



Cigarette smoke impairs airway epithelial barrier function and cell–cell contact recovery

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ABSTRACT: Cigarette smoking, the major cause of chronic obstructive pulmonary disease (COPD), induces aberrant airway epithelial structure and function. The underlying mechanisms are unresolved so far.

We studied effects of cigarette smoke extract (CSE) on epithelial barrier function and wound regeneration in human bronchial epithelial 16HBE cells and primary bronchial epithelial cells (PBEs) from COPD patients, nonsmokers and healthy smokers.

We demonstrate that CSE rapidly and transiently impairs 16HBE barrier function, largely due to disruption of cell–cell contacts. CSE induced a similar, but stronger and more sustained, defect in PBEs. Application of the specific epidermal growth factor receptor (EGFR) inhibitor AG1478 showed that EGFR activation contributes to the CSE-induced defects in both 16HBE cells and PBEs. Furthermore, our data indicate that the endogenous protease calpain mediates these defects through tight junction protein degradation. CSE also delayed the reconstitution of 16HBE intercellular contacts during wound healing and attenuated PBE barrier function upon wound regeneration. These findings were comparable between PBEs from smokers, healthy smokers and COPD patients.

In conclusion, we demonstrate for the first time that CSE reduces epithelial integrity, probably by EGFR and calpain-dependent disruption of intercellular contacts. This may increase susceptibility to environmental insults, e.g. inhaled pathogens. Thus, EGFR may be a promising target for therapeutic strategies to improve mucosal barrier function in cigarette smoking-related disease.

KEYWORDS: Cell adhesion molecules, cigarette smoking, epithelial cell biology, epithelial repair, lung epithelial permeability, pathogenesis of chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is an inflammatory airway disease associated with airway remodelling and/or lung emphysema, leading to accelerated lung function decline. Although smoking is the major risk factor for COPD, it has remained unclear how different COPD phenotypes develop from the same cigarette smoke exposure. Inhaled tobacco smoke first encounters the airway epithelium, which forms a highly regulated barrier. Under pathological conditions, disruption of this barrier may lead to increased access of pollutants and pathogens to the submucosa. Epithelial barrier function is maintained by tight junctions (TJs), which comprise the interacting proteins occludin, zona occludens (ZO)-1 and claudins. TJs restrict paracellular permeability and fulfil a fence function, keeping the basolateral region separated

from the apical region. Efficient TJ restoration upon wounding may be crucial for epithelial homeostasis.

Smoking may considerably disturb epithelial junctions by inducing structural changes, such as mucous hyperplasia in the airways of COPD patients [1]. Smoking is not only associated with increased numbers of mucus-producing cells [2, 3], but also with increased mucosal permeability [4] and increased permeability to allergens *in vitro* [5, 6]. Similarly, cigarette smoke may facilitate trans-epithelial crossing of viruses and bacteria, with crucial consequences, since bacterial infections have been implicated in nearly 50% of COPD exacerbations [7]. Therefore, it is important that the mechanisms involved in smoke-induced barrier dysfunction are understood.

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The smoke-induced increase in permeability was initially attributed to the cytotoxic effects of smoke. More recently, cigarette smoking, as well as 6-day exposure to cigarette smoke extract (CSE) *in vitro*, has been shown to cause downregulation of apical junctional complex genes in airway epithelium [8]. Furthermore, mainstream cigarette smoke reduces trans-epithelial resistance and induces tyrosine phosphorylation of occludin in human airway adenocarcinoma Calu-3 cells [9, 10]. Additionally, cigarette smoke condensate causes damage to human bronchial epithelial BEAS-2B cells by a mechanism involving activation of the epidermal growth factor receptor (EGFR)/extracellular signal-related kinase (ERK) pathway and subsequent delocalisation of TJ proteins [11]. This effect could be attributed to lipophilic compounds present in CSE (including phenolic compounds, aldehydes and polycyclic aromatic hydrocarbons) that are able to pass the epithelial plasma membrane [12]. Effects of CSE on epithelial resistance as a read-out for barrier function have not been investigated. Our goal was to evaluate the effect of a non-toxic concentration of CSE on TJ formation and epithelial barrier function and to elucidate the underlying mechanism of smoke-induced epithelial barrier dysfunction.

We studied effects of CSE exposure in human bronchial epithelial cell line 16HBE as well as in primary bronchial epithelial cells (PBECs) from COPD patients and healthy nonsmoking and smoking donors. We demonstrate that CSE induces a transient defect in epithelial barrier function and decreases reconstitution of 16HBE cell-cell contacts upon wounding, probably by EGFR-dependent disruption of TJs. To our knowledge, this study shows for the first time that CSE induces comparable defects in PBEC, but with a delayed onset and a prolonged period of recovery. No clear differences in the response to wounding and CSE were observed between PBECs from nonsmokers, healthy smokers and COPD patients.

METHODS

Epithelial cell culture

16HBE cells were kindly provided by D.C. Gruenert (University of California, San Francisco, CA, USA) and cultured as described previously [13]. PBECs were obtained from three severe COPD patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages III and IV disease [14] by bronchial brushings (inclusion based on ≥ 10 pack-yrs smoking, forced expiratory volume in 1 s (FEV₁)/forced vital capacity <70% and FEV₁ <50% of predicted and age 50–62 yrs; table 1) according to standard guidelines [15]. The medical ethics committee of the University Medical Center Groningen, Groningen, the Netherlands, approved the study and participants gave signed informed consent. Additionally, PBECs from four healthy smokers (age 40–48 yrs) and three healthy nonsmokers (age 52–56 yrs) were derived from Lonza (Walkersville, MD, USA). PBECs were grown in 2.5 mL hormonally supplemented bronchial epithelium growth medium (BEGM; Lonza) on collagen/fibronectin-coated flasks as described [16], and used for experimentation at passage 2. Before experimentation, cell viability was evaluated by Trypan blue staining (mean viability $95 \pm 0.6\%$ for brushed cells and $93 \pm 1.6\%$ for Lonza cells).

Cells were seeded in duplicates into electric cell substrate impedance sensing (ECIS) arrays, chamber slides (LabTek; Thermo Fisher Scientific Inc., Waltham, MA, USA) or 24-well

TABLE 1 Characteristics of the chronic obstructive pulmonary disease patients from whom epithelial cells were obtained

Subject	Age yrs	Sex	Smoking history pack-years	FEV ₁ % pred
1	62	Female	42	18
2	50	Male	11	20
3	61	Male	31	48

FEV₁: forced expiratory volume in 1 s; % pred: % predicted.

plates. At 80–90% confluency, Eagle's minimal essential medium (EMEM)/10% fetal calf serum (FCS) or BEGM (Lonza) was replaced with EMEM/0.5% FCS (for 16HBE cells) or basal medium (BEBM; Lonza)/0.5% FCS (for PBECs). Methods for epithelial resistance measurements, western blotting and immunofluorescence are described in the online supplementary material.

Preparation of CSE

Cigarette smoke extract (CSE) was prepared as described previously [17]. In short, Kentucky 2R4F research-reference cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) were used without filter. Smoke from two cigarettes was bubbled through 25 mL medium (100% CSE). The extract was prepared freshly, sterilised using a 0.22 μ m filter and used within 30 min.

Stimulation of epithelial cells

Cells were pretreated with or without specific EGFR tyrosine kinase inhibitor AG1478 (2 μ M; Calbiochem; La Jolla, CA, USA), calpain inhibitor II (ALLM, 10 μ M; Sigma-Aldrich, St Louis, MO, USA) or vehicle (dimethylsulfoxide (DMSO)) for 30 min. Subsequently, cells were treated with 5% CSE, epidermal growth factor (EGF) (10 ng·mL⁻¹; Sigma-Aldrich) or vehicle (medium/DMSO) for 0–24 h and monitored by ECIS, fixed for immunofluorescent staining or harvested for cell lysate preparation. Cell viability was evaluated by Trypan blue staining. 5% CSE did not affect cell viability (data not shown).

ECIS

Electrical properties of confluent or wounded epithelium were measured using ECIS (Applied Biophysics, Troy, NY, USA) as described previously [18, 19]. Upon inoculation, resistance and capacitance were measured at 400 Hz and 40 kHz, respectively. Cells were wounded immediately after addition of CSE/vehicle by electroporation (5 V, 40 kHz, 30 s).

Statistical analysis

Data were analysed using the nonparametric rank sum Mann-Whitney U-test for analysis between subject groups and the t-test for paired observations to compare conditions within groups or the 16HBE cell line. Two-way ANOVA was used to test for significant differences between the control and treatment time curves in the epithelial resistance measurements.

RESULTS

CSE induces a transient decrease in electrical resistance of 16HBE cells

We first examined the effect of CSE on barrier function in a monolayer of 16HBE cells. Addition of CSE induced a substantial transient fall in low-frequency resistance, the most sensitive parameter to monitor barrier tightness [18, 19]. Resistance levels started to decline immediately, with a maximal effect between 4

and 6 h (maximal mean $\pm$ SEM decrease $26 \pm 3\%$; $n=5$), followed by a slow return to control values over ~ 15 h (fig. 1a and b). Resistance of the vehicle-treated wells also decreased over 4–6 h (maximal mean $\pm$ SEM inhibition $14 \pm 3\%$), but a significantly stronger decrease was observed with CSE ($n=5$; $p=0.011$). We additionally measured the high-frequency capacitance, which is sensitive to changes in cell-matrix contacts, but relatively insensitive to changes in cell-cell contacts [18, 19]. Here, CSE

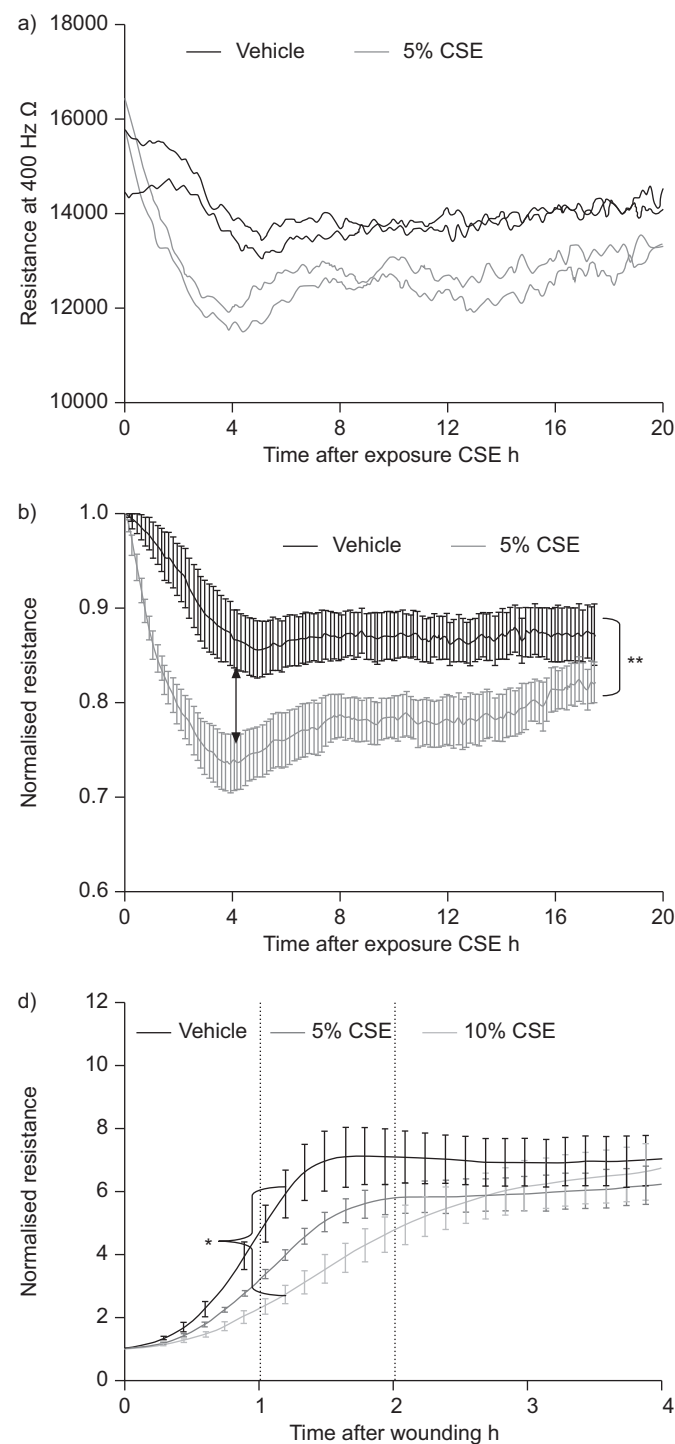


FIGURE 1. Effects of cigarette smoke extract (CSE) on electrical resistance in 16HBE cells. 16HBE cells were seeded in duplicates in electric cell substrate impedance sensing (ECIS) arrays, grown for 3–4 days, placed at low serum, exposed to 5% or 10% CSE or vehicle and monitored for 48 h or immediately wounded by electroporation. Resistance was measured at 400 Hz and capacitance was measured at 40 kHz using ECIS. a) Absolute resistance values of a representative experiment are shown. b) Normalised resistance values (mean $\pm$ SEM) are shown ($n=5$). Values were normalised to the levels prior to the addition of CSE/vehicle. **: $p<0.01$ between the control and CSE curve, two-way ANOVA. c) Normalised capacitance values (mean $\pm$ SEM) are shown ($n=5$). d) Normalised resistance values (mean $\pm$ SEM) are shown ($n=3$). Values were normalised to the levels immediately upon wounding. *: $p<0.05$ between the control and 10% CSE curve, two-way ANOVA. e) Normalised capacitance values (mean $\pm$ SEM) are shown ($n=3$).

exerted less pronounced effects (mean $\pm$ SEM maximal increase $9 \pm 1\%$), indicating that cell–matrix contacts were affected to a smaller extent than cell–cell contacts (fig. 1c).

CSE delays epithelial regeneration and reconstitution of cell–cell contacts of 16HBE cells upon wounding by electroporation

In addition to its effects on 16HBE barrier function, CSE impaired epithelial barrier reconstitution after wounding by electroporation. Using this type of wounding, the epithelial monolayer is able to recover within a few hours, as a result of cell migration/spreading, and not from proliferation [19]. Barrier function of the 16HBE cells completely recovered within 2 h (fig. 1d). Cells repopulated the electrode in 1 h, as reflected by the stabilisation of high-frequency capacitance (fig. 1e). CSE markedly delayed the recovery of epithelial resistance upon wounding. While a trend towards reduced epithelial resistance was observed when CSE was added in a concentration of 5%, the addition of 10% CSE significantly impaired recovery of the epithelial barrier (fig. 1d). Again, CSE (5 and 10%) displayed less dramatic effects on high-frequency capacitance than on low-frequency resistance. 1–2 hrs after wounding, low-frequency resistance was still markedly reduced compared with the control-treated wells, while capacitance

values had almost completely returned to levels of the confluent monolayer for both 5 and 10% CSE (fig. 1e). This indicates that CSE affects the reconstitution of epithelial cell–cell contacts rather than affecting epithelial migration, spreading and/or attachment upon wounding.

CSE-induced defects in barrier function are reversed by inhibition of EGFR activity

Since previous studies proposed a role for EGFR-dependent signalling in CSE-induced redistribution of TJ proteins [11], and that increased EGFR activity has been implicated in COPD [20], we next investigated whether EGFR activity was involved in the CSE-induced defects in epithelial barrier function. Treatment of the 16HBE cells with the EGFR tyrosine kinase inhibitor AG1478 significantly and almost completely prevented the CSE-induced changes in low-frequency resistance (fig. 2a and c). Similarly, AG1478 attenuated the CSE-induced increase in high-frequency capacitance, which was significant within 30–120 min (fig. 2b and d, but not at the time point where CSE exerted its strongest effects, *i.e.* 4 h after addition of CSE (fig. 1c).

In line with the involvement of EGFR activity in CSE-induced barrier dysfunction, we found that CSE increased phospho-EGFR

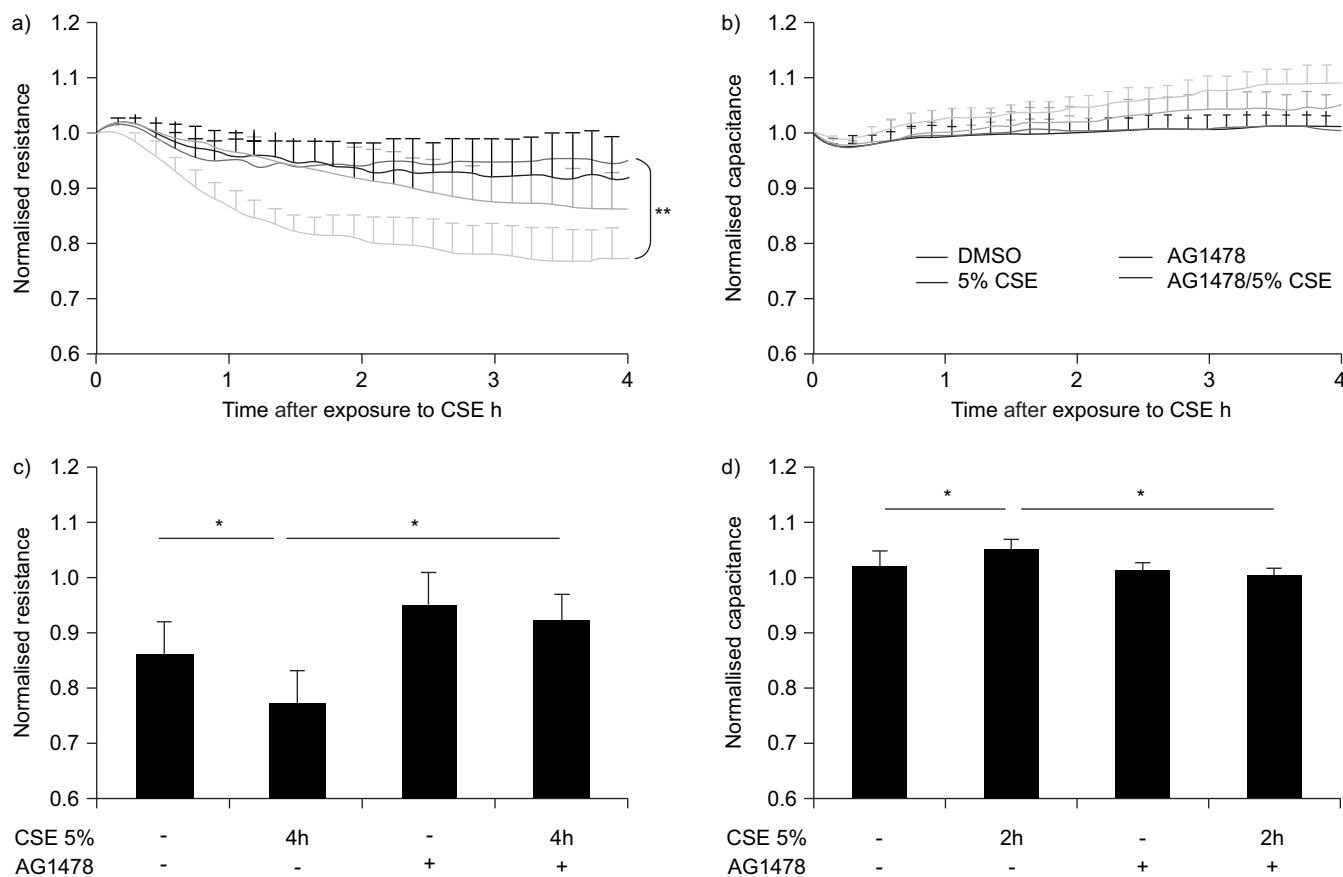


FIGURE 2. The effects of cigarette smoke extract (CSE) on barrier function in 16HBE cells are dependent on epidermal growth factor receptor activity. 16HBE cells were seeded in duplicates in electric cell substrate impedance sensing arrays, grown for 3–4 days, placed at low serum, pretreated for 30 min with or without AG1478 (1 μ M) and exposed to 5% CSE or vehicle. Resistance was measured at 400 Hz and capacitance was measured at 40 kHz. Values were normalised to the levels prior to the addition of CSE/vehicle. a and c) Normalised resistance values (mean $\pm$ SEM) are shown (n=5). **: $p < 0.01$ between the indicated curves (CSE and CSE+AG1478), two-way ANOVA; *: $p < 0.05$ between the indicated values. b and d) Normalised capacitance values (mean $\pm$ SEM) are shown (n=5). *: $p < 0.05$ between the indicated values.

(Tyr 1173) as well as EGFR downstream signalling molecule phospho-ERK-1/2, but not total EGFR levels, at 6 h (fig. 3a and b). Furthermore, addition of EGF transiently decreased epithelial resistance, with a maximum effect around 4–6 h, after which the cells recovered slowly (fig. 3c and d).

CSE induces EGFR-dependent disassembly of TJs

To underscore the mechanism of CSE-induced barrier dysfunction, we studied whether the changes in barrier function were paralleled by changes in intercellular junctions. We observed that extra- and intracellular TJ components, *i.e.* ZO-1 and occludin, respectively, were localised to intercellular junctions under resting conditions, forming a continuous ring (fig. 4a). Upon CSE exposure, staining for ZO-1 (fig. 4a) and also, to a lesser extent, occludin (fig. 4b) became more fragmented. The presence of AG1478 partially reversed these effects, resulting in more circumferential staining of ZO-1 and occludin (fig. 4a and b). In agreement with the hypothesis that EGFR activity

plays a role in CSE-induced TJ disassembly, EGF induced delocalisation of ZO-1 and occludin (fig. 4a and b).

Next, we investigated whether CSE and EGF also affected total levels of occludin and ZO-1. CSE induced partial ZO-1 cleavage, as demonstrated by the appearance of a smaller fragment (~ 192 kDa), and partial cleavage of occludin from its full length form (~ 65 kDa) into smaller cleavage fragments (~ 45 and ~ 30 kDa; fig. 4c–e). We did not observe cleavage of adherens junction (AJ) protein E-cadherin (data not shown). The presence of AG1478 reduced the appearance of smaller ZO-1 and occludin fragments (fig. 4c–e). Previously, activation of the endogenous cysteine protease calpain has been implicated in EGFR signalling [21] and calpain has been described to cleave junctional proteins [22]. The calpain inhibitor II also reduced the CSE-induced cleavage of occludin and ZO-1 into smaller fragments. Thus, exposure to CSE appears to disrupt TJs in an EGFR-dependent manner, and our data suggest this to involve calpain-mediated cleavage of TJ proteins.

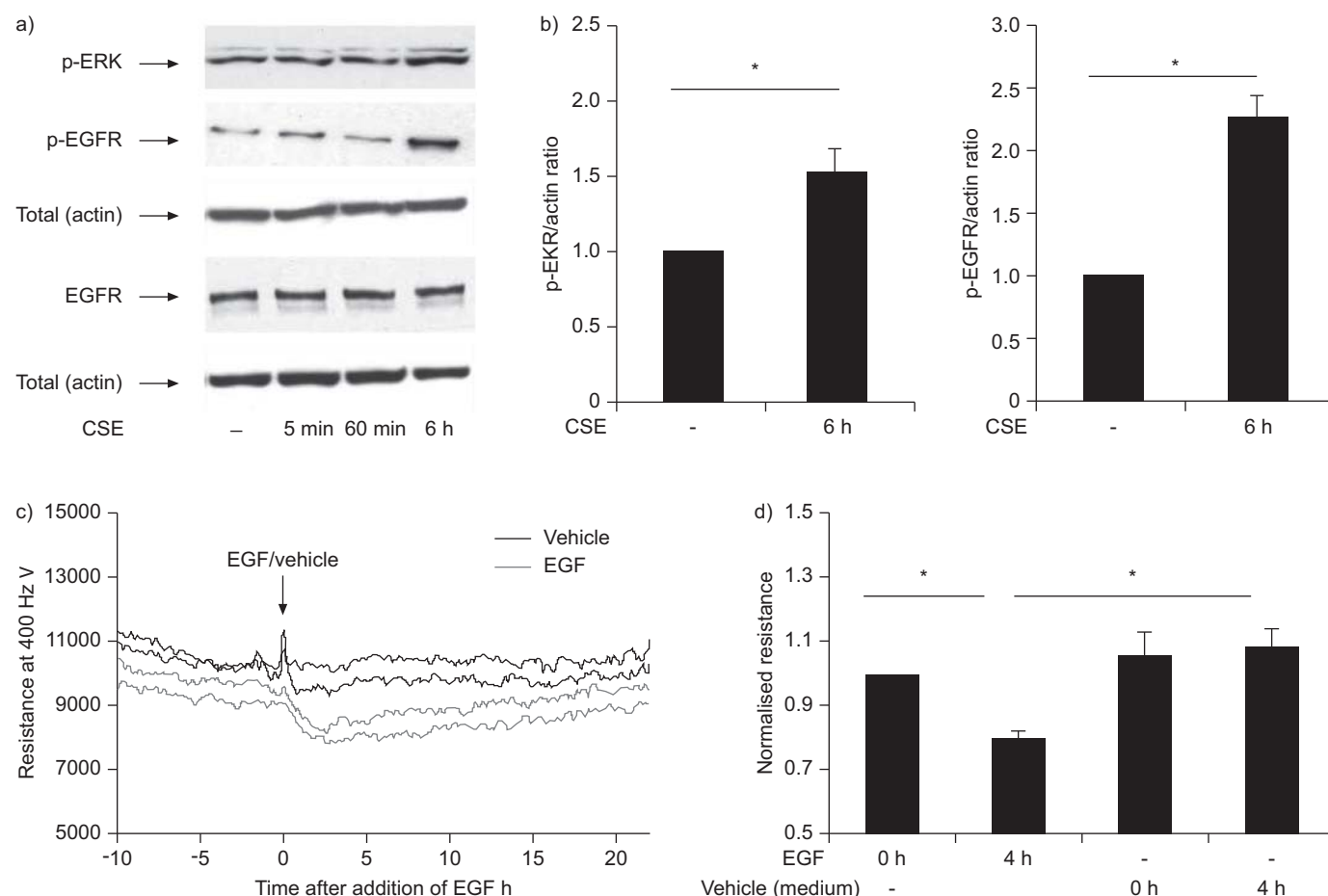


FIGURE 3. Cigarette smoke extract (CSE) induces epidermal growth factor receptor (EGFR)-dependent signalling and epidermal growth factor (EGF) affects electrical resistance of 16HBE cells. 16HBE cells were seeded in duplicates in 24-well plates or electric cell substrate impedance sensing arrays, grown to confluence for 3–4 days, placed at low serum and stimulated with or without 5% CSE or EGF ($10 \text{ ng}\cdot\text{mL}^{-1}$) for 5 min to 24 h, as indicated. **a)** Total cell lysates were prepared and phospho-extracellular signal-related kinase (phospho-ERK), phospho-EGFR and EGFR were detected by western blotting (arrows). Actin was used as loading control. Representatives of 4 independent experiments are shown. **b)** Densitometry was performed and phospho-ERK and phospho-EGFR levels were related to actin. Ratios (mean $\pm$ SEM; $n=4$) are depicted. **c)** Absolute resistance values of a representative experiment are shown. **d)** Resistance values normalised to the levels prior to the addition of EGF/vehicle are shown (mean $\pm$ SEM; $n=3$). *: $p<0.05$ between the indicated values.

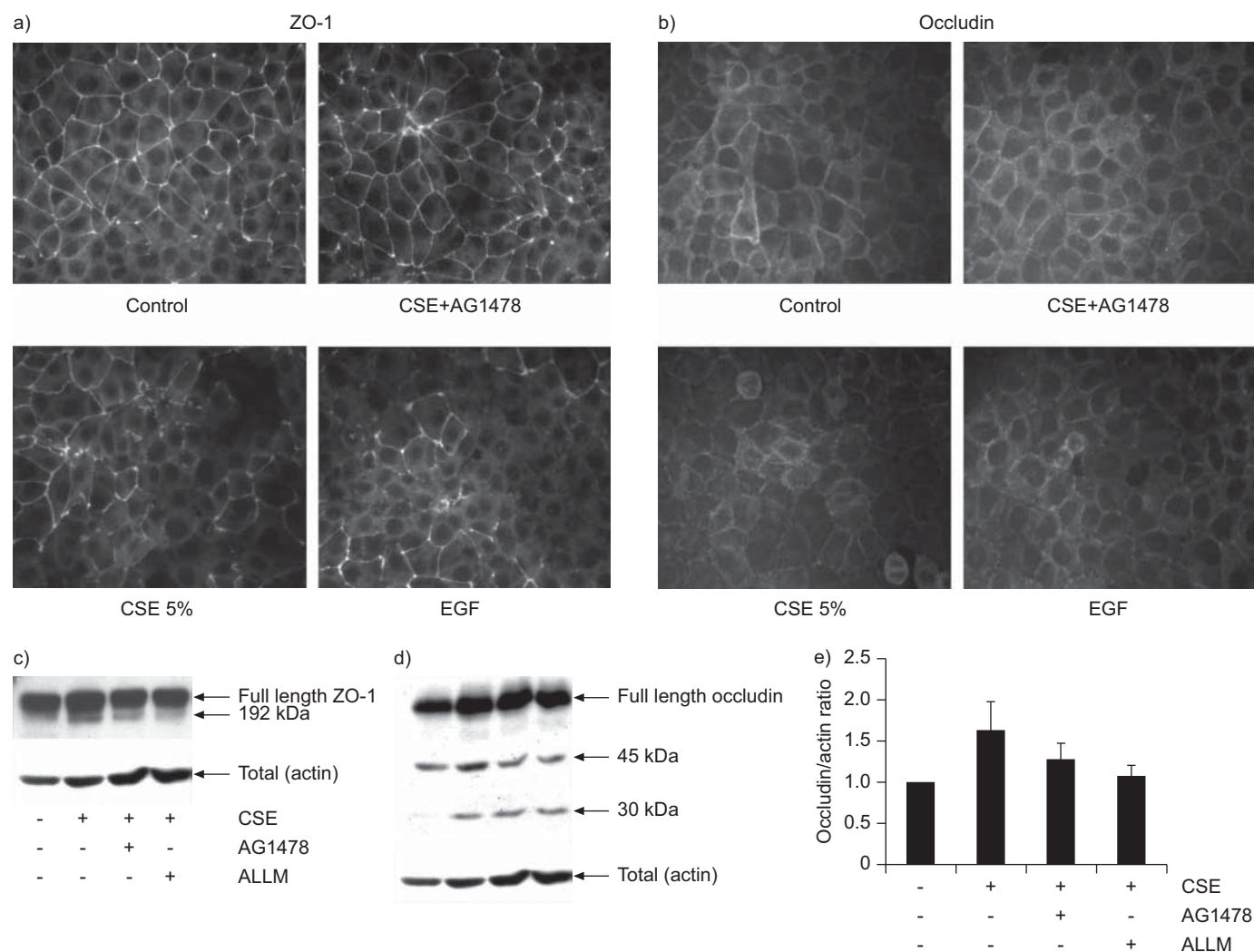


FIGURE 4. Cigarette smoke extract (CSE) and epidermal growth factor (EGF) induce disassembly of tight junctions. 16HBE cells were cultured in chamber slides or 24-well plates for 3 days, placed at low serum, pretreated for 1 h with and without AG1478 (1 μ M) or ALLM (10 μ M) and incubated with or without CSE (5%) or EGF (10 ng·mL⁻¹) for 4 h. a) Zona occludens-1 (ZO-1) was detected by immunofluorescence staining. b) Occludin was detected by immunofluorescence staining. c) Total cell lysates were prepared and ZO-1 was detected by western blotting (arrows). Representatives of four independent experiments are shown. d) Total cell lysates were prepared and occludin was detected by western blotting (arrows). Actin was used as loading control. e) Densitometry was performed on the 30 kDa fragment of occludin and levels were related to actin; ratios (mean $\pm$ SEM; n=4) are depicted.

CSE induces EGFR-dependent defects in barrier function of PBEC from nonsmokers and healthy smokers

Next, we were interested in the effects of CSE in primary epithelium and possible differences between nonsmoking individuals and current smokers, in order to assess whether epithelial abnormalities are present upon long-term smoking. PBECs showed considerably lower resistance levels upon establishment of the monolayer than 16HBE cells, with no significant differences between nonsmokers and smokers (mean $\pm$ SEM levels $4,257 \pm 1,422 \Omega$ and $5,238 \pm 1,350 \Omega$, respectively). Exposure to CSE induced a $\sim 50\%$ decrease in epithelial barrier function in both nonsmokers and smokers (fig. 5a and 5b), to levels of $1,937 \pm 563 \Omega$ and $2,942 \pm 955 \Omega$, respectively. The onset of the CSE-induced barrier dysfunction was delayed when compared to 16HBE cells, with the largest effect between 12–18 h, without recovery until 25–35 h after CSE exposure. No significant differences were observed between the two groups.

Next, we tested for the involvement of EGFR activity. Similar to 16HBE cells, AG1478 significantly reduced the CSE-induced defect in PBEC barrier function at 9–28 h (data shown for 12 h, the time point where most pronounced effects of CSE were observed; fig. 5c), and CSE was no longer able to significantly reduce epithelial resistance in the presence of AG1478. AG1478 also increased epithelial resistance in the absence of CSE; however, this effect did not reach statistical significance until 30 h upon its addition. Furthermore, AG1478 abolished the effect of CSE on high-frequency capacitance, although this effect of CSE was less pronounced than on low-frequency resistance, and only significant at 3–5 h (fig. 1 of the online supplementary material).

Additionally, we studied the response of PBECs to wounding by electroporation and observed that these cells regenerated more slowly than 16HBE cells, as it took cells ~ 3 h to restore

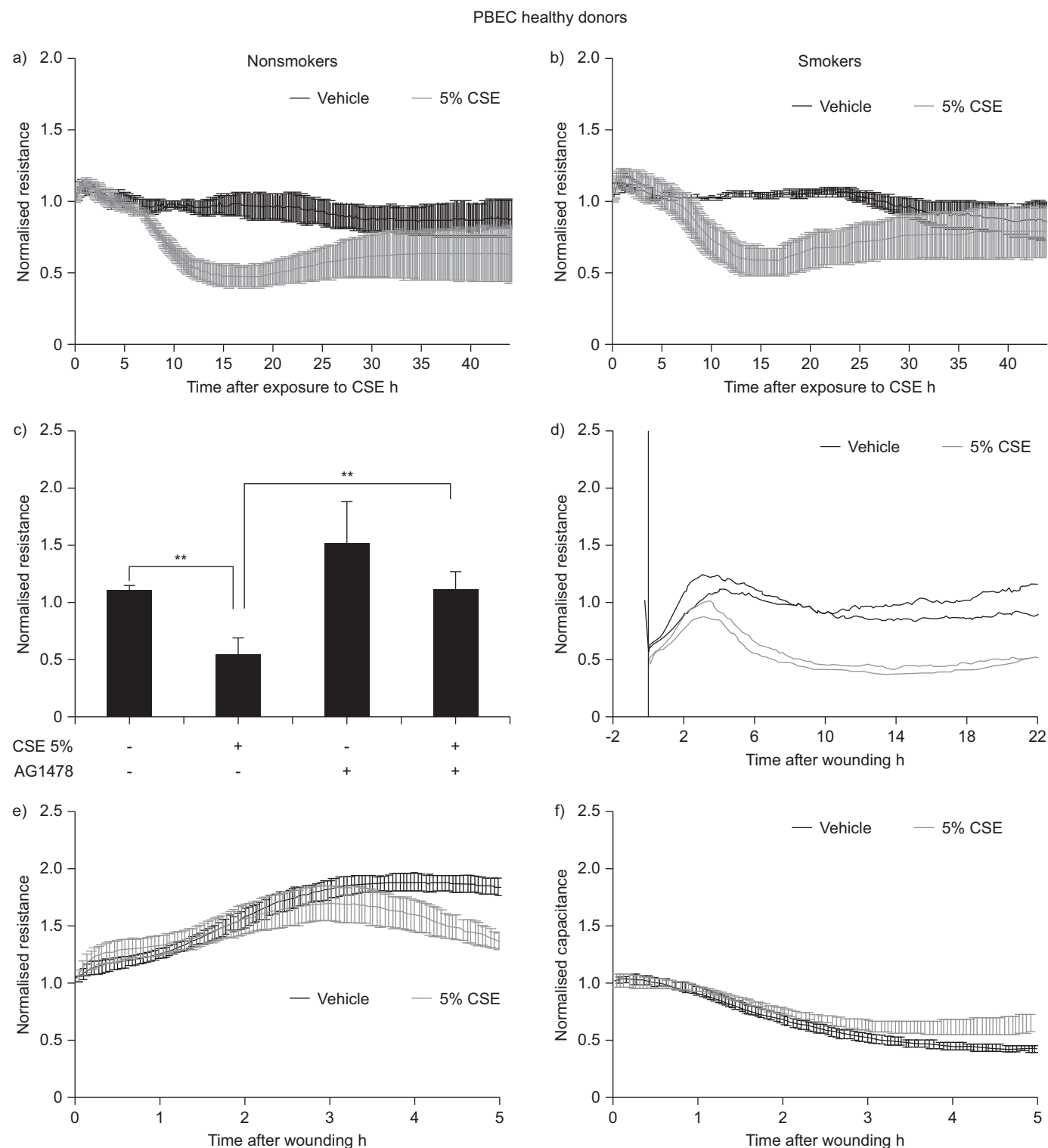


FIGURE 5. Primary bronchial epithelial cells (PBECs) from nonsmokers and smokers were seeded in duplicates in electric cell substrate impedance sensing arrays, grown to confluence for 3–4 days, growth factor/hormone-deprived overnight, exposed to 5% cigarette smoke extract (CSE) or vehicle and monitored for 48 h or immediately wounded by electroporation. Resistance was measured at 400 Hz and capacitance was measured at 40 kHz. Values were normalised to the levels prior to the addition of CSE/vehicle. When enough cells were available, cells were pretreated with or without AG1478 before CSE exposure. a) Normalised resistance values (mean $\pm$ SEM) of PBECs from nonsmokers ($n=3$). Values were normalised to the levels prior to the addition of CSE/vehicle. b) Normalised resistance values (mean $\pm$ SEM) of PBECs from smokers ($n=4$). c) Normalised resistance values (mean $\pm$ SEM) (PBECs from both smokers and nonsmokers; $n=4$). **: $p<0.01$ between the indicated values. d) Normalised resistance values of PBECs from a smoker (representative). Values were normalised to the levels prior to wounding. e) Normalised resistance values (mean $\pm$ SEM) of PBECs from smokers ($n=3$). Values were normalised to the levels immediately upon wounding. f) Normalised capacitance values (mean $\pm$ SEM) of PBECs from smokers ($n=3$).

their barrier function (shown for healthy smoker epithelium; fig. 5d and e). Again, we did not observe differences between epithelial cells from smokers and nonsmokers (fig. 2 of the online supplementary material). Exposure to CSE reduced the resistance of the newly established monolayer, with a further decline once the resistance values in untreated cells were stabilised (fig. 5e). Effects on high-frequency capacitance were again less pronounced (fig. 5f).

CSE induces similar defects in barrier function of PBECs from COPD patients

To further evaluate the relevance of our findings, we used PBECs from three patients with severe COPD (all ex-smokers). Monolayers of these cells established a similar low-frequency resistance to that of healthy PBECs, *i.e.* $3,281 \pm 926 \Omega$. In addition, CSE caused an equal $\sim 50\%$ decrease in barrier function (fig. 6a), resulting in levels of $1,516 \pm 163 \Omega$. In further agreement with the healthy PBECs, low-frequency resistance was affected more markedly by CSE than high-frequency capacitance (data not shown).

Furthermore, the response to wounding by electroporation was similar in PBECs from COPD patients and healthy PBECs, with resistance levels returning to their original values within ~ 3 h. Again, CSE reduced the low-frequency resistance of the

newly formed monolayer upon wounding (fig. 6b), with less pronounced effects on high-frequency capacitance (fig. 6c).

Together, our data indicate that CSE exposure impairs the integrity of airway epithelium, mainly by disruption of intercellular contacts, with a similar pattern in COPD and healthy epithelium.

DISCUSSION

Epithelial remodelling and aberrant epithelial repair responses may disturb mucosal barrier function in COPD. Cigarette smoke is known to increase epithelial permeability, although the underlying mechanism has remained incompletely understood. Our study shows that CSE causes a transient, but substantial, decrease in airway epithelial barrier function in both 16HBE cells and PBECs. This effect appears dependent on EGFR activation, and we provide evidence for a novel mechanism of barrier dysfunction involving calpain-mediated disruption of TJs. Furthermore, CSE decreased restoration of 16HBE cell-cell contacts during wound regeneration and attenuated barrier function of PBECs upon wounding, with no striking differences between COPD patients and healthy controls. Our data may have important implications, *e.g.* for exacerbations in smokers with COPD, since reduced epithelial

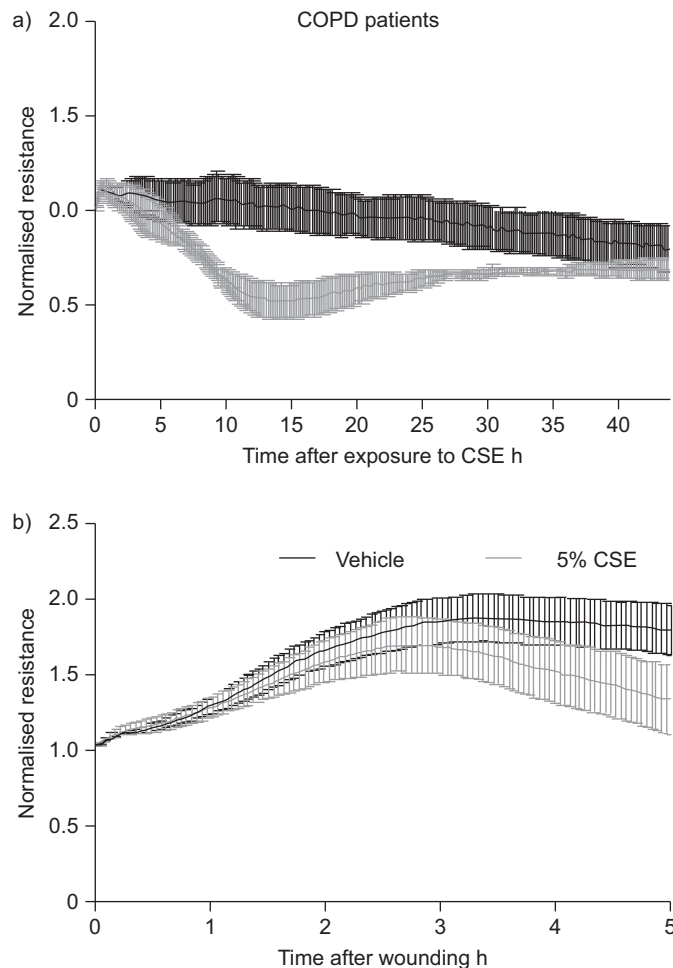
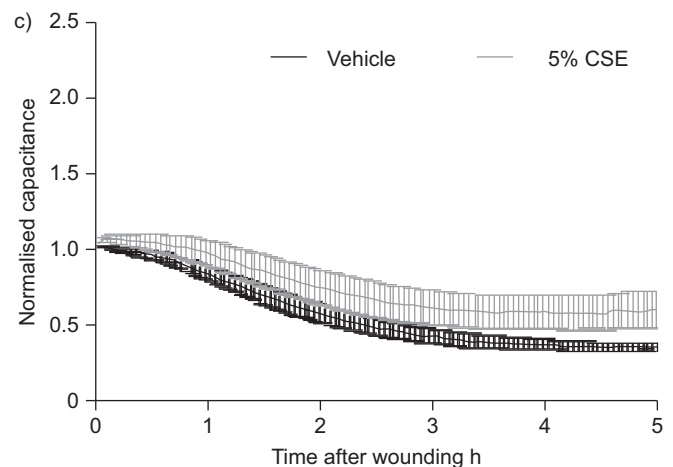


FIGURE 6. Bronchial epithelial cells were derived from bronchial brushings in Global Initiative for Chronic Obstructive Lung Disease stage IV chronic obstructive pulmonary disease patients. Cells were seeded in duplicates in electric cell substrate impedance sensing arrays, grown to confluence for 3–4 days, growth factor/hormone-deprived overnight, exposed to 5% CSE or vehicle and monitored for 48 h or immediately wounded by electroporation. Resistance was measured at 400 Hz and capacitance was measured at 40 kHz. a) Normalised resistance values (mean $\pm$ SEM) are shown ($n=3$). Values were normalised to the levels prior to the addition of CSE or vehicle. b) Normalised resistance values (mean $\pm$ SEM) are shown ($n=3$). Values were normalised to the levels immediately upon wounding. c) Normalised capacitance values (mean $\pm$ SEM) are shown ($n=3$). Values were normalised to the levels immediately upon wounding.



integrity may facilitate the transport of viruses and bacteria that make an important contribution to COPD exacerbations.

CSE affected epithelial resistance similarly in PBECs from COPD patients, healthy smokers and nonsmokers. Considering the role of EGFR in the CSE-induced defects, this is in line with our observation that EGFR levels did not differ between the subject groups (fig. 3 of the online supplementary material). The effects in PBECs were stronger and more sustained than in 16HBE cells. It is possible that PBECs are more susceptible to CSE-induced damage as result of weaker intercellular contacts [19]. Primary cells are able to form a tight barrier when cultured at an air-liquid interface (ALI), allowing for differentiation into polarised mucociliary epithelium [23]. Unfortunately, the ECIS system does not allow for measurements under ALI conditions. We have previously shown that culturing under ALI conditions substantially increases membrane expression of junctional proteins in PBECs, resulting in an epithelial resistance that equals the levels in 16HBE cells [24]. Therefore, we consider 16HBE cells as a reliable model when studying epithelial barrier function [19]. Indeed, 16HBE cells have been reported to polarise and form functional AJs and TJs under submerged conditions [25]. In both 16HBE cells and PBECs, EGFR activity appeared to contribute to the CSE-induced defects. Previously, EGFR activity has been shown to induce delocalisation of junctional proteins and calpain activation [21, 26, 27], which may lead to degradation of TJ proteins [28]. Our data support a role for EGFR in the CSE-induced disassembly of TJs, since the EGFR tyrosine kinase inhibitor AG1478 abrogated the CSE-induced defect on barrier function. Furthermore, CSE increased EGFR phosphorylation in 16HBE cells. Accordingly, activation of EGFR by its ligand EGF transiently decreased epithelial barrier function, and both CSE and EGF induced delocalisation as well as cleavage of the TJ proteins occludin and ZO-1 in 16HBE cells.

Several mechanisms may underlie this CSE-induced EGFR activation. First, increased access of apically secreted EGF ligands to basolaterally localised EGFR could be involved upon opening of tight junctions [29]. However, this does not seem to be the case, as EGFR activity itself was required for the disruption of TJs. Alternatively, smoke has been described to activate A disintegrin and metalloproteinase (ADAM)17, the main shed-dase of the EGFR ligand transforming growth factor (TGF)- α [30]. We observed that CSE induces ADAM17 and EGFR-dependent interleukin-8 production in 16HBE cells (fig. 4 of the online supplementary material), suggesting that CSE can indeed activate ADAM17. However, we were not able to detect TGF- α upon CSE exposure. Moreover, the presence of ADAM17/10 inhibitor GW280264 did not prevent the CSE-induced defect on barrier function nor the CSE-induced activation of EGFR (data not shown), rendering a role for ADAM17 less likely. Thus, a more plausible explanation might be the induction of Src kinase activity and subsequent ligand-independent EGFR phosphorylation, which has been described in A549 cells [31] and we will explore this possibility in future studies.

In addition to its effects on cell-cell contacts, EGFR activity can affect cell-matrix contacts by induction of calpain activity and cleavage of fibronectin and/or focal adhesion component talin [32–34]. In our setting, the modest CSE-induced increase in high-frequency capacitance reflected effects on cell-matrix contacts, and the presence of AG1478 abrogated these effects. In both

16HBE cells and PBECs, we observed more pronounced effects on low-frequency resistance than on high-frequency capacitance, suggesting that cigarette smoke-induced EGFR activity affects intercellular contacts more than cell-matrix contacts.

Our data show that CSE also affects the reconstitution of intercellular contacts in response to wounding, causing a delay in the recovery of barrier function in 16HBE cells. In PBECs, CSE impaired barrier function upon re-establishment of the monolayer rather than during its recovery. We do not have an explanation for the later onset of CSE-induced defects in PBECs. It may well be that this involves differential effects of CSE on intracellular signalling and/or on cell architecture in the two cell types. Again, we did not observe significant differences in the epithelial responses of nonsmokers and healthy smokers, in either presence or absence of CSE. This indicates that long-term smoking does not induce changes that remain present after the culture procedure, *e.g.* with respect to proteins involved in epithelial regeneration. Furthermore, PBECs from COPD patients did not show striking differences with regard to their responses to wounding and/or CSE exposure when compared to healthy PBECs. However, we cannot exclude the possibility that there are differences in ALI-polarised PBECs, between those in COPD patients and those in healthy individuals. It will be of interest to further explore possible intrinsic differences in COPD epithelium in a separate, future study including larger numbers of COPD patients and epithelial cells derived from bronchial brushings in healthy donors matched for age and smoking history.

In conclusion, we demonstrate that cigarette smoke induces EGFR-dependent disruption of TJs and reduces airway epithelial integrity upon wounding, possibly through a mechanism involving the endogenous protease calpain. We propose that cigarette smoking may have important implications for bacterial and viral infections in COPD, both by facilitating the access of pathogens as well as impairing recovery of the mucosal immune barrier upon pathogen-induced damage. Thus, targeting the EGFR may provide new avenues for the treatment of COPD exacerbations.

STATEMENT OF INTEREST

A statement of interest for this study can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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