

## **Neutrophil elastase upregulates Cathepsin B and Matrix Metalloprotease-2 expression**

**Running title:** NE activation of Protease Expression

Patrick Geraghty,<sup>\*§</sup> Mark P. Rogan,<sup>\*§</sup> Catherine M. Greene,<sup>\*</sup> Rachel M.M. Boxio,<sup>‡</sup> Tiphaine Poiriert,<sup>‡</sup> Michael O'Mahony,<sup>\*</sup> Abderazzaq Belaaouaj,<sup>‡</sup> Shane J. O'Neill,<sup>\*</sup> Clifford C. Taggart<sup>\*</sup> and Noel G. McElvaney<sup>\*</sup>

<sup>\*</sup>Pulmonary Research Division, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin, Ireland and <sup>‡</sup>Institut National de la Santé et de la Recherche Médicale UMRS 514, IFR 53, CHU Maison Blanche, Reims, France.

<sup>§</sup> These authors contributed equally to the study

Address correspondence: Dr Clifford Taggart, Pulmonary Research Division, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland. Tel 00353 1 809 3800; Fax 00353 1 809 3808; Email: [ctaggart@rcsi.ie](mailto:ctaggart@rcsi.ie)

**Grant support:** This work was supported by the Health Research Board, The Alpha One Foundation, The Program for Research in Third levels Institutes administered by HEA, Science Foundation Ireland, Cystic Fibrosis Hope Source, Cystic Fibrosis Research Trust, Cystic Fibrosis Association of Ireland and the Royal College of Surgeons in Ireland.

**Key words:** Monocytes/Macrophages, Inflammation, Transgenic/Knockout Mice, Gene Regulation and Transcription Factors.

## **Abstract**

Neutrophil elastase (NE) activity is increased in many diseases. Other families of proteases including cathepsins and matrix-metalloproteases (MMPs) are also present at elevated levels in similar disease conditions. We postulated that NE could induce expression of cathepsins and MMPs in human macrophages. NE exposure resulted in macrophages producing significantly greater amounts of cathepsin B and latent and active MMP-2. Cathepsin B and MMP-2 activities were decreased in *Pseudomonas*-infected NE knockout mice compared to wild-type littermates. We also demonstrate that NE can activate NF- $\kappa$ B in macrophages and inhibition of NF- $\kappa$ B resulted in a reduction of NE induced cathepsin B and MMP-2. Also, inhibition of toll-like receptor-4 (TLR-4) or transfection of macrophages with dominant negative IRAK-1 resulted in a reduction of NE induced cathepsin B and MMP-2. This study describes for the first time a novel hierarchy among proteases whereby a serine protease upregulates expression of MMPs and cathepsins. This has important implications for therapeutic intervention in protease-mediated diseases.

## **Introduction**

Proteases are pivotal in a wide range of disease processes including Alzheimer's disease, cancer, metastasis, atherosclerosis and acute and chronic lung diseases. An understanding of the role played by proteases in these processes and their regulation may provide the opportunity for therapeutic intervention. The primary families of proteases released into the extracellular space following cell activation include members of the serine protease, matrix metalloprotease (MMP) and cysteinyl cathepsin groups of proteases.

Neutrophil elastase (NE) is a 29-kDa serine protease stored in azurophil granules in its active form until it is released following neutrophil exposure to inflammatory stimuli. Once released, NE is potentially fully active because it functions optimally in a neutral environment. The main intracellular physiological function of NE is the degradation of foreign organic molecules phagocytosed by neutrophils (1). NE can degrade almost all extracellular matrix and key plasma proteins, protease inhibitors and several proteases (2, 3). One of the most prominent families of proteases cleaved by NE are the MMP group of proteases. Serine proteases (NE, cathepsin G and proteinase-3) have been shown to activate latent-MMP-2 involving membrane-type 1 matrix metalloproteinase (MT1-MMP) expression (4). MMP-2 activation by serine proteases was blocked by the elastase inhibitor  $\alpha$ 1-antitrypsin but not by an MMP inhibitor (4).

MMP's are produced by a wide variety of cell types including epithelium, fibroblasts, neutrophils and macrophages. MMP-2 is secreted as an inactive, 72-kDa zymogen and is extracellularly activated by proteolytic cleavage, involving MT1-MMP binding to MMP-2 on the cell membrane in a multimeric complex with TIMP-2 (5). The transcriptional regulation of MMP-2 is not well characterized but several factors have

been implicated in its regulation, e.g. TGF- $\beta$  (6), intracellular calcium levels (7, 8), insulin-like growth factor-I (9), laminin and vitronectin (10, 11).

Macrophages synthesise another group of destructive proteases called cysteinyl cathepsins (12-14). Several expression patterns for cathepsins have been identified in different tissues. Cathepsin B is abundant and widely expressed in various human tissues and cells including cancer cells (15, 16). We have shown previously that cathepsins cleave and inactivate key innate immunity proteins including human beta defensins (hBD) 2 and 3 (17), Secretory leucoprotease inhibitor (SLPI) (18) and lactoferrin (19). A number of cytokines including Interferon (IFN)- $\gamma$ , interleukin (IL)-6 and IL-13 as well as bacterial products activate cathepsin expression (20).

The ability of proteases to activate gene expression is well documented in the literature. NE, cathepsin G and proteinase 3 can activate human gingival fibroblasts to produce IL-8 and monocyte chemoattractant protein 1 (MCP-1) through protease-activated receptors (PAR) -2 *in vitro* (21). NE and Cathepsin G cleave the peptide corresponding to the N terminus of PAR-2 with exposure of its tethered ligand (21). In human lung epithelial cells, NE and cathepsin G deactivate PAR-2 by proteolysis of the extracellular domain downstream from the trypsin cleavage/activation site (22). However, NE does not activate PAR-1 in human blood mononuclear cells (23). We have previously demonstrated that NE upregulates IL-8 gene expression in human bronchial epithelial cells (HBEs) via a non-PAR-2 pathway (24). IL-1 receptor-associated kinase (IRAK-1), MyD88 and TRAF-6 were shown to be involved in NE-induced NF- $\kappa$ B activation and subsequent IL-8 expression. This pathway transduces signals of the IL-1 receptor (IL-1R)/toll-like receptor (TLR) superfamily but not PARs. There are 11 TLR family

members which recognise bacterial and viral antigens leading to an immune response (25) and we have further demonstrated that IL-8 up-regulation by NE occurs in part through the cell surface membrane bound TLR-4 (26).

In this study we describe for the first time a novel hierarchy among proteases whereby the serine protease, NE up-regulates expression of MMP-2 and the cysteinyl protease cathepsin B. Furthermore, knockout studies of NE demonstrated that during *Pseudomonas* infection the presence of NE is necessary for the activities of these other major protease groups. Inhibition of NF- $\kappa$ B or TLR-4 activity or transfection of macrophages with dominant-negative IRAK-1 causes a reduction of NE induced cathepsin B and MMP-2 expression. Such regulation by a protease of other proteases from different families implies the existence of a protease cascade that has important implications as to how proteases function in immune responses, tissue development, repair and disease with wide ranging implications for many health and disease states.

## **Methods**

### **Culture and stimulation of monocyte cells**

Myelomonocytic cells (U937) (European Collection of Cell Cultures Health Protection Agency, Salisbury, Wiltshire, UK) were cultured in RPMI 1640 medium (Gibco) and were differentiated to macrophage-like cells for 48 hours with phorbol myristic acetate (PMA). The macrophage-like cells were incubated in fresh medium for a further two days before stimulation. An hour prior stimulation, cells were washed and incubated in serum-free medium. Stimulation was performed with NE (Low-endotoxin elastase derived from human sputum (approx. 50% active), Elastin Products, Owensville, MO, USA) at doses of 0, 16, 66, 166, 333 and 500 nM for 30 minutes and cultured in fresh serum-free medium for either 3 h or 24 hours before harvesting, pending if needed for RNA or protein isolation, respectively. NE activity levels were examined before and following stimulation to cells, and serum-free media, antibodies, PBS and all buffers added to cells used in sub-sequential experiments were found not to reduce NE activity. Methoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethyl Ketone (CMK) treated NE was used as a negative control. Cells were also treated for 1 hour with SN50 and its inactive control, SN50M, (Calbiochem) or with mouse anti human CD284 antibody (AbD serotec) and mouse IgG2a (R&D Systems) prior to NE stimuli to block NF- $\kappa$ B activity or TLR-4, respectively. The SN50 peptide contains the nuclear localization sequence of NF- $\kappa$ B p50 and thereby inhibits translocation of the NF- $\kappa$ B active complex into the nucleus.

### **Isolation of PBMCs**

Mononuclear cells were also isolated from heparinised venous peripheral blood obtained from healthy volunteers as described (27). Briefly, density gradient centrifugation was carried out in Ficoll-Paque (Pharma Biotech, Uppsala, Sweden) to separate the red cell pellet containing the neutrophil population from the monolayer. The mononuclear cell band was aspirated and washed three times in serum-containing RPMI medium before culture. Monocytes were enriched from the mononuclear fraction by selectively attaching them to 24- or 12-well plates for 60 min at 37°C. Monocytes were purified to 97% purity using the EasySep human CD14 selection cocktail as recommended by manufactures (StemCell Technologies, London England). Monocytes were then cultured in RPMI containing 40% autologous serum, penicillin G (final concentration 100 U/ml), and streptomycin sulfate (final concentration 100 µg/ml) at 37°C in a 5% CO<sub>2</sub> atmosphere for 9 days (28). An hour prior to stimulation, cells were washed and incubated in serum-free medium. Stimulation was performed with NE (150 nM) for 30 minutes and cultured in fresh serum-free medium for 24 hours before harvesting.

### **Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)**

After treatment, cells were harvested in Tri reagent (Sigma-Ireland) and RNA was extracted as detailed in the manufacturer's protocol. RNA (2 µg) was reverse-transcribed at 37°C with 1 mM deoxynucleotide mix (Promega, Southampton, UK), 1.6 µg oligo-p[dT]15 primer (Roche, Lewes, UK) and 1 µl M-MLV reverse transcriptase (Promega, Southampton, UK) in a 20 µl volume as described in the manufacturer's protocol. 2 µl of each cDNA was amplified with 1.25 U Taq DNA polymerase, 1×PCR buffer and 10 mM dNTPs (Promega) in a 50 µl volume containing 100 pmol each of the following primers:

5'- ATG TGG CAG CTC TGG GCC T-3' and 5'-TAC TGA TCG GTG CGT GGA ATT-3' for cathepsin B; 5'-GCC CCC AAA ACG GAC AAA GA-3' and 5'-TCC CAA GGT CCA TAG CTC ATC G-3' for MMP-2; 5'-AAC TCT GGT AAA GTG GAT-3' and 5'-TAC TCA GCG CCA CCA GCA TCG-3' for GAPDH. PCR products were quantified densitometrically at cycle numbers between 10 and 40 to determine the appropriate cycle number at which exponential amplification of products was occurring, and to identify the cycle number at which sufficient discrimination was possible to accurately quantify increases or decreases in gene expression. After a hot start the amplification profile was 32 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C. RT-PCR amplification of cathepsin B, MMP-2 and GAPDH generated products of 1004 bp, 525 bp and 211 bp respectively. PCR products were commercially sequenced (MWG Biotech AG, Ebersberg, Germany) to verify gene identity. PCR products were resolved on a 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma). The ratio of PCR fragment intensities of cathepsin B and MMP-2 relative to GAPDH was determined by densitometry.

### **NE Knockout mouse analysis**

NE gene-targeted mice were generated as previously described (29). NE knockout mice and their wild type littermates (n=3/genotype) were intranasally challenged with PBS (50 µl) or PBS containing *P. aeruginosa* H103 ( $4.8 \times 10^6$  CFUs). Twenty four hours after, mouse lungs were lavaged with PBS and the protein concentration of the lavages was determined as previously described (30). The lungs were processed for histology and immunohistochemistry. Briefly, lungs were inflated with 10% formalin in PBS. The excised lungs were then immersion-fixed with 10% buffered formalin overnight,



dehydrated, embedded in paraffin, and cut into 5- $\mu$ m sections. Serial lung tissue sections were deparaffinized, rehydrated, and H&E stained. Lung sections were stained for cathepsin B and MMP-2 with anti-mouse cathepsin B (R&D Systems) and anti-mouse/rat MMP-2 antibody (R&D Systems), using the Cell and Tissue HRP-DAB system (R&D Systems).

### **Presence of cathepsin B**

Cathepsin B activity was determined from either medium taken from macrophage-like cells 24 hours after stimulation with or without NE or BAL. Cathepsin B activity was determined in 100  $\mu$ l of each sample using the substrate Z-Arg-Arg-AMC (0.1 mM). A cathepsin B inhibitor CA-074 (10  $\mu$ g/ml) was used as a control for the specificity of the cathepsin B substrate. The reaction buffer used for cathepsin B activity estimation was 0.2 M sodium acetate, 2mM EDTA, 1 mM DTT, 1  $\mu$ M pepstatin, and 2 mM Pefabloc, pH 5.5. The samples were incubated with substrate for 60 min at 37°C, and fluorescence (substrate turnover) was determined by excitation at 355 nm and emission at 460 nm. Results were expressed as a change ( $\Delta$ ) in fluorescence units over a 60-minute period (FU).

### **Zymography**

Gelatin zymography was performed on medium collected from unstimulated or NE stimulated cells and BAL samples. Samples were subjected to 7% SDS-polyacrylamide gel electrophoresis with a gel-containing gelatin (1mg/ml). After electrophoresis was performed gels were incubated in 50mM Tris (pH 7.5), 5mM CaCl<sub>2</sub>, 1 $\mu$ M ZnCl and 2.5% (v/v) Triton X-100 for 30 minutes. The gels were washed in the same buffer without the

Triton X-100 for 5 minutes and then 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. The presence of MMPs appears as transparent bands. Latent MMP-2 and active MMP-2 were observed at 72 and 66kDa, respectively. Densitometry was carried out to compare the intensity of the MMP transparent bands.

### **Preparation of Subcellular Fractions**

U937 cells were activated with NE and nuclear and cytoplasmic extracts were isolated. Briefly cells were washed and resuspended in 1 ml of ice-cold PBS and kept on ice for 5 min. Cells were lifted from plates with a cell scraper and pelleted by centrifugation at 10,000 rpm for 5 min at 4 °C. The supernatant was removed, and the cell pellet was resuspended in 1 ml of hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM dithiothreitol) (Sigma). Cells were pelleted by centrifugation at 14,000 rpm for 10 min at 4 °C and then lysed for 10 min on ice in 20 µl of hypotonic buffer containing 0.1% Igepal CA-630. Lysates were centrifuged as before, and the cytoplasmic extract was removed. The remaining nuclear pellet was lysed in 15 µl of lysis buffer (20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF) (Sigma) for 15 min on ice. After centrifugation at 14,000 rpm for 10 min at 4 °C, nuclear extracts were removed into 35 µl of storage buffer (10 mM Hepes (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 0.5 mM PMSF, and 0.5 mM dithiothreitol). Protein concentrations of cytoplasmic and nuclear extracts were determined, and extracts were stored at -80 °C until required for use.

## **Western blot**

Cytoplasmic fractions from macrophages were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Sigma-Aldrich) and this was probed using rabbit anti-GAPDH (Santa Cruz) and mouse anti-IRAK-1 antibody (BD Transduction Labs). Binding was detected using the appropriate horseradish peroxidase-conjugated secondary antibody and visualised by chemiluminescence (Pierce).

## **IL-8 and NF- $\kappa$ B activity ELISA**

IL-8 protein concentrations in the cell supernatants were determined by enzyme-linked immunosorbent assays (R&D Systems). The effect of NE on NF- $\kappa$ B activity was determined using the TransAM NF- $\kappa$ B ELISA (Active Motif), using nuclear protein fractions.

## **Dominant negative IRAK-1 transfection**

U937 cells were seeded at  $1 \times 10^5$  on 12-well plates in the presence of PMA for 48 h and were incubated in fresh medium for a further two days before transfection. Transfections were performed with JetPei transfection reagent (Polyplus-transfection) using 1  $\mu$ g of an *Renilla* luciferase reporter gene, pRLSV40. In combination with the luciferase reporter gene, dominant-negative expression vector IRAK-1 $\Delta$  (gift from Tularik), was cotransfected into the cells. IRAK-1 $\Delta$  is a truncated death domain-containing N terminus version of the IRAK-1 protein that lacks the kinase-binding domain. The total amount of DNA introduced into the cells was kept constant by supplementation with the relevant empty vectors. Transfection efficiencies were quantified using a *Renilla* luciferase vector

(Promega). Transfections were left untreated for 24 hours and were stimulated with NE (as before). After 24 h, supernatants were recovered for cathepsin B and MMP-2 activity estimation. Cells were lysed with Reporter Lysis Buffer (Promega), protein concentrations were determined, and reporter gene activity was quantified by luminometry on a Wallac Victor<sup>2</sup> 1420 multilabel counter (PerkinElmer) using the Promega luciferase assay system. Data are expressed as the relative luciferase activity  $\pm$  SE.

### **Densitometric analysis**

Gels were analysed by densitometry and compared in a semiquantitative manner using the GeneGenius Gel Documentation and analysis system (Cambridge, UK) and GeneSnap and GeneTools software. All expression values were verified by at least three independent experiments.

### **Statistical analysis**

Data were analyzed with the PRISM 3.0 software package (GraphPad, San Diego, CA). Results are expressed as the mean  $\pm$  SE and were compared by *t* test. When more than 2 groups were being compared an ANOVA test was used, followed by a Tukeys post hoc test. Differences were considered significant at  $p \leq 0.05$ .

## Results

### Neutrophil Elastase induces Cathepsin B and MMP-2 release from Macrophages

We hypothesised that NE could induce a protease cascade. To test this hypothesis, the effect of NE on macrophage protease gene expression was examined. U937 differentiated-cells were exposed to NE (0, 16, 66, 166, 333, 500 nM) for 30 minutes in serum-free medium before removing the NE and incubating the cells for a further 3 hours. Cathepsin B and MMP-2 mRNA expression levels were investigated by RT-PCR (Figure 1A) and were observed to significantly increase when cells were stimulated with NE at concentrations of 166nM and higher ( $P=0.02$ ,  $<0.01$  and  $<0.01$  for cathepsin B and  $P=0.02$ ,  $0.01$  and  $<0.01$  for MMP-2 expression between control cells (0nM) and cells stimulated with 166, 333 and 500nM NE, respectively). Cathepsin B and MMP-2 activities were measured in the supernatants 24 hours after NE stimulation and elevated cathepsin B and MMP-2 activity was observed in NE treated cell supernatants compared to non-stimulated control cells (Figure 1B and 1C). Both latent and active MMP-2 were significantly different to the control in the presence of NE (166nM or greater). NE was also observed to activate MMP-9 (data not shown), as described previously (31). NE treated with CMK prior to incubation with cells resulted in no increase in cathepsin B or MMP-2 activation (data not shown) showing that the effect by NE on cathepsin B and MMP-2 expression is dependent on its activity.

### Protease profile from NE-stimulated peripheral blood monocyte-derived macrophages

To investigate this increase in macrophage protease production, monocyte-derived macrophages (MDM) extracted from the blood of healthy volunteers was exposed to NE.

Increased cathepsin B and MMP-2 gene expression (figure 2A) were again observed following stimulation of MDM with NE in serum-free media, as before. Protease activity levels were also increased as before (cathepsin B -figure 2B, P=0.03 and MMP-2 - figure 2C) following stimulation of MDM.

### **Cathepsin B and MMP-2 activity in NE<sup>+/+</sup> and NE<sup>-/-</sup> mice**

A deficiency of NE could alter the production of cathepsin B and MMP-2 responses. To investigate this, cathepsin B and MMP-2 activities were measured in BAL fluid from NE<sup>+/+</sup> and NE<sup>-/-</sup> mice intravenously challenged with *P. aeruginosa*. Mice possessing NE (NE<sup>+/+</sup>) produced greater quantities of cathepsin B (Figure 3A, P=0.03) and MMP-2 (latent and active MMP-2, P= 0.02 and 0.02, respectively) than knockout mice (NE<sup>-/-</sup>) (Figure 3B). Analysis of lung tissue from NE<sup>+/+</sup> and NE<sup>-/-</sup> mice for cathepsin B and MMP-2 expression, by immunohistochemistry, further examined these protease levels confirmed greater levels of positive staining for cathepsin B and MMP-2 in NE<sup>+/+</sup> mice compared to NE<sup>-/-</sup> mice (Figure 3C-3F).

### **IRAK-1 degradation, NF- $\kappa$ B activation and IL-8 protein production in U937 macrophages stimulated with NE**

Time course studies demonstrated that 100 nM NE induced maximum NF- $\kappa$ B activation at 30 min (Figure 4B). NE-induced NF- $\kappa$ B nuclear translocation was increased 5-fold compared with control. Western blotting of cytoplasmic extracts was performed using anti-IRAK-1 antibody. Stimulation with NE resulted in degradation of IRAK-1 (Figure 4A). Our group has previously shown that NE induces IL-8 gene up-regulation in bronchial epithelial cells through an IRAK signaling pathway resulting in nuclear translocation of NF- $\kappa$ B (24). NE-induced IL-8 protein levels in cell supernatants from

U937s were quantified by enzyme-linked immunosorbent assay (Figure 4C). U937s produced a mean basal level of IL-8 of  $347.6 \pm 51.89$  pg/mg of protein. Dose-response experiments demonstrated that 100 nM NE induced maximal IL-8 protein production from U937 cells, increasing IL-8 levels to  $1002 \pm 122.9$  pg/mg of protein ( $P=0.01$ ).

**Inhibition of NF- $\kappa$ B, TLR-4 or transfection of dominant negative IRAK-1 leads to a reduction of NE induced cathepsin B and MMP-2**

SN50, a cell-permeable peptide that inhibits NF- $\kappa$ B nuclear translocation downstream of IKK, and its mutant peptide, NF- $\kappa$ B SN50M, were used to investigate whether inhibition of NF- $\kappa$ B could reduce NE-induced protease expression. SN50 was able to prevent the effects of NE on cathepsin B (Figure 5A,  $P=0.02$ ) and MMP-2 (Figure 5B) protein activity, demonstrating that NE signals via NF- $\kappa$ B to induce cathepsin B and MMP-2 expression in macrophages. Inhibition of TLR-4 with the aid of mouse anti human CD284 was also able to prevent the effects of NE on cathepsin B (Figure 5C,  $P=0.01$ ) and MMP-2 (Figure 5D) protein activity. Transfection of dominant negative IRAK-1 also lead to a reduction in NE-induced protease expression (Figure 6,  $P<0.01$  for cathepsin B). The empty vector had no effect on the effects of NE on cathepsin B and MMP-2.

## Discussion

Elevated levels of proteases are typically observed at many sites of inflammation leading to a multitude of effects including tissue destruction, tissue remodelling and cleavage of soluble innate factors. We have previously shown that NE can induce expression of IL-8 via the NF- $\kappa$ B pathway of activation in HBE (24). We postulated that increased extracellular NE activity may induce expression of other proteases such as cathepsins and MMPs, which have previously been demonstrated to be present along with NE in conditions such as emphysema and cystic fibrosis (CF) (17, 32). This study demonstrates that NE can induce increased cathepsin B and MMP-2 expression and activity in macrophages. Previous studies have observed increased levels of protease activation in the presence of raised NE levels (4, 33-36) but have not demonstrated corresponding increased protease gene expression. This study provides molecular and animal model data that supports the view that NE presides over a novel hierarchy in protease regulation. Cathepsin B and MMP-2 gene expression and activity were both increased in macrophages exposed to NE. Increased cathepsin B and MMP-2 levels were observed in wild-type mice compared to NE-knockout mice intravenously challenged with *P. aeruginosa*. This study illustrates a potential novel method for NE to cause tissue destruction particularly in diseases associated with high NE burden.

Increased levels of NE have been demonstrated in many disease processes characterised by an inflammatory response (34, 35). It is estimated that approximately 250mg of NE is turned over kilogram of body weight per day in normal individuals, demonstrating the requirement for a large anti-NE protective screen in the body (37). NE driven diseases also tend to exhibit reduced levels of anti-protease levels (18). Shapiro *et*



*al* (38) postulated that NE is meant to function within the cell or perhaps at the cell surface where it has a role in intracellular killing of Gram-negative bacteria and that free NE in the extracellular space is pathological. These investigators postulated that this could occur during inefficient apoptosis or due to the inability of macrophages to clear dead neutrophils. It is also possible that extracellular NE is released as a result of receptor-mediated degranulation responses in neutrophils. Our data demonstrating that NE can stimulate cathepsin B and MMP-2 gene expression and activation are supportive of the theory that free NE in the extracellular space can indeed be pathological.

Cathepsin B has previously been shown to induce emphysema in experimental models of emphysema (20, 39). Zheng *et al* (20) have shown that cathepsins are released in response to cigarette smoke. Cathepsin B release in smoking-related lung disease results in degradation of the extracellular matrix and emphysema. Our study demonstrated that extracellular NE is not only required to activate cathepsin B (33) but causes increased expression of the gene. The predominant form of cathepsin B (Mr 42,000) is converted to an active form (Mr 38,000) upon treatment with NE (31, 33). We have also previously shown that cathepsin B can inactivate important respiratory tract innate immune proteins such as SLPI, hBD-2/3 and lactoferrin (17, 19, 32). In this study we show a novel pathway for cathepsin upregulation. Cathepsin B released in response to stimulation by NE causes may cause degradation of the extracellular matrix, generating the emphysema seen in lung disease, as well as impact on the function of important antimicrobial proteins and peptides. It has been well documented that NE-burden conditions like cardiopulmonary bypass demonstrate increased plasma levels of NE and MMPs, which cause pulmonary injury. Inhibition of both NE and MMPs in this condition

can prevent pulmonary injury (35, 40) and, interestingly, increased MMPs levels correlate with NE levels in CF patients (34).

MMPs are up-regulated during allergic inflammation but participate in the formation of many lung diseases (20) (41). Previously, NE has been shown to activate MMP-9 (31). MMP-9 and MMP-12 have been implicated in the pathogenesis of chronic lung injury, particularly in emphysema. This is shown in MMP-12 knockout mice, which do not develop air space enlargement in response to smoke exposure (42). We observed an increase in active MMP-9 but MMP-9 gene expression was unchanged (data not shown) unlike NE activation of MMP-2 was observed to occur at the level of gene expression. MMP-2 has an important anti-inflammatory role, playing a central role in the IL-13-dependent regulatory loop that has been shown to be responsible for dampening airway inflammation (43). Parenchymal inflammatory cells egress into the airway lumen in an MMP2-dependent manner and MMP-2<sup>-/-</sup> mice are also more susceptible to lethal asphyxiation using a model of allergic inflammation, indicating the importance of MMP-2 in leukocyte infiltration (43).

Previously, NF- $\kappa$ B has been shown to mediate cathepsin B and MMP-2 activation by doxorubicin treatment (44) and LPS (45), respectively. Interestingly, our study shows that inhibition of the NF- $\kappa$ B pathway (with SN50) will result in decreased cathepsin B and MMP-2 expression. Furthermore, a TLR-4 neutralizing antibody or transfection of macrophages with dominant negative IRAK-1 abrogates NE-induced cathepsin B and MMP-2 expression. We have therefore demonstrated that NE induces IL-8, cathepsin B and MMP-2 production through an IRAK-1/TLR-4 mediated pathway in macrophages. PAR-2 has been shown to cause activation of NF- $\kappa$ B in human keratinocytes resulting in

upregulation of cell adhesion molecules such as ICAM-1 (46). However PAR-2 has not been shown to interact with TLR-4/IRAK-1 pathway, thereby indicating that PAR-2 does not play a role in the NE-induced protease production observed in this study. The role of TLR-4 in the NE activation of cathepsin B and MMP-2 by macrophages is still unclear and further research into this area may be beneficial.

The data in this manuscript demonstrates that extracellular NE can induce a protease cascade involving cathepsin B and MMP2 expression. Elucidation of such a hierarchy in protease control and regulation coupled with identification of key protease/proteases central to direct tissue destruction or activation of other proteases represents an important advancement in protease biology. This would greatly enhance our understanding of these proteases and could lead to potential new therapeutic strategies to treat protease-mediated diseases. Neutralization of NE activities may be sufficient to lessen the overall protease burden without the need for inhibition of all proteases. Investigating the effect of other serine proteases on expression levels of different protease families may highlight other areas of interest.

### **Disclosures**

The authors have no conflicting financial interests.

## References

1. Janoff, A., and J. Scherer. 1968. Mediators of inflammation in leukocyte lysosomes. IX. Elastinolytic activity in granules of human polymorphonuclear leukocytes. *J Exp Med* 128:1137-1155.
2. Abe, H., K. Okajima, H. Okabe, K. Takatsuki, and B.R. Binder. 1994. Granulocyte proteases and hydrogen peroxide synergistically inactivate thrombomodulin of endothelial cells in vitro. *J Lab Clin Med* 123:874-881.
3. Klingemann, H.G., R. Egbring, F. Holst, M. Gramse, and K. Havemann. 1982. Degradation of human plasma fibrin stabilizing factor XIII subunits by human granulocytic proteinases. *Thromb Res* 28:793-801.
4. Shamamian, P., J.D. Schwartz, B.J. Pocock, S. Monea, D. Whiting, S.G. Marcus, and P. Mignatti. 2001. Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. *J Cell Physiol* 189:197-206.
5. Toth, M., M.M. Bernardo, D.C. Gervasi, P.D. Soloway, Z. Wang, H.F. Bigg, C.M. Overall, Y.A. DeClerck, H. Tschesche, M.L. Cher, S. Brown, S. Mobashery, and R. Fridman. 2000. Tissue inhibitor of metalloproteinase (TIMP)-2 acts synergistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (Membrane type 1)-MMP-dependent activation of pro-MMP-2. *J Biol Chem* 275:41415-41423.
6. Graham, C.H., I. Connelly, J.R. MacDougall, R.S. Kerbel, W.G. Stetler-Stevenson, and P.K. Lala. 1994. Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. *Exp Cell Res* 214:93-99.
7. Kermer, P., N. Klocker, M. Labes, and M. Bahr. 2000. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 In vivo. *J Neurosci* 20:2-8.
8. Luca, M., S. Huang, J.E. Gershenwald, R.K. Singh, R. Reich, and M. Bar-Eli. 1997. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am J Pathol* 151:1105-1113.
9. Yoon, A., and R.A. Hurta. 2001. Insulin like growth factor-1 selectively regulates the expression of matrix metalloproteinase-2 in malignant H-ras transformed cells. *Mol Cell Biochem* 223:1-6.
10. Reich, R., M. Blumenthal, and M. Liscovitch. 1995. Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells. *Clin Exp Metastasis* 13:134-140.
11. Seftor, R.E., E.A. Seftor, K.R. Gehlsen, W.G. Stetler-Stevenson, P.D. Brown, E. Ruoslahti, and M.J. Hendrix. 1992. Role of the alpha v beta 3 integrin in human melanoma cell invasion. *Proc Natl Acad Sci U S A* 89:1557-1561.
12. Turk, D., M. Podobnik, R. Kuhelj, M. Dolinar, and V. Turk. 1996. Crystal structures of human procathepsin B at 3.2 and 3.3 Angstroms resolution reveal an interaction motif between a papain-like cysteine protease and its propeptide. *FEBS Lett* 384:211-214.

13. Guncar, G., M. Podobnik, J. Pungercar, B. Strukelj, V. Turk, and D. Turk. 1998. Crystal structure of porcine cathepsin H determined at 2.1 Å resolution: location of the mini-chain C-terminal carboxyl group defines cathepsin H aminopeptidase function. *Structure* 6:51-61.
14. Nagler, D.K., R. Zhang, W. Tam, T. Sulea, E.O. Purisima, and R. Menard. 1999. Human cathepsin X: A cysteine protease with unique carboxypeptidase activity. *Biochemistry* 38:12648-12654.
15. Schaschke, N., I. Assfalg-Machleidt, W. Machleidt, D. Turk, and L. Moroder. 1997. E-64 analogues as inhibitors of cathepsin B. On the role of the absolute configuration of the epoxysuccinyl group. *Bioorg Med Chem* 5:1789-1797.
16. Heidtmann, H.H., U. Salge, M. Abrahamson, M. Bencina, L. Kastelic, N. Kopitar-Jerala, V. Turk, and T.T. Lah. 1997. Cathepsin B and cysteine proteinase inhibitors in human lung cancer cell lines. *Clin Exp Metastasis* 15:368-381.
17. Taggart, C.C., C.M. Greene, S.G. Smith, R.L. Levine, P.B. McCray, Jr., S. O'Neill, and N.G. McElvaney. 2003. Inactivation of human beta-defensins 2 and 3 by elastolytic cathepsins. *J Immunol* 171:931-937.
18. Taggart, C.C., G.J. Lowe, C.M. Greene, A.T. Mulgrew, S.J. O'Neill, R.L. Levine, and N.G. McElvaney. 2001. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J Biol Chem* 276:33345-33352.
19. Rogan, M.P., C.C. Taggart, C.M. Greene, P.G. Murphy, S.J. O'Neill, and N.G. McElvaney. 2004. Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. *J Infect Dis* 190:1245-1253.
20. Zheng, T., Z. Zhu, Z. Wang, R.J. Homer, B. Ma, R.J. Riese, Jr., H.A. Chapman, Jr., S.D. Shapiro, and J.A. Elias. 2000. Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J Clin Invest* 106:1081-1093.
21. Uehara, A., K. Muramoto, H. Takada, and S. Sugawara. 2003. Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. *J Immunol* 170:5690-5696.
22. Dulon, S., C. Cande, N.W. Bunnett, M.D. Hollenberg, M. Chignard, and D. Pidard. 2003. Proteinase-activated receptor-2 and human lung epithelial cells: disarming by neutrophil serine proteinases. *Am J Respir Cell Mol Biol* 28:339-346.
23. Roche, N., R.G. Stirling, S. Lim, B.G. Oliver, and K.F. Chung. 2003. Regulation of protease-activated receptor-1 in mononuclear cells by neutrophil proteases. *Respir Med* 97:228-233.
24. Walsh, D.E., C.M. Greene, T.P. Carroll, C.C. Taggart, P.M. Gallagher, S.J. O'Neill, and N.G. McElvaney. 2001. Interleukin-8 up-regulation by neutrophil elastase is mediated by MyD88/IRAK/TRAF-6 in human bronchial epithelium. *J Biol Chem* 276:35494-35499.
25. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immunity. *N Engl J Med* 343:338-344.

26. Devaney, J.M., C.M. Greene, C.C. Taggart, T.P. Carroll, S.J. O'Neill, and N.G. McElvaney. 2003. Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Lett* 544:129-132.
27. McCutcheon, J.C., S.P. Hart, M. Canning, K. Ross, M.J. Humphries, and I. Dransfield. 1998. Regulation of macrophage phagocytosis of apoptotic neutrophils by adhesion to fibronectin. *J Leukoc Biol* 64:600-607.
28. Punturieri, A., S. Filippov, E. Allen, I. Caras, R. Murray, V. Reddy, and S.J. Weiss. 2000. Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J Exp Med* 192:789-799.
29. Belaaouaj, A., R. McCarthy, M. Baumann, Z. Gao, T.J. Ley, S.N. Abraham, and S.D. Shapiro. 1998. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med* 4:615-618.
30. Hirche, T.O., J.J. Atkinson, S. Bahr, and A. Belaaouaj. 2004. Deficiency in neutrophil elastase does not impair neutrophil recruitment to inflamed sites. *Am J Respir Cell Mol Biol* 30:576-584.
31. Ferry, G., M. Lonchamp, L. Pennel, G. de Nanteuil, E. Canet, and G.C. Tucker. 1997. Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. *FEBS Lett* 402:111-115.
32. Taggart, C.C., C.M. Greene, N.G. McElvaney, and S. O'Neill. 2002. Secretory leucoprotease inhibitor prevents lipopolysaccharide-induced IkappaBalpha degradation without affecting phosphorylation or ubiquitination. *J Biol Chem* 277:33648-33653.
33. Burnett, D., M. Abrahamson, J.L. Devalia, R.J. Sapsford, R.J. Davies, and D.J. Buttle. 1995. Synthesis and secretion of procathepsin B and cystatin C by human bronchial epithelial cells in vitro: modulation of cathepsin B activity by neutrophil elastase. *Arch Biochem Biophys* 317:305-310.
34. Sagel, S.D., R.K. Kapsner, and I. Osberg. 2005. Induced sputum matrix metalloproteinase-9 correlates with lung function and airway inflammation in children with cystic fibrosis. *Pediatr Pulmonol* 39:224-232.
35. Steinberg, J., G. Fink, A. Picone, B. Searles, H. Schiller, H.M. Lee, and G. Nieman. 2001. Evidence of increased matrix metalloproteinase-9 concentration in patients following cardiopulmonary bypass. *J Extra Corpor Technol* 33:218-222.
36. Grayson, R., C.W. Douglas, J. Heath, A. Rawlinson, and G.S. Evans. 2003. Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and Porphyromonas gingivalis. *J Clin Periodontol* 30:542-550.
37. Zimmer, M., R.L. Medcalf, T.M. Fink, C. Mattmann, P. Lichter, and D.E. Jenne. 1992. Three human elastase-like genes coordinately expressed in the myelomonocyte lineage are organized as a single genetic locus on 19pter. *Proc Natl Acad Sci U S A* 89:8215-8219.
38. Shapiro, S.D., and R.M. Senior. 1999. Matrix metalloproteinases. Matrix degradation and more. *Am J Respir Cell Mol Biol* 20:1100-1102.
39. Wang, Z., T. Zheng, Z. Zhu, R.J. Homer, R.J. Riese, H.A. Chapman, Jr., S.D. Shapiro, and J.A. Elias. 2000. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* 192:1587-1600.

40. Carney, D.E., C.J. Lutz, A.L. Picone, L.A. Gatto, N.S. Ramamurthy, L.M. Golub, S.R. Simon, B. Searles, A. Paskanik, K. Snyder, C. Finck, H.J. Schiller, and G.F. Nieman. 1999. Matrix metalloproteinase inhibitor prevents acute lung injury after cardiopulmonary bypass. *Circulation* 100:400-406.
41. Cataldo, D., C. Munaut, A. Noel, F. Frankenne, P. Bartsch, J.M. Foidart, and R. Louis. 2000. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 123:259-267.
42. Hautamaki, R.D., D.K. Kobayashi, R.M. Senior, and S.D. Shapiro. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 277:2002-2004.
43. Corry, D.B., K. Rishi, J. Kanellis, A. Kiss, L.Z. Song Lz, J. Xu, L. Feng, Z. Werb, and F. Kheradmand. 2002. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat Immunol* 3:347-353.
44. Bien, S., C.A. Ritter, M. Gratz, B. Sperker, J. Sonnemann, J.F. Beck, and H.K. Kroemer. 2004. Nuclear factor-kappaB mediates up-regulation of cathepsin B by doxorubicin in tumor cells. *Mol Pharmacol* 65:1092-1102.
45. Kim, H., and G. Koh. 2000. Lipopolysaccharide activates matrix metalloproteinase-2 in endothelial cells through an NF-kappaB-dependent pathway. *Biochem Biophys Res Commun* 269:401-405.
46. Buddenkotte, J., C. Stroh, I.H. Engels, C. Moormann, V.M. Shpacovitch, S. Seeliger, N. Vergnolle, D. Vestweber, T.A. Luger, K. Schulze-Osthoff, and M. Steinhoff. 2005. Agonists of proteinase-activated receptor-2 stimulate upregulation of intercellular cell adhesion molecule-1 in primary human keratinocytes via activation of NF-kappa B. *J Invest Dermatol* 124:38-45.

## Figure legends

**FIGURE 1. Cathepsin B and MMP-2 gene expression and activity from macrophages (U937) exposed to increasing concentrations of NE.** (a) RT-PCR was carried out on mRNA from differentiated U937 cells treated with varying concentrations of NE (0, 16, 66, 166, 333 and 500nM activity) to amplify regions of the cathepsin B, MMP-2 and GAPDH genes. The quantification of the expression of cathepsin B and MMP-2 was assessed compared to GAPDH. \* P= 0.02, <0.01 and <0.01 when cells were exposed to NE at concentrations 166, 333 and 500nM, respectively, versus 0nM NE. # P= 0.02, <0.01 and <0.01 when cells were exposed to NE at concentrations 166, 333 and 500nM, respectively, versus 0nM NE. (b) Cathepsin activity was determined using the Z-Arg-Arg-AMC substrate 24 hours after stimulation with NE. \*, # and ~ P=0.01, P<0.01 and P<0.01 when cells are exposed to NE concentrations of 166, 333 and 500nM, respectively, versus 0nM NE. (c) MMP-2 was determined using gelatin zymography and by densitometry. Bands at 72 and 66kDa are representative of latent MMP-2 and active MMP-2, respectively. \* P= 0.02, 0.01, <0.01 and <0.01, respectively, for latent MMP-2 when cells are exposed to NE concentrations of 66, 166, 333 and 500nM versus 0nM NE. # P<0.01, <0.01, <0.01 and P<0.01, respectively, for active MMP-2 when cells are exposed to NE concentrations of 66, 166, 333 and 500nM versus 0nM NE. **The band observed at 78 kDa is active MMP-9.** Experiments were performed at least 3 times and representative data and SE are shown.

**FIGURE 2. Protease profile from peripheral blood monocytes.** (a) RT-PCR was carried out on mRNA from PBM treated with varying concentrations of NE (250 nM) to



amplify regions of the cathepsin B, MMP-2 and GAPDH genes. The quantification of the expression of cathepsin B and MMP-2 was assessed compared to GAPDH. \* P <0.01 when cells were exposed to NE, versus 0nM NE. # P <0.01 when cells were exposed to NE versus 0nM NE. (b) Cathepsin activity in supernatant of macrophages from healthy volunteers following stimulation with NE (250nM) compared to cells incubated in medium only. \* P=0.03 versus control. MMP-2 activity was determined in supernatants from control (Con) and NE-treated (NE) PBM using gelatin zymography (c). Bands at 72 and 66kDa are representative of latent MMP-2 and active MMP-2, respectively. Experiments or analyses of results were performed at least 3 times and representative data and SE are shown.

**FIGURE 3. NE<sup>-/-</sup> mice have less cathepsin B and MMP-2 activity than wild-type mice following *P. aeruginosa* lung function.** (a) Cathepsin activity was determined using the Z-Arg-Arg-AMC substrate in BAL from NE<sup>+/+</sup> and NE<sup>-/-</sup> mice. \* P=0.03 versus NE<sup>+/+</sup> BAL. (b) MMP-2 activity was determined from 1µg of BAL protein using gelatin zymography and by densitometry. Bands at 72 and 66kDa are representative of latent MMP-2 and active MMP-2, respectively. \* and ~ P=0.02 and P=0.03 for latent and active MMP-2, respectively, between the NE<sup>+/+</sup> and NE<sup>-/-</sup> mice. **Histologic sections from the lungs of NE<sup>-/-</sup> (C and D) and NE<sup>+/+</sup> (E and F) mice were stained for cathepsin B (C and E) and MMP-2 (D and F) following *P. aeruginosa* infection.** Scale bar represents 50 µm. Analyses of results were performed at least 3 times and representative data and SE are shown.

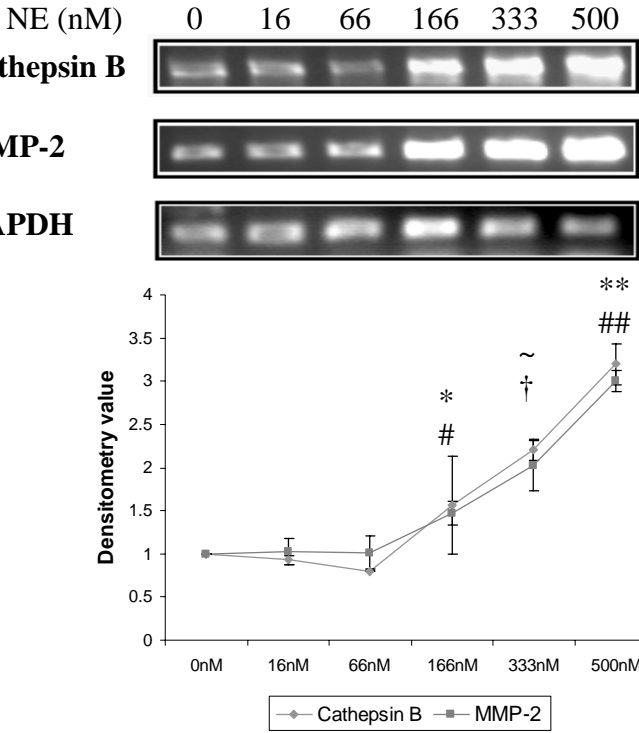
**FIGURE 4. IRAK-1 degradation, NF- $\kappa$ B activation and IL-8 protein production in U937 macrophages stimulated with NE.** (a) IRAK degradation was analyzed by Western blot using an anti-IRAK antibody and cytosolic extracts (10  $\mu$ g) from control (-) and NE-treated macrophages (100 nM, 30, 60, 90 and 120 minutes). (b) NF- $\kappa$ B activation was measured using the TransAM NF- $\kappa$ B activity ELISA in nuclear extracts (2  $\mu$ g) from control (-) and NE-treated macrophages (100 nM, 5, 15, 30, 60, 90 and 120 minutes). \* P<0.01 versus control. (c) Levels of IL-8 in supernatants were measured by enzyme-linked immunosorbent assay, and values were corrected to pg/mg of total protein. \* P<0.01 versus control. Experiments were performed at least 3 times.

**FIGURE 5. Inhibition of NF- $\kappa$ B and TLR-4 leads to a reduction of NE induced cathepsin B and MMP-2.** Cells were treated for 60 minutes with SN50 (1  $\mu$ g/ml) and its inactive control (Calbiochem), or with mouse anti human CD284 antibody (1  $\mu$ g/ml) and mouse IgG2a (isotype control IgG) prior to NE (100 nM) stimulation to block NF- $\kappa$ B and TLR-4 activity. (a & c) - Cathepsin activity was determined using the Z-Arg-Arg-AMC substrate 24 hours after stimulation with NE. \* P=0.02 and P=0.01 for NE compared to NE with SN50 and TLR-4 antibody, respectively. (b & d) - MMP-2 activity was determined using gelatin zymography and by densitometry. Bands at 72 and 66kDa are representative of latent MMP-2 and active MMP-2, respectively. **The band observed at 78 kDa is active MMP-9.** Experiments or analyses of results were performed at least 3 times and representative data and SE are shown.

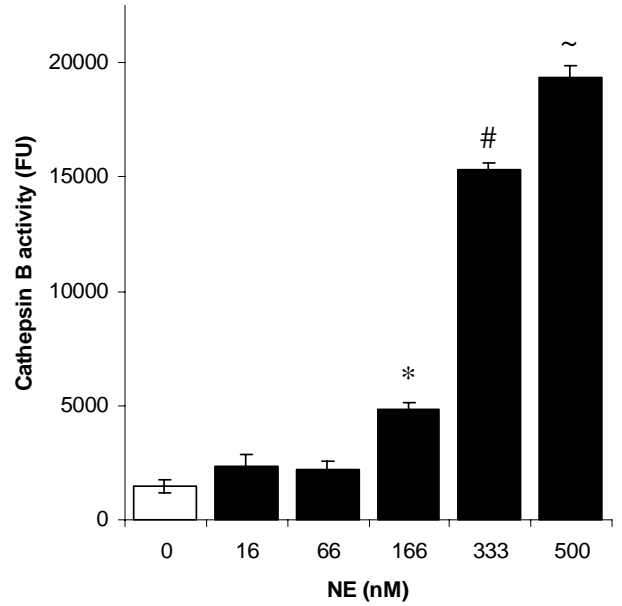
**FIGURE 6.** Transfection of dominant negative IRAK-1 leads to a reduction of NE induced cathepsin B and MMP-2. U937 cells were transfected with dominant-negative expression vector IRAK-1 $\Delta$  and the relevant empty vector 24 hours prior to NE stimulation. (a) Cathepsin B activity was determined using the Z-Arg-Arg-AMC substrate 24 hours after stimulation with NE. \* P<0.01 for cells transfected with IRAK-1 compared to transfection with the empty vector when both are stimulated with NE. (b) MMP-2 activity was determined using gelatin zymography and by densitometry. Bands at 72 and 66kDa are representative of latent MMP-2 and active MMP-2, respectively. **The band observed at 78 kDa is active MMP-9.** Experiments or analyses of results were performed at least 3 times and representative data and SE are shown.

Figure 1

**A**



**B**



**C**

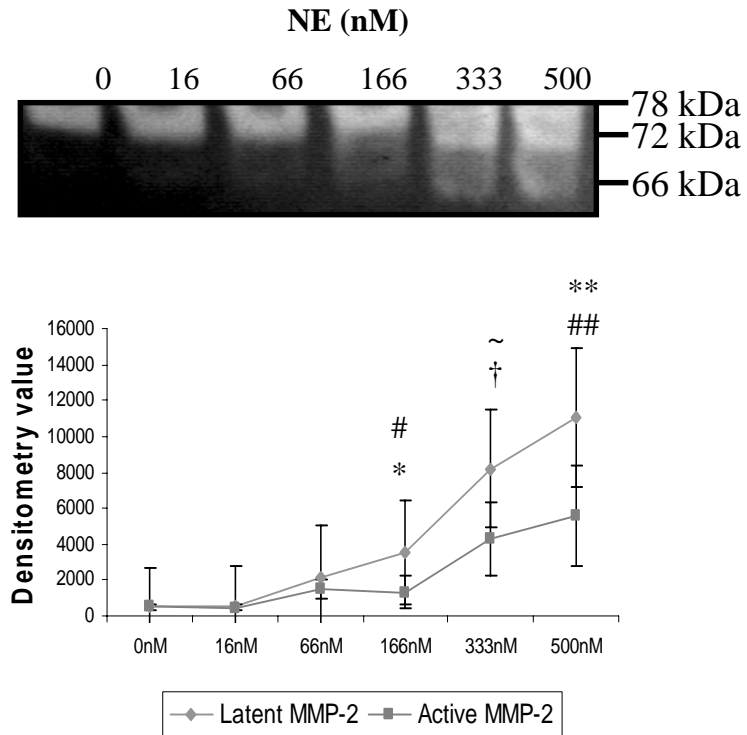
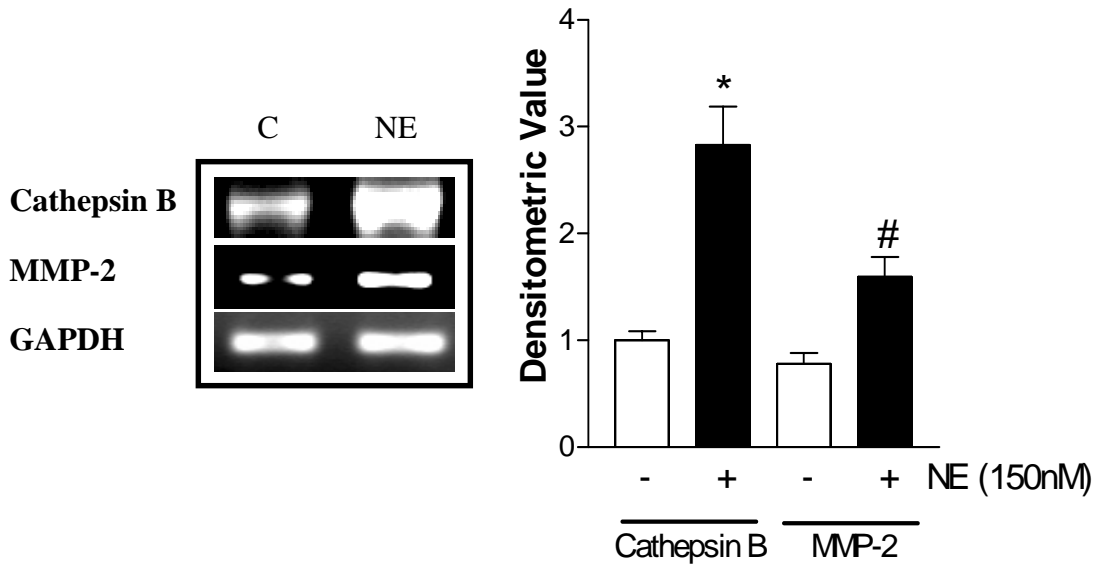
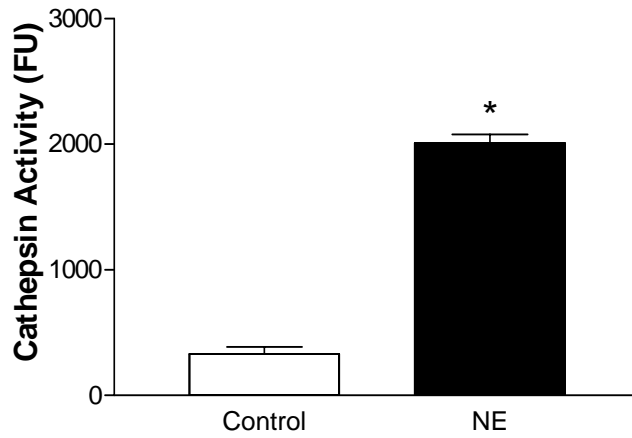


Figure 2

**A**



**B**



**C**

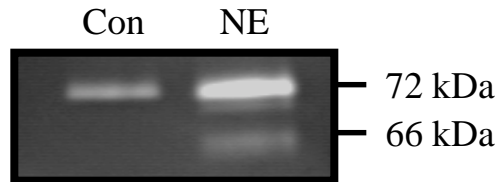


Figure 3

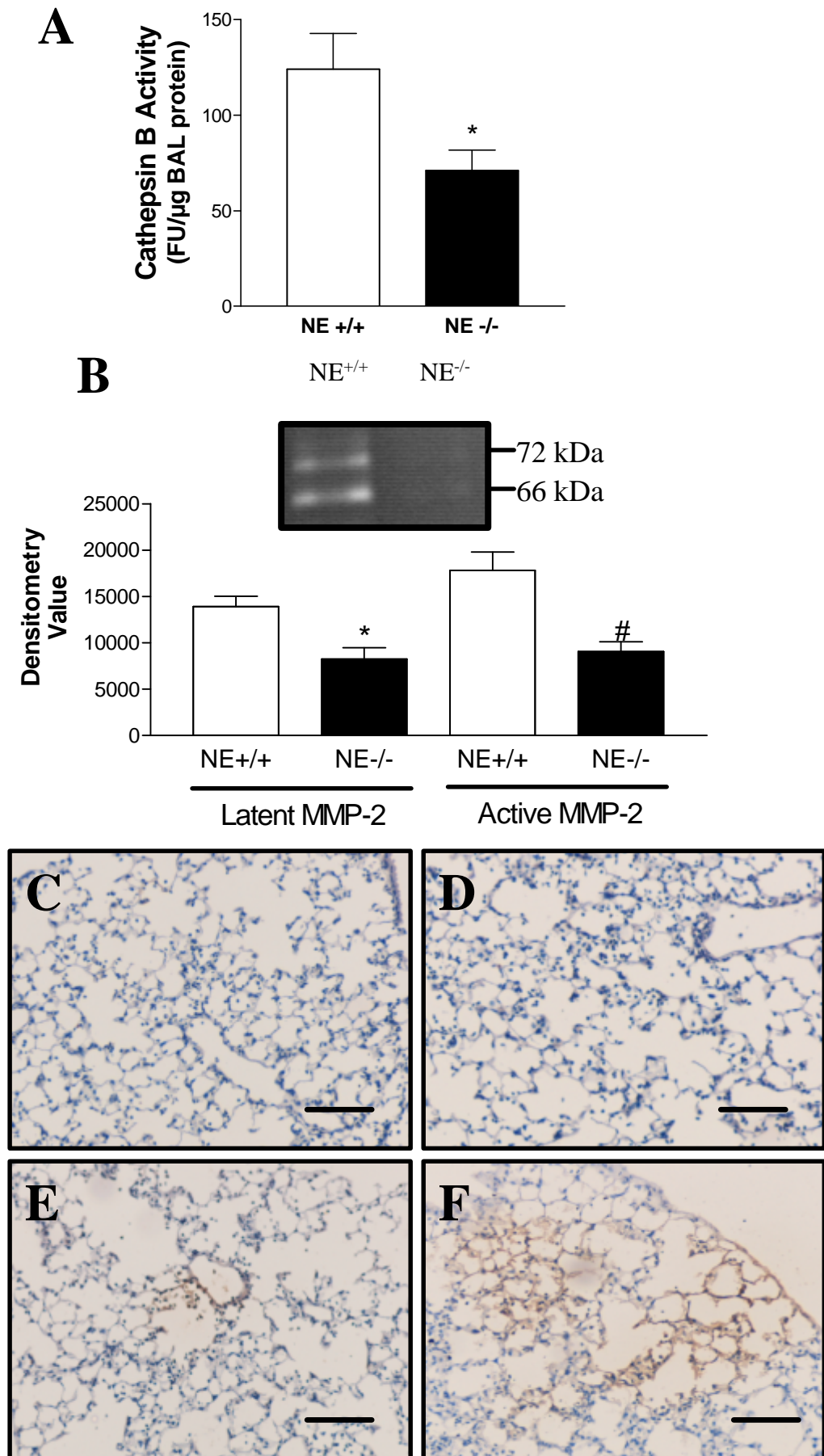


Figure 4

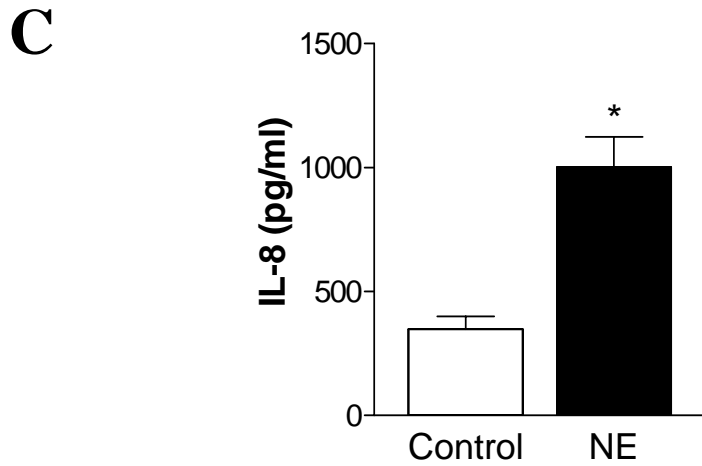
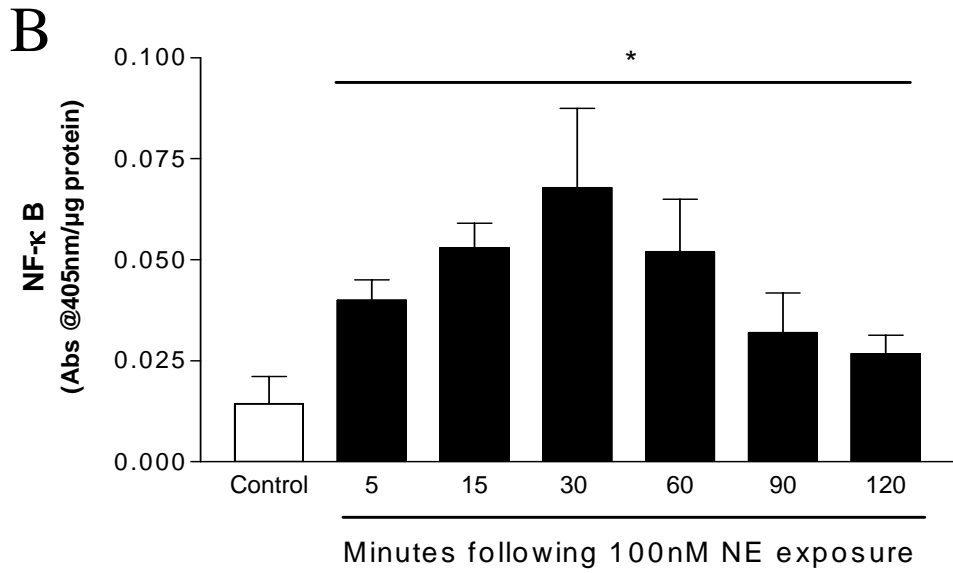
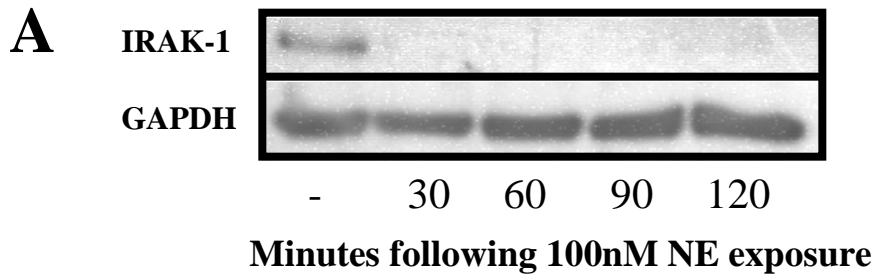
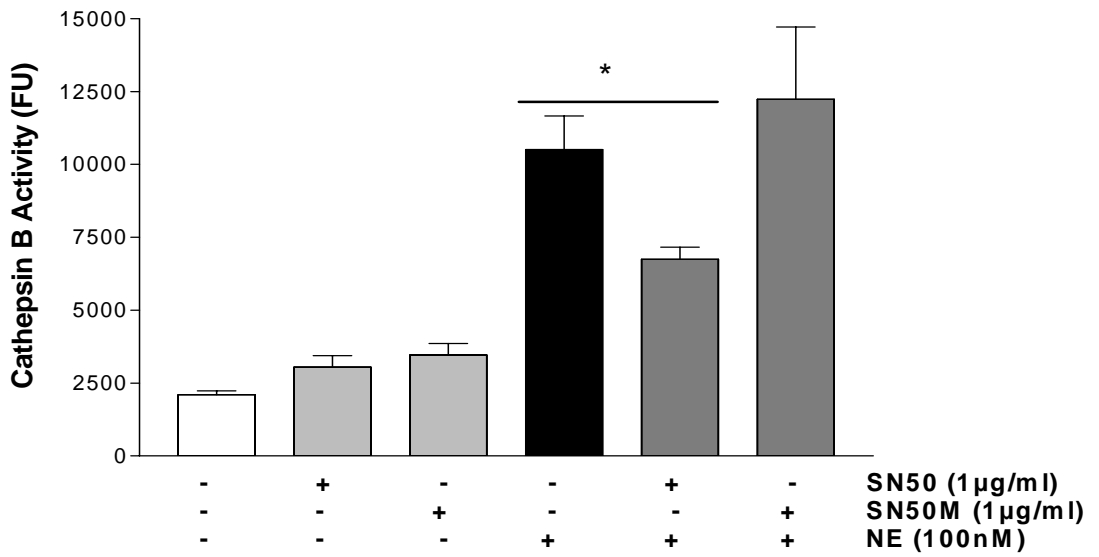
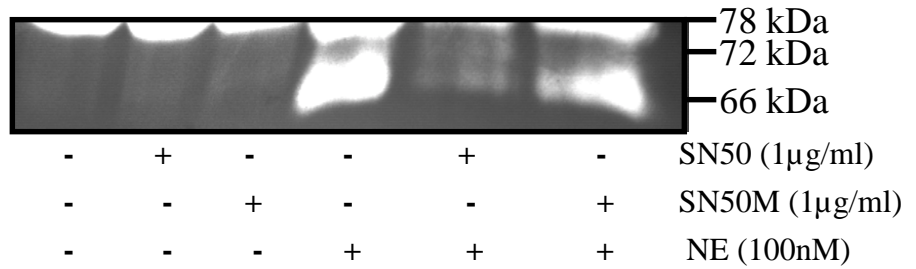


Figure 5

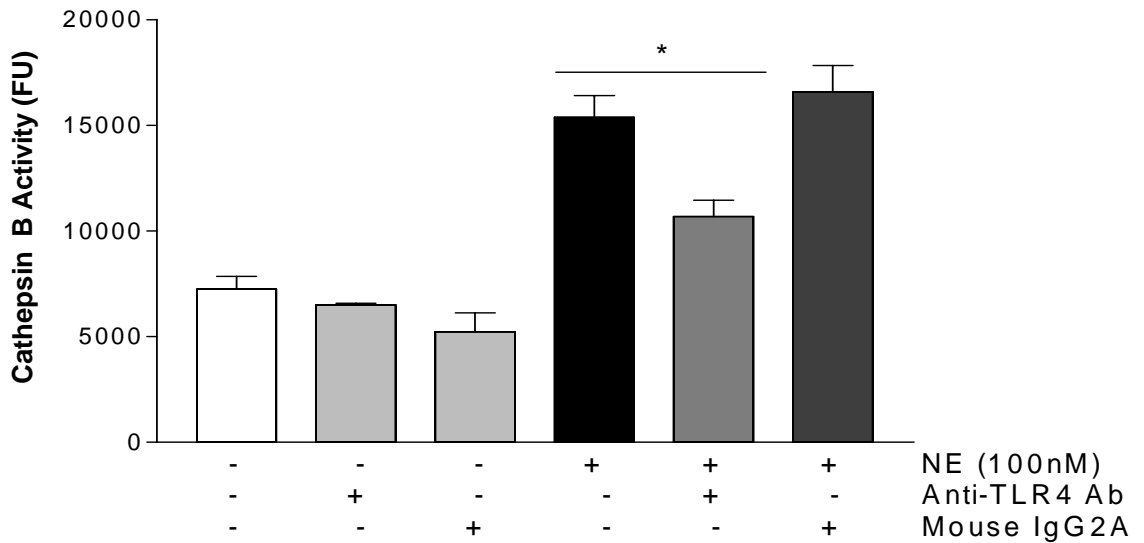
**A**



**B**



**C**



**D**

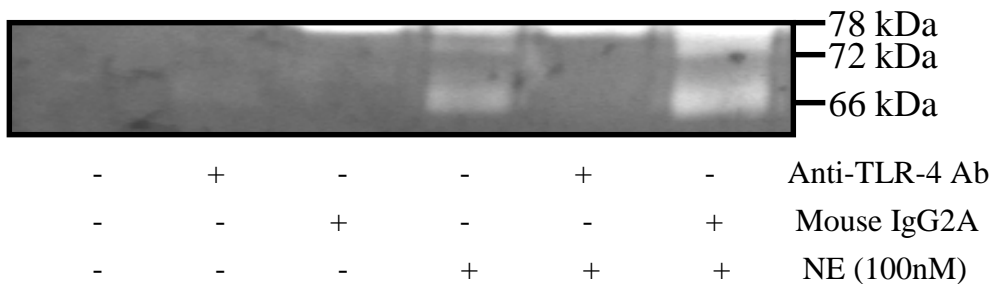
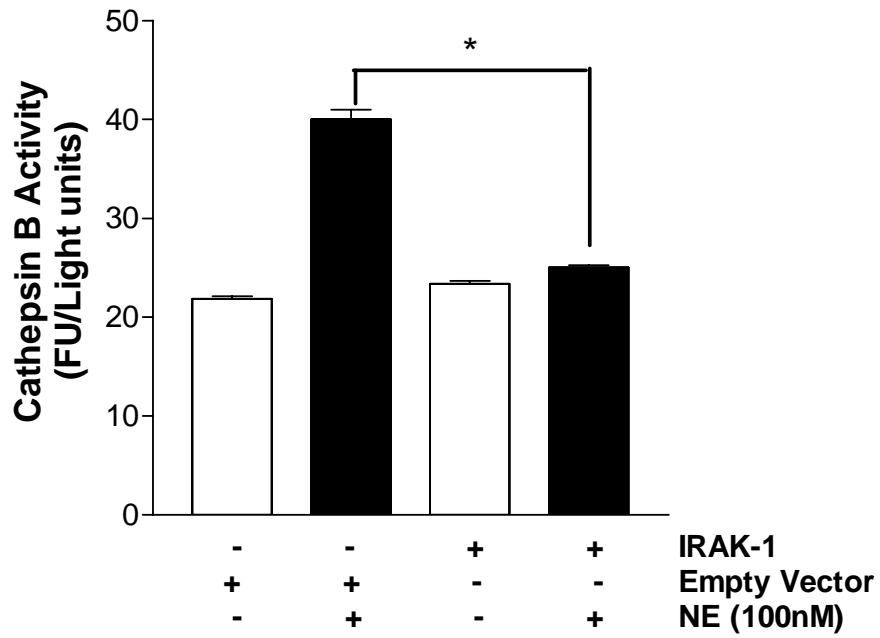




Figure 6

**A**



**B**

