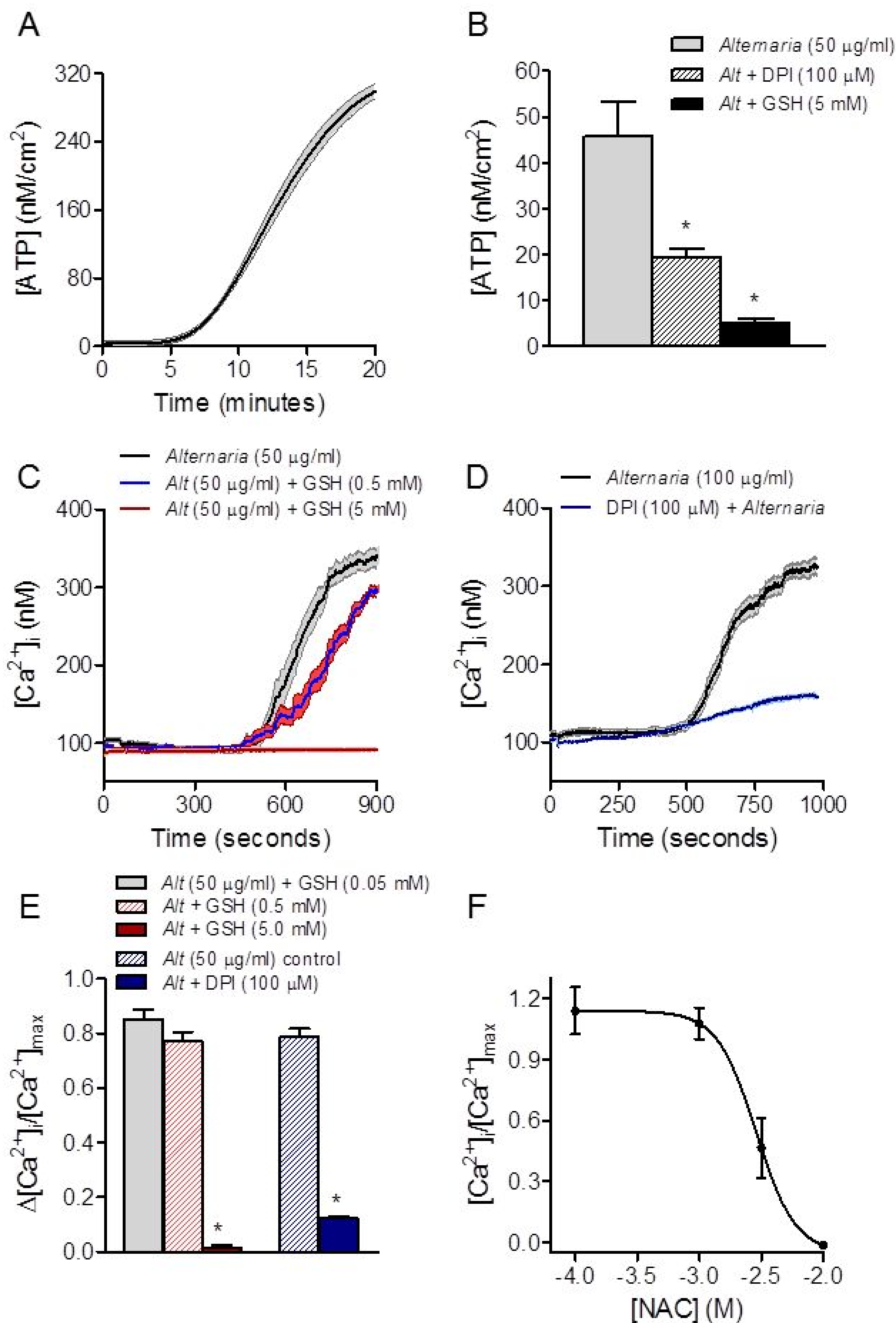

Figure 5

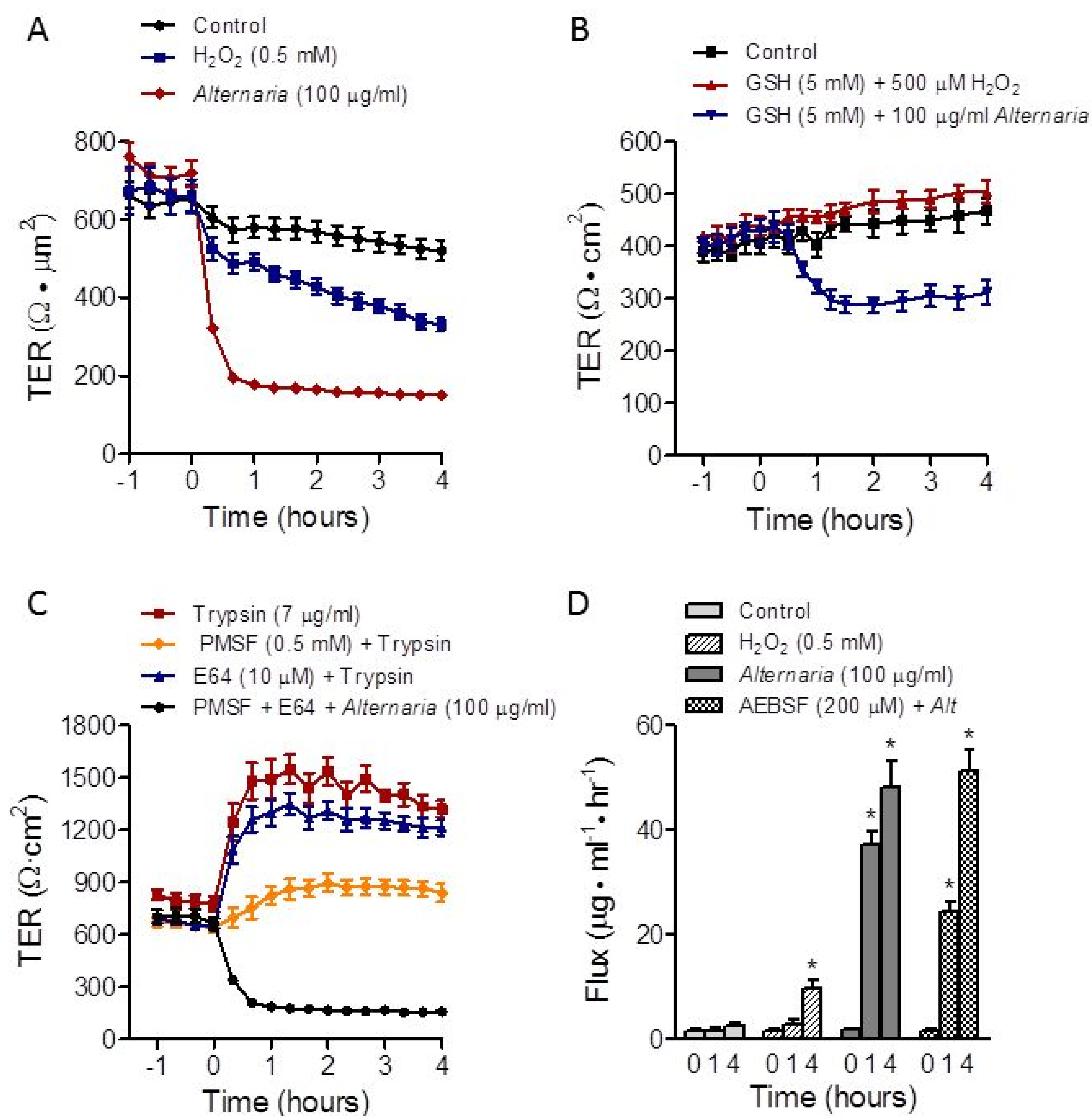

Figure 6

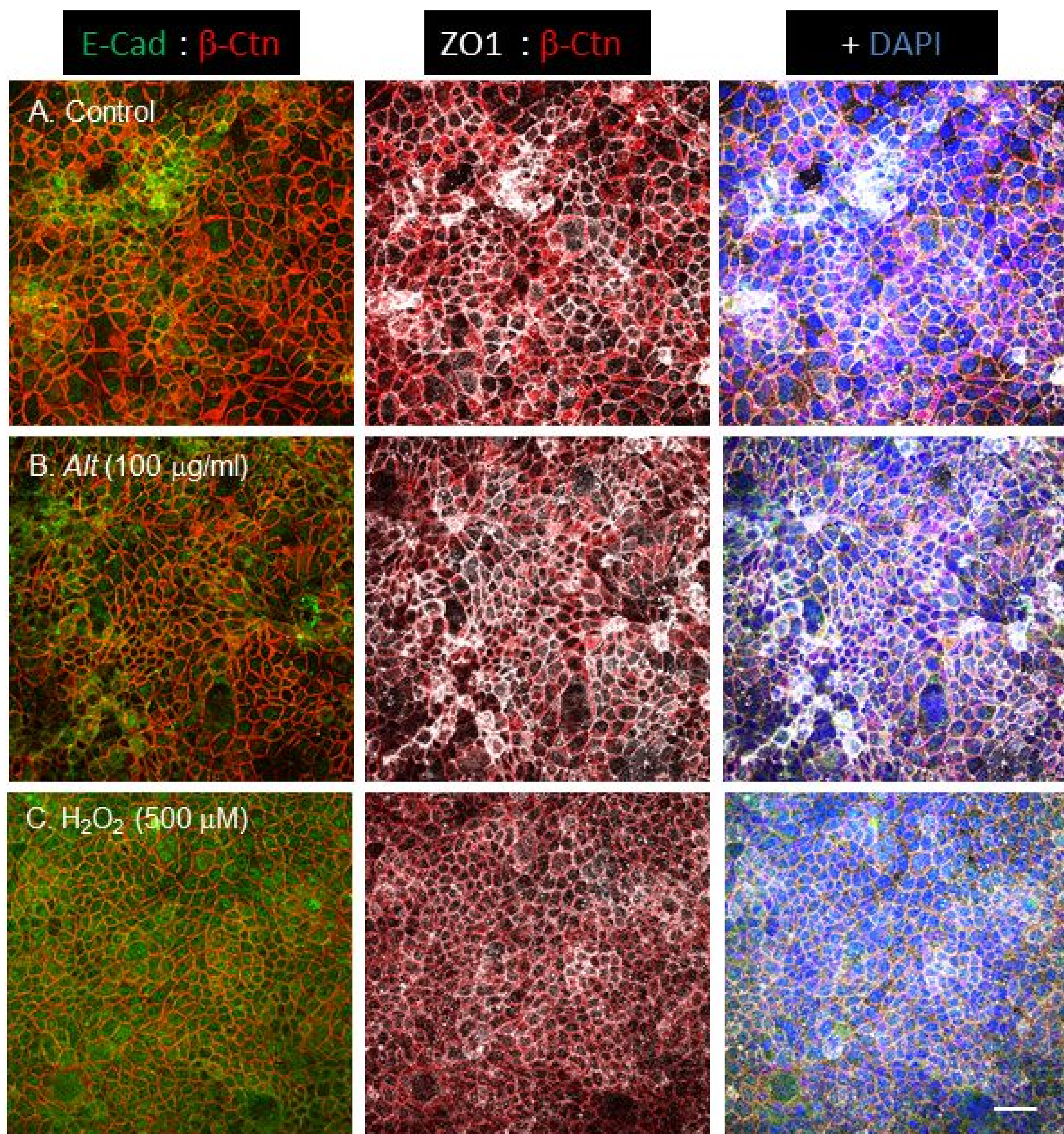

Figure 7