Elastase/anti-elastase imbalance is a hallmark of emphysema, a chronic obstructive pulmonary disease associated with the rupture and inefficient repair of interstitial elastin. We report that neutrophil elastase (NE) at low physiologic concentrations, ranging from 35 nm to 1 μM, invokes transient, peaking at 15 min, activation of extracellular signal-regulated kinases 1 and 2 (ERK) in elastogenic lung fibroblasts. ERK activation is preceded by the release of soluble 25–26-kDa forms of epidermal growth factor (EGF) and transactivation of EGFR receptor (EGFR) in NE-exposed cells. The stimulatory effect of NE on ERK is abrogated in the presence of anti-EGF-neutralizing antibodies, EGFR tyrosine kinase inhibitor (AG1478), and ERK kinase inhibitor (PD98059), as well as abolished in both EGFR-desensitized and endocytosis-arrested fibroblasts. Nuclear accumulation of activated ERK is associated with transient, peaking at 30 min, induction of c-Fos and sustained, observed at 24–48 h, decrease of tropoelastin mRNA levels in NE-challenged cells. Pretreatment and sustained, observed at 24–48 h, decrease of tropoelastin mRNA levels in NE-challenged cells. Pretreatment of fibroblasts with AG1478 or PD98059 abrogates the NE-initiated tropoelastin mRNA suppression. We conclude that proteolytically released EGF signals directly via EGFR and ERK to down-regulate tropoelastin mRNA in NE-challenged lung fibroblasts.

Neutrophil elastase (NE) is a serine protease involved in host defense against bacterial pathogens (1, 2). However, NE released by polymorphonuclear neutrophils is capable of hydrolyzing a broad spectrum of extracellular matrix (ECM) and cell surface proteins, such as elastin, interstitial collagens, proteoglycans, fibronectin, laminin, and others, leading to tissue damage (3, 4). In the lungs, under normal physiological conditions, the proteolytic activity of elastase secreted by recruited neutrophils is tightly regulated by anti-proteases, such as α1-proteinase inhibitor (α1-PI) and secretory leukoprotease inhibitor. Genetic deficiency of α1-PI in humans and tobacco smoking are some of the known risk factors for pulmonary emphysema. This progressive disabling disorder in humans is characterized by the destruction of the alveolar walls resulting in enlargement of the peripheral airspaces in the lung (5–9).

Elastolytic injury is associated with detachment and apoptosis of endothelial cells and neutrophils (10–12), morphological changes in airway epithelial cells (13), contraction of lung fibroblasts (14), and proliferation of pulmonary artery smooth muscle cells (15). NE is capable of provoking a variety of cellular responses by affecting multiple cell surface and ECM molecules. For instance, NE up-regulates the fibrinogen binding activity of α1bβ3 integrin through a restricted proteolysis of the ε180 subunit, and this process is relevant to the potentiation of platelet aggregation (16). In lung fibroblasts, elastase is known to initiate shedding of heparan sulfate proteoglycans (17), extracellular molecules serving as docking sites for multiple growth factors, such as basic fibroblast growth factor (bFGF) and transforming growth factor β (18, 19). This shedding, in turn, leads to reduction of bFGF binding to its high affinity receptors (17). Some of the cell surface proteoglycans were thought to affect enzymatic activity of NE. For example, tight binding of NE to syndecan-1 reduces NE affinity for α1-PI (20). The bFGF released by elastase and added to lung fibroblasts or vascular wall smooth muscle cells is capable of repressing elastin gene transcription (21) or inducing cell proliferation (15), respectively. NE also is capable of shedding the ligand-binding fragment from the tumor necrosis factor-α receptor, providing a mechanism for the attenuated neutrophil response to pro-apoptotic tumor necrosis factor-α at sites of inflammation (22). On the other hand, by splitting secreted tyrosyl-tRNA synthetase into two fragments with distinct cytokine activities, NE may stimulate production of tumor necrosis factor-α and provoke apoptosis under inflammatory conditions (23). In addition, it has been demonstrated that NE as part of a stable complex with the leukocyte-derived inhibitor is translocated from the cytoplasm to the nucleus and functions as a part of a DNase II machinery degrading genomic DNA during apoptosis (24).

Epidermal growth factor receptor (EGFR) belongs to the family of receptor tyrosine kinases (25). EGFR signaling is crucial for branching morphogenesis of lung to occur during
Northern Blot Analysis—Total RNA was extracted from cell cultures using a guanidinium thiocyanate/phenol/chloroform single-step method (32) (TRIZol, Invitrogen). Samples of total RNA (10 μg/lane) were electrophoresed through 1.0% agarose-formaldehyde gels, capillary-transferred to nylon membrane (Micron Separations), and cross-linked to the membrane ultraviolet irradiation (Ultra-Link UV cross linker model 350). To ensure integrity and correct RNA, loading blots were stained with 0.4% methylene blue in 0.5 M sodium acetate. Membranes were prehybridized at 42 °C for 2 h in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA and then hybridized overnight in a solution containing 50% formamide, 5× SSC, 10% dextran sulfate, 0.5% SDS, and 32P-labeled 1.1-kb rat tropoelastin cDNA EcoRI fragment (33) or 32P-labeled mouse c-Fos cDNA probe (provided by Dr. M. Birrer), generated using a random primer labeling kit (Invitrogen). After hybridization, membranes were washed (two times in 1× SSC and 0.1% SDS at 55 °C for 1 h) and exposed from 2 h to overnight at −80 °C with an intensifying screen to X-Omat film (Eastman Kodak Co.). Hybridization signals were quantitated with the use of a Molecular Dynamics laser scanning densitometer.

Western Blot Analysis—After challenge with the ligands, cell cultures in 12-well cluster plates, 35-, 60-, or 100-mm dishes were quickly rinsed twice with PBS at room temperature and lysed with gentle rocking at 4 °C for 10 min in 0.1, 0.25, 1.0, or 2.0 ml, respectively, of ice-cold RIPA buffer, containing 10 mM Tris/Cl, pH 7.4; Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DFP, and 0.2 mM sodium orthovanadate. The lysates were centrifuged at 4 °C for 30 min at 15,000 × g, and clear supernatants were kept at −80 °C. Forty-μl aliquots of supernatants were mixed with 20 μl of 3× SDS-PAGE sample buffer with 2-mercaptoethanol and heated for 10 min at 100 °C, and 40-μl aliquots (±25 μg of total protein) were loaded on 4% stacking, 9 or 12% separating SDS-PAGE mini-gel (34). Electrophoresis was performed at constant current (20 mA/0.75-mm-thick gel). After electrophoresis, the proteins were electroblotted (16 h, 4 °C, 65 V) onto a 0.45-μm pore size nitrocellulose membrane (Schleicher & Schuell) according to Ref. 35. Subsequent steps were performed at room temperature, unless specifically indicated. Transferred proteins were stained briefly with 0.1% Ponceau S (w/v) in 5% acetic acid (Sigma) to check for even loading and transfer. Membranes were blocked in 5% nonfat milk powder (w/v) in TBST (10 mM Tris/Cl, pH 7.4, 150 mM NaCl, and 0.05%Tween 20) for 1 h, washed three times for 5 min with TBST, treated with 1 h with 1:100 dilution of peroxidase-conjugated secondary IgG (Sigma). Immunodetection of proteins was visualized by using a Lumigen chemiluminescence detection kit (Kirkgaard & Perry Laboratories). Routinely, blots were stripped of bound antibodies in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/Cl, pH 6.8, at 70 °C for 30 min, washed with TBST, and re-probed with 1:1000 diluted anti-ERK antibody (Upstate Biotechnology Inc.) and 1:1000 diluted peroxidase-conjugated secondary IgG (Sigma). Immunodetection of proteins was visualized by using a LumiGlo chemiluminescence detection kit (Kirkgaard & Perry Laboratories). Routinely, blots were stripped of bound antibodies in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/Cl, pH 6.8, at 70 °C for 30 min, washed with TBST, and re-probed with 1:1000 diluted anti-ERK antibody (Upstate Biotechnology Inc.) and 1:1000 diluted peroxidase-conjugated secondary IgG (Sigma).

In-gel Kinase Assay—An in-gel myelin basic protein kinase assay was performed as described previously (36) with minor modifications. Briefly, after challenge with the ligands, cell cultures in 35-mm dishes were quickly rinsed twice with PBS at room temperature and lysed with gentle rocking for 10 min at 4 °C in 250 μl of ice-cold buffer containing 10 mM Tris/Cl, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DFP, and 0.2 mM sodium orthovanadate. The lysates were centrifuged at 4 °C for 30 min at 15,000 × g. Clear supernatants were frozen at −80 °C. Forty μl from each supernatant were mixed with 20 μl of 3× SDS-PAGE sample buffer containing 2-mercaptoethanol and heated for 10 min at 100 °C. Then-40-μl aliquots (±25 μg of total protein) were loaded on 4% stacking and 12% separating SDS-PAGE mini-gel that had been polymerized with 0.4 mg/ml myelin basic protein. After electrophoresis, the gel was washed with 20% isopropl alcohol in 100 mM Tris/Cl, pH 8.0, followed by a wash in 100 mM Tris/Cl, pH 8.0, containing 5 mM 2-mercaptoethanol. The gel was then denatured in 6 M guanidinium hydrochloride followed by renaturation in 0.4% Tween 40. The gel was incubated at 30 °C in 250 μl of kinase buffer containing 25 μM HEPEs, pH 7.2, 10 mM MgCl2, and 2 mM 2-mercaptoethanol for 30 min, followed by another incubation in kinase buffer containing 50 μM ATP and 50 μCi of [α-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) for 60 min at room temperature. The gel was washed with 1% sodium pyrophosphate in 5% trichloroacetic acid, stained with Coomassie Blue R-250 to check for even protein loading, and dried. Autoradiography was performed for 6–12 h at −80 °C with an intensifying screen and X-Omat film (Kodak).
Phosphorylation signals were quantitated with the use of a Molecular Dynamics laser scanning densitometer.

**Immunoprecipitation of EGFR**—After challenge with the ligands, cell cultures in 60-mm dishes were immediately rinsed twice with PBS at room temperature and lysed with gentle rocking for 10 min at 4 °C in 1.0 mM cold Triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 mM DFP, and 0.2 mM sodium vanadate. Lysates were pre-cleared by mixing with 50 μl of protein A-Sepharose slurry (50% v/v in PBS) for 30 min and centrifuged at 4 °C for 15 min at 16,000 g. Supernatants were incubated with 10 μg of anti-EGFR IgG (Upstate Biotechnology, Inc.) at 4 °C for 12–14 h with gentle rocking. The samples were centrifuged at 4 °C for 30 min at 16,000 × g. Clear supernatants were mixed with 75 μl of protein A-Sepharose slurry (50% v/v in PBS), incubated with gentle rocking at 4 °C for 3 h, and then washed twice for 15 min with 1 ml of ice-cold lysis buffer and once for 15 min with 1 ml of cold PBS. Thirty μl of 2× SDS-PAGE sample buffer with 2-mercaptoethanol were added to the final pellets; the samples were heated at 100 °C for 10 min and centrifuged, and the supernatant was loaded onto 4% stacking, 9% separating SDS-PAGE mini-gels. After electrophoresis, the proteins were electroblotted onto nitrocellulose membrane and stained briefly with 0.1% Ponceau S (w/v) in 5% acetic acid (Sigma) to check for IgG recovery and transfer. Membranes were first blocked in 5% nonfat milk powder and then probed sequentially first with 1:1000 diluted phosphotyrosine-specific PY-99 antibody (Santa Cruz Biotechnology) and then with 1:1000 diluted peroxidase-conjugated secondary IgG (Sigma). Immunodetection of proteins was visualized by using a Lumiglo chemiluminescence detection kit (Kirkegaard & Perry Laboratories). Blots were stripped of bound antibodies, washed, and re-probed with 1:1000 diluted anti-EGFR (Upstate Biotechnology, Inc.) and 1:1000 diluted peroxidase-conjugated secondary IgG (Sigma).

**Cellular Immunofluorescence Imaging**—The intracellular localization of activated ERK in control and NE-treated lung fibroblasts was studied by indirect immunofluorescence. For this purpose, cells were seeded on the glass coverslips placed into 12-well cluster plates with density 50,000 cells per well and grown for 5 days in the presence of 5% serum to reach the confluent stage. Then cells were grown in complete serum-free medium, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, and blocked with blocking solution (3% normal donkey serum and 1% bovine serum albumin in PBS) for 1 h at room temperature. The cells were then incubated overnight at 4 °C with 1:250 diluted anti-activated phospho-ERK (New England Biolabs) in blocking solution. After the primary antibody incubation, cells were washed in PBS (0.1% Triton X-100) and incubated for 1 h at room temperature with 1:800 diluted indocarbocyanine (Cy3)-conjugated secondary IgG (Jackson ImmunoResearch) in blocking solution. SlowFade-Light Antifade kit (Molecular Probes) was used for mounting covers on slides. Intracellular distribution of phospho-ERK was examined using a confocal laser scanning microscope (Zeiss LSM 510). Control cells, not incubated with primary antibody, were used to establish microscope voltage settings such that no fluorescence is visible within the control cultures, and these same settings were used to analyze the primary antibody-treated cells.

**RESULTS**

Exogenously added bFGF activates the ERK cascade leading to prominent repression of elastin gene transcription in lung fibroblasts (36). Because treatment of these elastogenic cell cultures with NE or concentric elastase (PE) resulted in the release of the endogenous, ECM-bound bFGF (17, 21) we hypothesized that the liberated growth factor might signal to ERK activation and tropoelastin mRNA down-regulation in protease-challenged cells. Addition of NE to lung fibroblasts indeed led to ERK activation in both a concentration- and time-dependent manner (Fig. 1). The initial increase of ERK phosphorylation was observed at concentrations of serine protease as low as 1 μg/ml (35 ng/ml) reaching its maximal level (20-fold increase) at 10 μg/ml NE (Fig. 1A). ERK activation was detectable at 5–6 min, peaked at 15 min, and decreased to basal levels by 240 min (Fig. 1B). Total intracellular levels of ERK were unaffected by NE treatment. The activation of ERK in NE-challenged cells was also detectable by a direct in-gel kinase assay using myelin basic protein (MBP) and [γ-32P]ATP as substrates (Fig. 1C, control). Thus, lung fibroblasts respond to NE challenge by pronounced transient activation of ERK.

To examine whether the ERK activating property of NE requires its proteolytic activity, we tested specific serine protease inhibitors in this assay. The ability of NE to initiate ERK activation was completely abolished in the presence of its natural inhibitor, serpin α1-PI (Fig. 2A). Inactivation of NE with disopropyl fluorophosphate (DFP), a synthetic serine protease inhibitor, totally abrogated the NE-initiated ERK response, as well as ERK activation initiated by other serine proteinases, such as PE, thrombin, and trypsin (Fig. 2B). In contrast, α1-PI and DFP did not affect ERK activation by bFGF or platelet-derived growth factor (PDGF) (Fig. 2A). These results demonstrate that NE-initiated ERK activation requires the proteolytic activity of this enzyme.

The NE-initiated ERK activation was accompanied by nuclear translocation of phosphorylated ERK (Fig. 3). Nuclear accumulation of ERK was evident at 7.5 min and progressed by 15 min of NE treatment. It is noteworthy that phosphorylated ERK were unaffected by NE treatment. The activation of ERK in NE-challenged cells was also detectable by a direct in-gel kinase assay using myelin basic protein and [γ-32P]ATP as substrates (Fig. 1C, control). Thus, lung fibroblasts respond to NE challenge by pronounced transient activation of ERK. The NE-initiated ERK activation was accompanied by nuclear translocation of phosphorylated ERK (Fig. 3). Nuclear accumulation of ERK was evident at 7.5 min and progressed by 15 min of NE treatment. It is noteworthy that phosphorylated ERK was also localized to fibril-like structures in the cytoplasm of NE-challenged cells (Fig. 3, B and C).
peak of ERK activation. In comparison, thrombin-dependent induction of c-Fos mRNA was more sustained (Fig. 4A) and correlated with the extended ERK activation by this protease (Fig. 4B). Taken together, these data indicate that NE-initiated ERK activation is associated with the induction of c-Fos.

In order to examine whether the NE-initiated ERK activation is mediated by the released bFGF, the ability of NE to activate ERK was assessed in the presence of anti-bFGF neutralizing antibodies. As evident from Fig. 5, the anti-bFGF IgG were not able to abrogate the NE-initiated activation of ERK, despite their specific inhibitory activity against exogenously added bFGF. Elastase is known to release ECM-bound PDGF from the elastogenic lung fibroblast cultures,2 as well as being able to shed the bioactive cell surface TGF-α. We examined whether the NE-initiated ERK activation might be associated with the release and signaling of PDGF and/or TGF-α. The addition of anti-PDGF or anti-TGF-α neutralizing antibodies to lung fibroblasts did not affect the NE-initiated ERK activation in these cells. At the same time, both anti-PDGF and anti-TGF-α antibodies specifically abrogated ERK activation by exogenously added PDGF and TGF-α, respectively, and did not neutralize ERK-activating properties of other growth factors (Fig. 5). It is important to note that exposure of each neutralizing antibody to ERK-activating concentrations of NE did not inhibit their individual neutralizing activities against specific growth factors.

2 J. Buszek-Thomas and M. A. Nugent, unpublished data.
Elastase-released EGF Down-regulates Tropoelastin mRNA

Transient tyrosine phosphorylation of EGFR leading to ERK activation has been shown to be induced by a wide variety of extracellular stimuli, which do not belong to the family of canonical EGFR ligands. This, so-called transactivation of EGFR can be initiated by multiple ligands signaling via G-protein-coupled receptors (GPCRs) (25, 43, 44), as well as by several cytokines and growth factors, including growth hormone, hepatopoietin, insulin-like growth factor 1, and PDGF (45–48). Moreover, cellular response to integrin clustering (49–51), as well as hyperosmotic shock (52), ultraviolet, ionizing radiation, membrane depolarization, and oxidative stress (53–57) also led to EGFR transactivation. It is well known that rapid and transient transactivation of the EGFR requires its intrinsic tyrosine kinase activity (25, 58). To ascertain whether NE-initiated ERK activation is mediated by EGFR transactivation, we examined the effect of tyrphostin AG1478, a potent and selective inhibitor of EGFR tyrosine kinase activity. Treatment of cells with AG1478 effectively abrogated, by 80–90%, the NE-initiated ERK activation as determined by the in-gel kinase activity (Fig. 1C) and Western blot (Fig. 6B) assays. As expected, AG1478 selectively prevented EGF-dependent activation of ERK and did not interfere with PDGF- or bFGF-dependent ERK activation (Fig. 6B). These data suggest that engagement of EGFR might be a central signaling event triggering ERK activation in NE-treated lung fibroblasts.

Serine proteases, such as thrombin and trypsin, are reported to stimulate ERK by acting on a subfamily of protease-activated GPCR (59). The level of elastase-initiated ERK activation in lung fibroblasts was comparable with that induced by protease-activated GPCR agonists, thrombin or trypsin (Fig. 2B). NE also is known to proteolytically process different integrin ligands (3–4) as well as up-regulate the ligand binding activity of integrins (16). Thus, we examined whether NE might initiate the EGFR-mediated ERK response via activation of GPCR and/or integrin signaling. For this purpose, several pharmacological compounds known to inhibit integrin- and/or GPCR-mediated ERK activation have been screened on NE-initiated ERK activation. Treatment of cells with cytochalasin D, an F-actin depolymerization and focal adhesion disassembly agent, induced dramatic morphological changes in adherent lung fibroblasts (data not shown) but did not affect their NE-initiated ERK response (Fig. 6C). Also, incubation of lung fibroblasts with PP2, a specific inhibitor of non-receptor protein tyrosine kinase, BAPTA, or BAPTA-AM, chelators of extracellular or intracellular Ca\(^{2+}\), U73122, a specific inhibitor of hormone-sensitive phospholipase C\(\beta\) activity, or with Ro31-8220, a specific inhibitor of protein kinase C, did not affect NE-initiated ERK activation (Fig. 6C). Moreover, long term treatment of cells with PMA, known to deplete PMA-sensitive isoforms of protein kinase C (60), did not affect NE-initiated ERK activation but did result in complete desensitization of the PMA-dependent ERK response (Fig. 6D). In sum, the panel of inhibitors suggests that GPCR- and/or integrin-mediated pathways do not play a significant role in the NE-initiated EGFR-mediated ERK activation in lung fibroblasts.

We next examined whether NE-initiated ERK activation is mediated by tyrosine phosphorylation of EGFR. For this purpose EGFR was immunoprecipitated from control and NE-challenged cells, and levels of phospho-EGFR and total EGFR were determined by Western blot analysis with the corresponding antibodies. As a positive control we studied immunoprecipitates isolated from EGF-treated cells. The 170-kDa protein band corresponding to the tyrosine-phosphorylated form of EGFR was detectable at 2 min, reaching a maximum at 5–10 min, and declining 20 min after NE addition (Fig. 7A). It is noteworthy that the peak of EGFR tyrosine phosphorylation
preceded by 5 min the peak of ERK activation in NE-challenged cells (Fig. 7B). The levels of NE-initiated tyrosine phosphorylation of EGFR in NE-challenged cells were significantly less pronounced than that achieved in EGF-treated cells (Fig. 7A, control). It is important to stress that AG1478 effectively inhibited NE- and EGF-induced EGFR tyrosine phosphorylation, as well as ERK activation in lung fibroblasts (Fig. 7). Elastase has been shown to remove an ~30-kDa peptide at the C-terminal intracellular portion of the native 170-kDa EGFR to generate 150-kDa form of the receptor (61). We did not observe any significant accumulation of the 150-kDa receptor form of EGFR in NE-challenged versus control or EGF-treated cells (Fig. 7A). We also demonstrated that AG1478 did not inhibit the proteolytic activity of NE, as determined by insoluble [3H]elastin degradation assay (data not shown). In sum, these data indicate that NE triggers transactivation of the EGFR which, in turn, results in ERK activation in NE-challenged lung fibroblasts.

To establish further the role of EGFR signaling in NE-initiated ERK activation, we took advantage of EGFR down-regulation (internalization and degradation) in response to EGF (25, 62). We examined whether EGF-induced EGFR down-regulation might lead to desensitization of ERK activation by NE. The long term, 24 h, treatment of cells with EGF led to depletion of EGFR levels by more than 90% and abolished the NE-initiated as well as EGF-dependent ERK activation (Fig. 7A). AG1478, a metalloproteinase inhibitor, did not induce significant down-regulation of the EGFR and did not affect the NE- or EGF-initiated ERK activation but effectively desensitized the ERK response to the second addition of bFGF (Fig. 8A). It should be noted that EGFR desensitization induced by long term treatment of the cells with other EGFR ligands, such as TGF-α, heparin-binding EGF-like growth factor (hbEGF), betacellulin, or amphiregulin, also led to complete abrogation of the NE-initiated ERK response. However, the long term challenge of cells with heregulin, the EGF family member that does not bind to EGFR (63), did not deplete EGFR levels and did not interfere with the NE-initiated or EGF-induced ERK responses (data not shown). Taken together, these data indicate that the presence of ligand-competent EGFR on the cell surface is required for NE-initiated ERK activation in lung fibroblasts.

Internalization and endocytic trafficking of EGFR and some other known components of the EGFR/ERK pathway are believed to be crucial for the initiation of EGFR-mediated ERK activation in many types of cells (62, 64). We examined whether the arrest of cellular endocytosis might affect NE-initiated ERK activation in our cell system. Challenging lung fibroblasts with endocytosis-arresting conditions (65), such as treatment with hypertonic or K+ -depleted media, abrogated NE-initiated ERK activation (Fig. 8B). It should be noted that the endocytosis-arresting media did not inhibit the proteolytic activity of NE against insoluble [3H]elastin (data not shown). These data suggest that endocytosis is required for NE-initiated EGFR-mediated ERK activation.

It has been discovered that cell-surface metalloproteinases trigger EGFR transactivation via proteolytic processing of the transmembrane form of the hbEGF precursor into the mature soluble form of the ligand (47, 66). We examined whether metalloproteinase(s) are involved in NE-initiated ERK activation. Treatment of lung fibroblasts with potent broad-spectrum metalloproteinase inhibitors, such as hydroxamic acid-based GM6001 or the Zn2+-chelating agent 1,10-phenanthroline, did not affect the NE-initiated ERK activation (Fig. 6A), thus arguing against metalloproteinase involvement in NE signaling.

We hypothesized that NE might signal toward ERK via direct release of EGF. First, we examined whether treatment of lung fibroblasts with NE resulted in release of EGF into conditioned media. Western blot analysis with EGF antibodies revealed NE-initiated time-dependent accumulation of soluble 25- and 26-kDa proteins in the conditioned media. Both EGF-like proteins appeared in similar amounts as early as 2–5 min after NE treatment. After 10–15 min the accumulation of the
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**DISCUSSION**

EGFR activation occurs in the plasma membrane in response to ligand-induced dimerization and results in a pronounced tyrosine phosphorylation of the receptor complex. Transient tyrosine phosphorylation of EGFR, so-called EGFR transactivation, leading to ERK activation is invoked in different types of cells in response to a wide variety of external stimuli unrelated to primary EGFR ligands (25, 58). Recent studies (47, 66) established that at least some of those stimuli transactivate EGFR via metalloproteinase-catalyzed processing of cell-surface pro-hbEGF into mature soluble hbEGF, which, in turn, activates EGFR and signals to ERK activation in target cells. Constitutive shedding of membrane-anchored precursors of TFG-α, hbEGF, and amphiregulin was demonstrated in different cell types and can be stimulated in response to mobilization of intracellular Ca^{2+}, PMA-sensitive form(s) of protein kinase C, as well as by protein kinase C-independent mechanisms (69–71). However, current knowledge of the proteases involved in this process is very limited. Several members of the ADAM family of metalloprotease disintegrins, such as TACE/ADAM 17 and ADAM 9, as well as serine proteases, such as NE and PE, have been implicated in proteolytic processing of cell-sur-

Fig. 9. NE initiates ERK activation by releasing EGF. A, serum-starved lung fibroblasts (in 100-mm plates) were incubated with or without 5 μg/ml NE or 5 μg/ml PE for the indicated times, and then conditioned medium samples, as well as total cell lysates, were harvested in the presence of 1 mM DFP. Conditioned media samples (initial volume 12 ml) were concentrated (final volume 500 μl) and dialyzed against 10 mM Tris-HCl, pH 7.5, at 4 °C on microconcentrators (Amicon), and 50 μl of each sample were analyzed by Western blot (in non-reducing conditions) with anti-EGF-neutralizing antibodies (upper part). The electrophoretic mobility of protein standards (Bio-Rad) is shown on the right upper part. The EGF-neutralizing antibody was highly selective, as it recognized the 25- and 26-kDa doublet of EGF-like molecules in NE-conditioned medium (Fig. 9A, left panel). Because we demonstrated that treatment of lung fibroblasts with PE also results in ERK activation (Fig. 2B), we examined whether this proteinase can release EGF. Treatment of cells with PE resulted predominantly in accumulation of the 25-kDa EGF form (Fig. 9A). It should be noted that the NE-initiated release of EGF molecules was blocked by α1-PI but was not affected by GM6001 (data not shown). We examined whether the NE-released forms of EGF possess any biological activity. The addition of conditioned media samples collected from NE-treated cultures to control lung fibroblasts invoked ERK activation. The levels of ERK activation were proportional to the amount of the NE-released 25-kDa form of EGF (Fig. 9A). Importantly, the ERK-activating properties of NE-conditioned media, or NE itself, were effectively abrogated in the presence of the EGF-neutralizing antibody that recognized the 25- and 26-kDa doublet of EGF-like molecules in NE-conditioned media. The EGF-neutralizing antibody was highly selective, as it blocked ERK activation by EGF but not by other members of the EGF family that were examined such as TFG-α, hbEGF, or betacellulin (Fig. 9B). As expected, the ERK-activating effect of NE-conditioned medium on control lung fibroblasts was blocked by AG1478 (Fig. 9B). The results obtained clearly implicate the 25-kDa form of EGF as a predominant EGFR ligand activating ERK in NE-challenged lung fibroblasts.

It has been shown that EGF and TGF-α can both decrease the steady-state tropoelastin mRNA levels in elastogenic vascular smooth muscle cells and skin fibroblasts (67, 68). We decided to investigate the effect of EGF-releasing NE on tropoelastin mRNA expression in our cell system. Cells were treated in the presence or absence of NE for 15 min to allow maximal ERK activation, and then protease activity was quenched by the addition of a 10-fold molar excess of α1-PI. Samples were analyzed for tropoelastin mRNA expression at 3, 5, 24, and 48 h after injury. This acute treatment of cells with NE resulted in time-dependent suppression of tropoelastin mRNA expression, as evident at 24 and 48 h after initial addition of protease (Fig. 10A). Challenge of lung fibroblasts with EGF or bFGF also led to tropoelastin mRNA down-regulation (Fig. 10B, control). We examined whether the down-regulation of tropoelastin mRNA in NE-challenged cells depends on EGFR transactivation and ERK activation. The NE-initiated as well as EGF-dependent tropoelastin mRNA responses were abrogated in the presence of AG1478, whereas the bFGF-dependent repression of tropoelastin mRNA expression was unaffected (Fig. 10B). On the other hand, PD98059 effectively abrogated tropoelastin mRNA repression induced by all three stimuli (Fig. 10B). These results indicate that the EGFR-mediated ERK activation signals to repress tropoelastin mRNA expression in NE-treated lung fibroblasts.
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![Fig. 10. NE initiates down-regulation of tropoelastin mRNA, effect of AG1478 and PD98059 compounds. A, serum-starved lung fibroblasts (in 35-mm plates) were incubated for 15 min with or without 5 μg/ml NE, and then α1-PI was added to all cultures in a final concentration 100 μg/ml. Total RNA samples were isolated 3, 5, 24, and 48 h after addition of α1-PI. B, serum-starved lung fibroblasts (in 35-mm plates) were incubated in the presence or absence of 10 μM AG1478 or 50 μg/ml PD98059 for 30 min and challenged for 15 min with or without 10 μg/ml NE, 10 ng/ml EGF, or 10 ng/ml bFGF as indicated. Then α1-PI was added to all samples to a final concentration of 100 μg/ml, and total RNA samples were isolated 24 h after addition of α1-PI. Tropoelastin mRNA expression was determined by Northern blot (upper part). Before hybridization nylon membranes were stained with methylene blue to ensure integrity and even RNA loading (bottom part).](http://www.jbc.org/)

face EGFR ligands (38–40, 69–73). Our efforts to uncover the “metalloproteinase-trace” within the NE-mediated release of EGF molecules leading to EGFR-mediated ERK activation failed. None of the established approaches used to inhibit metalloproteinase-mediated EGFR transactivation in other cell systems, such as metalloproteinase inhibitors, PMTA treatment, as well as neutralizing antibodies against TGF-α or bFGF (data not shown), interfered with NE-induced signaling in our cellular model. We propose that NE-initiated EGFR/ERK signaling in lung fibroblasts is induced via EGF molecule(s) directly released by this serine protease. Exposure of lung fibroblast cultures to elastase resulted in shedding of the 25–26-kDa forms of EGF rather than the mature 6-kDa EGF molecules. Nevertheless, the elastase-released form(s) of EGF is bioactive, as was clearly demonstrated by its potential to activate ERK in naive lung fibroblasts. Our observation is consistent with the concept that proteolytic processing of high molecular weight cell surface-associated EGF species into lower molecular weight soluble ligands actually changes the mode of EGF signaling from juxtamembrane to diffusible (38, 40, 72, 74).

Previously we identified bFGF released by elastase treatment of lung fibroblast cultures as a repressor of elastin gene transcription when added to lung fibroblasts that had not been treated with elastase. Interestingly, it has been shown that this elastase injury model is associated with the loss of cell-surface heparan sulfate proteoglycans and concatenated reduction of the effective affinity of bFGF receptors. It has been suggested that the elastogenic phenotype of elastase-treated cells might not be immediately affected by the released bFGF (17, 21). In our current study, we confirmed the lack of immediate signaling in the elastase-injured cells in response to bFGF and identified elastase-released EGF as an immediate growth factor initiating the down-regulation of elastogenic phenotype in the elastase-injured cells. It is important to note that both bFGF and EGF signal to repress tropoelastin mRNA levels in control and injured lung fibroblasts via activation of the ERK cascade. Thus, nuclear accumulation of activated ERK in bFGF-challenged cells leads to phosphorylation of the transcription factor Elk-1, inducing c-Fos, which, in turn, up-regulates Fra-1. Finally, the Fra-1/c-Jun heterodimer binds to an AP-1-like sequence within the distal promoter of the elastin gene, inhibiting elastin gene transcription, which results in down-regulation of elastin mRNA expression in targeted cells (36). The elastase-initiated EGFR-mediated activation and nuclear accumulation of ERK also resulted in c-Fos induction in lung fibroblasts, suggesting that the initiation of the Elk1/c-Fos/Fra-1-mediated route to inhibit elastin gene transcription might be a general mechanism to suppress the elastogenic phenotype of NE-challenged cells. Nevertheless, because NE might also trigger internalization and trafficking of the activated EGFR, we cannot exclude the scenario that placing the activated EGFR complex into the appropriate intracellular location might be required to down-regulate the elastogenic phenotype of lung fibroblasts. In this regard, the EGFR complex might also be targeted into the nucleus and function as a transcription factor or co-activator regulating AT-rich consensus sequence-dependent transcription of genes (75), suggesting an alternative route for EGFR-mediated modulation of elastin gene transcription in elastase-challenged cells. The NE-initiated suppression of tropoelastin mRNA levels may also reflect changes in the half-life of tropoelastin mRNA. Tropoelastin mRNA stability is known to contribute significantly to high levels of tropoelastin mRNA expression in a variety of elastogenic models (76), including lung fibroblasts (77, 78). Future studies of transcription rate and mRNA stability of the elastin gene in NE-challenged lung fibroblasts should unravel the detailed mechanism(s) underlying the suppression of the elastogenic phenotype of these cells.

Elastin, a major extracellular matrix constituent of the lung, which maintains the structural integrity of airways, serves as an important morphogenic factor required for distal airway branching and alveologenesis in the lung during development and repair (79–81). EGFR, by playing an essential role in epithelial cell proliferation and differentiation, positively controls branching morphogenesis during lung development (26, 27). Proteolytic degradation of interstitial elastic fibers by serine proteases and metalloproteinases primarily of neutrophil and macrophage origin as well as insufficient re-synthesis of tropoelastin are thought to contribute to inadequate repair and result in airspace enlargement and progression of pulmonary emphysema in experimental animal models and humans (6–9, 82). Discordant signaling of EGFR results in a variety of lung pathologies, including emphysema. Thus, surfactant protein C promoter-driven expression of TGF-α in lungs of transgenic mice resulted in the absence of...
Elastase-released EGF Down-regulates Tropoelastin mRNA

elastin fiber formation in alveolar septae and concomitant loss of alveoli in the lung parenchyma (83, 84). Importantly, airspace enlargement in lungs was corrected in bitransgenic animals ob-

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