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Activation of the Epidermal Growth Factor Receptor (EGFR) by a Novel Metalloprotease Pathway*

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Neutrophil Elastase (NE) is a pro-inflammatory protease present at higher than normal levels in the lung during inflammatory disease. NE regulates IL-8 production from airway epithelial cells and can activate both EGFR and TLR4. TACE/ADAM17 has been reported to *trans*-activate EGFR in response to NE. Here, using 16HBE14o-human bronchial epithelial cells we demonstrate a new mechanism by which NE regulates both of these events. A high molecular weight soluble metalloprotease activity detectable only in supernatants from NE-treated cells by gelatin and casein zymography was confirmed to be meprin alpha by Western immunoblotting. *In vitro* studies demonstrated the ability of NE to activate meprin alpha, which in turn could release soluble TGF α and induce IL-8 production from 16HBE14o- cells. These effects were abrogated by actinonin, a specific meprin inhibitor. NE-induced IL-8 expression was also inhibited by meprin alpha siRNA. Immunoprecipitation studies detected EGFR/TLR4 complexes in NE-stimulated cells overexpressing these receptors. Confocal studies confirmed colocalization of EGFR and TLR4 in 16HBE14o-cells stimulated with meprin alpha. NF κ B was also activated via MyD88 in these cells by meprin alpha. In bronchoalveolar lavage fluid from NE knock-out mice infected intra-tracheally with *Pseudomonas aeruginosa* meprin alpha was significantly decreased compared with control mice, and was significantly increased and correlated with NE activity, in bronchoalveolar lavage fluid from individuals with cystic fibrosis but not healthy controls. The data describe a previously unidentified lung metalloprotease meprin alpha, and its role in NE-induced EGFR and TLR4 activation and IL-8 production.

intracellular physiological function of NE is the degradation of foreign organic molecules phagocytosed by neutrophils. NE is well documented as one of numerous neutrophil proteases responsible for destroying microbes (2, 3). Its principal target is elastin (4); however, it can also degrade extracellular matrix proteins, collagen types I-IV, proteoglycan, fibronectin, platelet IIb/IIIa receptor, complement receptor, thrombomodulin (5), lung surfactant proteins (6), cadherin (7), and cleave complement factors, immunoglobulin, protease inhibitors, and several proteases (8). Neutrophils are the primary cell lineage that produces NE and are a key immune cell involved in pro inflammatory lung disorders. A high neutrophil burden has been linked to an increase in inflammation within the lung (9), and NE has been implicated in several diverse inflammatory lungs disorders including cystic fibrosis (10).

Previous studies have identified in part the intracellular signaling pathway regulated by NE (11) and established that NE can induce IL-8 via TLR4 (12). Other studies have demonstrated partial inhibition of NE-induced IL-8 production by using specific inhibitors of TLR signaling in airway epithelial cells (12, 13). NE is also likely to regulate expression of IL-8 and other genes by alternative mechanisms.

In addition to TLR4, another receptor of interest which NE has been shown to *trans*-activate, is EGFR (14). EGFR plays a key role in many cellular processes (15) and is important for mucin production from airway epithelial cells (16) and in sustaining neutrophil inflammation (17). EGFR is a transmembrane protein consisting of an extracellular ligand-binding domain to which EGFR ligands such as transforming growth factor α (TGF α) and epidermal growth factor (EGF) bind for activation. Its intracellular domain consists of a tyrosine kinase domain. Upon activation via ligand binding, EGFR is phosphorylated on key tyrosine residues and docking of signal transducers can occur (18). In addition to direct activation by EGFR ligands, EGFR can also be *trans*-activated by other stimuli such as lipoteichoic acid (19), other TLR agonists (20) and NE (16, 21). The mechanism of NE-induced Mucin (MUC5AC) production via EGFR in NCI-H292 cells, an epithelial line derived from a mucoepidermoid pulmonary carcinoma has been documented (16) with TNF- α -converting enzyme (TACE/

Neutrophil elastase (NE)⁴ is a 29-kDa serine protease that is stored in the azurophilic granules of neutrophils (1). The main

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⁴ The abbreviations used are: NE, neutrophil elastase; BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; EGF, epidermal growth factor; EGFR, EGF receptor; HBEGF, heparin-binding epidermal growth factor; MMP, matrix

metalloprotease; TACE, TNF- α -converting enzyme; TGF, transforming growth factor; TLR, toll-like receptor; IL, interleukin; FCS, fetal calf serum; PBS, phosphate-buffered saline; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

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ADAM17) shown to be involved in EGFR ligand generation. EGFR has also been implicated in NE-induced IL-8 production in lung epithelial cells (22); however, the *in vivo* biological significance of TACE in these events remains to be elucidated. Interestingly other metalloproteases in addition to TACE are reported to have a role in activation of EGFR (23).

We set out to understand in greater detail how NE *trans*-activates EGFR in human bronchial epithelial cells and to integrate this new knowledge with our current understanding of the NE mechanism of action on IL-8 expression. We postulated that a novel metalloprotease that shares redundancy with TACE is activated by NE in bronchial epithelial cells. Our aim was to identify such a protease, determine its biological activity against EGFR and TLR and elucidate its role in NE-induced activation of NF κ B and regulation of IL-8 gene expression. Here we demonstrate the involvement of meprin alpha, a previously unidentified target of NE, in the NE-induced EGFR/TLR4 inflammatory pathway in human bronchial epithelial cells.

MATERIALS AND METHODS

Chemicals—Chemicals and reagents were purchased from Sigma unless indicated otherwise.

Cell Culture and Treatment—16HBE14o- cells are an SV40-transformed human bronchial epithelial cell line and were obtained as a gift from D. Gruenert (University of Vermont). The cells were cultured in minimal essential medium (MEM) supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, (Invitrogen). The human embryonic kidney cell line, HEK293, (ECACC-85120602) was cultured in Eagle's Minimal Essential Medium (EMEM, Invitrogen) supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 1% NEAA (Invitrogen). 293-hTLR4-HA cells, an isolated clone of HEK293 cells stably transfected with human HA-tagged TLR4 (InvivoGen, San Diego), were cultured in DMEM, 10% FCS, 10 μ g/ml blastocidin S (InvivoGen), and 1% penicillin/streptomycin.

NE Activity Assay—NE activity was quantified prior to all experiments using the NE substrate (0.5 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide). Δ absorbance units (2 min) at 405 nm were recorded and compared with an NE standard of known activity.

Zymography—Zymography was performed on 150 μ l serum-free medium from untreated, EGF- or NE-treated cells (1×10^6). Samples of human or murine BALF containing 400 ng or 2 μ g of protein, respectively, were treated for 10 min with sample buffer (0.25% bromophenol blue, 50 mM Tris, pH 7.5, 40% glycerol, and 1% SDS) and were electrophoresed on a 7% SDS-polyacrylamide gel containing gelatin or casein (1 mg/ml). After electrophoresis, the gels were incubated in 50 mM Tris, pH 7.5, 5 mM CaCl₂, 1 μ M ZnCl₂, and 2.5% (v/v) Triton X-100 for 30 min, washed in the same buffer without Triton X-100 for 5 min and incubated at 37 °C overnight in the same buffer supplemented with 1% (v/v) Triton X-100. The gels were stained with 0.125% Coomassie Blue and washed with 10% acetic acid and 40% methanol in water.

RT-PCR Analysis for Meprin Gene Expression—RNA was isolated from 16HBE14o- and HEK293 cells in TRI reagent, and RNA was extracted. RNA (2 μ g) was reverse-transcribed into

cDNA, and 2 μ l was amplified with 1.25 units of TaqDNA polymerase, $1 \times$ PCR buffer, and 10 mM dNTPs (Promega) in a 50- μ l volume containing 100 pmol each of the following primers: Meprin alpha: 5'-GAAATCCAGAAATGGCCTGA-3', 5'-TGGAAATGTTCTGTCCCACA-3'; β -actin: 5'-GGGTACA-TGGTGGTGCCG-3', 5'-GCCGGGAAATCGTGCGTG-3'.

After a hot start, the amplification profile was 45 cycles of 1 min denaturation at 95 °C, 1 min annealing at 50 °C, and 1 min extension at 72 °C. RT-PCR amplification of meprin A and β -actin generated products of 242 bp and 402 bp, respectively. PCR products were resolved on a 1% agarose gel containing 0.5 μ g/ml ethidium bromide.

Microscopy—Immunofluorescence microscopy was performed as described previously (24) with some alterations. 16HBE14o- cells (1×10^5) were cultured overnight in chamber slides (Lab-Tek). Cells were washed and fixed with 4% (w/v) paraformaldehyde for 10 min, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 10 min and blocked with 10 mM NaBH₄ for 1 h. Cells were incubated with rabbit anti-meprin alpha (25) overnight at 4 °C, or mouse anti TLR4 FITC labeled (Abcam) and EGFR (SCBT) antibody for 1 h, washed, and incubated with goat anti-rabbit IgG secondary antibody Rhodamine labeled (Abcam) at 5 μ g/ml for 1 h. Controls for this experiment included cells alone, Rabbit IgG isotype control (R&D Systems) and those exposed to secondary antibody only.

Meprin Activity Assay—Activity assays were carried out using the fluorogenic peptide substrate Mca-YVADAPK-(Dnp)-OH according to the manufacturer's direction (R&D Systems). Briefly, to activate meprin alpha, the enzyme (100 ng) was incubated with 0.1 ng of trypsin or 100 nM of NE in TCNB (50 mM Tris, 10 mM CaCl₂, 0.15 M NaCl, 0.05% Brij 35, pH 7.5) for 3 h at 37 °C. Trypsin and NE were inactivated with AEBSE (1 mM) or CMK (5 μ M), respectively, to prevent turnover of the meprin substrate, and fluorescence was quantified at 320 nm excitation and 450 nm emission after 60 min.

IL-8 ELISA—16HBE14o- cells (1×10^5 /well) were left untreated or treated for 1 h with 5 μ g/ml EGFR ligand neutralizing antibody (R&D Systems), 500 nM AG1478 (Calbiochem), 2 μ g of EGFR neutralizing antibody (Oncogene Ab-3), or 100 μ M actinonin followed by EGF, TGF α , HBEGF, or NE for 4 h. Medium was removed and used in an IL-8 ELISA (R&D Systems). 5 μ M MetOSuc-Ala-Ala-Pro-Val-chloromethyl ketone (CMK) was added to NE-treated media to prevent active NE from interfering with IL-8 measurement.

TGF α ELISA—Cells were untreated or treated with vehicle (DMSO) or actinonin (100 μ M) for 1 h followed by NE, activated recombinant meprin alpha, or media for 4 h. For all experiments EGFR was blocked for 30 min with 2 μ g/ml anti-EGFR neutralizing antibody (Calbiochem) prior to agonist treatment. Supernatants were collected, and TGF α was measured via ELISA (R&D Systems) (14).

Bronchoalveolar Lavage Fluid (BALF)—NE gene-targeted mice were generated, as previously described (2). NE knock-out mice ($n = 3$) and their wild-type littermates ($n = 3$) were intranasally challenged with PBS (50 μ l) or PBS containing *Pseudomonas aeruginosa* H103 (4.8×10^6 CFUs). Twenty-four hours later, BALF was collected (26).

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BALF from individuals with CF ($n = 11$) or healthy controls ($n = 10$) was collected following informed consent using a protocol approved by Beaumont Hospital Ethics Committee (13). Samples were filtered through gauze, centrifuged at $1,000 \times g$ for 10 min, and cell-free supernatants were aliquoted and stored at -80°C .

EGFR trans-Activation—16HBE14o- cells were seeded at 1×10^6 on a 6-well plate for 24 h in supplemented MEM. Cells were serum-starved in serum-free MEM for 2 h prior to stimulation and then left untreated (control) or stimulated with EGF (R&D systems) (10 ng/ml) or 100 nM NE (Elastin Products, MO) for 10 min. Medium was removed, centrifuged, and stored at -20°C . Cells were washed with PBS and lysed using a radioimmune precipitation assay buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate) containing protease inhibitor tablets (Roche UK) and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were clarified by centrifugation and were used in EGFR and EGFR [pY1173] ELISAs (BIOSOURCE CA) according to the manufacturer's instructions.

Transfections—16HBE14o- cells were uniformly co-transfected for 24 h with a constitutive luciferase reporter gene construct, pRLSV40 (100 ng, Promega) and an empty vector pCDNA3.1 (150 ng, Invitrogen) or the same vector carrying a gene encoding a dominant negative functionally inactive MyD88 (150 ng) as previously reported (12, 13) using Gene Juice (Novagen). Cells were left untreated or stimulated as indicated. NF κ B activation was assessed (see below) or supernatants were retained after 24 h for IL-8 ELISA, and cells were lysed with Reporter Lysis buffer (Promega) and quantified by luminometry to determine transfection efficiencies (Wallac Victor², 1420 multilabel counter). IL-8 protein production was calculated as pg per light unit (L.U.).

293-hTLR4-HA cells (5×10^6) were transfected for 48 h with an empty vector or a plasmid expressing human EGFR (1 μg , Upstate Cat. 21-176) and left untreated or treated with agonists (as described) for 10 min prior to immunoprecipitation with an anti-HA tag (InvivoGen) or an anti-EGFR monoclonal antibody (sc-120, Santa Cruz Biotechnology) and immunodetection with either anti-HA tag or anti-EGFR monoclonal antibodies.

NF κ B Assays—NF κ B activation was assessed by three methods. (i) Cells (5×10^5) were co-transfected as described above with an (NF κ B)₅-luciferase reporter plasmid and pRLSV40 for 24 h then stimulated with meprin alpha for 6 h. Relative luciferase production was quantified by luminometry. (ii and iii) Cells were co-transfected with pCDNA3 or the Δ MyD88 expression plasmid for 24 h, then stimulated with meprin for 1 h. Nuclear extracts were prepared and examined by Western blotting using p65 and TBP antibodies (Santa Cruz Biotechnology), and p65/TBP ratios were quantified by densitometry or assessed 1 h post-stimulation with meprin using TransAM NF κ B p65 (Activ Motif) as recommended by the manufacturer.

RNAi—16HBE14o- cells (5×10^5) were transfected with 50 nM of a siRNA-targeting Meprin alpha (siRNA ID 104082, Ambion) or a scrambled control duplex (Silencer GAPDH siRNA Control Ambion) for 24 h. Cells were placed in serum-free medium and stimulated with NE (100 nM for 24h). Total

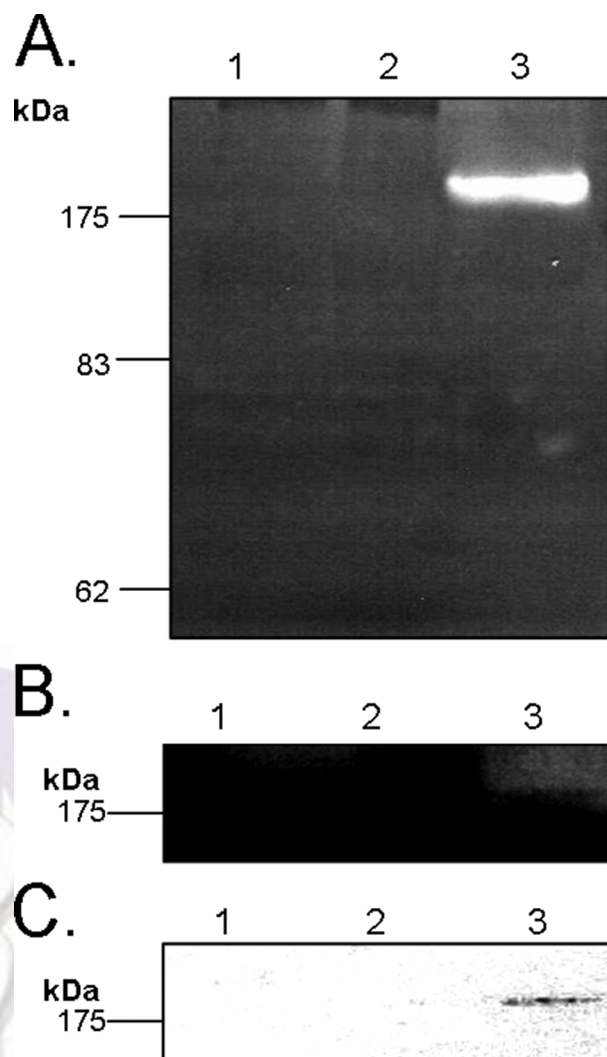


FIGURE 1. Identification of metalloproteases in media of control and stimulated bronchial epithelial cells. 16HBE14o- cells (1×10^6) were untreated (control) or treated with EGF (20 ng/ml) or NE (100 nM) in serum-free media for 10 min. Medium from treated cells (5 μg) was electrophoresed on: A, a gelatin zymogram; B, a casein zymogram; or C, electrophoresed under non-reducing conditions in SDS-PAGE, transferred to nitrocellulose, and probed with an anti-meprin alpha antibody.

RNA was extracted for qRT-PCR using the Roche LC480 and SYBR Green I Master with Meprin alpha or GAPDH (Forward: 5'-CATGAGAAGTATGACAACAGCCT-3', Reverse: 5'-AGTCCCTCCACGATACCAAAGT-3') primers (MWG Biotech) to quantify knockdown. Cell supernatants were retained for IL-8 ELISA.

Statistical Analysis—Data were analyzed with the PRISM 3.0 software package (GraphPad, San Diego, CA). Results are expressed as the mean \pm S.E. and were compared by Student's *t* test or analysis of variance. Differences were considered significant at $p \leq 0.05$.

RESULTS

Identification of Novel Protease Activity from 16HBE14o- Cells Treated with NE—In our search for novel metalloproteases activated by NE, we analyzed supernatants from untreated, EGFR ligand- and NE-treated 16HBE14o- cells by zymography. Fig. 1 shows that a high molecular weight gelatin-

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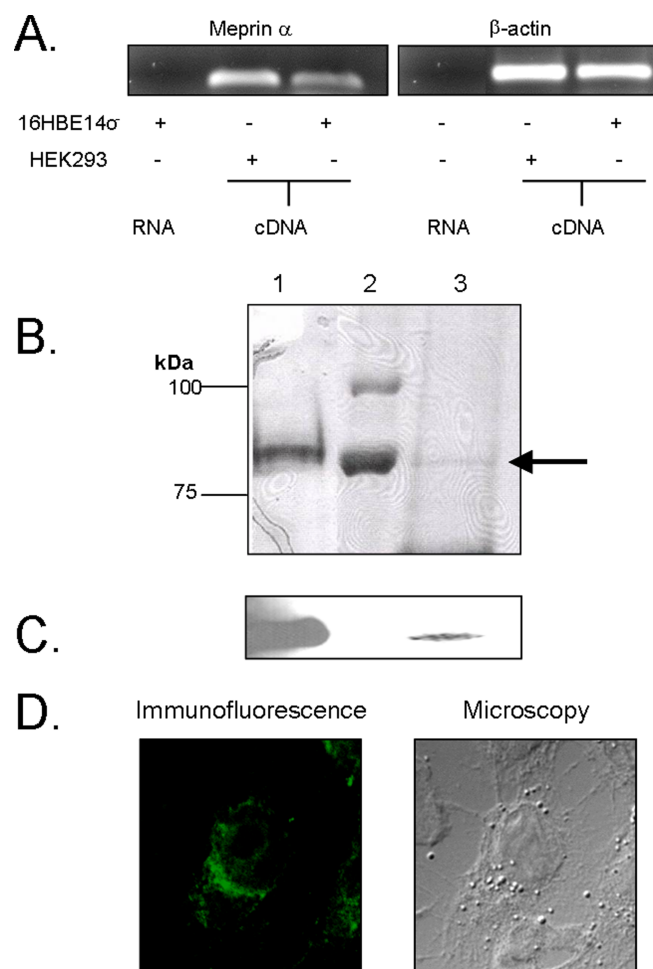
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FIGURE 2. Detection of meprin alpha mRNA and protein in 16HBE14o-cells. A, total RNA or cDNA from 16HBE14o- and HEK293 cells was used in PCR reactions with meprin alpha or β -actin primers, as indicated. Products were visualized on a 1% agarose gel. B, Coomassie Blue-stained SDS-PAGE of meprin alpha (lane 1), molecular weight marker (lane 2), and a meprin alpha immunoprecipitate from a whole cell lysate of 16HBE14o-cells (lane 3, arrow indicates IP). C, Western blot analysis of a duplicate gel probed with rabbit anti-meprin polyclonal antibody. D, fixed and permeabilized 16HBE14o-cells were incubated with primary antibody against human meprin alpha and visualized by fluorescence confocal microscopy.

ase activity, above 175 kDa, was detectable in the NE-treated supernatants but was absent from the control and EGF-treated samples (Fig. 1A).

Examination of protease activity using a casein zymogram showed this high molecular weight NE-induced gelatinase also had caseinase activity of the same size, which was not present in the control, or EGF-treated samples (Fig. 1B). Based on these properties and its migration in non-reducing PAGE, this indicated that it may be meprin; therefore, we performed immunoblot analysis using an anti-meprin alpha antibody. An immunoreactive band of the same size was detected in the NE-treated samples but was not detectable in the control or EGF-treated samples (Fig. 1C). No signals were detected in any of the samples at >175 kDa using MMP2 or MMP9 antibodies (data not shown).

Detection of Meprin Alpha Expression in 16HBE14o-Cells—To verify meprin alpha expression by 16HBE14o-cells RT-PCR, Western blotting, and immunofluorescence confocal

microscopy was performed (Fig. 2). Meprin alpha mRNA was detected in 16HBE14o-cells and HEK293 cells, a human embryonic kidney cell line known to express meprin alpha (Fig. 2A). Meprin alpha has a M_r of 84.4 kDa but because of glycosylation migrates with a molecular weight of ~90 kDa in reducing SDS-PAGE (27). Fig. 2B shows a Coomassie Blue-stained gel of meprin alpha immunoprecipitated from 16HBE14o-cells using a monoclonal antibody (lane 3). Recombinant meprin alpha was used as a control (lane 1) and molecular weight markers are in lane 2. A duplicate gel was transferred to a nitrocellulose membrane and probed with a rabbit polyclonal anti-meprin alpha antibody and an immunoreactive signal of the correct molecular weight was evident in the immunoprecipitate (Fig. 2C). Immunofluorescence and confocal microscopy were performed to visualize the location of meprin alpha in 16HBE14o-cells using a goat polyclonal anti-human meprin alpha antibody. As shown in Fig. 2D, meprin alpha expression was detected peri-nuclear and also on the cell membrane.

Meprin Alpha Activated by NE Can Induce IL-8 Expression and Release TGF α from 16HBE14o-Cells—Trypsin is a potent activator of meprin alpha. We examined whether NE, like trypsin, could activate meprin alpha using a fluorimetric activity assay. A recombinant inactive form of meprin alpha was incubated with trypsin or NE, the serine proteases were inactivated, and meprin activity was measured using a substrate that had meprin specificity. Fig. 3A shows that both trypsin and NE can activate meprin to similar levels ($p = 0.01$ and $p = 0.02$, respectively).

Having established that NE can activate meprin alpha we next evaluated whether meprin alpha, like NE, can induce IL-8 production from 16HBE14o-cells. Here cells were either untreated, treated with NE or active meprin, and IL-8 levels were quantified in cell supernatants. Fig. 3B shows that meprin alpha induced greater than 2-fold increase in IL-8 secretion compared with control cells ($p < 0.001$) similar to NE ($p < 0.001$ versus control).

NE can generate soluble TGF α from 16HBE14o-cells (Fig. 3C, $p = 0.03$). We investigated the effect of meprin alpha on proTGF α . Cells were left untreated or treated with NE, meprin alpha, or supernatants from NE-treated 16HBE14o-cells (from which TGF α had been depleted and that had also been treated with a specific NE inhibitor). These supernatants had gelatinase activity (data not shown). TGF α levels in the cell supernatants were quantified by ELISA. Fig. 3C shows that like NE, meprin alpha or NE-treated supernatants leads to release of soluble TGF α ($p = 0.01$ and 0.01 , respectively).

Inhibition of Meprin Alpha Can Impair IL-8 Production in NE-stimulated Bronchial Epithelial Cells—NE-induced IL-8 expression is TGF α -dependent and can be inhibited using a TGF α -neutralizing antibody (Fig. 4A). Neutralizing antibodies to EGF and HBEGF have no effect. EGFR is also involved in NE-induced IL-8 expression. NE can *trans*-activate EGFR (Fig. 4B) and inhibition of EGFR using AG1478 or an EGFR antibody impairs NE-induced IL-8 expression (Fig. 4C).

Actinonin is a naturally occurring hydroxamate that is the most effective inhibitor of meprin alpha (35–38). We investigated its effects on NE-induced IL-8 and TGF α production. We performed a dose response of actinonin (0–100 μ M) investigat-

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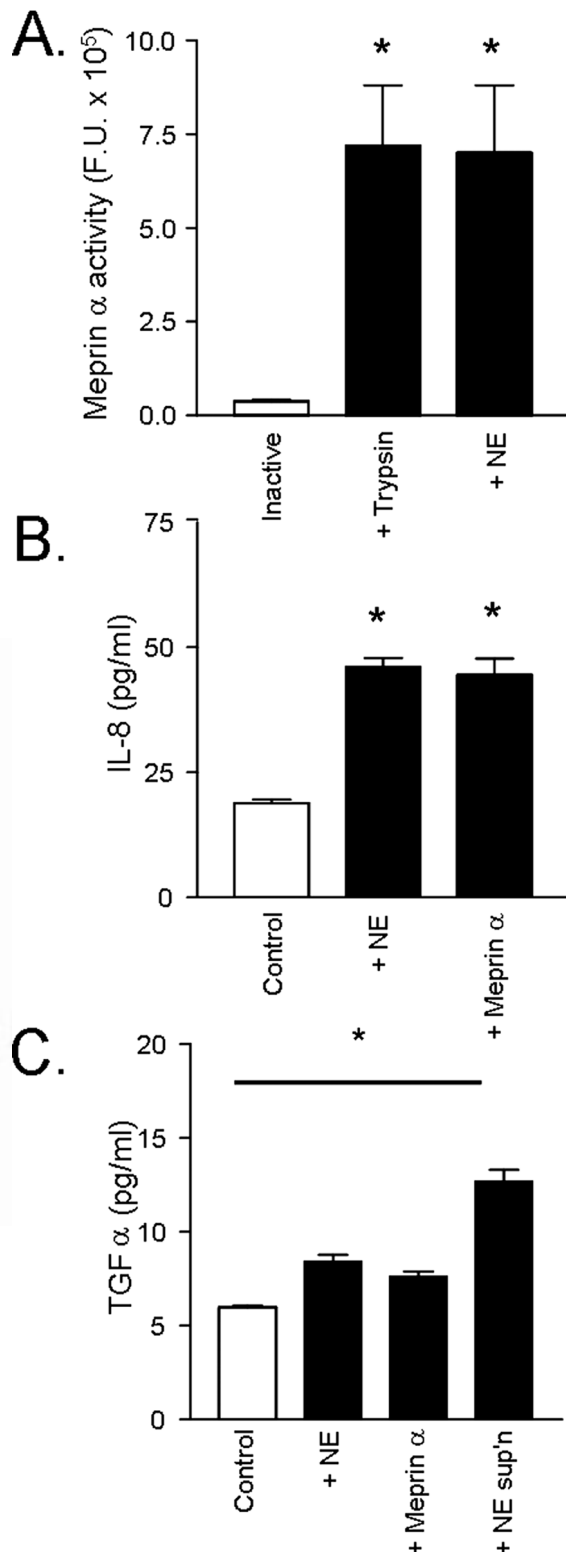


FIGURE 3. Activation of meprin alpha and meprin alpha induction of TGF α and IL-8. A, inactive recombinant meprin alpha (100 ng) was exposed to trypsin (0.1 ng) or NE (100 nM) for 3 h. Meprin activity was quantified at 320 nm/405 nm (* versus control). B, 16HBE14o- cells (1×10^5) were stimulated with NE (100 nM, 4 h) or activated meprin alpha (100 nM, 4 h). IL-8 levels in supernatants were quantified by ELISA (* versus control). C, CMK (5 μ M) was added to cell-free media from 16HBE14o- cells treated with NE (100 nM, 10 min) and used to stimulate fresh 16HBE14o- cells (1×10^5 , treated with EGFR ligand-neutralizing antibody (2 μ g/ml, 1 h)). TGF- α levels in supernatants were quantified by ELISA. NE (100 nM, 4 h) and meprin alpha (100 nM, 4 h) were also used (* versus control).

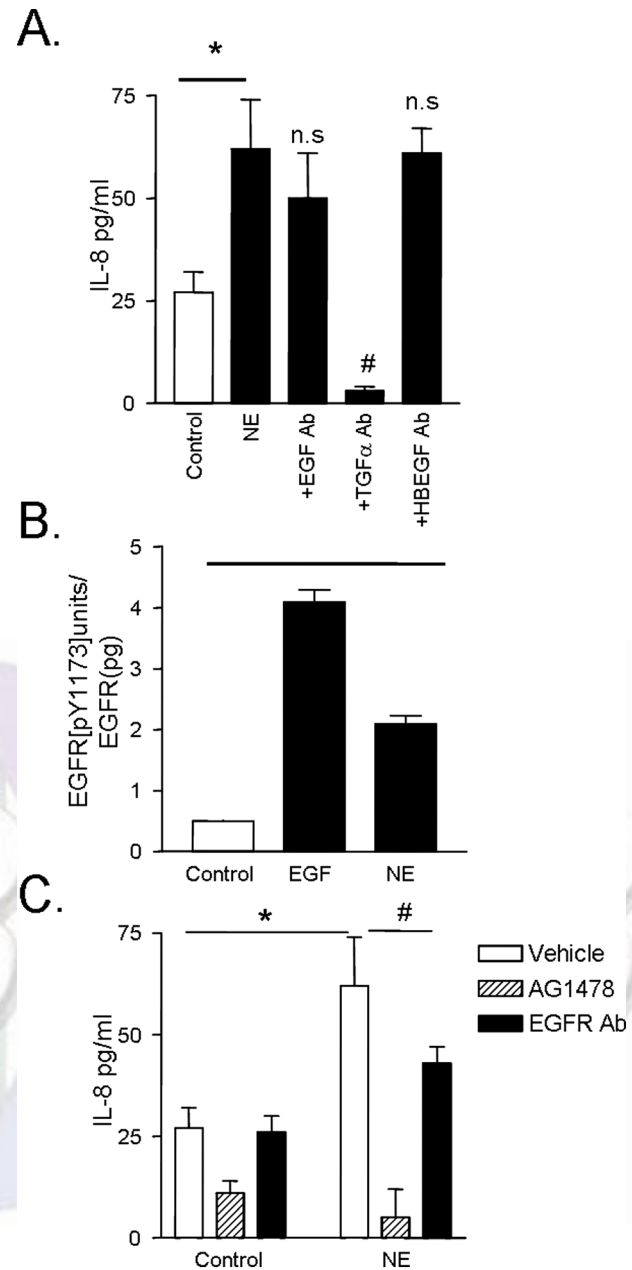


FIGURE 4. NE-induced IL-8 occurs via trans-activation of EGFR generation of TGF α in human bronchial epithelial cells. 16HBE14o- cells were (A) incubated with EGF, TGF α , or HBEGF neutralizing antibodies (2 μ g, 1 h), then stimulated with NE (100 nM, 4 h) in serum-free media. IL-8 levels in supernatants were quantified by ELISA (* versus control, # versus agonist), B, stimulated with EGF (10 ng/ml) or NE (100 nM) for 10 min and total and phospho-Y1173 levels were quantified by ELISA (* versus control). C, 16HBE14o- cells were treated with medium or DMSO (vehicle), AG1478 (500 nM, hatched bar), or an EGFR-neutralizing antibody (2 μ g, black bar) for 1 h prior to treatment with NE (100 nM, 4 h) and IL-8 levels in supernatants were quantified by ELISA (* versus control, # versus agonist).

ing its effect on NE-induced IL-8 expression in 16HBE14o- cells (data not shown), and from this selected 100 μ M as the most effective inhibitory dose. Actinonin effectively inhibited meprin alpha activity induced by either trypsin or NE ($p < 0.001$ for both) (Fig. 5A) and also significantly inhibited NE-induced IL-8 (Fig. 5B) and TGF α (Fig. 5C) production from 16HBE14o- cells (#, $p < 0.001$). To confirm that inhibition of NE-induced IL-8 expression by actinonin is mediated via block-

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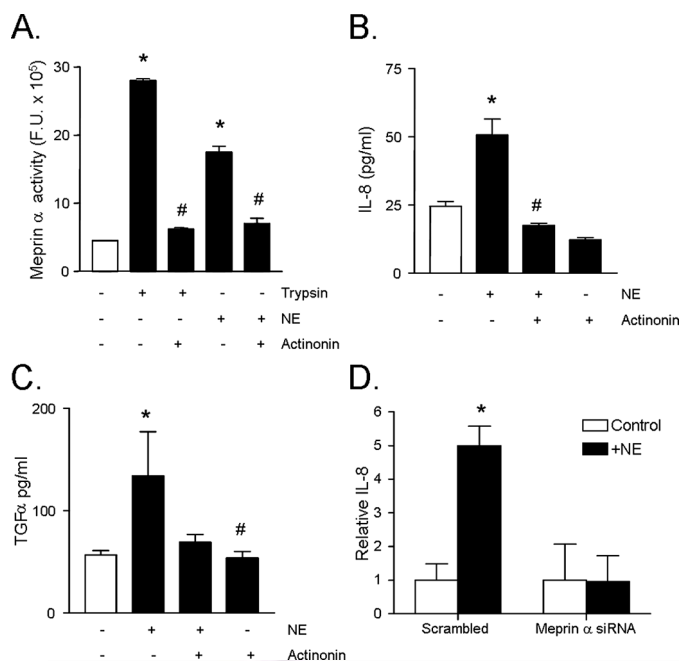


FIGURE 5. Actinonin and knockdown of meprin alpha inhibits NE-induced TGF α and/or IL-8 production. A, inactive recombinant meprin alpha (100 ng) was exposed to trypsin (0.1 ng) or NE (100 nM) for 3 h. Actinonin (100 μ M) was added as indicated. Meprin activity was quantified at 320 nm/405 nm (* versus control, # versus without actinonin). Cells (B, 1×10^5 or C, 1×10^6) were stimulated with NE (100 nM) and/or 100 μ M actinonin (30 min) and (B) IL-8 levels or (C) TGF α levels in supernatants were quantified by ELISA (* versus control, # versus agonist). D, cells (5×10^5) transfected with meprin alpha or scrambled (50 nM) siRNAs were stimulated with NE (100 nM, 6 h). RNA was isolated and used for qRT-PCR and relative IL-8 levels in supernatants are shown (* versus untreated cells). Data are representative of three independent experiments performed in triplicate.

ing meprin alpha, we next performed siRNA experiments knocking down meprin alpha (98.6% knockdown). Fig. 5D shows that NE-induced IL-8 expression is abrogated by a meprin alpha siRNA.

Presence of Meprin Alpha in Bronchoalveolar Lavage Fluid (BALF) in Lung Inflammation Correlates with NE—We next assessed the role of meprin alpha *in vivo*. Meprin activity was examined in BALF of NE knock-out and wild-type mice infected with *Pseudomonas aeruginosa*. Gelatin zymography detected a high molecular weight gelatinase activity above 175 kDa that was present in higher quantities in wild type (Fig. 6A, lanes 1–3) versus NE knock-out mice (lanes 4–6).

We also evaluated bronchoalveolar lavage fluid samples from individuals with cystic fibrosis (CF) and healthy controls for meprin activity. CF is an inflammatory lung condition associated with high levels of active NE in the lung. Fig. 6B shows evidence of a high molecular weight gelatinase activity indicative of meprin alpha in 11 of 11 BALF samples from CF individuals. No gelatinase activity was detectable in 10 control BALF samples under the same conditions. Fig. 6C shows the corresponding NE activities per μ g of protein in each of the BALF samples ($p = 0.0002$ for normal versus CF BALF). The gelatinase activity in 2 representative CF BALF samples was confirmed to be meprin alpha by Western immunoblotting (Fig. 6D).

Meprin Induces Co-localization of EGFR and TLR4—We investigated how the NE-Meprin-TGF α -EGFR-IL-8 pathway reported here integrates with our previous reports demonstrat-

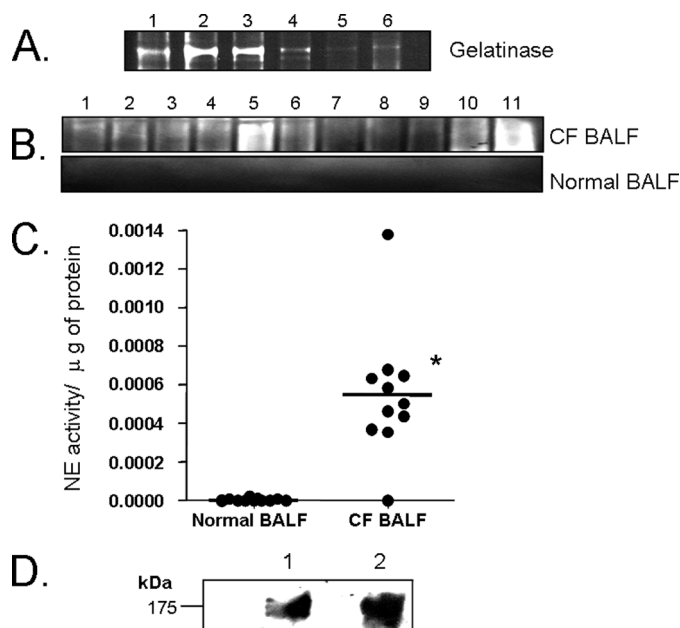


FIGURE 6. Analysis of meprin in BALF samples. A, wild-type BALF (lanes 1–3) and NE^{-/-} BALF (lanes 4–6) was applied to a gelatin zymogram (meprin activity). B, CF ($n = 11$) and normal BALF ($n = 10$) samples (400 ng/lane) were applied to a gelatinase zymogram. Lane 11 in normal BALF is blank. C, NE activity per μ g of protein was quantified in CF BALF and normal BALF samples. D, two representative CF BALF samples were electrophoresed under non-reducing conditions on SDS-PAGE, transferred to nitrocellulose, and probed with an anti-meprin alpha antibody (* versus normal).

ing the NE-TLR4-MyD88-IL-8 pathway (11, 28). In Fig. 7A we evaluated the effect of Δ MyD88, an inhibitor of TLR4 signaling, on NE- and TGF α -induced IL-8 production. 16HBE14o- cells were transfected with an empty vector or a Δ MyD88 transgene, stimulated with NE, TGF α , or LPS, and IL-8 protein production per transfected cell was quantified. NE, TGF α , and LPS all significantly increased IL-8 expression. Δ MyD88 inhibited not only NE-induced IL-8 expression but also that induced by TGF α and LPS, suggesting that EGFR and TLR4 may interact. We investigated this by performing immunoprecipitations from HEK293 cells expressing TLR4 and EGFR. Fig. 7B shows TLR4 expression (lane 1) in untreated 293-hTLR4-HA cells and EGFR expression only in those transfected with an EGFR expression plasmid (lanes 2 and 3). Immunoprecipitations using an anti-EGFR antibody followed by Western immunoblotting with either anti-HA (to detect TLR4) or anti-EGFR antibodies detected a weak interaction between TLR4 and EGFR in untreated cells (Fig. 7C, lanes 1 and 4) as indicated by the weak EGFR signal (lane 4). Treatment with NE (lanes 2 and 5) or EGF (lanes 3 and 6) enhanced the formation of this complex confirming that EGFR can be co-immunoprecipitated in a complex with TLR4 in agonist-treated cells. We confirmed the observations of these overexpression studies in real cells by confocal microscopy. Fig. 7D shows that in 16HBE14o- cells stimulated with meprin alpha endogenously expressed TLR4 and EGFR co-localize. NE induces a similar effect but also damages the integrity of the monolayer. LPS treatment did not induce co-localization of EGFR and TLR4 in 16HBE14o- cells (data not shown).

Meprin Activates NF κ B via MyD88—Finally we investigated the ability of meprin alpha to activate NF κ B. Fig. 8A shows

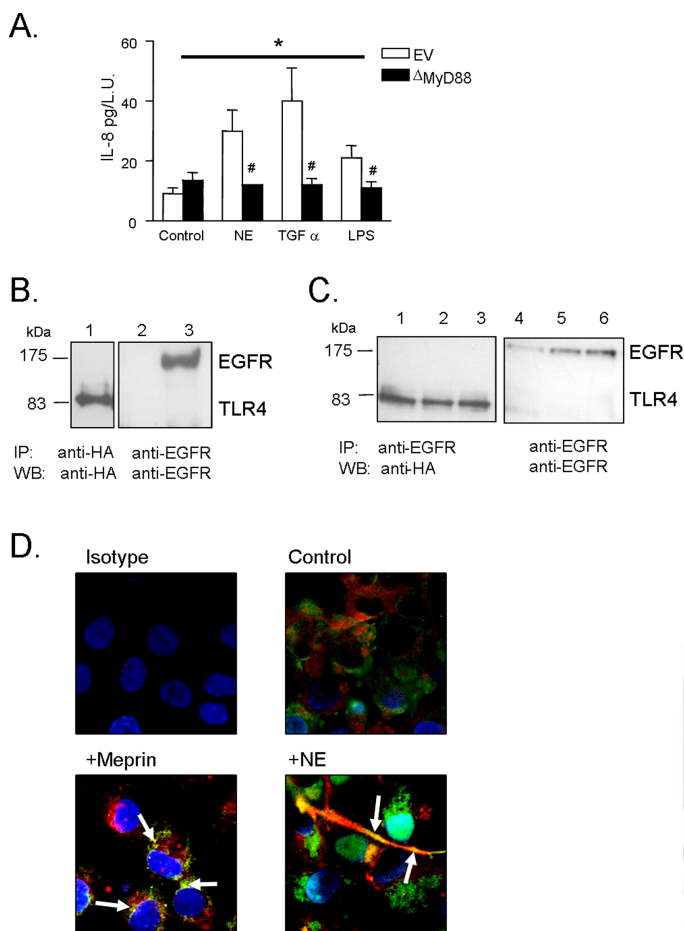
NE Activates EGFR and TLR4 via Meprin α 

FIGURE 7. Meprin and NE induce co-localization of EGFR and TLR4. A, 16HBE140 cells (1×10^5) were co-transfected for 24 h with pCDNA3.1 (EV) or a Δ MyD88 expression plasmid and a constitutive luciferase reporter plasmid. Cells were stimulated for 24 h with NE (100 nM), TGF α (10 ng/ml), or LPS (10 μ g/ml) and IL-8 protein production was quantified by ELISA. Units are represented as IL-8 pg/light unit. B, 293-hTLR4-HA cells (5×10^6) were left untreated (lane 1) or transfected for 48 h with an empty vector (lane 2) or a plasmid expressing human EGFR (lane 3). Immunoprecipitations and Western immunoblotting were performed as indicated. C, 293-hTLR4-HA cells (5×10^6) transfected with a plasmid expressing human EGFR were left untreated (lanes 1 and 4) or treated for 10 min with NE (100 nM, lanes 2 and 5) or EGF (10 ng/ml, lanes 3 and 6) prior to immunoprecipitation with an anti-EGFR monoclonal antibody followed by immunodetection with anti-HA (lanes 1–3) or anti-EGFR (lanes 4–6) monoclonal antibodies for TLR4 and EGFR, respectively. D, 16HBE140 cells were left untreated or stimulated with meprin alpha (75 nM) or NE (100 nM) for 30 min, labeled with anti-TLR4 (red) and anti-EGFR (green) or isotype antibodies, and visualized by fluorescence confocal microscopy. White arrows indicate co-localization of EGFR and TLR4.

that meprin alpha (75 nM) induces expression from an NF κ B-luciferase reporter plasmid as potently as NE (100 nM). Meprin alpha also induced nuclear localization of NF κ B in a MyD88-dependent manner. Fig. 8B shows relative p65 nuclear localization, normalized to the nuclear marker TATA-binding protein (TBP), in cells transfected with an empty vector or a Δ MyD88 transgene. p65 nuclear localization was inhibited by Δ MyD88. Fig. 8C shows the dose-dependent effect of meprin alpha on NF κ B activation using a p65 nuclear localization ELISA. The meprin alpha effect is inhibited by Δ MyD88. Meprin alpha-induced IL-8 expression is also inhibited in a similar way (Fig. 8D).

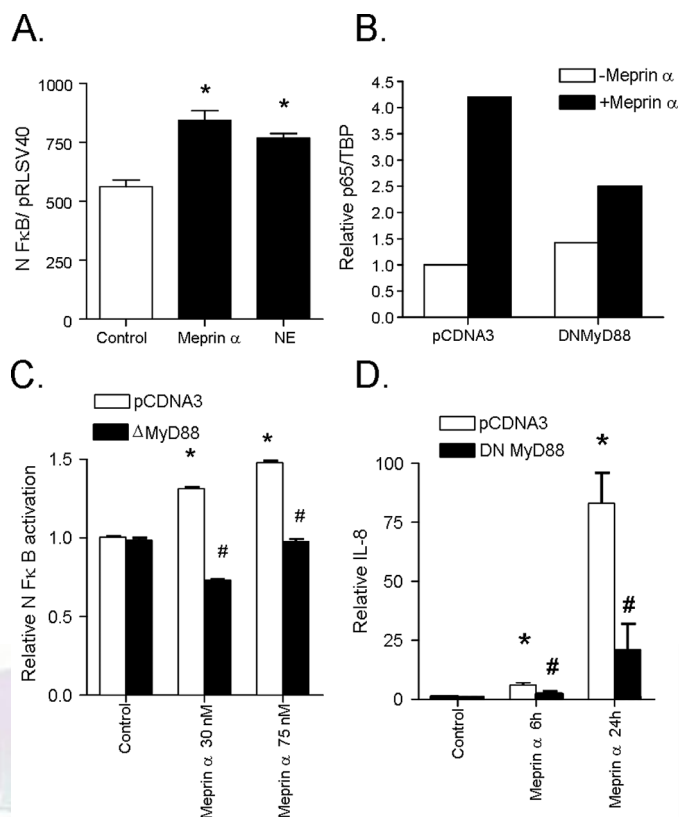


FIGURE 8. Δ MyD88 inhibits meprin-induced NF κ B activation and IL-8 expression. 16HBE140 cells (1×10^6) were co-transfected for 24 h with a constitutive luciferase reporter plasmid pRLSV40 and an inducible (NF κ B) $_5$ -luciferase reporter plasmid then stimulated for 6 h with meprin alpha (75 nM) or NE (100 nM). Relative NF κ B activity is shown (* versus unstimulated cells) (B–D) (1×10^6) were co-transfected for 24 h with a constitutive luciferase reporter plasmid pRLSV40 and pCDNA3.1 (EV) or a Δ MyD88 expression plasmid. Cells were stimulated for 1 h (B and C) or 6 or 24 h (D) with meprin alpha (75 nM in B and D or as indicated in C). Nuclear extracts were analyzed for p65 by Western immunoblotting using p65 and TBP antibodies (B), densitometry was performed, and p65/TBP ratios were calculated ($n = 3$, representative results shown) or TransAM p65 ELISA (C) (* versus control, # versus agonist). Data are representative of two independent experiments performed in triplicate. D, IL-8 levels were quantified in supernatants and normalized for transfection efficiency. * versus unstimulated cells, # versus pCDNA3. Assays were performed in triplicate ($n = 2$).

DISCUSSION

In this study, we set out to identify a new airway epithelial cell-derived metalloprotease that can participate in NE-induced IL-8 production via EGFR. Our data demonstrate that human airway epithelial cells express meprin alpha and that NE, an abundant serine protease in inflamed and infected airways, can activate meprin alpha rendering it capable of cleaving proTGF α . This in turn leads to *trans*-activation of EGFR inducing it to co-localize with TLR4. We also show that meprin alpha can activate NF κ B in a MyD88-dependent manner and induce IL-8 expression in 16HBE140 cells. Overall we establish the presence and activity of meprin alpha in NE-rich samples both *in vitro* and *in vivo* and demonstrate that meprin alpha has the capability of inducing IL-8 via EGFR. Together, the data identify meprin alpha as a new biologically active target of NE in the airways and implicate it as a potential therapeutic target for inflammatory lung disease.

The principal finding from this study is the identification of meprin alpha as an NE-activated metalloprotease capable of

NE Activates EGFR and TLR4 via Meprin α

generating soluble TGF α . TGF α is synthesized as a precursor membrane-bound protein. The mature TGF α protein is cleaved from the membrane-bound form to allow binding to EGFR (29). Previously TGF α has been implicated as a key mediator of EGFR activation via NE in mucin secretion (14, 16). Through the use of a range of EGFR ligand-neutralizing antibodies, we demonstrated that TGF α is also the key ligand involved in NE induction of IL-8 via EGFR in 16HBE14o- cells in this study. This is likely to be of particular importance in the CF lung where it is known that levels of TGF α , but not EGFR, are increased (30). Our findings complement the work of others studying the mechanism by which NE induces mucin gene expression in the NCI-H292 mucoepidermoid epithelial cell line (14, 16, 22), with NE signaling via PKC-duox1-ROS-TACE-EGFR prior to downstream signaling via EGFR.

It has been demonstrated in a TACE $^{-/-}$ cell line that EGFR activation can still occur indicating that enzymes sharing redundancy with TACE can cause EGFR activation (23). Indeed it is known that a number of proteases other than TACE are capable of controlling ectodomain shedding of membrane-bound factors that are members of the TNF family (31). With this as background, we examined the possibility that other metalloproteases can participate in NE-induced IL-8 production via EGFR. We noted the presence of a high molecular weight gelatinase present in NE-treated bronchial epithelial cell supernatants. This activity was significant when compared with the relative activities of other gelatinases in the samples assayed. Previous studies using epithelial and other cell lines have reported the presence of high molecular weight gelatinases evident on zymograms (32–34) thought to represent MMP-9 dimers. We tested this using non-reducing PAGE and Western blot analysis and failed to detect immunoreactive signals for MMP-9 above 175 kDa. Casein zymography of the samples indicated caseinase activity in NE-treated bronchial epithelial cell supernatants at the molecular weight similar to the gelatinase activity, indicating dual activity from this enzyme. Using an antibody against meprin alpha, an epithelial-derived metalloprotease with dual caseinase and gelatinase activity (39) we confirmed that the high molecular weight NE-induced activity in our samples was indeed meprin alpha (25). Indeed, the concept of a serine protease activating a metalloprotease, and thus EGFR is not unprecedented given the recent report by Rafiq *et al.* (40) describing cathepsin G regulation of EGF shedding in cardiomyocytes.

Meprin is a zinc endopeptidase belonging to the astacin family, which is composed of either homo- or heterodimers of meprin alpha, termed meprin A, or homodimers of meprin beta, termed meprin B. Meprin expression has been detected on a variety of epithelial cells (41–43), salivary glands (44), leukocytes (45), in certain cancer cells and tumor tissues (46–48) and more recently in epidermis (27). Meprins have the ability to cleave growth factors and cytokines (49, 50) and in the past were shown to have the ability to cleave TGF α on renal microvillar membranes (51). We have shown meprin alpha, similar to TACE, is capable of causing TGF α release in bronchial epithelial cell and suggest that in 16HBE14o- cells meprin alpha plays a key role in NE-induced IL-8 expression given that actinonin and meprin alpha siRNA could both inhibit induction of IL-8 by NE in these cells. In other cell

types redundancy may exist between meprin alpha and TACE. These two proteases are closely related and the TACE inhibitor Ro 32–7315 is known to also inhibit meprin alpha (37).

Meprin is a proenzyme requiring activation by removal of its N-terminal pro-peptide. In the gut trypsin is believed to perform this function (25). However, other activating proteases are likely to exist *in vivo*, since trypsin is not expressed in all meprin-expressing tissues. For example, plasmin has been shown to process pro-meprin alpha in a cell culture model of colorectal cancer (25) and kallikrein-related peptidase 4 activates meprin β (27). Here we report for the first time that NE has the potential to process pro-meprin alpha in bronchial epithelial cells suggesting that this process may occur *in vivo* at sites associated with a high NE burden. Our animal and human studies support this. NE knock-out mice produced less meprin alpha compared with wild-type mice following intratracheal instillation with *Ps. aeruginosa*. Our clinical data support this and showed that high NE levels in CF BALF correlated well with meprin alpha activity.

Previously, we have implicated the TLR4-Mal/MyD88-IRAK-NF κ B pathway in NE-induced IL-8 expression in 16HBE14o- cells (11–13, 28). The data presented here now provide direct evidence that EGFR and TLR4 engage in cross-talk not only in an overexpression system but also in human bronchial epithelial cells to activate intracellular signals leading to a change in IL-8 gene expression. Our evidence implicates both EGFR and TLR4 as members of an NE-activated signaling complex, with MyD88 participating in signaling leading to IL-8 production. We show that meprin alpha can activate NF κ B in a MyD88-dependent fashion and suggest that in response to NE, meprin alpha and/or other metalloproteases generate EGFR ligands that can then signal via EGFR and TLR4 to NF κ B activation via the MyD88 pathway. Cross-talk between EGFR and IL-1R, a member of the IL-1R/TLR family, has previously been reported in human keratinocytes (53), while a role for TLR4 in EGFR *trans*-activation has also been reported (54). Our previous studies have demonstrated TGF α and EGFR involvement in TLR2-, TLR4-, and TLR9-mediated mucin expression (52), a phenomenon that is supported by the observations of Koff *et al.* (20). Further evidence is emerging, from studies in tissues other than the lung, of TLR/EGFR co-operativity suggesting a broader role for both receptors in orchestrating cellular responses to *trans*-activating signals.

In summary, this study has demonstrated the previously unreported presence of meprin alpha, in human bronchial epithelial cells. Our data highlight meprin as a new target of NE with important functional effects and emphasizes the key role of NE as a primary therapeutic target for inflammatory lung disease including CF.

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