TNF-α–Converting Enzyme/A Disintegrin and Metalloprotease–17 Mediates Mechanotransduction in Murine Tracheal Epithelial Cells

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Bronchoconstriction applies compressive stress to airway epithelial cells. We show that the application of compressive stress to cultured murine tracheal epithelial cells elicits the increased phosphorylation of extracellular signal–regulated kinase (ERK) and Akt through an epidermal growth factor receptor (EGFR)–dependent process, consistent with previous observations of the bronchoconstriction-induced activation of EGFR in both human and murine airways. Mechanotransduction requires metalloprotease activity, indicating a pivotal role for proteolytic EGF-family ligand shedding. However, cells derived from mice with targeted deletions of the EGFR ligands *Tgfα* and *Hb-egf* showed only modest decreases in responses, even when combined with neutralizing antibodies to the EGFR ligands epiregulin and amphiregulin, suggesting redundant or compensatory roles for individual EGF family members in mechanotransduction. In contrast, cells harvested from mice with a conditional deletion of the gene encoding the TNF-α–converting enzyme (TACE/ADAM17), a sheddase for multiple EGF-family proligands, displayed a near-complete attenuation of ERK and Akt phosphorylation responses and compressive stress–induced gene regulation. Our data provide strong evidence that TACE plays a critical central role in the transduction of compressive stress.

Keywords: asthma; airway remodelling; TACE

Cells in the lung are normally subjected to a variety of mechanical forces as a result of the dynamic nature of lung function (1). These forces are small, however, compared with the transepithelial pressure gradients generated during bronchoconstriction in patients with asthma. We previously showed that the application of transepithelial pressure gradients, similar in magnitude to those found during asthmatic bronchoconstriction, generates diverse cellular responses in primary bronchial epithelial cells derived from rats or humans and grown in air–liquid interface (ALI) culture conditions. Many of these responses mimic the molecular events characteristic of asthmatic airway remodeling (2–6).

Based on our previous observations, we deduced that stimulation of the epidermal growth factor receptor (EGFR) is a key component of events linking the mechanical compression of cells to subsequent biochemical responses. Importantly, we also observed an enhanced activation of EGFR directly resulting from bronchoconstriction in the murine airway, linking cell culture and the *in situ* response to mechanical loading (7). Here, we adopted our ALI culture model for two distinct purposes, using mice. First, uncontrolled genetic heterogeneity occurs among human cell donors, which contributes to variability in responses when studying human cells. Second, mice with targeted deletions of key components of the pathways under consideration are available for probing the mechanisms of response, which constitutes an alternative approach to using pharmacologically based or antibody-based interventions. For example, we previously showed that in normal human bronchial epithelial (NHBE) cells, the application of compressive stress elicits a robust increase in EGFR activation that is both ligand-dependent and metalloprotease-dependent (7, 8). However, because of the limitations inherent in pharmacologically based or antibody-based interventions, considerable ambiguity remains regarding the specific ligands and metalloproteases involved in mechanotransduction (7, 9).

EGFR ligands are produced as membrane-spanning propeptides, and are cleaved by cell-surface proteases (termed "sheddases") to release mature growth factors that bind EGFR (10). Among known sheddases, TNF-α–converting enzyme (TACE/ADAM17) is a major sheddase for TGF-α, amphiregulin, epiregulin, and heparin-binding epidermal growth factor–like growth factor (HB-EGF) (11–16). In the experiments described here, we first showed that primary cultures of murine tracheal epithelial cells (MTECs) transduce mechanical compression in a manner similar to that in rat and human airway epithelial cells. We then studied primary ALI cultures of murine tracheal epithelial cells harvested from genetically manipulated mice, to elucidate the molecular mechanisms controlling responses to mechanical stress. Our results demonstrate redundancy in EGF-family ligand contributions to mechanical stress–driven signal transduction, and also demonstrate a critical role for TACE/ADAM17 in the transduction of compressive stress by airway epithelial cells.

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MATERIALS AND METHODS

Animals

Wild-type C57BL/6 mice and B6129/SV F1 mice were purchased from Taconic (Hudson, NY). *R26Cre^{+} ER* (B6;129-*Gt(ROSA)26Sor$^{tm1(Cre/Esr1)Nat}$*/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Heparin-binding EGF (*Hb-egf*) knockout mice and TGF-α (*Tgfα*) knockout mice were prepared as described elsewhere (15, 17). *Tace$^{flox/flox}$* mice were previously described (18). *Tace$^{flox/flox}$* mice were crossed with *R26Cre^{+} ER* mice to generate *Tace$^{flox/flox}$ R26Cre^{+} ER* mice. For some experiments,

TABLE 1. REAL-TIME PCR PRIMERS

Gene Name	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
GAPDH	CATGGCCTTCCGTGTTCCTA	TGCTTCACCACCTTCTTGATG
AREG	TTAGGCTCAGGCCATTATGCA	TCCCCAGAAAGCGATTCG
EREG	GGGTCTTGACGCTGCTTTG	GATCACGGTTGTGCTGATAACTG
HB-EGF	GCCTCAGGAAATACAAGGACTACTG	ACACCTGTGTCCGTGGTAACC
BTC	CAACCAGAACACCAGAAACCAA	GGTGGTACCTGTGCAGTTTTCC
TGF-α	GGTTTTTGGTGCAGGAAGAGAA	TCACAGCGAACACCCACGTA
EGF	GACTGGATTGGCCGGAGAA	CGCTCCCTCCAACAACAGA

Definition of abbreviations: AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EREG, epiregulin; GAPDH, glyceraldehyde phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor–like growth factor.

B6129/SV F1 mice were crossed with *R26Cre*$^{+}$ *ER* mice to generate *Tace*$^{wild/wild}$ *R26Cre*$^{+}$ *ER* mice. Mice of an appropriate genotype were crossed with *Tace*$^{flox/flox}$ mice to produce littermates that were *Tace*$^{flox/flox}$ *R26Cre*$^{+}$ *ER* mice or *Tace*$^{flox/flox}$ *R26Cre*$^{-}$ *ER* mice. All mice were age-matched and sex-matched, and when appropriate, littermate-matched. At 6–10 weeks of age, mice were killed by carbon dioxide narcosis, their tracheas were removed, and cells were harvested. All procedures and protocols using mice were approved by the Harvard Medical Area Standing Committee on Animals.

Cell Culture

MTECs were isolated from mouse trachea and cultured on Transwell membranes, establishing an ALI according to the method of You and colleagues (19), with minor modifications. We seeded cells on 12-mm-diameter, 0.4-μm-pore polycarbonate semipermeable membrane Transwell inserts (Corning, Cambridge, MA) in a 12-well plate format at 7–8 $\times$ 10^4 cells per well. Cells sufficient for six inserts were obtained from 3–4 mice. Cells were cultured using MTECs plus medium (see details in the online supplement) and 0.01 μM retinoic acid (Sigma-Aldrich, St. Louis, MO) for 10–12 days, until they appeared confluent according to phase microscopy. When confluence was achieved after 10–14 days of culture, the medium was removed from the upper chamber of the Transwell to establish an ALI. The lower part of the Transwell was provided with fresh MTEC/NS (NuSerm) medium (see details in the online supplement) and 0.01 μM retinoic acid (Sigma-Aldrich) every 2 days. On Day 14 after establishing the ALI, we performed the experiments (see details in the online supplement). PCR was performed with primers as indicated in Table 1.

Experimental Apparatus

MTECs grown on Transwells were exposed to transcellular compression, as previously described (2). At the onset of an experiment, compressed cells were exposed to a transcellular gradient. Control cells were similarly manipulated, but were exposed only to atmospheric pressure. Approximately 20 hours before each experiment, the culture medium was changed to MTEC basic medium.

Statistical Analysis

Results are expressed as means ± SE. Two groups were compared using the Student *t* test. Between-group comparisons of means were examined using one-way ANOVA, followed by Tukey *post hoc* analysis or two-way ANOVA. $P < 0.05$ was regarded as indicative of a significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

MTEC Responses to Compressive Stress

MTECs exposed to compressive stress (*see* Figures E1A and E1B) displayed an early and robust increase in ERK1/2 phosphorylation. This was an increase of 15.1 ± 3.3-fold above baseline, 20 minutes after applying 30 cm H_2O compressive stress (Figure 1A, $P < 0.0001$, ANOVA). The phosphorylation of ERK1/2 displayed a prolonged peak response with the maximal effect evident between 20 minutes and 4 hours. By 8 hours, the effect had begun to diminish, but was still present. These results are similar to those in our previous report on NHBE cells (5), except that in MTEC ERK1/2, the phosphorylation was sustained for a longer period than in NHBE cells. To examine the magnitude of dependence in this response, cells were exposed to transcellular pressure gradients of 0, 7.5, 15, 30, or 45 cm H_2O for 20 minutes, and the phosphorylation of ERK1/2 was used as the outcome measure (Figure 1B). In these experiments, cells were derived from two different mouse strains (i.e., C57BL6 and B6129F1). Because the genetically manipulated mice used below were from a mixed C57BL6 and SV129 background, we tested the B6129F1 mice to determine whether strain-related differences in the response to compressive stress would confound our analysis (Figure 1B). Cells from the two strains were quite similar in terms of their response to mechanical stress at transcellular pressures up to 15 cm H_2O. At 30 and 45 cm H_2O, the magnitude of the response was significant, compared with no pressure in both strains, and was lower by approximately 20% in cells derived from B6129F1 mice, but no statistically significant differences were evident between strains. We found that the magnitude-dependence of mechanical stress–induced ERK1/2 phosphorylation mirrored that observed in NHBE cells (5).

We extended our findings on the response of MTECs in ALI culture beyond that established in rat and human cells by examining the phosphorylation of Akt in MTECs exposed to compressive stress (30 cm H_2O). The phosphorylation of Akt increased, with a time course similar to that of ERK1/2. The maximal effect was a 3.5 ± 0.3-fold increase in the phosphorylation of Akt at 30 minutes (Figure 1C; $P < 0.0001$, ANOVA). We also examined the magnitude-dependence of the Akt phosphorylation response after exposure to transcellular gradients of 0, 7.5, 15, 30, or 45 cm H_2O for 30 minutes in cells from C57BL6 mice. As was the case for ERK1/2, the increase in the phosphorylation of Akt was dependent on the magnitude of transcellular pressure gradient (Figure 1D; $P < 0.0001$, ANOVA).

Because we previously found that compressive stress increased transcript levels for a panel of EGFR ligands in NHBE cells (8), we tested whether similar changes occurred in MTECs. Compressive stress (30 cm H_2O) applied for 8 hours led to an increase in expression of mRNA for multiple EGFR ligands (Figure 1E). At 1 hour after the onset of compression, message levels for HB-EGF were the most up-regulated (i.e., 3.2 ± 0.4-fold above baseline; $P < 0.01$, ANOVA). At 4 hours, amphiregulin was the most up-regulated (i.e., 7.5 ± 0.7-fold above baseline), followed by statistically significant increases in epiregulin, HB-EGF, and betacellulin. TGF-α and EGF transcript levels were not significantly up-regulated by exposure to compressive stress at any time point tested (Figure 1E).

Compressive Stress Drives Signal Transduction through EGFR Signaling

To examine whether compressive stress induced the phosphorylation of ERK1/2 and Akt through EGFR signaling, we used AG1478, an inhibitor of the EGFR tyrosine kinase. Cells were

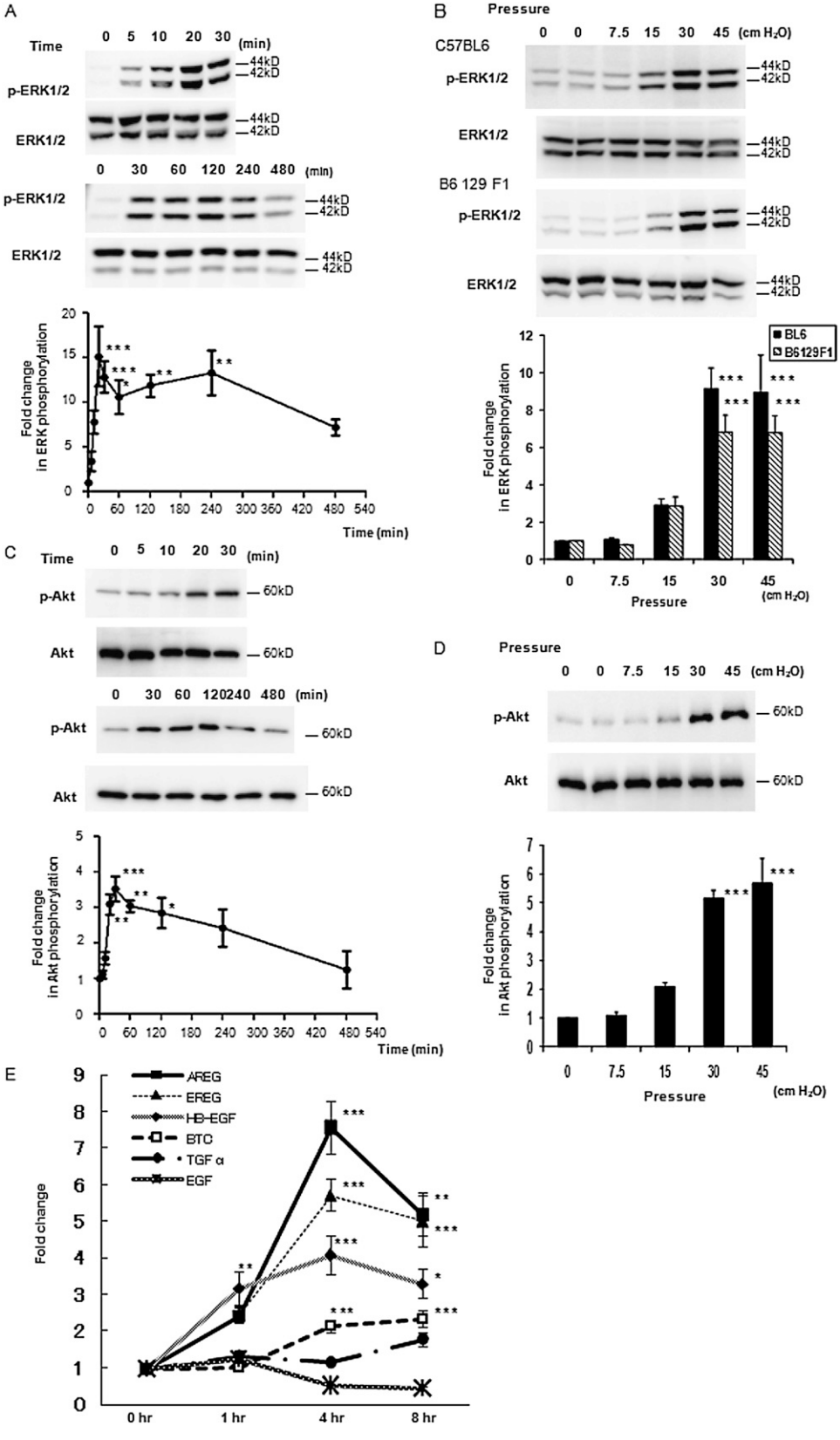

Figure 1. Murine tracheal epithelial cells (MTECs) respond to compressive stress. (*A*) Application of 30 cm H_2O transcellular pressure significantly increased extracellular signal–regulated kinase (ERK) 1/2 phosphorylation, with a peak 15.1 ± 3.3-fold increase observed 20 minutes after the onset of transcellular pressure ($n = 4$; $P < 0.0001$, ANOVA). The increase in ERK1/2 phosphorylation by compressive stress was sustained for 4 hours. (*B*) The increase in the phosphorylation of ERK1/2 at 20 minutes was dependent on the magnitude of the transcellular pressure gradient. Statistically significant increases occurred at 30 cm H_2O and 45 cm H_2O, relative to no pressure ($n = 4$ for each strains; $P < 0.0001$, ANOVA). Representative blots are shown from four independent experiments. (*C*) The application of 30 cm H_2O transcellular pressure significantly increased the phosphorylation of Akt, with a peak 3.5 ± 0.3-fold increase at 30 minutes after the onset of transcellular pressure ($n = 4$; $P < 0.0001$, ANOVA). The increase in the phosphorylation of Akt by compressive stress was sustained for 2 hours. (*D*) The phosphorylation of Akt at 30 minutes increased in proportion to the applied pressure ($n = 4$; $P < 0.0001$, ANOVA). Representative blots are shown from four independent experiments. (*E*) Compressive stress led to an increase in multiple epidermal growth factor receptor (EGFR) ligand gene expression. Among the six ligands examined, amphiregulin (AREG), epiregulin (EREG), heparin-binding epidermal growth factor–like growth factor (HB-EGF), and betacellulin (BTC) were significantly altered ($n = 4$; $P < 0.001$ for four EGFR ligands, ANOVA). TGF-α and epidermal growth factor (EGF) were not significantly up-regulated. Values are expressed as mean fold changes with standard errors. Significant differences, as determined by Tukey *post hoc* analysis, are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Results are presented as means ± SE. p-Akt, phosphorylated Akt; p-ERK, phosphorylated ERK.

exposed to a compressive stress of 30 cm H_2O for 20 minutes to 4 hours in the presence or absence of AG1478 (500 nM). We found that the presence of AG1478 abolished both the early and sustained compressive stress–induced phosphorylation of ERK1/2 and Akt (Figures 2A and 2B). Thus we confirmed in MTECs that compressive stress drives signal transduction through EGFR signaling.

Taken together, the results in Figures 1 and 2 demonstrate that murine tracheal epithelial cells respond to mechanical stress with the activation of the same pathway that we identified

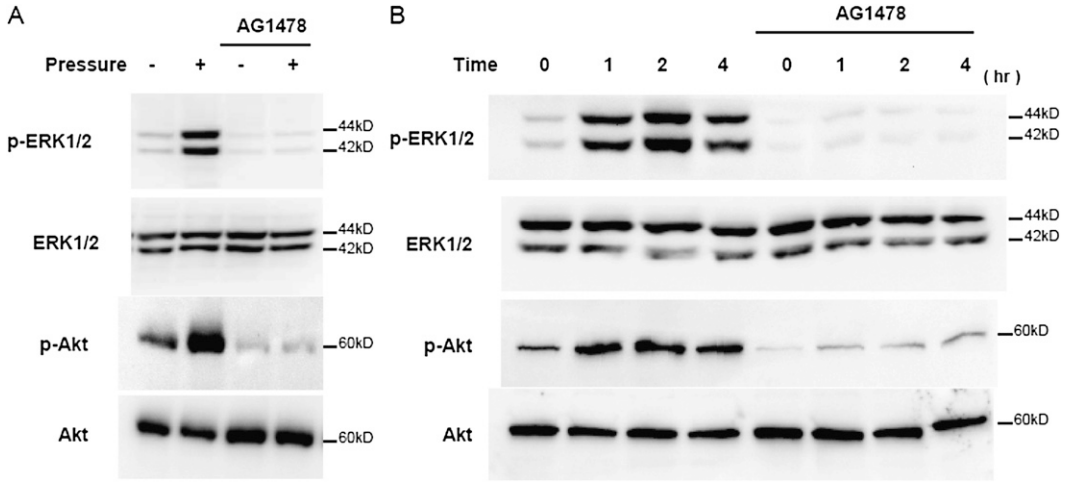

Figure 2. Compressive stress drives signal transduction through EGFR signaling. (*A*) Cells were exposed to compressive stress (30 cm H_2O) for 20 minutes in the absence or presence of AG1478 (500 nM). AG1478 strongly attenuated the compressive stress–induced phosphorylation of ERK1/2 and Akt. (*B*) Cells were exposed to 30 cm H_2O for 4 hours in the absence or presence of AG1478. AG1478 strongly inhibited the compressive stress–induced phosphorylation of ERK1/2 and Akt.

in rat and human cells, and in constricted murine airways (2, 3, 5–7). This similarity provided the basis for our further study of this model, to better understand how mechanical stress is linked to the biological activation of airway epithelial cells in ALI culture, using genetic manipulations available only in mice.

EGFR Ligands Play Redundant Roles in Mechanotransduction

To identify which EGFR ligands were responsible for the compressive stress–induced phosphorylation of ERK1/2, we tested tracheal epithelial cells derived from mice with targeted deletions of various EGFR ligands. Our first experiments were performed using cells from mice with a targeted deletion of *Hb-egf*. We had anticipated that this would be a key experiment, because we previously found in NHBE cells that HB-EGF was the EGFR ligand regulating the response to mechanotransduction (7). Cells derived from *Hb-egf*–deficient mice, along with littermate control mice, were exposed to a transcellular pressure gradient of 0, 7.5, 15, 30, or 45 cm H_2O for 20 minutes, and the phosphorylation of ERK1/2 was examined. Surprisingly, the effect of compressive stress on the phosphorylation of ERK1/2 in these *Hb-egf*$^{-/-}$ cells was not distinguishable from that observed in cells derived from wild-type littermates (Figure 3A). These data indicate a substantial functional difference in the response to compressive stress between NHBE cells and MTECs. Therefore, we explored the role of other EGFR ligands.

Based on evidence that TGF-α signaling is involved in the phosphorylation of EGFR in human airway epithelial cells (20), we performed experiments using cells from mice with a targeted deletion of *Tgf*α. Cells derived from mice with a deletion of *Tgf*α were exposed to compressive stress (30 cm H_2O) for 20 minutes, alongside cells derived from wild-type littermate controls. We found that the cells derived from mice with a targeted deletion of *Tgf*α showed a diminished ERK1/2 phosphorylation response relative to the response in cells derived from wild-type littermate controls. The ratio of the ERK1/2 phosphorylation response in cells with the *Tgf*α deletion to the phosphorylation response of cells derived from mice without the deletion was 0.64 ± 0.07 ($P < 0.001$) (Figure 3B).

To investigate the involvement of additional EGFR ligands in compressive stress–induced ERK1/2 phosphorylation, we applied compressive stress (30 cm H_2O) for 20 minutes to cells derived from mice with the deletion of *Tgf*α in the presence of mouse-specific neutralizing antibodies against epiregulin (2.5 μg/ml) and amphiregulin (5 μg/ml), and examined the phosphorylation of ERK1/2. We used those antibodies at a concentration that was shown to be more than adequate to prevent the activation of EGFR in other experiments (21). No further difference was evident in the magnitude of the compressive stress–induced ERK1/2 phosphorylation response in the presence or absence of these neutralizing antibodies (Figure 3C).

These observations may be explained by proposing that in mice with the deletion of *Tgf*α, other EGFR ligands are recruited to compensate for the loss of TGFα signaling. To test this idea, cells from mice with or without the deletion of *Tgf*α were exposed to compressive stress (30 cm H_2O) for 4 hours, and the levels of transcripts for amphiregulin, epiregulin, HB-EGF, betacellulin, and EGF were measured. Cells from mice with the deletion of *Tgf*α exhibited no differences compared with wild-type mice in their transcripts for HB-EGF, amphiregulin, epiregulin, betacellulin, and EGF before the application of compressive stress. These data indicate no baseline compensation in ligand gene expression (Figure 3D). We found that the basal level of threshold cycle values for EGF was much higher than for other EGFR ligands, indicating that the expression of EGF was much lower than that of other EGFR ligands. Surprisingly, cells derived from mice deficient in *Tgf*α exhibited a significantly greater induction of amphiregulin in response to compressive stress than did wild-type mice, that is, a 1.8 ± 0.1-fold increase compared with the response observed in mice without the deletion of *Tgf*α ($P < 0.05$) (Figure 3E). This amplified response was mirrored in the epiregulin response, although the magnitude of the differential effect did not reach statistical significance. The responses for transcript levels of HB-EGF, betacellulin, and EGF were essentially the same in wild-type and *Tgf*α knockout mice exposed to compressive stress.

Proteolytic Ligand Shedding Is Essential for Mechanotransduction

Because all EGFR ligands are produced as membrane-spanning proforms that are cleaved by cell-surface sheddases (10), we examined whether proteolytic ligand shedding was required for mechanotransduction in our model. Cells in ALI culture from C57BL/6 mice were exposed to a compressive stress of 30 cm H_2O for 20 minutes in the absence or presence of one of two sheddase inhibitors. In one set of experiments (Figure 4A) (1), we used TAPI-2 (5 μM or 50 μM), a hydroxamate-based inhibitor of matrix metalloproteases including TNF-α–converting enzyme (TACE, also referred to as a disintegrin and metalloprotease 17, ADAM17) (22, 23). In the other set, we used GM6001 (10 μM), a broad-spectrum inhibitor of matrix metalloproteases. The phosphorylation of ERK1/2 and Akt was measured as the outcome indicator in both experiments. The concentrations of

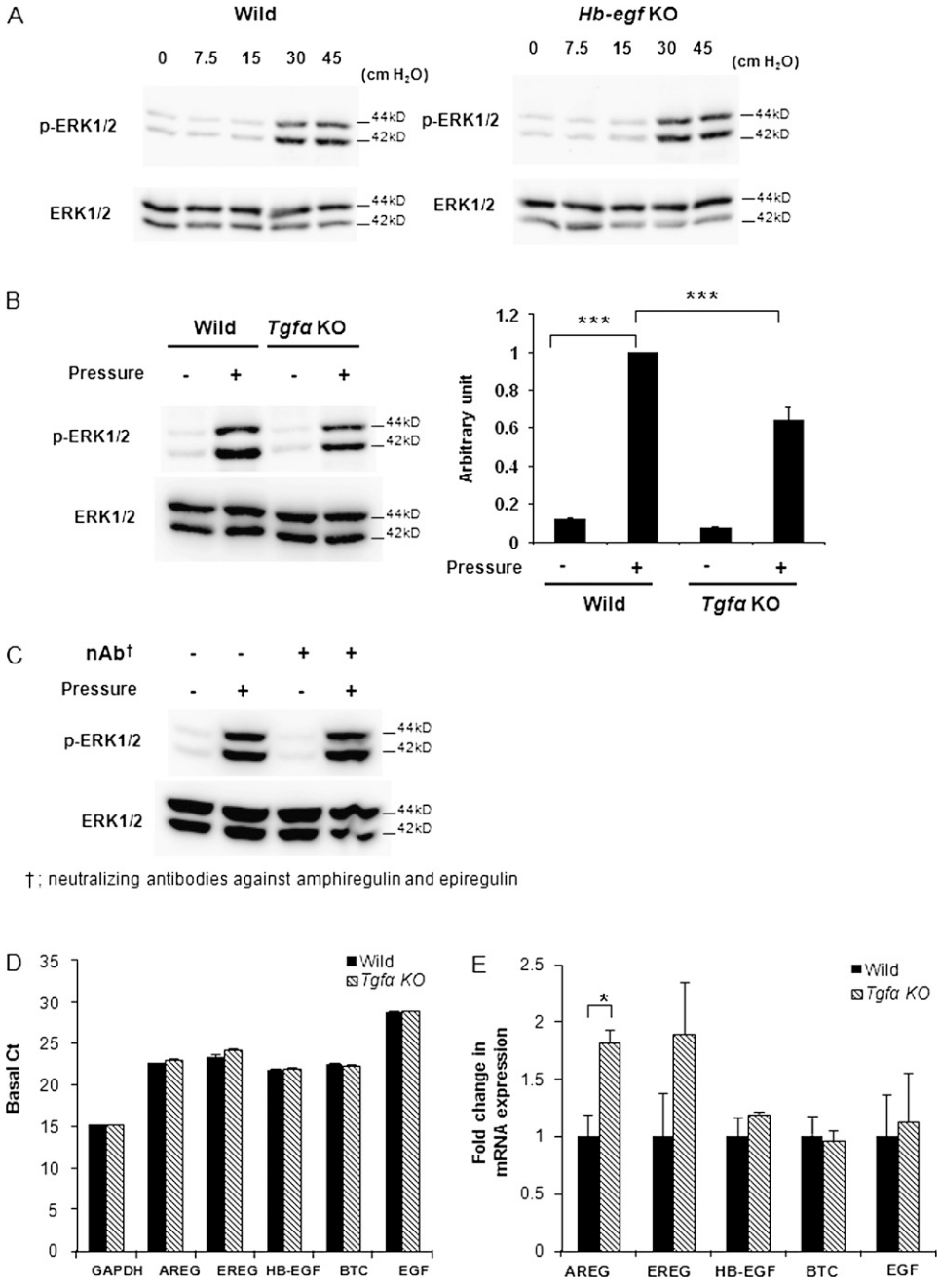

Figure 3. Cells derived from EGFR ligand knockout mice remain responsive to compressive stress. (*A*) Cells derived from mice with or without the deletion of *Hb-egf* were exposed to a transcellular gradient of 0, 7.5, 15, 30, and 45 cm H_2O for 20 minutes. A representative blot from two independent experiments indicates no difference between these groups in their response to compressive stress in ERK1/2 phosphorylation. (*B*) Cells derived from mice with or without the deletion of *Tgfα* were exposed to compressive stress (30 cm H_2O) for 20 minutes. Cells derived from mice with the deletion of *Tgfα* showed a significant decrease in the phosphorylation of ERK1/2, compared with those without the deletion of *Tgfα* ($n = 3$; $P < 0.0001$, ANOVA). Representative blots are shown from three independent experiments. Significant differences according to Tukey *post hoc* analysis are indicated by an asterisk (***$P < 0.001$). Results are presented as means ± SE. (*C*) Cells derived from mice with the deletion of *Tgfα* were exposed to compressive stress (30 cm H_2O) for 20 minutes in the presence of both mouse-specific neutralizing antibodies (nAb) against EREG and AREG ($n = 2$). Neutralizing antibodies against EREG and AREG did not inhibit the compressive stress–induced phosphorylation of ERK1/2. (*D*) Basal-level threshold cycle (Ct) values for the expression of EGFR ligands in wild-type (Wild) and *Tgfα* knockout (KO) mice. No differences were evident in basal-level Ct values for the expression of EGFR ligands in wild-type and *Tgfα* knockout mice. (*E*) Cells derived from mice with or without the deletion of *Tgfα* were exposed to compressive stress (30 cm H_2O) for 4 hours. EREG, AREG, HB-EGF, BTC, and EGF gene expression was examined at 4 hours. Cells from mice with the deletion of *Tgfα* showed an increased expression of AREG in response to compressive stress, compared with those from mice without the deletion of *Tgfα* ($n = 3$; *$P < 0.05$).

TAPI-2 were chosen on the basis of published reports (24, 25), but we also tested the effect of TAPI-2 at 50 μM on the viability of MTECs. In these experiments, incubation with 0.1% Triton-X100 increased the concentration of LDH in the medium over 100-fold, whereas incubation with 50 μM TAPI-2 increased the concentration of lactic dehydrogenase (LDH) in the medium less than 0.1-fold. These data indicate a lack of toxicity for TAPI-2 at 50 μM. Moreover, we found that the treatment of MTECs with TAPI-2 attenuated the compressive stress–induced phosphorylation of ERK1/2 and Akt in a dose-dependent manner. GM6001 also inhibited the compressive stress–induced phosphorylation of ERK1/2 and Akt (Figure 4A).

Next, the transcript levels for EGFR ligand genes were examined in the presence and absence of TAPI-2 (50 μM; see protocol outlined in Figure E1, 2b). The treatment of cells with TAPI-2 significantly ablated the compressive stress–induced expression of epiregulin, amphiregulin, and HB-EGF ($P = 0.0001$ for all three EGFR ligands, ANOVA) (Figure 4B). Thus, in contrast to the weak effects of removing individual ligands on mechanotransduction (Figure 3), these data suggest an absolute requirement for the membrane cleavage of ligand precursors from the cell membrane by a sheddase to initiate mechanical signal transduction.

TACE Mediates Mechanotransduction

We hypothesized that among sheddases, TACE was the major, if not only, enzyme responsible for the cleavage of EGFR ligand precursors from the cell membrane in our system. Because a targeted deletion of *Tace* leads to perinatal lethality (16), we bred $Tace^{flox/flox}$ mice with mice that widely expressed Cre recombinase under control of a tamoxifen-specific promoter (*R26CreER*) (B6;129-$Gt(ROSA)26Sor^{tm1(cre/Esr1)Nat}$/J; Jackson Laboratory). We isolated MTECs from the floxed mice, and

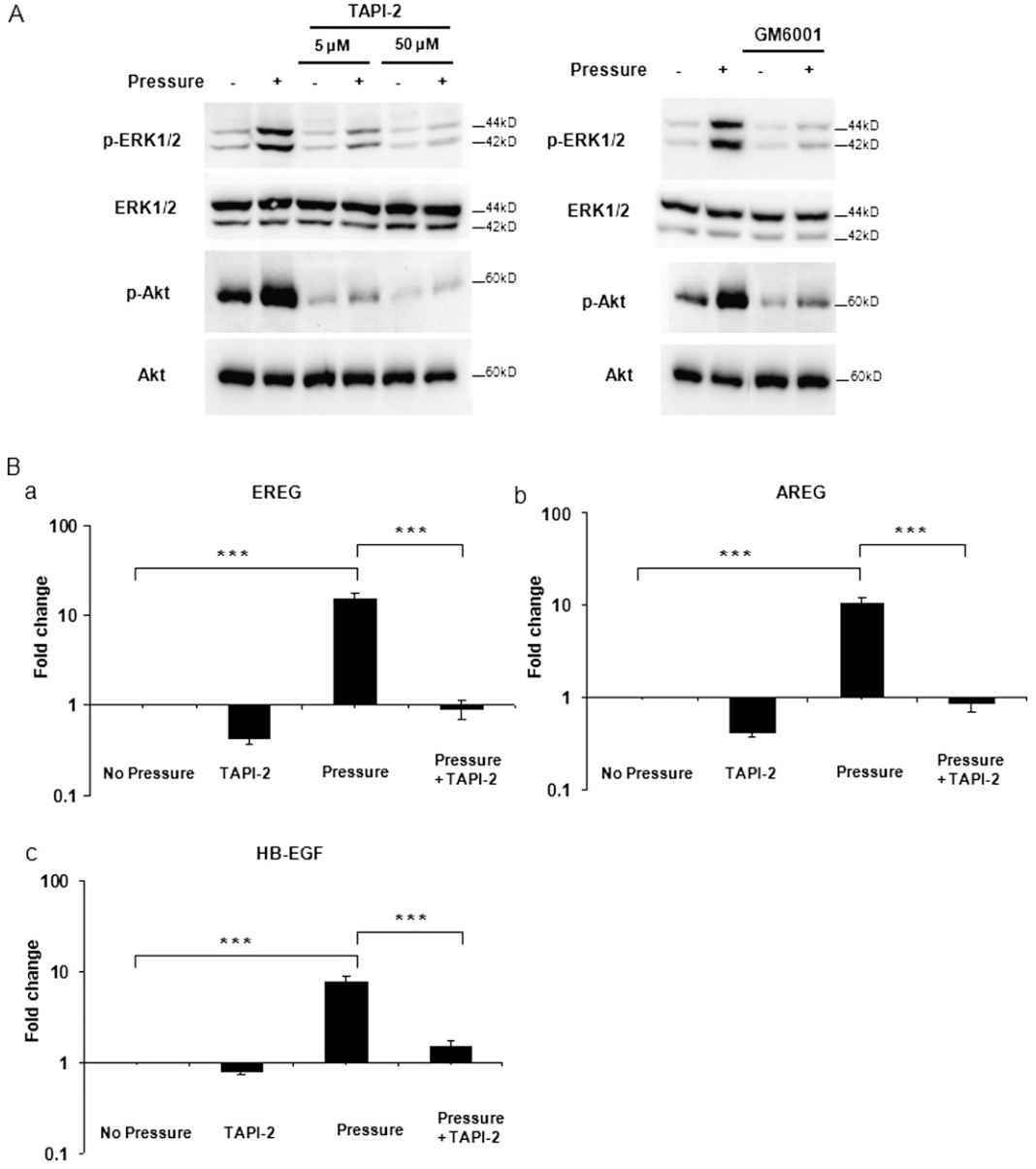

Figure 4. Compressive stress drives signal transduction through a metalloprotease-dependent process. (*A*) Cells were exposed to compressive stress (30 cm H_2O) for 20 minutes in the absence or presence of TAPI-2 (5 μM or 50 μM), a hydroxamate-based inhibitor of matrix metalloprotease and tumor necrosis factor α–converting enzyme (TACE), or GM6001 (10 μM), a broad-spectrum matrix metalloprotease inhibitor. TAPI-2 attenuated the compressive stress–induced phosphorylation of ERK1/2 and Akt in a dose-dependent manner. GM6001 also inhibited the compressive stress–induced phosphorylation of ERK1/2 and Akt. (*B*) Cells were exposed to compressive stress (30 cm H_2O) for 4 hours. EREG, AREG, and HB-EGF gene expression was examined at baseline, in the presence of TAPI-2 (50 μM), after 4 hours of compression, and after 4 hours of compression with TAPI-2. Values are expressed as mean fold change ± SE. ANOVA indicated significant differences between groups ($n = 3$; $P = 0.0001$ for all three EGFR ligands). Asterisks indicate significant differences according to Tukey *post hoc* analysis. ***$P < 0.001$.

cultured them at an ALI with and without 4-OH tamoxifen in the culture medium for various periods of time (Figure 5A). We confirmed the deletion of TACE protein in tamoxifen-treated MTECs from *Tace$^{flox/flox}$ Cre^{+} ER* mice by Western blot analysis (Figure 5B). When cells were cultured in the presence of tamoxifen and exposed to compressive stress, the phosphorylation of ERK1/2 increased 13.7 ± 2.9-fold in cells from *Tace$^{flox/flox}$ R26Cre^{-} ER* mice, compared with 2.7 ± 0.9-fold in cells from *Tace$^{flox/flox}$ R26Cre^{+} ER* mice ($P < 0.01$) (Figure 5C). The phosphorylation of Akt increased 3.9 ± 0.5-fold in cells from *Tace$^{flox/flox}$ R26Cre^{-} ER* mice, compared with 1.5 ± 0.4-fold in cells from *Tace$^{flox/flox}$ R26Cre^{+} ER* mice ($P < 0.01$) (Figure 5D). These results indicate that in the presence of tamoxifen, cells derived from *Tace$^{flox/flox}$ R26Cre^{+} ER* mice showed a substantial (>80%) decrease in the response of ERK1/2 and Akt to compressive stress relative to Cre^{-} cells, consistent with a dominant role for TACE in the compressive stress–induced activation of EGFR.

The incomplete attenuation of the mechanotransduction response may have resulted from the presence of residual TACE protein, or from the contributions of another metalloprotease to EGF-family shedding. To investigate the first possibility, we measured mRNA for TACE in MTECs treated with tamoxifen from *Tace$^{flox/flox}$ R26Cre^{+} ER* mice. TACE was present at less than 0.2% of the concentration found in *Tace$^{flox/flox}$ R26Cre^{-} ER* mice (data not shown). To test whether residual TACE protein might account for the remaining ERK phosphorylation response, we prolonged the period of tamoxifen exposure from 14 days to 19 days. This amount of time was based on the reasoning that longer exposure to tamoxifen would lead to a more complete deletion of TACE protein. However, even with the longer period of incubation, no change was evident in the phosphorylation of residual ERK induced by compressive stress in cells from *Tace$^{flox/flox}$ R26Cre^{+} ER* mice (data not shown), suggesting that contributions from other proteolytic shedding mechanisms are likely to account for the residual mechanotransduction.

Because the mechanoactivation of EGFR in human cells appears to initiate an autocrine amplification loop (8), we examined the effects of compressive stress on transcripts for EGFR ligands in cells with or without the tamoxifen-sensitive Cre promoter. Hence tamoxifen was included in all cell cultures. A

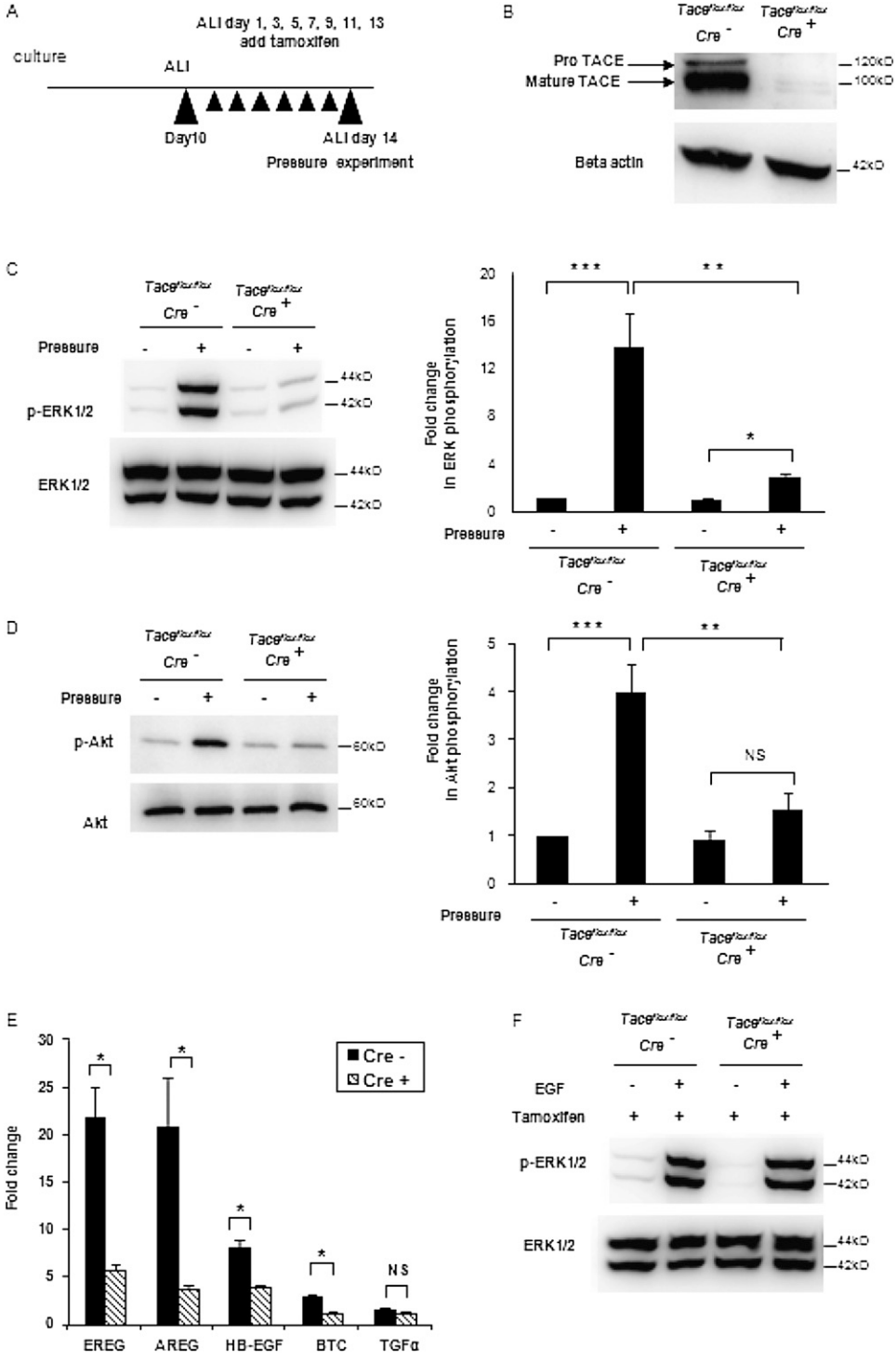

Figure 5. The ablation of TACE in MTECs decreases responses to compressive stress. (*A*) 4OH-tamoxifen (1 μM) was applied to culture media to induce the expression of Cre recombinase at indicated times. (*B*) Western blots of cell lysates of MTECs from *Tace*$^{flox/flox}$/*R26Cre*$^{+}$ *ER* and *Tace*$^{flox/flox}$/*R26Cre*$^{-}$ *ER* mice, when cultured in the presence of tamoxifen (1 μM). TACE protein is not evident in cells cultured from Cre^{+} mice. Representative blots are shown from four independent experiments. (*C*, *D*) Cells derived from *Tace*$^{flox/flox}$ *R26Cre*$^{+}$ *ER* mice and littermates *Tace*$^{flox/flox}$ *R26Cre*$^{-}$ *ER* mice, cultured in the presence of tamoxifen (1 μM), were exposed to compressive stress (30 cm H_2O) for 20 minutes. Cells derived from *Tace*$^{flox/flox}$/*R26Cre*$^{+}$ *ER* mice showed a significant decrease in the phosphorylation of ERK1/2 and Akt induced by compressive stress ($n = 4$; $P < 0.0001$ and $P < 0.0001$, respectively, ANOVA). Representative blots are shown from four independent experiments. (*E*) The ablation of TACE in MTECs decreases the mRNA expression of EGFR ligands induced by compressive stress. Cells derived from *Tace*$^{flox/flox}$ *R26Cre*$^{+}$ *ER* mice and *Tace*$^{flox/flox}$ *R26Cre*$^{-}$ *ER* mice were exposed to compressive stress (30 cm H_2O) for 4 hours. The expression of EGFR ligand genes was examined at 4 hours. Values are expressed as mean fold change ± SE. A Student *t* test showed significant differences between groups ($n = 4$; $^{*}P < 0.05$; NS, not significant). Significant differences according to Tukey *post hoc* analysis are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; NS, not significant). Results are presented as means ± SE. (*F*) Cells derived from *Tace*$^{flox/flox}$ *R26Cre*$^{+}$ *ER* mice and *Tace*$^{flox/flox}$ *R26Cre*$^{-}$ *ER* mice, cultured in the presence of tamoxifen, transduced signals stimulated by EGF in the same fashion. Representative blots are shown from three independent experiments.

significant decrease was evident in the compressive stress–induced up-regulation of multiple EGFR ligands in cells from *Tace*$^{flox/flox}$ *R26Cre*$^{+}$ *ER* mice compared with those from *Tace*$^{flox/flox}$ *R26Cre*$^{-}$ *ER* mice (Figure 5E). The differential response to compressive stress between cells derived from these strains was lost when cells were cultured in the absence of tamoxifen (data not shown), confirming the specific and pivotal role of TACE in mechanoregulated gene expression. Because Cre recombinase can be toxic (26, 27), we performed experiments to determine if EGFR stimulation using recombinant EGF (thus bypassing sheddases in the signaling cascade) had an impact on EGFR-mediated signaling. In these experiments, the exogenous EGF induced a similar degree of ERK1/2 phosphorylation in the presence of the tamoxifen induction of Cre recombinase (Figure 5F).

DISCUSSION

Our study demonstrates that MTECs in ALI cultures respond to compressive stress in a manner similar to, but not identical with, human airway epithelial cells cultured under similar conditions. During bronchoconstriction, as may occur in asthma, the constriction of smooth muscle generates a compressive force on the order of 30 cm H_2O on airway epithelial cells (28). We found that a transepithelial cell layer pressure gradient of 30 cm H_2O

induced a peak response in the phosphorylation of ERK1/2 through EGFR signaling in MTECs. This finding is similar to our previous findings in NHBE cells (5). We also found that mechanical stress up-regulated the gene expression of EGF-family ligands in MTECs, with a peak at 4 hours.

Some differences were evident in the response of MTECs and NHBE cells. In MTECs, amphiregulin was most highly up-regulated by compressive stress, whereas in NHBE cells, HB-EGF was most highly up-regulated. However, both MTECs and NHBE cells exhibited the activation of the same EGFR-dependent pathways in response to compressive stress. This similarity (Figures 1 and 2) provided a basis for using this model to elucidate how mechanical stress is linked to the biological activation of airway epithelial cells, using cells from genetically manipulated mice.

We first examined mice with a deletion of *Hb-egf*, based on previous experiments in human cells where a neutralizing antibody to HB-EGF appeared effective at attenuating mechanotransduction (7). Although we expected that cells derived from these animals would not respond to compressive stress, we found that the signal transduction response was not distinguishable from that observed in cells derived from littermate control mice without the deletion. We considered that species differences might exist between humans and mice. In fact, *Hb-egf* was not up-regulated in response to mechanical stress in MTECs. We also speculate that mice born without *Hb-egf* may develop compensatory pathways for signaling that replace *Hb-egf*.

Based on evidence that TGF-α plays a critical role in autocrine EGFR phosphorylation in airway epithelial cell lines (20) and that human bronchial epithelial cells shed TGF-α (9), we explored mechanotransduction responses in cells harvested from mice with a targeted deletion of *Tgfα*. We found that the ERK1/2 phosphorylation response to compressive stress was partially attenuated in these cells ($\sim$ 36% reduction). This attenuation was not further modified by the addition of neutralizing antibodies against the EGF-family ligands epiregulin and amphiregulin.

Our study was limited insofar as blocking all EGF-family ligands is impossible, because of the lack of availability of neutralizing antibodies for all murine EGF family members. Taken together, our data allow us to conclude that TGF-α plays a contributing role in the phosphorylation of ERK1/2 induced by compressive stress in MTECs, but the genetic deletion of TGF-α alone only partly attenuates the mechanotransduction response.

However, we know that other (likely multiple) EGFR ligands could also contribute to mechanotransduction responses, based on the abundant expression of amphiregulin, epiregulin, and betacellulin. EGF is unlikely to play as important a role, because the EGF mRNA in MTECs was minimally detectable compared with that for TGFα, amphiregulin, epiregulin, HB-EGF, and betacellulin, and the expression of EGF mRNA was not up-regulated by mechanical stress. We attempted to measure the direct release of EGFR ligands in our system, but were unsuccessful. This failure likely occurred because after EGFR ligands are released, the soluble ligands are captured by EGFR, making detection in the cell supernatant difficult (29, 30). In our previous study of NHBE cells, to detect EGFR ligands (EGF and TGF-α) in the cell-culture medium, we needed to add a neutralizing EGFR antibody (9). Because no mouse-specific neutralizing EGFR antibody was available, we could not detect any EGFR ligands in our cell supernatants.

Although we were unable to pinpoint a specific EGFR ligand responsible for linking mechanical compression to biological signal transduction, all EGFR ligands are released from cell membranes by sheddases (13, 31), that is, enzymes that cleave membrane-bound precursors of a ligand, leading to signal transduction in the microenvironment of its release. We reasoned that an individual sheddase could impose a rate-limiting step in the mechanotransduction cascade. Our initial data were consistent with a pivotal role for shedding in mechanotransduction, in that the inhibition of metalloproeases with the small molecule inhibitors GM6001 and TAPI-2 abrogated the ERK1/2 and Akt phosphorylation responses to mechanical stress (Figure 4). These findings were in agreement with results from NHBE cells (7). However, because these inhibitors are not specific for any single metalloprotease or class of metalloproteases, our results did not allow us to determine which specific sheddase links mechanical stimulation to downstream biological events.

Among known sheddases, tumor necrosis factor-α–converting enzyme (TACE/ADAM17), we hypothesized, was the major enzyme responsible for the cleavage of EGFR ligand precursors from the cell membrane. TACE is a member of the ADAM family, a group of zinc-dependent transmembrane metalloproteases (16, 32) and a major sheddase for TGF-α, amphiregulin, epiregulin, and HB-EGF (11–13, 31). *Tace* knockout mice are perinatal-lethal, and their phenotype resembles that of mice with a targeted deletion of *EGFR*, sharing features with *Tgfα*-deficient, *Hb-egf*–deficient, or *amphiregulin*-deficient mice (11, 14, 16, 33). The overlapping phenotypes of mice lacking these growth factors and those lacking TACE support a critical role for the soluble forms of EGFR ligands, and imply that the proteolytic release of EGFR ligands is fundamental in EGFR signaling.

To test the role of TACE in mechanotransduction, we bred floxed *Tace* mice (18) with mice expressing a tamoxifen-inducible Cre recombinase (34). We induced Cre activity after cells were isolated and in culture, based on the knowledge that the activation of autocrine EGFR plays a key role in airway epithelial proliferation. Thus we anticipated that cells harvested from mice in which *Tace* was already deleted by the activation of Cre *in vivo* might not be viable for our cell-culture experiments.

In control experiments, we found that incubating MTECs derived from normal mice in ALI culture with 1 μM 4-OH tamoxifen for 14 days (35, 36) had no impact on responses to compressive stress. Therefore, the duration of tamoxifen exposure needed for the removal of floxed *Tace* alleles would not affect our results. Cells derived from mice with the floxed *Tace* allele, when activated by tamoxifen, exhibited markedly decreased ($>$ 80%) responses to compressive stress (Figure 5). In contrast, cells from the same mice cultured in the absence of tamoxifen transduced compressive stress in a normal fashion. These results demonstrate that TACE is the dominant sheddase necessary for the transduction of compressive stress through the activation of EGFR. Our data do not allow us to distinguish between an increased activation of TACE by compressive stress or simply greater access of already activated TACE to membrane-bound precursor ligands. Further research will be needed to determine the specific mechanisms at work.

The modest residual response of tamoxifen-treated MTECs derived from $Tace^{flox/flox}$ $R26Cre^{+}$ *ER* mice could involve the manifestations of at least two possible mechanisms, residual TACE protein activity or the contributions of an alternative sheddase. Extending tamoxifen exposure to 19 days did not resolve the residual mechanotransduction response, arguing against a measurable contribution from residual TACE protein, which should diminish with prolonged tamoxifen exposure. In a murine model of asthma, both ADAM10 and TACE were overexpressed in the lungs (37). Because ADAM10 can shed EGFR ligands, including TGF-α (38) and HB-EGF (39), ADAM 10 could be responsible for the residual response to compressive

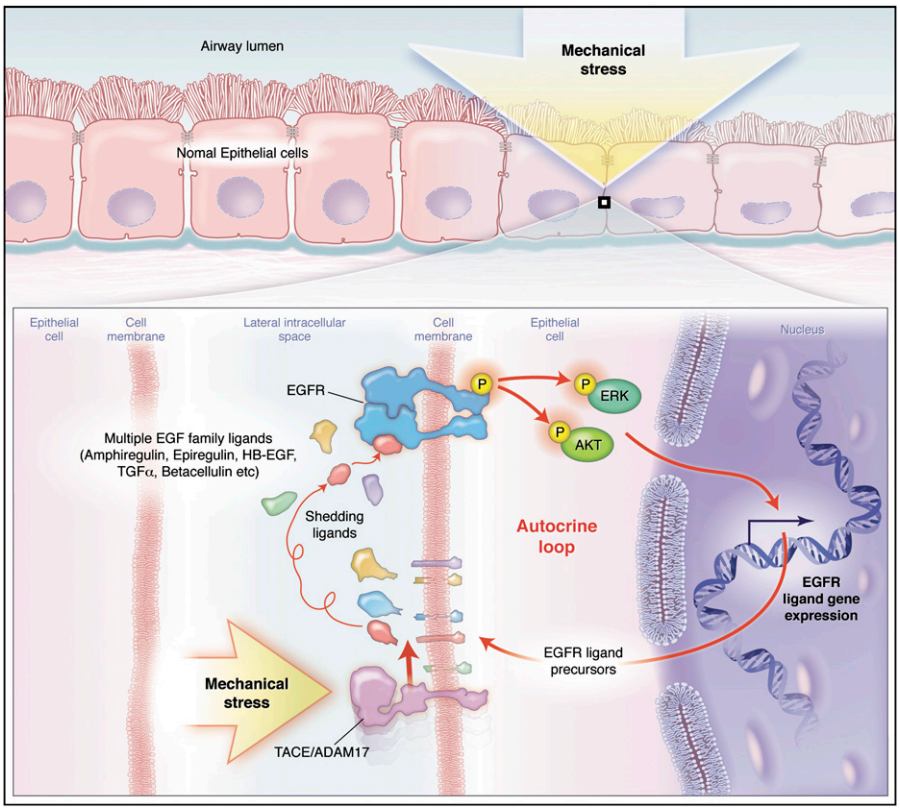

Figure 6. Proposed mechanism of mechanical stress–induced activation of EGFR pathway in murine tracheal epithelial cells. The compressive stress–induced activation of EGFR depends on the TACE-dependent shedding of EGF-family ligands. The activation of EGFR, signaling via MAP kinase and the Akt pathway, leads to up-regulated gene expression, including select EGF-family ligands. These ligands can initiate a positive feedback loop in an autocrine manner, promoting an amplified and prolonged activation of EGFR. ADAM17, a disintegrin and metalloprotease 17; P, phosphorylation.

stress. Consistent with this explanation, recent studies demonstrated that ADAM10 plays a role in the TGF-α processing of *Tace*-deficient cells (40), and ADAM10 is a major sheddase for betacellulin (31, 41), which was one of the EGF-family ligands up-regulated by compressive stress in MTECs.

Recent evidence suggests that a variety of stimuli can trigger the activation of EGFR in airway epithelium, and that EGFR plays important roles in airway biology. Thus the demonstration of a dominant role for TACE as a sheddase in airway epithelium has potentially broad implications. Several lines of evidence link the activation of EGFR and asthma, and in particular, the remodeling of airway epithelium in animals (42) and human cells (43, 44). We reported that EGFR-dependent mechanosignaling contributes to mucous cell hyperplasia in response to repeated bouts of compressive stress (45). TACE was previously linked to the regulation of mucin gene expression in cultured human epithelial cells (20), although the link was only definitively documented in the NCI-H292 cell line. Another study demonstrated that TACE mediates cell proliferation via the shedding of amphiregulin in response to cigarette smoke, again in NCI-H292 cells (46). Our study (to the best of our knowledge, for the first time) definitively identified a dominant role for the TACE-dependent activation of EGFR in primary airway epithelial cells grown under well-differentiated conditions in ALI cultures.

Our data extend previous findings by demonstrating that compressive stress also initiates the phosphorylation of Akt in a pressure-dependent manner (Figure 1). Studies of the ovalbumin-induced allergic asthma murine model showed that the serine phosphorylation of Akt is part of the "asthma-like" response observed in these mice (47). The finding that mechanical stress can provoke this asthma-related signal echoes previous observations that mechanical stress responses in airway epithelial cells mimic many of the molecular events characteristic of asthmatic airway remodeling (2–6). Although redundant EGFR ligands appear to occur in the signaling cascade, the cleavage of these ligands is largely restricted to TACE, making it a potential target for limiting airway remodeling, as is known to occur in patients with asthma. (Figure 6). Further work will be needed to determine if TACE activity is regulated biochemically or by variations in the access of EGFR ligands to TACE.

In conclusion, we demonstrate in MTECs that the compressive stress–induced phosphorylation of ERK1/2 and Akt through EGFR signaling is a metalloprotease-dependent process (Figure 6). Our data indicate a dominant role for TACE as the key sheddase involved in mechanically driven signal transduction in MTECs. These findings provide new insights into the mechanisms regulating mechanotransduction in MTECs.

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