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Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction

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ABSTRACT: Impaired airway epithelial barrier function has emerged as a key factor in the pathogenesis of allergic asthma. We aimed to discern the involvement of the epidermal growth factor receptor (EGFR) in allergen-induced epithelial barrier impairment, as we previously observed that house dust mite (HDM) signals through EGFR.

We investigated the junctional integrity of human bronchial epithelial cells using electric cell-substrate impedance sensing and immunofluorescent staining.

HDM induced a rapid, transient fall in epithelial resistance, concomitant with delocalisation of E-cadherin and zona occludens (ZO)-1, and proteolytic cleavage of the latter. EGFR inhibition by AG1478 reduced the HDM-triggered decrease in epithelial resistance and improved restoration of epithelial junctions. Similarly, AG1478 increased epithelial barrier recovery upon electroporation-induced injury, although it delayed the migration phase of the wound healing response. HDM-promoted redistribution of E-cadherin was mediated *via* EGFR-dependent activation of protease-activated receptor (PAR)-2, while the concomitant ZO-1 degradation was PAR-2/EGFR-independent. Importantly, the fibrogenic cytokine transforming growth factor (TGF)-β prolonged HDM-induced EGFR phosphorylation and inhibited ligand-induced EGFR internalisation/degradation, which resulted in sustained E-cadherin and ZO-1 redistribution.

Thus, allergen-induced, PAR-2/EGFR-mediated signalling decreases epithelial resistance and promotes junction disassembly. The TGF-β-enhanced EGFR signalling may be an important contributor to barrier dysfunction and increased epithelial vulnerability in response to HDM.

KEYWORDS: E-cadherin, epithelial junctions, protease-activated receptor-2, transforming growth factor-β, zona occludens-1

Allergic asthma is characterised by allergen-induced airway inflammation, hyperresponsiveness (AHR) and remodelling. The respiratory epithelium forms the first barrier against deposited aeroallergens and is a central player in the initiation of allergic responses, as well as airway remodelling. Breakdown of intercellular junctions not only facilitates transport of allergens, but may also promote pro-inflammatory responses [1] and growth factor release [2] by the epithelium. Therefore, strategies directed towards the maintenance and/or restoration of epithelial barrier function may be crucial. Intercellular junctions are the structural basis of epithelial barrier function and include tight and adherens junctions. Adherens junctions are composed of the adhesion molecule E-cadherin, which regulates epithelial architecture through homophilic interactions and is connected to the cytoskeleton by catenins [3]. The more apically localised tight junctions are the main contributors to epithelial resistance, which restrict paracellular permeability, are comprised of interacting transmembrane proteins, *e.g.* occludin and claudins, and are anchored to the cytoskeleton by zona occludens (ZO)-1 and cingulin.

Airway epithelial integrity is often compromised in asthma, with detachment of ciliated cells, decreased E-cadherin expression at these sites, disruption of tight junctions and increased permeability to allergens [4–6]. Many allergens, including house dust mite (HDM), contain proteolytic activity, causing epithelial damage [7, 8]. In addition, proteases act on the protease-activated receptor (PAR)-2, a receptor expressed by airway epithelium and linked to intracellular pro-inflammatory signalling pathways [9]. Recently, PAR-2 activation has been described as disrupting E-cadherin-mediated contacts [10] and impacting on epithelial integrity, as well as pro-inflammatory activity [1]. Additionally, E-cadherin downregulation is a key

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component of epithelial-to-mesenchymal transition (EMT), a process involved in tissue repair and remodelling. Importantly, recent studies suggest that EMT contributes to the development of the abnormal phenotype of the asthmatic epithelium [11, 12].

Nonetheless, the mechanism underlying structural changes to proteolytically active, but normally harmless, allergens in asthma is still undefined. Asthma epithelium may be more vulnerable [6] and/or inefficient in regeneration upon injury, as supported by increased levels of the repair markers transforming growth factor (TGF)-β and epidermal growth factor receptor (EGFR) in the asthmatic airways [2, 13, 14]. Despite the evidence that EGFR is upregulated in asthma [15], the functional significance is not clear. We have previously observed that EGFR is crucial in HDM-induced signalling in bronchial epithelium [16]. Herein we sought to determine whether EGFR is also involved in structural changes upon HDM exposure. We observed that HDM transiently disrupts epithelial junctions, to which EGFR activation significantly contributes. Moreover, the presence of TGF-β sustained the EGFR-mediated disruption of epithelial junctions and, thus, may increase vulnerability to HDM.

METHODS

Cell culture and stimulation

The human bronchial epithelial cell line 16HBE14o- (16HBE) was kindly provided by D.C. Gruenert (University of California, San Francisco, CA, USA) and cultured in EMEM medium/10% fetal calf serum (Biowhittaker, Verviers, Belgium) with 100 $U \cdot mL^{-1}$ penicillin/100 $\mu g \cdot mL^{-1}$ streptomycin on collagen-coated flasks as previously described [1]. Primary bronchial epithelial cells were derived from Lonza (Walkersville, MD, USA) as cryopreserved passage 1 normal human bronchial epithelium (NHBE). Cells were cultured in hormonally supplemented bronchial epithelium growth medium (Lonza), in collagen/fibronectin-coated flasks [17] and used for experiments at passage 2. Cells were seeded at 50×10^3 cells$\cdot mL^{-1}$ in plates or 75×10^3 cells$\cdot array^{-1}$ for Electric Cell-Substrate Impedance Sensing (ECIS) and grown to confluence. EGFR inhibitor AG1478 (Sigma, St Louis, MO, USA), ADAM inhibitor TAPI-2 (Calbiochem, San Diego, CA, USA), and cycloheximide (CHX; Sigma) were added 60–120 min prior to stimulation with HDM (Greer Laboratories, Lenoir, NC, USA), TGF-β (Sigma), PAR-2 activating peptide or epidermal growth factor (EGF; Sigma). Serine peptidase inhibitor AEBSF was added to the mite extract for 60 min at 37°C. Furthermore, primary epithelial cells derived from bronchial brushings in asthma patients were cultured and used for experimentation as described previously [16].

ECIS

Electrical properties of confluent or wounded epithelium were measured using electric ECIS as described previously [18]. Cell adhesion measurements were based on changes in resistance/capacitance to current flow applied at different frequencies (Applied Biophysics, Troy, NY, USA). Cells were inoculated at 75×10^3 cells$\cdot well^{-1}$ in 400 μL in duplicates and resistance/capacitance was measured at 400 and 40,000 Hz. Wounding was performed by electroporation using voltage pulses of 5 V and 40 kHz for 30 s.

Immunodetection by Western blotting

Total cell lysates were obtained by resuspension of the cells in Laemmli buffer. Immunodetection was performed as described previously [1] using anti-EGFR, anti-phospho-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-extracellular-regulated kinase (ERK; Cell Signalling Technology, Hitchin, UK), anti-occludin and anti-ZO-1 (Zymed Laboratories, San Francisco, CA, USA).

Immunofluorescent staining

Cells grown on LabTeks were washed with PBS/$CaCl_2$, fixed in ice-cold aceton (90%) for 30 min, blocked in PBS/5% bovine serum albumin for 60 min, incubated for 60 min with primary antibodies (1:200) against EGFR, E-cadherin (Santa Cruz) and ZO-1 (Zymed) and subsequently incubated for 60 min with FITC-labelled anti-rabbit (1:200; DAKO Diagnostics, Mississauga, ON, Canada) or Rhodamine-labelled anti-mouse immunoglobulin G conjugates (1:400; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and analysed as described previously [19].

Transmission electron microscopy

Cells were fixed for 1 h at room temperature and overnight at 4°C in 2% PFA, 2.5% glutaraldehyde and 0.1 $mol \cdot L^{-1}$ sodium cacodylate, pH 7.4 and prepared for TEM as described previously. TEM was performed at the Mount Sinai Hospital core facility (Toronto, ON, Canada).

Cell surface biotinylation

Cells were washed four times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and incubated with 1.5 $mg \cdot mL^{-1}$ sulfosuccinimidyl 2-(biotinamido) ethyl-dithioproprionate (sulfo-NHS-SS-biotin) twice for 20 min, followed by washing with 50 mM NH_4Cl in PBS/$MgCl_2$/$CaCl_2$ to quench free sulfo-NHS-SS-biotin, followed by four additional washes in PBS/$MgCl_2$/$CaCl_2$. Cells were then washed with ice-cold PBS containing 1 mM sodium orthovanadate (Na_3VO_4) and 1 mM PMSF and subsequently lysed in 200 μL Triton lysis buffer (30 $mmol \cdot L^{-1}$ HEPES (pH 7.4), 100 $mmol \cdot L^{-1}$ NaCl, 1 $mmol \cdot L^{-1}$ EGTA, 20 $mmol \cdot L^{-1}$ NaF, 1% Triton X-100, 1 $mmol \cdot L^{-1}$ PMSF, 20 $\mu l \cdot mL^{-1}$ protease inhibitory cocktail (BD Biosciences, San Diego, CA, USA), and 1 $mmol \cdot L^{-1}$ Na_3VO_4) for 20 min on ice. Cell lysates were centrifuges at $10{,}000 \times g$ for 15 min and incubated with streptavidin beads to collect bound biotinylated proteins. Following this, immune complexes were washed three times with lysis buffer. The precipitates were subjected to SDS 10% PAGE, immunoblotted on nitrocellulose membrane and E-cadherin and EGFR were detected using enhanced chemoluminescence, which was performed according to the manufacturer's guidelines. In all cases, membranes were stained with Ponceau S (Sigma) to ensure equal loading and transfer of the proteins.

Statistical analysis

We used the student Wilcoxon-signed rank test for paired observations for before–after analysis or the Mann-Whitney U-test to analyse for significance between different conditions in the epithelial resistance measurements.

RESULTS

HDM induces short-term changes in epithelial barrier formation

Junctional loss of E-cadherin with compromised intercellular contacts, as observed in asthma, may have important consequences for airway epithelium function. Accordingly, contact injury is a key component of EMT [20], a process that may contribute to airway epithelium remodeling in allergic asthma [11, 12]. Therefore, we aimed to investigate the effect of HDM on intercellular junctions. We observed that both ZO-1 and E-cadherin, markers of tight and adherens junctions, respectively, were localised to intercellular junctions in resting conditions, forming a continuous ring. Upon HDM exposure, E-cadherin adopted a jagged arrangement and ZO-1 staining became discontinuous with occasionally complete loss from the adjacent cell borders within 15 min (fig. 1a–f). After 60 min, the epithelial barrier was restored, as observed by the re-localisation of ZO-1 and E-cadherin to the membrane (fig. 1a–f).

Following this we tested whether these changes in barrier structure were paralleled by changes in barrier function. We measured epithelial resistance at 400 Hz (by ECIS), since low-frequency resistance has been demonstrated to be most sensitive for changes in barrier tightness [18]. In contrast, measurement of capacitance at 40 kHz is the most sensitive parameter to monitor changes in cell attachment to the matrix, being relatively insensitive to intercellular contact formation [18]. We observed that the addition of HDM induced a rapid and substantial (~20%) fall in low-frequency resistance (fig. 1g, h), but not high-frequency capacitance (fig. 1i), indicating selective disruption of cell-to-cell adhesion [18]. This effect was temporary: resistance returned to its original values within ~1 h, concomitant with the re-localisation of ZO-1 and E-cadherin to the membrane. It is noteworthy that within a few hours, a trend towards a second, less steep and longer-lasting decrease in resistance could be observed ($p=0.07$ for HDM-treated *versus* the control values).

HDM activates EGFR in a protease dependent manner leading to redistribution of E-cadherin and delocalisation of ZO-1

To unravel the mechanisms underlying HDM-induced contact injury, we considered that EGFR signalling may contribute to this type of damage, based on our previous findings showing that EGFR activity plays a crucial role in HDM-induced signalling in bronchial asthma epithelium [16]. Indeed, we observed that HDM activated the EGFR as well as downstream signalling, as detected by increased levels of phospho-EGFR (Tyr 1173) and phospho-ERK, with a maximum effect of 5–20 min upon HDM exposure (fig. 2a). This time course is in accordance with the transient decrease in barrier function. This may involve the activation of PAR-2, resulting in ADAM17-dependent shedding of heparin-bound-EGF [16, 21–23] or TGF-α [24]. The allergen Der p 1 is known to act as a cysteine peptidase, whereas Der p 3, Der p 6 and Der p 9 contain serine protease activity. Notably, serine proteases present in HDM extract have been shown to activate PAR-2 [22]. The extract we used was proteolytically active, with detectable serine, but not cysteine, peptidase activity (data not shown). It is of interest to note that upon heat-inactivation, as well as treatment with serine and cysteine protease inhibitors, we still detected substantial levels of proteolytic activity (data not shown). This indicates that additional, heat-insensitive and yet to be defined proteases may also contribute to the effect of HDM. Accordingly, the HDM-induced EGFR phosphorylation was reduced upon pre-treatment with serine protease inhibitor AEBSF, indicating that this is at least partly dependent on serine protease activity (fig. 2a). To assess whether a serine protease-dependent/PAR2-mediated mechanism could be involved, we used PAR2-activating peptide (PAR2-ap) and observed a similar increase in phospho-EGFR. This depended on ADAM activity, as it was blocked by ADAM inhibitor TAPI-2 (fig. 2b). Furthermore, PAR-2 downstream signalling was dependent on EGFR, as the PAR2-ap-induced phospho-ERK-1/2 response was suppressed by EGFR inhibitor AG1478 (fig. 2b). Additionally, both PAR2-ap and EGF were able to mimic the HDM-induced redistribution of E-cadherin. In line with this, both AEBSF and AG1478 blocked the HDM-induced E-cadherin redistribution (fig. 2c). TAPI-2 exerted a similar effect (data not shown). These results suggest involvement of a protease/PAR-2/ADAM/EGFR-dependent pathway in the HDM-induced destabilisation of E-cadherin. To find further support for ADAM-dependent shedding of EGFR ligands by HDM, we used neutralising α-EGFR to block ligand-dependent activation of the receptor. We observed that HDM was no longer able to increase EGFR phosphorylation in the presence of α-EGFR (fig. 2c). Additionally, AG1478 increased the membrane localisation of ZO-1 upon HDM exposure (fig. 2d). This was reflected by changes in epithelial barrier function; AG1478 significantly reduced (although could not completely block) the HDM-induced fall in epithelial resistance (fig. 2e, f).

EGFR is involved in epithelial reorganisation upon wounding by electroporation

Similar to HDM-induced injury, we observed that EGFR inhibition improved epithelial reconstitution upon injury by electroporation. Under these circumstances, with a relative small area of wounding, epithelial integrity is expected to recover within a few hours, *i.e.* by cell migration/spreading, and not proliferation. Once the monolayer is restored, resistance may further increase due to restoration of intercellular contacts. Indeed, cells repopulated the electrode within 2 h, as observed by the stabilisation of high-frequency capacitance (fig. 3b). Subsequently, resistance levels slowly continued to increase, indicating intercellular contact formation (fig. 3a). AG1478 markedly delayed the repopulation of the electrode within 2 h upon wounding, with a $19\pm7\%$ *versus* a $51\pm10\%$ increase. This delay was also observed for high-frequency capacitance (fig. 3b) and is probably due to the inhibition of cell spreading/migration. On the contrary, AG1478 increased the recovery of the epithelial barrier after 2 h, as reflected by an additional $42.2\pm9\%$ increase in resistance *versus* a $10\pm12\%$ increase in its absence ($n=4$). The addition of EGF prior to wounding had an opposite effect; it promoted the migration phase (fig. 3c, d), but impaired the restoration of the epithelial barrier (fig. 3c), indicating that EGFR activity facilitates the migration phase and inhibits contact reorganisation during epithelial regeneration.

HDM induces EGFR-independent cleavage of junctional proteins

The initial HDM-induced fall in resistance, which appeared partly independent of EGFR, might be caused by short-term changes in tight junction sealing. Besides redistribution of

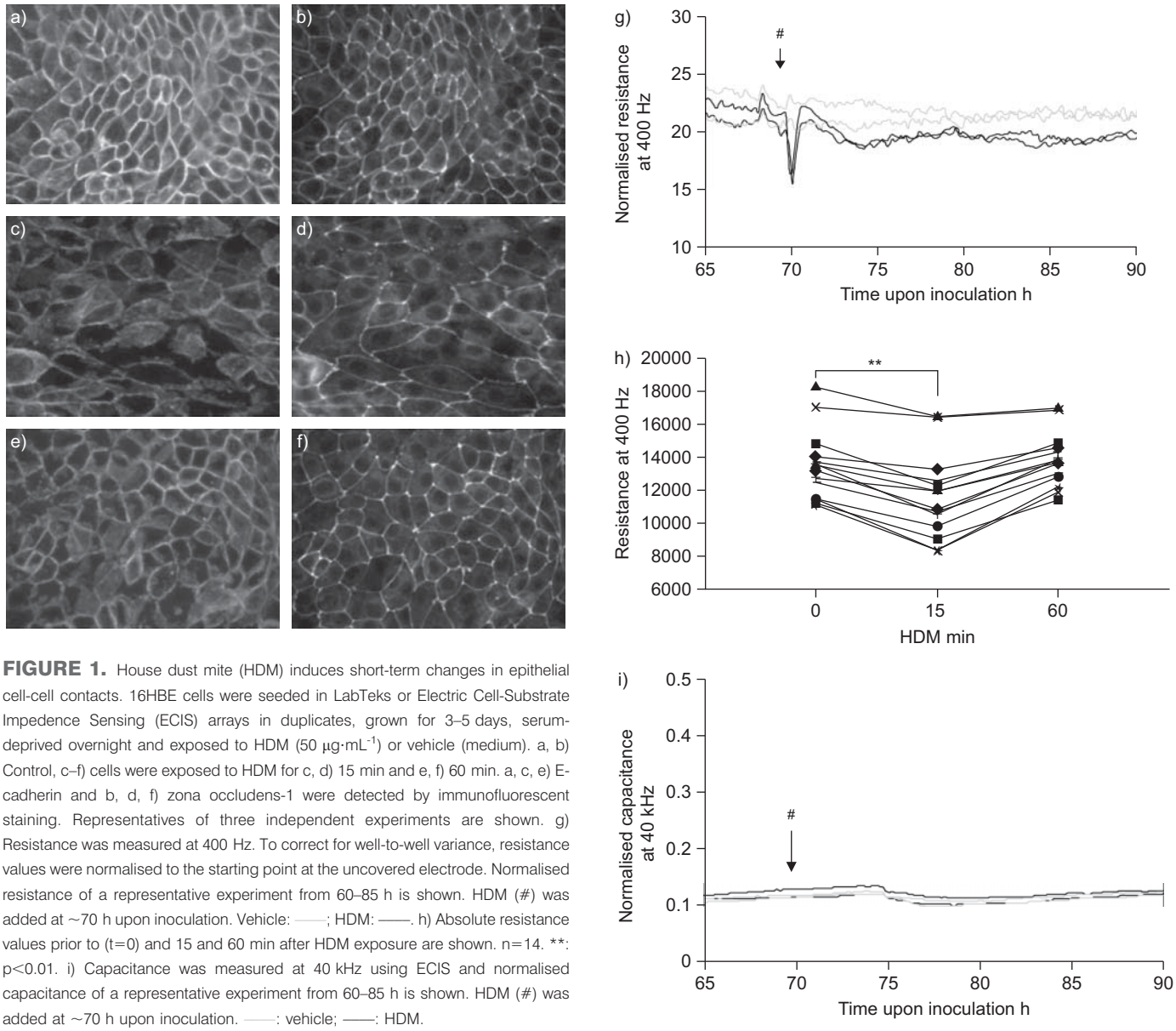

FIGURE 1. House dust mite (HDM) induces short-term changes in epithelial cell-cell contacts. 16HBE cells were seeded in LabTeks or Electric Cell-Substrate Impedence Sensing (ECIS) arrays in duplicates, grown for 3–5 days, serum-deprived overnight and exposed to HDM (50 $\mu g \cdot mL^{-1}$) or vehicle (medium). a, b) Control, c–f) cells were exposed to HDM for c, d) 15 min and e, f) 60 min. a, c, e) E-cadherin and b, d, f) zona occludens-1 were detected by immunofluorescent staining. Representatives of three independent experiments are shown. g) Resistance was measured at 400 Hz. To correct for well-to-well variance, resistance values were normalised to the starting point at the uncovered electrode. Normalised resistance of a representative experiment from 60–85 h is shown. HDM (#) was added at ~70 h upon inoculation. Vehicle: ——; HDM: ——. h) Absolute resistance values prior to (t=0) and 15 and 60 min after HDM exposure are shown. n=14. **: $p<0.01$. i) Capacitance was measured at 40 kHz using ECIS and normalised capacitance of a representative experiment from 60–85 h is shown. HDM (#) was added at ~70 h upon inoculation. ——: vehicle; ——: HDM.

ZO-1, degradation of tight junction proteins might be involved [25, 26]. We investigated whether HDM affects extra- and/or intracellular tight junction components, *e.g.* occludin and ZO-1, through such mechanisms. Indeed, HDM induced partial occludin cleavage, as demonstrated by the reduction in its 80 kD hyperphosphorylated form [27] the and appearance of smaller cleavage fragments (~45 and ~30 kD). We also observed partial cleavage of ZO-1 upon HDM exposure into smaller products (~192 and ~165 kD). As expected, the presence of AG1478 could not prevent ZO-1 or occludin degradation (fig. 4a). Furthermore, the cleavage of neither protein was prevented by AEBSF (data not shown), suggesting the involvement of other proteolytic/enzymatic activities or HDM-activated proteases. In addition to serine peptidase activity, the extract contained chitinase activity (~20 $U \cdot \mu L^{-1}$ β-N-acetylglucosaminidase), but it is currently unknown whether occludin can serve as substrate for chitinases. HDM extracts are often contaminated with endotoxin. However, endotoxin levels in our extract were relatively low (*i.e.* 0.5 $EU \cdot \mu L^{-1}$, as determined by the Limulus Amebocyte Lysate endotoxin test, rendering a final concentration of 2.5 $EU \cdot mL^{-1}$ in our cells). Lipopolysaccharide levels up to 50,000 $EU \cdot mL^{-1}$ did not affect epithelial integrity (fig. 4b). Therefore, involvement of endotoxins seems highly unlikely.

Signalling through the EGFR and subsequent changes in cell-cell contacts formation are promoted in a TGF-β-primed epithelium

Together, the above data suggest that signals which curtail EGFR signalling, *e.g.* ligand-induced EGFR internalisation/lysosomal degradation [28], may improve epithelial recovery from injury. Conversely, sustained EGFR signalling may attenuate restoration of the epithelial barrier. Since our previous findings have shown that TGF-β prolongs ligand-induced EGFR activation [11], we examined whether TGF-β is able to postpone termination of EGFR signalling and thereby

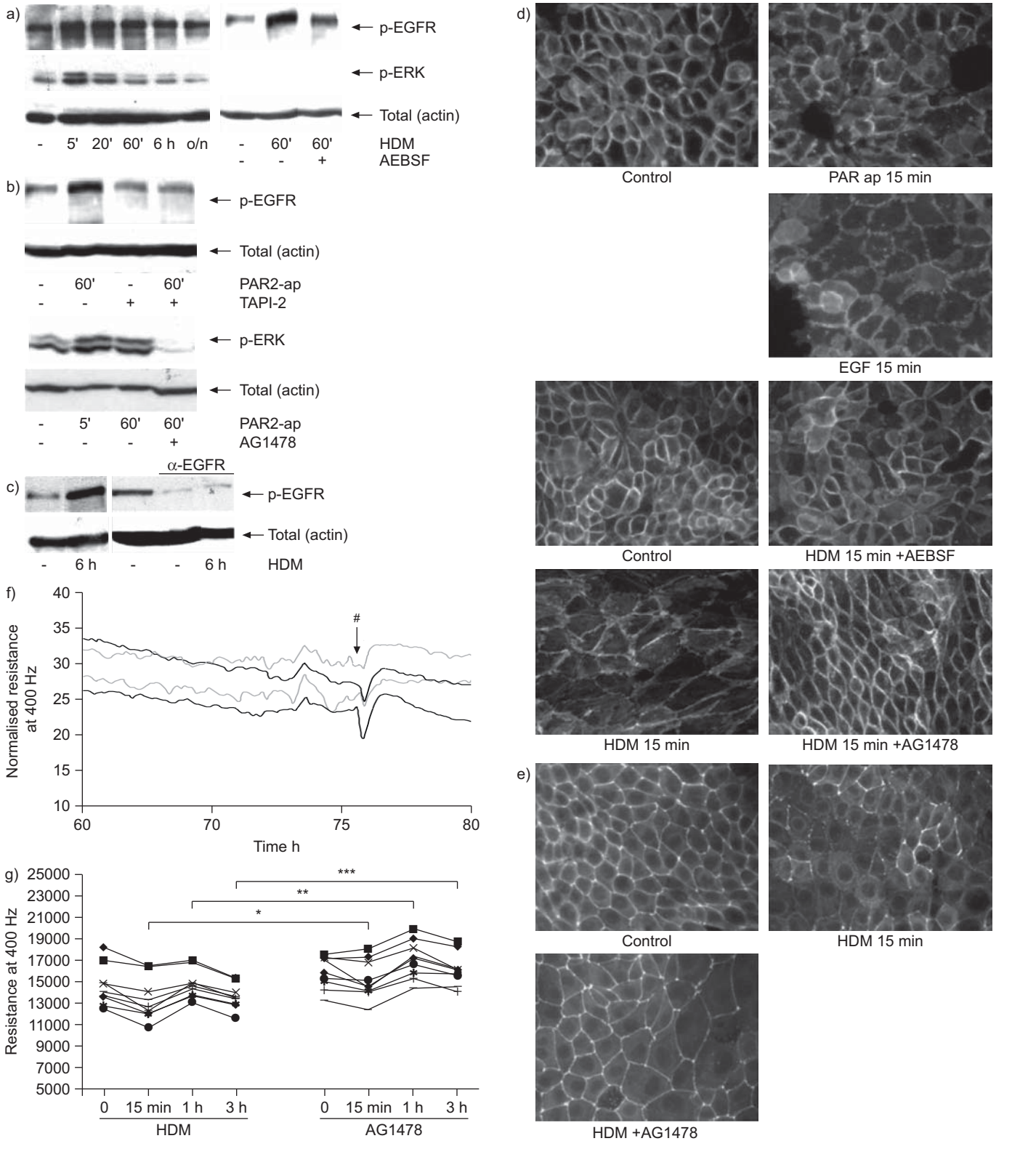

FIGURE 2. Protease-dependent activation of epidermal growth factor receptor (EGFR) by house dust mite (HDM) is involved in the disruption of intercellular contacts. 16HBE cells were grown for 3–5 days in 24-well plates, LabTeks or Electric Cell-Substrate Impedance Sensing arrays, serum deprived overnight, incubated with or without EGFR inhibitor AG1478 (1 μM), TAPI-2 (22.5 μM), AEBSF (1 nM) or α-EGFR (1 μg·mL^{-1}) for 60–120 min and exposed to HDM (50 μg·mL^{-1}), protease-activated receptor-2 activating peptide (PAR2-ap; 50 μM) or epidermal growth factor (EGF; 10 ng·mL^{-1}) as indicated. a–c) Total cell lysates were prepared and phospho-extracellular-regulated kinase (ERK) or phospho-EGFR was detected by Western blotting (arrow). β-actin was used as a control for equal loading. Representatives of three independent experiments are shown. d) E-cadherin and e) zona occludens-1 were detected by immunofluorescent staining. Representatives of three independent experiments are shown.

FIGURE 2. (cont.) f) Normalised resistance of a representative experiment from 60–70 h. HDM (#) was added at ~75 h upon inoculation. AG1478: ——; control: ——. g) Absolute resistance values prior to (t=0) and 15 min, 1 h and 3 h after HDM exposure are shown. n=9. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. Resistance was measured at 400 Hz. AG1478 (1 μM) was added 2 h prior to the addition of HDM/vehicle.

impact on epithelial barrier function. We first studied whether TGF-β alters the cellular fate of EGFR through effects on endocytosis and degradation. Prolonged treatment with EGF (6 h) resulted in downregulation of EGFR expression (fig. 5a). Importantly, TGF-β pre-treatment substantially mitigated this (fig. 5a), while TGF-β alone did not have such an effect, and even increased protein expression of EGFR (fig. 5b). When new protein synthesis was inhibited by CHX, exposure of the cells to EGF caused a dramatic decrease in EGFR expression, unmasking robust EGF-promoted receptor degradation. Again, pre-treatment with TGF-β almost completely abolished the EGF-induced EGFR degradation (fig. 5c). Furthermore, EGF caused complete internalisation of EGFR into an endosomal vesicular compartment. In TGF-β-treated cells, besides vesicular accumulation, distinct cytoplasmic and peripheral EGFR labelling was still visible. This was also demonstrated by higher levels of biotinylated EGFR on the cell surface *versus* treatment with EGF alone (fig. 5d, e). Together, these findings suggest that TGF-β may contribute to the maintenance of EGFR by upregulating EGFR expression, reducing EGFR internalisation and preventing degradation of internalised EGFR.

Consistent with the reduced phosphorylation upon prolonged HDM exposure, HDM also induced downregulation of EGFR expression (fig. 5f) and caused EGFR internalisation, which was prevented by TGF-β pre-treatment (fig. 5d, e). TGF-β increased EGFR phosphorylation (fig. 5g), and both basal and TGF-β-induced EGFR phosphorylation were further enhanced by HDM, an effect that persisted for prolonged periods in TGF-β-treated cells (fig. 5g).

Following this we tested the consequences of sustained EGFR signalling upon TGF-β treatment. TGF-β itself had no major impact on E-cadherin localisation (fig. 6a, b), however, it prolonged the HDM-induced delocalisation of E-cadherin as well as ZO-1, which could still be observed after 60 min (fig. 6a, b). AG1478 blocked the redistribution of ZO-1 (fig. 6a). Furthermore, electron microscopic analysis revealed a major reduction in intercellular tight junctions in cells exposed to the combination of TGF-β and HDM *versus* control cells (fig. 6c).

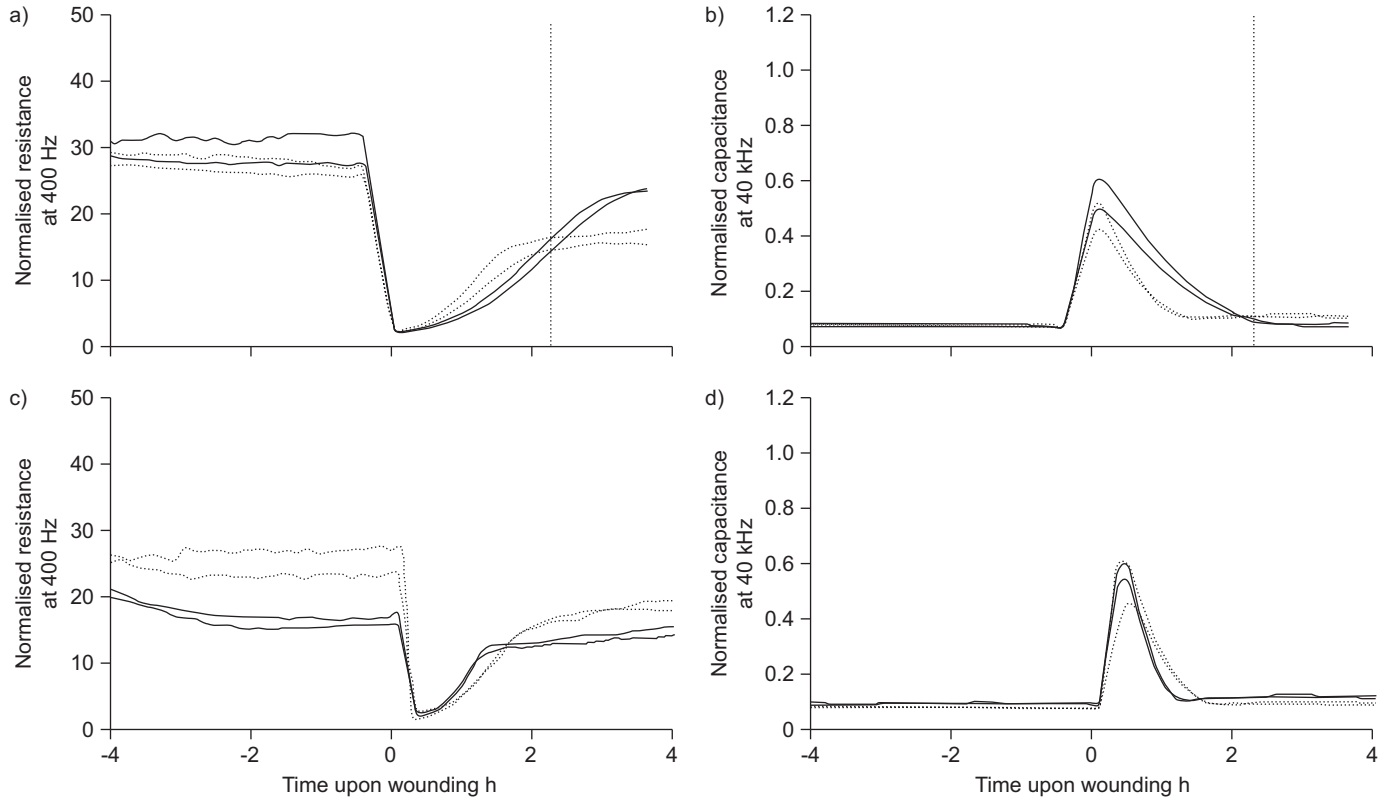

FIGURE 3. Inhibition of epidermal growth factor (EGF) receptor activity increases spreading/attachment and reduces formation of intercellular contacts upon wounding by electroporation. 16HBE cells were seeded in duplicates in Electric Cell-Substrate Impedance Sensing (ECIS) arrays, grown for 3 days, serum-deprived overnight, pre-treated with (——) or without (·····) a, b) AG1478 (1 μM) or c, d) EGF (10 ng·mL^{-1}) for 6–8 h and wounded by electroporation. a, c) Resistance was measured at 400 Hz and b, d) capacitance was measured at 40 kHz using ECIS. Normalised resistance and capacitance of representative experiments are shown 4 h prior to wounding to 4 h after wounding.

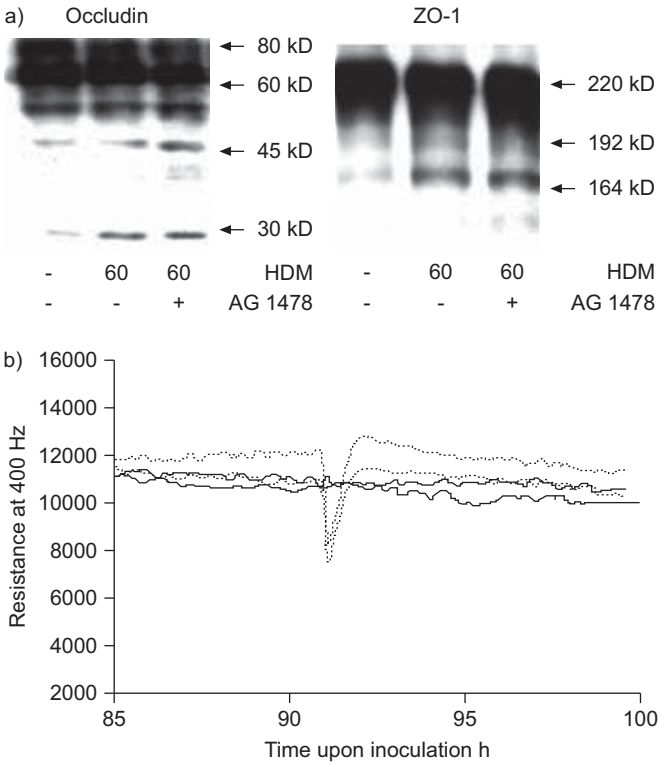

FIGURE 4. a) House dust mite (HDM) induces short-term cleavage of TJ proteins in an epidermal growth factor receptor-independent manner. 16HBE cells were for 3–5 days in 24-well plates, serum-deprived overnight and exposed to HDM (50 μg·mL^{-1}) for 60 min. AG1478 (1 μM) was added 2 h prior to stimulation with HDM. Full length occludin (60–80 kD), zona occludens-1 (ZO-1; 220 kD) and their cleavage products were detected by Western blotting and indicated by arrows. b) Exposure to lipopolysaccharide (LPS; 500 EU·10^{-1} μL) does not reduce epithelial barrier function. 16HBE cells were grown for 3–5 days in Electric Cell-Substrate Impedance arrays, serum deprived overnight and exposed to HDM (50 μg·mL^{-1}; ·····) or LPS (——). Resistance was measured at 400 Hz. Resistance of a representative experiment from 85–100 h is shown. HDM and LPS were added at ~90 h upon inoculation.

Accordingly, while TGF-β alone did not significantly affect epithelial resistance, the HDM-induced reduction in epithelial resistance was potentiated by TGF-β, which became significant after prolonged times of exposure (as shown for the 8-h time-point) (fig. 6d).

Finally, to test the relevance of our findings, we studied NHBE. EGF also induced E-cadherin and ZO-1 delocalisation in NHBE cells and the combination of HDM plus TGF-β resulted in prolonged junctional loss of E-cadherin, while HDM alone did not exert a clear effect at 6 h (fig. 6e). Moreover, we observed that exposure to HDM alone induced sustained delocalisation of E-cadherin in bronchial asthma epithelium (fig. 6e). In line with the effects in 16HBE (fig. 4a) and NHBE (not shown), HDM caused ZO-1 cleavage in asthma epithelium (fig. 6f).

DISCUSSION

Epithelial junctions have emerged as important targets in allergic sensitisation. Their breakdown may facilitate transport of allergens, promote pro-inflammatory responses by the epithelium [1] and allow access of growth factors to basolateral receptors [29]. This may serve normal re-epithelisation, but may also contribute to aberrant repair and ongoing tissue remodelling. Therefore, efficient restoration of tight junctions upon injury may be crucial in patients suffering from allergic asthma.

Previously, HDM has been described to increase epithelial permeability [30]. Herein, we show that this may be due to a temporary loss of epithelial junctions, which is probably mediated by the HDM-induced redistribution of junctional proteins, *e.g.* E-cadherin and ZO-1, as well as proteolytic degradation of tight junction proteins, followed by rapid re-localisation of these molecules to the membrane. Our results also provide insight into the underlying mechanism: HDM induces EGFR activation in a serine protease/PAR2-dependent manner, which is critical for the ensuing transient disruption of intercellular junctions. As previously described, this may involve EGFR-induced tyrosine phosphorylation and delocalisation of junctional proteins, as well as attenuated redistribution of these proteins to epithelial junctions [31–35]. Inhibition of EGFR reduced the disassembly of epithelial junctions and the subsequent migration/spreading, and improved the restoration of barrier function upon both HDM-induced injury and wounding. In addition, HDM affected junctional integrity through EGFR-independent mechanisms, causing the cleavage of ZO-1 and occludin. The inhibition of serine peptidase activity did not block cleavage of TJ molecules, suggesting the involvement of other enzymatic activities, either contained within HDM (*e.g.* chitinases) or by the induction of intercellular proteases. The latter likely contributes to ZO-1 degradation, since this is an intracellular process. It is of interest to note that chitins and microbial glucose structures present in HDM extract can activate C-type lectin receptors [36], causing Ca^{2+} fluxes [37], which may lead to the activation of calpains and subsequent cleavage of junctional proteins [38]. In line with this, HDM allergens can induce Ca^{2+} influx in airway epithelial cells [39]. Although further investigation is required, we observed that calcium ionophore induced redistribution and cleavage of ZO-1 (data not shown).

Given the effects of AG1478 on E-cadherin distribution we speculated that sustained EGFR activity may impair epithelial barrier function, since adherens junctions are thought to provide the architecture required for proper assembly of tight junctions. We have shown that EGFR activity is increased/prolonged upon pre-treatment of the epithelial cells with TGF-β, a fibrogenic cytokine with elevated levels in the asthmatic airways [13]. TGF-β appeared to do so by upregulation of EGFR expression, as well as partial prevention of ligand-induced degradation and endocytosis of EGFR. In conjunction with this, TGF-β increased HDM-provoked EGFR signalling, leading to ongoing HDM-induced contact injury. This may offer a possible explanation for the inadequate repair of epithelial barrier function as observed in asthma, where HDM exposure may occur in the presence of increased TGF-β levels [13]. The latter may be related to a TGF-β promoter polymorphism associated with asthma [40]. Notably, HDM exposure modifies the effect of *TGF*-β single nucleotide polymorphisms on AHR in children [41]. Furthermore, increased EGFR activity may be relevant for asthma given the aberrant EGFR activity/expression observed in asthmatic airways [13, 15]. The importance of aberrant EGFR activity is

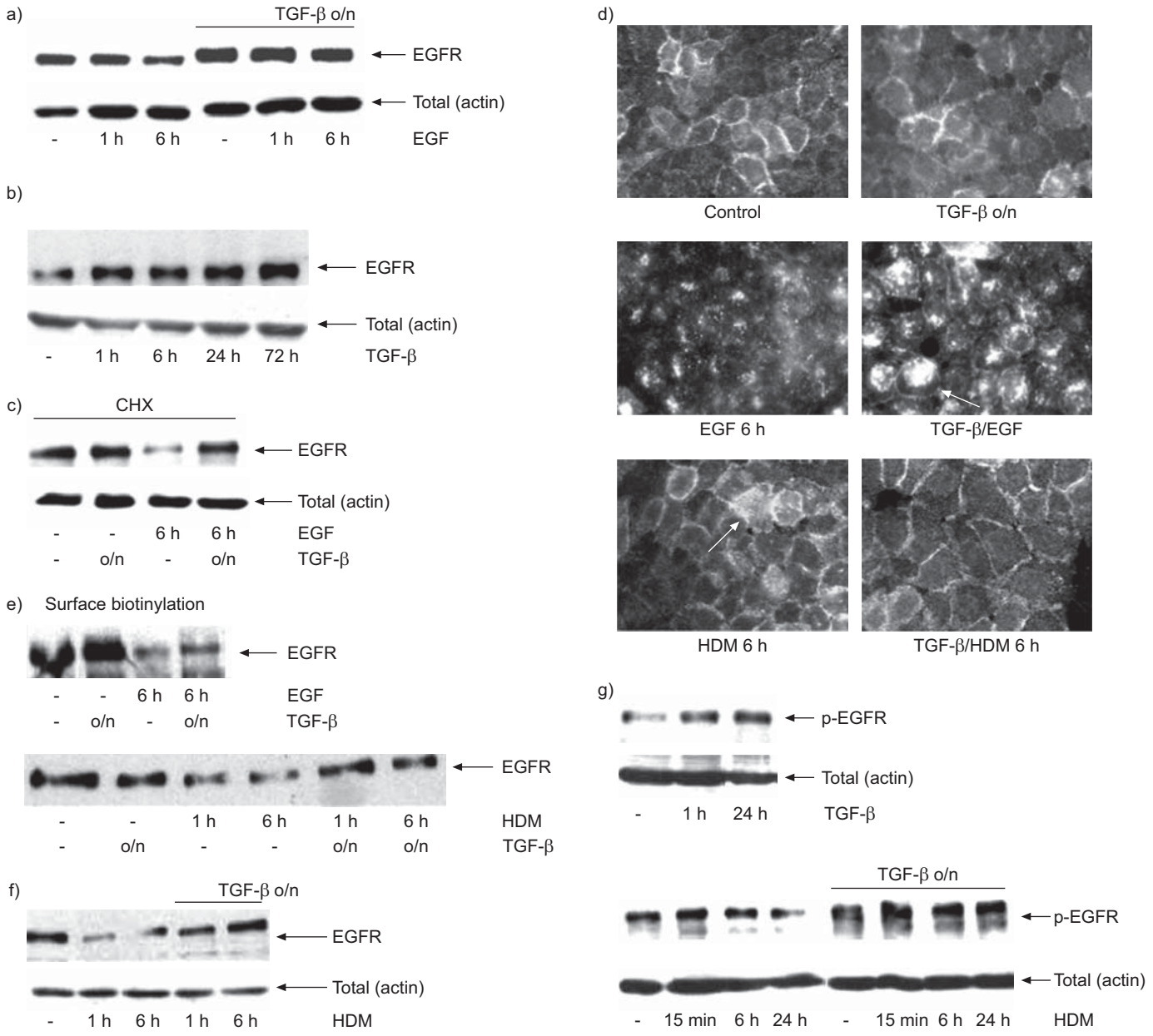

FIGURE 5. Transforming growth factor (TGF)-β prevents epidermal growth factor receptor (EGFR) degradation, reduces endocytosis of EGFR and prolongs house dust mite (HDM)-induced EGFR signalling. 16HBE cells were grown for 3 days in 24-well plates or LabTeks, serum deprived for 4 h, pre-treated overnight with or without TGF-β (2 ng·mL^{-1}) and subsequently stimulated with TGF-β (2 ng·mL^{-1}), epidermal growth factor (EGF; 10 ng·mL^{-1}) or HDM (50 μg·mL^{-1}) as indicated, in the presence and absence of cycloheximide (CHX; 10 μg·mL^{-1}). a–c) Total cell lysates were prepared and EGFR was detected by Western blotting (arrow). β-actin was used as a control for equal loading. Representatives of three independent experiments are shown. d) Cells were grown on LabTeks and EGFR was detected by immunofluorescent staining. EGFR localised at the cell membrane is indicated by the arrows. e) Cells were surface biotinylated and lysed. Biotinylated proteins were recovered using streptavidin beads and analysed by SDS-PAGE. EGFR was detected by Western blotting (arrow). f, g) Total cell lysates were prepared and EGFR and phospho-EGFR were detected by Western blotting (arrow). β-actin was used as a control for equal loading. Representatives of three independent experiments are shown.

further emphasised by a CA-repeat polymorphism in the EGFR gene, which is associated with both the presence and the severity of asthma [42]. Importantly, our study shows that HDM induces prolonged E-cadherin delocalisation in asthma epithelium, indicating that our data may indeed be of relevance to asthma. This is further supported by our preliminary histological findings in asthmatic patients (data unpublished), where we observed delocalisation of E-cadherin in the bronchial epithelium. In future studies we aim to perform a more extended comparison between healthy and asthma subjects. The sustained junctional loss of E-cadherin upon increased/prolonged EGFR activation may have important consequences, as it may contribute to epithelial remodelling, *e.g.* by EMT [11]. In addition, the TGF-β and HDM-induced loss of epithelial junctions may promote the expression of growth factors as well as pro-allergic factors [1, 16] by the airway epithelium.

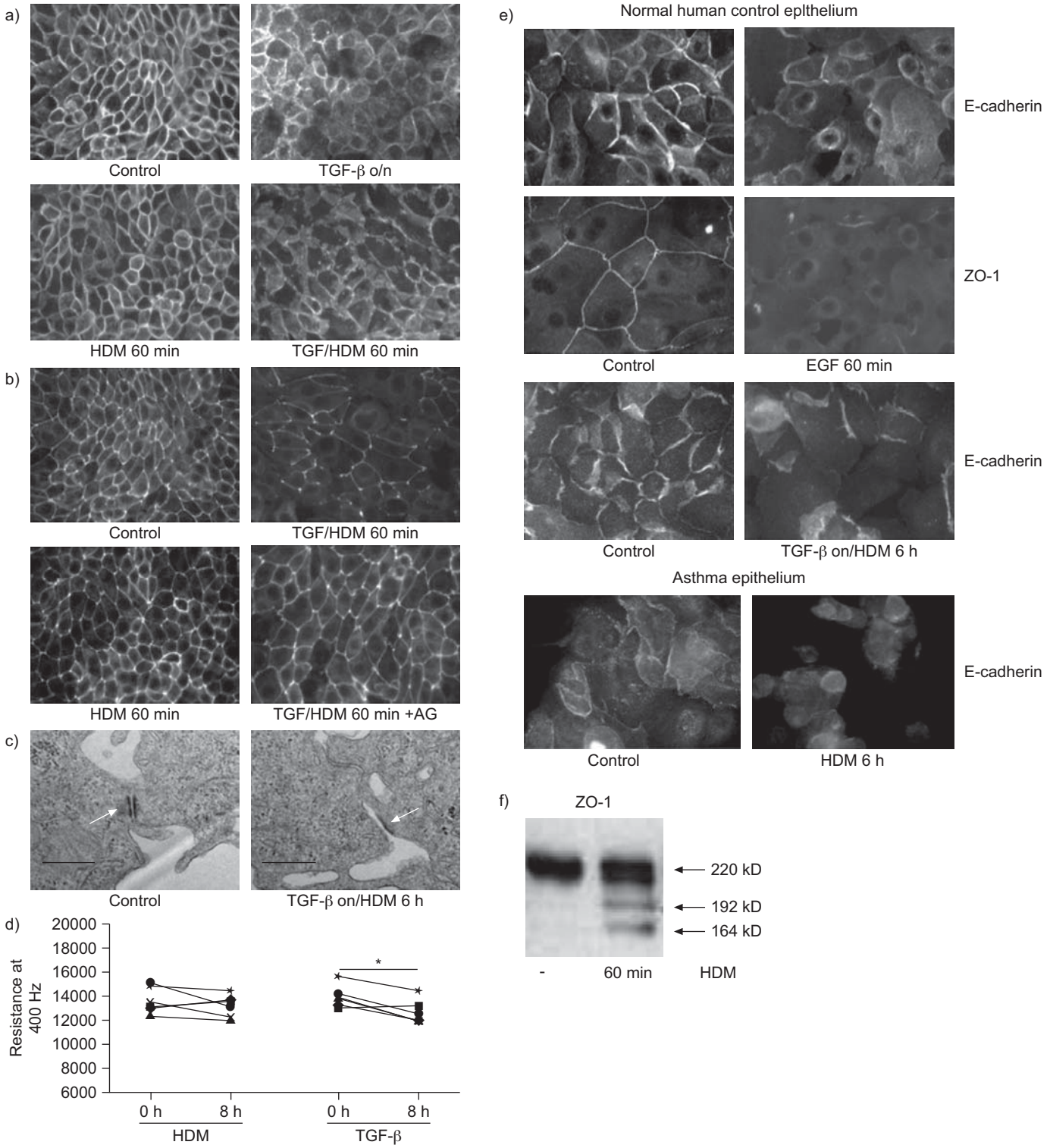

FIGURE 6. Transforming growth factor (TGF)-β pre-treatment prolongs house dust mite (HDM)-induced disruption of epithelial junctions. a, b) 16HBE cells were cultured in LabTeks for 3 days, serum deprived for 4 h, pre-treated overnight with or without TGF-β (2 ng·mL^{-1}) and subsequently stimulated with HDM (50 μg·mL^{-1}) as indicated, in the presence and absence of AG1478 (1 μM). a) E-cadherin and b) zona occludens (ZO)-1 were detected by immunofluorescent staining. Representatives of three independent experiments are shown. c) 16HBE cells were grown on cover slips for 3 days, serum deprived for 4 h, pre-treated overnight with or without TGF-β (2 ng·mL^{-1}) and subsequently stimulated with HDM (50 μg·mL^{-1}) for 6 h. Electron microscopy analysis of tight junctions was performed as indicated (white arrows). Representative electron microscopy images are shown. d) Cells were grown on Electric Cell-Substrate Impedance Sensing (ECIS) arrays in duplicates for 3 days, serum deprived for 4 h, pre-treated overnight with or without TGF-β (2 ng·mL^{-1}) and subsequently stimulated with HDM (50 μg·mL^{-1})/vehicle. Resistance was measured at 0 and 8 h upon HDM treatment at 400 Hz using ECIS. n=6. *: $p<0.05$. Median values are indicated. Normal primary bronchial epithelial cells obtained from Lonza (Walkersville, MD, USA)

FIGURE 6. (cont.) e) and epithelial cells derived from bronchial brushings in asthma [16] were grown to confluence in LabTeks for 3–5 days, growth factor/hormone-deprived for 4 h, pre-treated overnight with or without TGF-β (2 ng·mL^{-1}) and subsequently stimulated with HDM (50 μg·mL^{-1}) or epidermal growth factor as indicated. E-cadherin and ZO-1 were detected by immunofluorescent staining. Representatives of three independent experiments are shown. f) Total cell lysates were prepared from bronchial asthma epithelial cells [16] and ZO-1 was detected by Western blotting (arrow). Representatives of three independent experiments are shown.

Furthermore, disruption of the barrier may facilitate airway inflammation through activation of the epithelium by cytokines released from inflammatory cells in the airway lumen with receptors restricted to the basolateral surface of the epithelium.

In conclusion, we have shown that HDM induces temporary reduction of barrier function, which is sustained when EGFR signalling is facilitated, *e.g.* by the presence of TGF-β. Thus, despite facilitated migration/spreading, increased EGFR signalling may reduce epithelial integrity, impair epithelial repair and enhance vulnerability of the epithelium to HDM.

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STATEMENT OF INTEREST

None declared.

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