β -Tryptase Regulates IL-8 Expression in Airway Smooth Muscle Cells by a PAR-2-Independent Mechanism

Charlotte S. Mullan¹, Michael Riley¹, Deborah Clarke¹, Amanda Tatler¹, Amy Sutcliffe¹, Alan J. Knox¹, and Linhua Pang¹

¹Division of Respiratory Medicine, City Hospital, University of Nottingham, Nottingham, United Kingdom

Mast cells are central in the development of several allergic diseases and contain a number of pre-formed mediators. β -tryptase, the most abundant mast cell product, is increasingly recognized as a key inflammatory mediator, as it causes the release of cytokines, particularly the chemokine IL-8, from both inflammatory and structural cells. The molecular mechanisms, however, remain largely unknown. In this study we sought to investigate whether β -tryptase could induce IL-8 expression in human airway smooth muscle (ASM) cells and to explore the molecular mechanisms involved. We found that purified human β -tryptase stimulated IL-8 production in a time- and concentration-dependent manner, which was inhibited by protease inhibitors and mimicked by recombinant human β -tryptase, but not by the protease-activated receptor-2 (PAR-2) agonist SLIGKV-NH₂, consistent with the low-level expression of PAR-2 protein in these cells. β -tryptase also up-regulated IL-8 mRNA expression, as analyzed by RT-PCR and real-time PCR, which was abolished by the transcription inhibitor actinomycin D. Reporter gene assay showed that β -tryptase-induced IL-8 transcription was mediated by the transcription factors activator protein-1, CCAAT/enhancer binding protein, and NF- κ B, and chromatin immunoprecipitation assay demonstrated that β -tryptase induced *in vivo* binding of these transcription factors to the IL-8 gene promoter. Furthermore, β -tryptase stabilized IL-8 mRNA, suggesting additional post-transcriptional regulation. Collectively these findings show that β -tryptase up-regulates IL-8 expression in ASM cells through a PAR-2-independent proteolytic mechanism and coordinated transcriptional and post-transcriptional regulation, which may be of particular importance in understanding the role and the mechanisms of action of β -tryptase in regulating chemokine expression in mast cell-related disorders.

Keywords: mast cell; β -tryptase; human airway smooth muscle cell; IL-8

Mast cells are widely recognized as critical effector cells in various allergic disorders and other immunoglobulin E-associated acquired immune responses (1). However, it is still unclear how they contribute to the respective conditions. One clear possibility is that one or more of the compounds present in the mast cell secretory granule, which are released after mast cell stimulation, contribute to a pathologic response (2). Tryptase (EC.3.4.21.59) is the most abundant and a unique mast cell product (3). Human tryptase is a serine protease and exists as three isoforms (the soluble α and β isoforms and the membrane anchored γ isoform), with β -tryptase predominating in lung mast cells (2, 3). β -tryptase is stored in mast cell granules as enzymatically active tetramers in a complex with heparin, which facilitates the conversion of mature β -tryptase monomers to tetramers and stabilizes them (4).

Because β -tryptase is the main type of tryptase that is released during mast cell degranulation and isolated from normal human lung tissues, the enzymatically active lung tryptase preparations as well as β -tryptase produced recombinantly will be subsequently referred to as "tryptase."

Research on tryptase so far has revealed a large number of potential functions for this enzyme and identified it as a key inflammatory mediator in mast cell-related diseases and a potential target for therapeutic intervention (2). Tryptase has been shown to stimulate the release of chemokine IL-8 from various inflammatory and structural cells such as eosinophils (5), neutrophils (6), endothelial cells (7, 8), and epithelial cells (9), but the mechanisms involved are poorly defined. Since IL-8 is a potent chemoattractant for a number of granulocytes, particularly neutrophils and eosinophils, and to a lesser extent mast cells, tryptase may play a key role in neutrophilic and eosinophilic inflammatory diseases.

Work performed by our group and others has shown that human ASM cells have important synthetic functions, which contribute to chronic airway inflammation and remodeling in airway diseases such as asthma (10). Recent studies by Brightling and associates (11) have demonstrated a selective localization of mast cells within the airway smooth muscle (ASM) layer in asthma, suggesting that mast cell products, including tryptase, may influence the synthetic functions of human ASM cells. Indeed, tryptase has been shown to stimulate human ASM cells to attract mast cells largely through the production of functionally active transforming growth factor β 1 (TGF- β 1) (12). We have also reported recently that tryptase selectively cleaves human ASM cell-derived eotaxin and regulated on activation, normal T cell expressed and secreted (RANTES) and abrogates their eosinophil chemotactic activities (13).

It has been shown that tryptase exerts several of its cellular effects through cleavage of protease-activated receptor-2 (PAR-2). PAR-2 belongs to a family of four G protein-coupled receptors (PAR-[1-4]). The cleavage of the extracellular part of PAR-2, as for all of the PARs, leads to the exposure of a "tethered" ligand, which subsequently binds to the receptor, thereby inducing signaling events and receptor/ligand internalization (14). Studies so far have demonstrated that tryptase-induced IL-8 production is mediated by PAR-2 in eosinophils and fibroblasts (5), neutrophils (6), endothelial cells (8), and epithelial cells (9). However, it is interesting to note that some effects of tryptase, notably ASM cell proliferation, are mediated via a PAR-2-independent nonproteolytic mechanism (15).

The mechanisms involved in IL-8 regulation have been studied with respect to cytokines in several cell types, but less information is available for other agents and none for tryptase. IL-8 can be regulated at both transcriptional and post-transcriptional levels. The nucleotide sequence -1 to -162 in the 5' flanking region of the IL-8 gene promoter contains binding sites for transcription factors activator protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP), and nuclear factor (NF)- κ B. These are essential for IL-8 gene transcription in response to cytokines in some cells but not others (16, 17). We have previously demonstrated that human ASM cells are a rich source of IL-8

(Received in original form March 9, 2007 and in final form November 19, 2007)

This study was supported by the Wellcome Trust (Grant No. 078227), Novartis, and the Institute of Clinical Research of the University of Nottingham.

Correspondence and requests for reprints should be addressed to Linhua Pang, MB, PhD, Division of Respiratory Medicine, Clinical Sciences Building, City Hospital, University of Nottingham, Hucknall Road, Nottingham NG5 1PB, UK. E-mail: linhua.pang@nottingham.ac.uk

Am J Respir Cell Mol Biol Vol 38, pp 600-608, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0082OC on December 13, 2007

Internet address: www.atsjournals.org

(18–20) and that transcription factors AP-1, C/EBP, and NF- κ B are all involved in bradykinin-induced IL-8 transcription in these cells (21). Although tryptase has been shown to induce IL-8 production in other cells through PAR-2 activation, the molecular mechanisms through which tryptase carries out its actions on IL-8 gene expression remains unclear.

In the present study we sought to determine whether tryptase could up-regulate IL-8 expression in human ASM cells and to characterize the mechanisms involved. We found that IL-8 was released in large quantities from human ASM cells stimulated by tryptase and that the effect of tryptase was via a PAR-2-independent proteolytic mechanism. The regulation of IL-8 gene expression was partly transcriptional and partly post-transcriptional. Furthermore, transcriptional regulation was dependent on activation of transcription factors AP-1, C/EBP, and NF- κ B.

MATERIALS AND METHODS

Materials

Purified human lung tryptase dissolved in tryptase buffer (50 mM NaOAc, 1 M NaCl, 0.05 mM heparin [pH 5.0], and 0.01% NaN₃) was obtained from Europa Bioproducts (Cambridge, UK) (specific activity: 12,870 mU/mg protein using *n*-benzyl-DL-arginine-pNA as substrate). Recombinant human lung tryptase (rh-tryptase) was from Promega (Southampton, UK) (specific activity: 1,000 Units/mg protein using α -CBZ-L-lysine thiobenzyl ester as substrate). Peptide corresponding to the tethered ligand of human PAR-2 SLIGKV-NH₂ and its partially reversed control peptide LSIGKV-NH₂ were from Bachem (Merysey-side, UK). IL-8 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Europe (Abingdon, Oxon, UK) using substrate solutions A and B, which were purchased from BD Biosciences Pharmingen, (San Diego, CA). RNeasy mini kit was from Qiagen (West Sussex, UK). Excite real-time mastermix and SYBR green were from Biogene (Cambridge, UK). Polyclonal antibodies against NF- κ B (p65), AP-1 (c-Jun), and C/EBP (C/EBP β) and monoclonal antibody against PAR-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Serotec Ltd (Oxford, UK). Polyclonal rabbit anti-mouse immunoglobulins/HRP was from Dakocytomation (Glostrup, Denmark). Rainbow colored protein molecular weight markers, ECL Western blotting detection reagent, and Hyperfilm-ECL from Amersham (Little Chalfont, Bucks, UK). Pure nitrocellulose membrane from Gelman Sciences (Northampton, Northants, UK). The Bio-Rad protein assay reagent from Bio-Rad Laboratories Ltd (Hemel Hempstead, Herts, UK). FuGene 6 transfection reagent was from Roche Molecular Biochemicals (East Sussex, UK). The internal control *Renilla* luciferase reporter construct pSRV40 and the dual-luciferase reporter assay system were from Promega (Southampton, UK). Chromatin immunoprecipitation kit (ChIP-IT) was purchased from Active Motif Europe (Rixensart, Belgium). FUT-175 (nafamostat mesylate) was from Biomol (Exeter, UK). Leupeptin, protease inhibitor cocktail (Cat. No. P8340), bradykinin, and all other chemicals were from Sigma (Poole, Dorset, UK).

Cell Culture

Human ASM were obtained from post-mortem tracheal specimens from four individuals with no history of respiratory diseases and no evidence of airway abnormalities as previously reported (22). Cells were used at passage 6–7. When grown in this manner they depict the phenotypical characteristics of typical human ASM cells (22).

Normal human bronchial/tracheal epithelial (NHBE) cells were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and were cultured using the company's own Bronchial Epithelial Cell Medium. Cells at passage 4 were used.

Cells were grown to confluence in 24-well plates and growth arrested for 24 hours before treatment. Purified tryptase, recombinant tryptase, SLIGKV-NH₂, and bradykinin were diluted in serum-free medium. Vehicle controls were used in the inhibitor studies. The effect of drugs and drug vehicles on cell viability was assessed by thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay as

previously described (22); no cytotoxic effect was observed (data not shown).

IL-8 Assay

The concentration of IL-8 in the supernatants was quantitated by ELISA as previously described (19, 20).

RNA Isolation and RT-PCR

Confluent human ASM cells were serum-starved for 24 hours. After treatment with β -tryptase for times indicated in the text, total RNA was extracted using the RNeasy mini kit and transcribed in to cDNA using Moloney murine leukemia virus reverse transcriptase. The following primers were used for IL-8 and the internal control GAPDH cDNA amplification. IL-8 sense: 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3', and IL-8 antisense: 5'-TCT CAG CCC TCT TCA AAA ACT TC-3'; GAPDH sense: 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3', and GAPDH antisense: 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'. The amplification and PCR conditions used were as follows: initial denaturation at 94°C for 3 minutes, 35 cycles of the following time and temperature profile: denaturation at 94°C for 1 minute, primer annealing at 58°C for 2 minutes, primer extension at 72°C for 1 minute, and a final extension of 72°C for 5 minute. The PCR products were visualized on a 2% agarose gel and density analysis of the bands was conducted using GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

Quantitative Real-Time PCR

IL-8 expression was determined using primer sequences described above. β_2 -microglobulin was used as the house keeping gene (23). Quantitative PCR was performed on an ABI prism 7700 (Applied Biosystems, Foster City, CA) using Excite real-time Mastermix with SYBR green as described previously (23).

IL-8 mRNA Stability

Confluent and serum-starved cells were treated with or without tryptase for 4 hours before the addition of actinomycin D (5 μ g/ml) for the times indicated to block new transcript generation. Total RNA were then extracted and RT-PCR was performed.

Western Blotting

Confluent and growth-arrested human ASM cells and NHBE cells in 6-well plates were washed twice with ice-cold PBS and incubated for 2 minutes with a total cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl, and Sigma Protease inhibitor cocktail [1:100]) with gentle shaking. The samples were collected and centrifuged, and the protein concentration in the supernatant was determined using the Bio-Rad protein assay reagent.

PAR-2 protein expression was analyzed by Western blotting as described before (22). Briefly, protein samples (55 μ g/track) were subject to electrophoresis in 10% SDS-polyacrylamide gel. Separated proteins were electroblotted to methanol-activated PVDF membranes, and the blot was blocked for 2 hours at room temperature with blocking buffer (wash buffer with 5% fat-free dried milk powder). The blot was then incubated with monoclonal anti-PAR-2 antibody (1:250 dilution with blocking buffer) at 4°C overnight, washed with wash buffer (PBS pH 7.4 with 0.3% Tween-20), and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000 dilution with blocking buffer) for 1 hour. The blot was washed again and then incubated with ECL Western blotting detection reagent for 1 minute and finally exposed to Hyperfilm-ECL.

DNA Constructs

The vectors encoding either the wild-type IL-8 promoter (–162/+44)/luciferase reporter, or site mutations of one of the binding sites (AP-1, C/EBP, or NF- κ B) in the IL-8 promoter region were kindly provided by Dr. A. R. Brasier (Sealy Centre for Molecular Science, Galveston, TX) and were described in detail in Dr. Braiser's publications (24, 25). The NF-

κ B-dependent reporter 6 κ Btkluc, which contains six copies of the consensus NF- κ B-binding site (5'-GGG ACT TTC C-3'), was a gift from Dr. R. Newton (Calgary, AB, Canada) and has been previously described (26). The AP-1 reporter construct pRTU14 was a gift from Dr. A. Kieser (Hanover, Germany) and consists of a luciferase gene under the control of a minimal promoter and four TREs (12-*O*-tetradecanoate-13-acetate responsive element), to which the AP-1 transcription factor binds (27). The C/EBP reporter construct containing a promoter construct of four repeated consensus binding sites for C/EBP in tandem, upstream of a luciferase reporter gene pTK18-Luc, was obtained from Dr. H. Yang (Harvard University, MA) and has previously been described (28).

Reporter Gene Assay

Transient transfections were performed using FuGene 6 transfection reagent as described previously (19, 23, 29) with slight modification. Briefly, human ASM cells were seeded into 24-well plates, grown to 60% confluence, and growth-arrested for 8 hours in serum- and antibiotic-free medium before being co-transfected with the firefly luciferase and the internal control *Renilla* luciferase plasmids (0.4 μ g firefly + 4 ng *Renilla* + 1.2 μ L FuGene 6 per well) for 16 hours. Cells were then treated with tryptase (0–15 mU/ml) for the indicated times and lysed. Luciferase activities were measured using the dual-luciferase reporter assay system and relative light units (RLU) recorded. Relative luciferase activity was calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity.

Chromatin Immunoprecipitation

To detect the binding of transcription factors to the IL-8 promoter, chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT kit as per the manufacturer's instructions. Eighty to ninety percent confluent and serum-deprived human ASM cells were treated with tryptase (15 mU/ml) for 0, 1, or 2.5 hours. Cells were then washed in ice-cold PBS and incubated for 10 minutes with 1% formaldehyde to fix protein–DNA complexes, and reaction quenched using 0.1 M glycine. Cells were collected in 2 ml PBS supplemented with phenylmethylsulfonyl fluoride (PMSF), re-suspended in lysis buffer containing PMSF and a protease inhibitor cocktail, and incubated on ice for 30 minutes before homogenization to aid nuclear release. Samples were subsequently sonicated (five 10-s pulses with incubation on ice between cycles) after being resuspended in shearing buffer supplemented with protease inhibitors. The soluble chromatin fragments (average 500–800 bp) were pre-cleared by incubation with protein G–sepharose at 4°C for 2 hours. After centrifugation the pre-cleared supernatant was subdivided into aliquots of equal volume. One aliquot was used as DNA input control and the remaining aliquots were incubated with antibodies directed against NF- κ B (p65), AP-1 (c-Jun), or C/EBP (C/EBP β) (4 μ g each) on a rotating platform overnight at 4°C. The immunoprecipitated complexes of antibody–protein–DNA were washed nine times with wash buffer. Cross-linking of protein–DNA was reversed by incubating samples in 200 mM NaCl and 10 μ g of RNase A at 65°C for 4 hours and the protein was digested by incubation with proteinase K in the presence of 0.5 M EDTA and 1 M Tris-HCl (pH 6.5) at 42°C for 90 minutes. The DNA from input control and immunoprecipitated samples was then extracted and purified with the DNA spin columns provided, and eluted with 100 μ L H₂O. The DNA was then subjected to PCR amplification with the sense primer 5'-TTCACCAAATTGTGGAG-CTT-3' and the antisense primer 5'-GAAGCTTGTGTGCTCTGCTG-3', which were specifically designed for the IL-8 promoter and were described previously (19). The PCR products were then visualized and analyzed with the GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

Statistics

Data were expressed as the mean $\pm$ SE. Data from ELISA and luciferase reporter assays were analyzed by ANOVA and an unpaired two-tailed Student's *t* test using GraphPad Prism (version 4) to determine the significant differences between the means. A value of $P \leq 0.05$ was accepted as significant.

RESULTS

Tryptase Stimulates IL-8 Protein Production from Human ASM Cells

To examine the effect of tryptase on IL-8 production, human ASM cells were treated with or without purified human lung tryptase (15 mU/ml) for up to 24 hours. As shown in Figure 1A, IL-8 was produced constitutively from tryptase buffer-treated control ASM cells, but tryptase time-dependently enhanced IL-8 protein production from human ASM cells, with significant increases observed 8 and 24 hours after incubation. Tryptase also induced a concentration-dependent increase in IL-8 production above basal level, reaching significance at 3.25 mU/ml and peaking at 30 mU/ml (Figure 1B). To confirm that the effect of

Figure 1. Effects of tryptase on IL-8 production from human airway smooth muscle (ASM) cells. Confluent and growth-arrested human ASM cells in 24-well plates were incubated with tryptase buffer (same dilution factor as 15 mU/ml purified tryptase) as control or with purified tryptase (15 mU/ml) for the times indicated (A), or with increasing concentrations of purified tryptase for 24 hours (B), or with increasing concentrations of purified tryptase or rh-tryptase with or without heparin for 24 hours (C) (ratio of heparin:rh-tryptase, 1:0.148 in molar concentration; concentration for heparin alone, 29.14 nM, equivalent to heparin concentration with 7.5 mU/ml purified tryptase). IL-8 released into the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). Each point represents the mean $\pm$ SEM of three to eight replicates from two to four independent experiments. ** $P < 0.01$ and *** $P < 0.001$ compared with control cells.

purified tryptase was truly due to the activity of tryptase, rather than any other contaminating proteases or heparin, we compared the effect of purified and recombinant human tryptase (rh-tryptase). In the absence of heparin, rh-tryptase caused very little IL-8 production; however, in the presence of heparin, rh-tryptase enhanced IL-8 production in a similar concentration-dependent manner to that of purified tryptase (Figure 1C). Heparin alone was without effect (data not shown). Since purified tryptase was in a solution containing heparin, the results therefore indicate that the induction of IL-8 by tryptase requires the presence of heparin and that the effect of purified tryptase on IL-8 production is indeed due to the activity of tryptase. Purified tryptase (tryptase) was then used throughout the study.

Tryptase Increases IL-8 mRNA Levels via both Transcriptional and Post-Transcriptional Mechanisms

To explore whether the effect of tryptase on IL-8 production from ASM cells was due to its induction of IL-8 mRNA, the semi-quantitative RT-PCR and the quantitative real-time PCR were used to determine IL-8 mRNA levels. IL-8 mRNA was constitutively expressed in untreated ASM cells, as analyzed by RT-PCR, and the expression was concentration-dependently increased by tryptase, with marked enhancement observed after treatment for 1 hour and a 5-fold increase achieved at 8 and 24 hours (Figures 2A and 2B). Tryptase also induced IL-8 mRNA expression concentration-dependently, as analyzed by real-time PCR; significant increase was observed with 3.25 mU/ml tryptase, and the maximum effect was achieved with 15 mU/ml tryptase (Figure 3A). The increase of IL-8 mRNA by tryptase (15 mU/ml for 8 h) was abolished by pre-treatment with the general transcription inhibitor actinomycin D (5 μ g/ml for 1 h) (Figure 3B), suggesting involvement of transcriptional regulation. To further determine if the effects of tryptase on IL-8 mRNA expression were solely transcriptional or whether post-transcriptional mechanisms were also involved, transcription arrest studies with actinomycin D were performed and IL-8 mRNA stability was assessed by RT-PCR. As shown in Figure 4, in the absence of tryptase there was a natural decay of IL-8 mRNA over 24 hours after the addition of actinomycin D; however, this was almost abolished by pre-treatment of the cells with tryptase (15 mU/ml for 4 h) before the addition of actinomycin D, suggesting involvement of post-transcriptional regulation in tryptase-induced IL-8 mRNA expression in ASM cells.

Tryptase Acts Proteolytically via a Non-PAR-2 Mechanism

To determine if the effect of tryptase on IL-8 production from ASM cells was due to its proteolytic properties, we studied the effect of protease inhibitors. We found that leupeptin (2.5 μ g/ml), an inhibitor of serine and cysteine proteases, and a broad-spectrum protease inhibitor cocktail (containing aprotinin, bestatin, E-64, leupeptin and pepstatin A), caused significant ($P < 0.001$) and near-total inhibition of tryptase-induced IL-8 production (Figure 5A). The effect of tryptase on IL-8 production was also markedly inhibited by the selective tryptase inhibitor FUT-175 (FUT, $K_i = 0.095$ nM for tryptase [30]) (Figure 5B). In contrast, IL-8 production induced by bradykinin (10 μ M), a peptide inflammatory mediator, was not affected by these protease inhibitors (Figure 5C). The results strongly suggest that the effect of purified tryptase on IL-8 production is due to the proteolytic activities of tryptase.

As tryptase has been shown to act through the PAR-2 receptor in other cell types (5, 6, 8, 9), we used synthetic peptide SLIGKV-NH₂, which is a specific agonist for the PAR-2 receptor, to determine if it would mimic the effect of tryptase on IL-8 production in ASM cell, thereby implicating the involvement of PAR-2 receptor in this process. NHBE cells are known to express PAR-2. Treatment of these cells with SLIGKV-NH₂ (500 μ M, 24 h) caused a marked increase of IL-8 production over basal level in these cells (Figure 5D); however, treatment with the partially reversed control peptide LSLGKV-NH₂ (500 μ M, 24 h) had no effect (data not shown). In contrast, SLIGKV-NH₂ (500 μ M, 24 h) did not show any effect on IL-8 production in ASM cells (Figure 5D). Western blotting analysis confirmed a high level expression of PAR-2 protein in NHBE cells, whereas PAR-2 was barely detectable in ASM cells (Figure 5E), consistent with the respective effects of the PAR-2 agonist in the two types of cells. Taken together, the results strongly suggest that the effect of tryptase on IL-8 production from human ASM cells is not mediated by PAR-2 receptors.

Tryptase Induces IL-8 Gene Transcription via Transcription Factors AP-1, NF- κ B, and C/EBP

The IL-8 gene promoter contains regulatory elements for transcription factors AP-1, NF- κ B, and C/EBP. To identify the regulatory elements responsible for tryptase-induced IL-8 gene transcription, human ASM cells were co-transfected with the internal control *Renilla* luciferase plasmids and firefly luciferase

Figure 2. Effect of tryptase on IL-8 mRNA expression in human ASM cells. (A) Confluent and growth-arrested human ASM cells were incubated with tryptase (15 mU/ml) for the times indicated. mRNA levels of IL-8 and GAPDH were measured by RT-PCR. The gel is representative of three experiments with similar results. (B) Density analysis of IL-8 bands for the gels of the above experiment normalized against GAPDH bands. Data were expressed as fold increase over control. Each point represents the mean $\pm$ SEM of three experiments.

Figure 3. Effect of tryptase on IL-8 mRNA expression in human ASM cells. Confluent and growth-arrested human ASM cells were incubated with increasing concentrations of tryptase for 24 hours (A), or were pre-incubated with actinomycin D (5 μ g/ml) for 1 hour before being stimulated with tryptase (15 mU/ml) for 8 hours (B). The levels of IL-8 mRNA were measured by real-time PCR and normalized to the house-keeping gene β_2 -microglobulin. Each point represents the mean $\pm$ SEM of three independent experiments performed in triplicate.

reporter plasmids containing the wild-type IL-8 promoter sequence (–162) or its mutations. Tryptase (7.5 mU/ml), after 6 hours of incubation, caused a marked increase (3.3-fold) in IL-8 promoter activity compared with the control in cells transfected with plasmids containing the wild-type IL-8 promoter sequence (Figure 6A). The effect of tryptase was significantly reduced when cells were transfected with plasmids containing the IL-8 promoter

Figure 4. Effect of tryptase on IL-8 mRNA stability. Confluent and growth-arrested human ASM cells were pre-incubated without (control) or with tryptase (15 mU/ml) for 4 hours before incubation with or without actinomycin D (5 μ g/ml) for the times indicated. mRNA levels of IL-8 and GAPDH were measured by RT-PCR. Density analysis was conducted and data for IL-8 were normalized against GAPDH. Each point represents the mean $\pm$ SEM of three experiments.

sequence mutated at the AP-1-, NF- κ B-, or C/EBP-binding sites (Figure 6A), suggesting that all three transcription factors are concurrently necessary for maximum IL-8 induction by tryptase.

To confirm that tryptase activated these transcription factors, cells were transfected with plasmids containing multiple repeat of AP-1-, NF- κ B-, or C/EBP-binding sequences as described in MATERIALS AND METHODS. Tryptase caused 4.7-, 5.8-, and 4.8-fold increases in AP-1, NF- κ B, and C/EBP reporter activity, respectively, compared with control (Figure 6B), confirming that tryptase indeed activates these transcription factors in human ASM cells.

Tryptase Induces *In Vivo* Binding of Transcription Factors AP-1, NF- κ B, and C/EBP to the IL-8 Promoter

The semiquantitative ChIP assay was then applied to analyze transcription factor binding to the IL-8 promoter in a chromatin context (*in vivo*) using antibodies against a typical subunit of AP-1 (c-Jun), CEBP/P (C/EBP β), and NF- κ B (p65), respectively. A fixed amount of immunoprecipitated DNA was amplified by PCR with primer pairs spanning the IL-8 promoter segment containing AP-1-, NF- κ B-, and C/EBP-binding sites. After tryptase treatment (15 mU/ml), immunoprecipitates with all three antibodies showed a marked enrichment of IL-8 promoter DNA segment in a time-dependent manner compared with control. The kinetics was slightly different for each transcription factor, with significant increases occurring 1 hour after treatment for c-Jun and 2.5 hours after treatment for p65 and C/EBP β (Figure 7). The results indicate that tryptase induces *in vivo* binding of c-Jun, C/EBP β , and p65 to the IL-8 promoter in human ASM cells.

DISCUSSION

The main findings of our studies are that tryptase up-regulates IL-8 gene expression in human ASM cells through a PAR-2-independent proteolytic mechanism, with coordinated regulation at both transcriptional and post-transcriptional levels, and that the transcriptional regulation of IL-8 expression is dependent upon the activation and promoter binding of the transcription factors NF- κ B, AP-1, and C/EBP to the IL-8 gene promoter. This is the first systematic study to explore the molecular mechanisms of the transcriptional regulation of chemokine genes by tryptase in any cell system. These findings are important in understanding the role of β -tryptase in the regulation of inflammatory gene expression and the signal transduction mechanisms of β -tryptase.

We found that tryptase time- and concentration-dependently increased IL-8 release from human ASM cells. We used purified tryptase from a commercial source for these studies. To ensure that the effects of tryptase on IL-8 were indeed due to tryptase, we confirmed these effects using recombinant human tryptase (rh-tryptase). Since the specific activities of these two tryptase preparations were measured with different substrates, even though the same concentrations of the two tryptases in terms of specific activities were used in the experiment, different quantities of these two tryptases were actually applied. For instance, 7.5 mU/ml of tryptase was the equivalent of 583 ng/ml, whereas 7.5 mU/ml of rh-tryptase was the equivalent of 7.5 ng/ml, the difference was approximately 78-fold. There may be a number of main reasons for this difference: different substrates in measuring specific activities, lost activity in the purified preparation, and the contaminating proteins in the purified preparation. However, since the aim of this experiment was to confirm the effect of tryptase on IL-8 production with rh-tryptase, different quantities of the two tryptase should not affect the final conclusion, particularly when the quantities of rh-tryptase were smaller than those of purified tryptase. Indeed, we found that rh-tryptase increased IL-8 production in a manner similar to that of the purified preparation,

Figure 5. Effect of protease inhibitors on trypsin-induced IL-8 production and effect of SLIGKV-NH₂ on IL-8 production. (A, B, and C) Trypsin (7.5 mU/ml, A and B) and bradykinin (10 μ M, C) were pre-treated with or without protease inhibitor leupeptin (2.5 μ g/ml), or protease inhibitor cocktail (1:200 dilution), or the selective trypsin inhibitor FUT-175 (FUT), at 37°C for 60 minutes. Confluent and growth-arrested human ASM cells were then incubated with or without the above preparations for 24 hours. (D) Confluent and growth-arrested NHBE cells and human ASM cells were incubated with or without SLIGKV-NH₂ (500 μ M) for 24 hours. IL-8 concentration in the media was measured by ELISA. Each point represents the mean $\pm$ SEM of four to eight determinations from two to four separate experiments. * P < 0.05, *** P < 0.001 compared with the effect of trypsin. (E) Total cell lysates were prepared from confluent and growth-arrested NHBE cells and human ASM cells, and protease-activated receptor 2 expression was analyzed by Western blotting.

although (as would be expected) it required the presence of heparin, which had no effect on its own. This is consistent with our recent finding that recombinant human trypsin mimicked the effect of purified trypsin on the cleavage of eotaxin and RANTES only in the presence of heparin (13). This is also consistent with the findings that rh-trypsin induces IL-8 production from eosinophils (5) and neutrophils (6). Purified trypsin did not require exogenous heparin to induce IL-8 production because heparin was present in the trypsin preparation.

As trypsin has proteolytic properties we performed experiments to determine if these were involved in its mechanism of action on IL-8 gene expression. We applied leupeptin, a protease inhibitor cocktail, and the selective trypsin inhibitor FUT-175 to inhibit the proteolytic activity of trypsin and found that all inhibited trypsin-induced IL-8 production but not bradykinin-induced IL-8 production, strongly suggesting that the effect of trypsin is mediated through a proteolytic mechanism. FUT-175 is a serine protease inhibitor. Although it inhibits other serine proteases such as trypsin (K_i = 15 nM) and thrombin (K_i = 900 nM), it is most potent against trypsin (K_i = 0.095 nM) (30). The fact that trypsin-induced IL-8 production from human ASM cells was significantly inhibited by FUT-175 at 0.01 nM and 0.1 nM in the current study also strongly suggests the proteolytic effect is trypsin specific. It is well documented that some of the cellular effects of trypsin are mediated by cleaving PAR-2 through its proteolytic activity (2, 14). PAR-2 has been shown to be expressed in human ASM cells (31), and the PAR-2-activating peptide SLIGKV-NH₂, which activates PAR-2 nonproteolytically, has been shown to mimic the effects of trypsin on IL-8 production in eosinophils (5), neutrophils (6), endothelial cells (8), and epithelial cells (9) and other effects of trypsin in various cell types including human ASM cells (31–33). We therefore compared the action of trypsin on IL-8 with SLIGKV-NH₂. The findings that SLIGKV-NH₂ (500 μ M) caused a marked increase in IL-8 production in NHBE cells but not from ASM cells despite the use of a higher concentration (1,000 μ M; data not shown) and that the partially reversed control peptide LSLIGKV-NH₂ had no effect in NHBE cells demonstrate that the PAR-2 agonist induces IL-8 production in NHBE cells but not in human ASM cells. The different responses of these two types of cells to the PAR-2-activating peptide is consistent with the high-level PAR-2 protein expression in NHBE cells and the low-level PAR-2 protein expression in ASM cells. The results therefore strongly suggest that the effect of trypsin on IL-8 gene expression in human ASM cells is not mediated through PAR-2 activation. This is, to our best knowledge, the first study to show the PAR-2-independent proteolytic mechanism of trypsin in cellular functions of human

Figure 6. Identification of transcription factor involvement in tryptase-induced IL-8 transcription and activation of transcription factors activator protein (AP)-1, CCAAT/enhancer binding protein (C/EBP), and NF-κB by tryptase. (A) Sixty percent confluent human ASM cells were cotransfected with *Renilla* luciferase internal control plasmid (4 ng/well) and firefly luciferase plasmid (0.4 μg/well) containing either the wild-type IL-8 promoter sequence with AP-1, C/EBP, and NF-κB binding sites or IL-8 promoter with mutated AP-1, C/EBP, or NF-κB binding sites (denoted by x), respectively. (B) Sixty percent confluent human ASM cells were cotransfected with *Renilla* luciferase internal control plasmid (4 ng/ml) and firefly luciferase plasmid (0.4 μg/well) containing repeated sequences for the binding sites of AP-1, NF-κB, or C/EBP. Transfection was conducted using FuGene 6 transfection reagent for 16 hours. The transfected cells were then incubated with or without tryptase (7.5 mU/ml) for 6 hours. Luciferase activities were assayed using the dual luciferase reporter assay system, and the relative luciferase activity was obtained by normalizing firefly luciferase activity against *Renilla* luciferase activity. Data were expressed as fold increase over the control. Each point represents mean ± SEM of 15 to 18 determinations from three independent experiments.

ASM cells and is in line with similar observations by other investigators using ASM cells of other species, in which PAR-2 is expressed. For instance, Brown and associates have recently found that tryptase activates PI3-kinases proteolytically independently from PAR-2 (PAR-2 activating peptide has no effect in cultured dog airway smooth muscle cells) (34); Corteling and coworkers have also demonstrated that tryptase exerts its mitogenic effect on guinea-pig tracheal smooth muscle cells via a PAR-2-independent proteolytic mechanism (35). In addition to ASM cells, this phenomenon has also been observed in other cells. For instance, the mitogenic effect of tryptase in cultured human dermal fibroblasts is also via a proteolytic mechanism independent from PAR-2 (36). It has been speculated that tryptase may activate PAR-4, rather than PAR-2, in ASM cells to stimulate PI-3 kinases (34). This may apply to tryptase effect on IL-8 gene expression in human ASM cells. It is also possible that tryptase may activate new receptors yet to be discovered either within or outside of the PAR family or may activate other known receptors whose functions have yet to be associated with tryptase.

Previous studies have shown that tryptase can stimulate IL-8 production from other cells (5–9), but ours is the first in human ASM cells. The ability of tryptase to induce IL-8 expression in human ASM cells could be important for the recruitment of inflammatory cells, such as neutrophils and eosinophils, to the sites of inflammation. We have previously demonstrated that human ASM cells can generate IL-8 in response to inflammatory stimuli, such as bradykinin and TNF-α, in human ASM cells (18–20), and that IL-8 expression by bradykinin involves prostanoid-dependent activation of transcription factors AP-1 and C/EBP and prostanoid-independent activation of transcription factor NF-κB (21). Although AP-1 and NF-κB have been shown to be activated by tryptase in eosinophils (5) and endothelial cells (8), respectively, the involvement of transcription factors in tryptase-induced IL-8 transcription has not been clearly defined in any cell system. Thus, to explore the molecular mechanisms of tryptase regulation of IL-8 gene expression in these cells, we then investigated whether the effect was transcriptional. Real-time and semi-quantitative RT-PCR showed that tryptase increased IL-8

Figure 7. Effect of tryptase on AP-1, C/EBP, and NF-κB *in vivo* binding to the IL-8 promoter. Eighty to ninety percent confluent and serum-deprived human ASM cells were treated with tryptase (15 mU/ml) for 0, 1, or 2.5 hours. AP-1, C/EBP, and NF-κB binding to the IL-8 promoter was analyzed by chromatin immunoprecipitation assay with specific antibodies against AP-1 (c-Jun; open bars in B), C/EBP (C/EBPβ; shaded bars in B) and NF-κB (p65; solid bars in B). Results are representative of three independent experiments with similar results (A). Density analysis for the PCR gels of the above experiment was normalized against DNA input control, and data were expressed as fold increase over 0 hours (untreated). Each point represents the mean ± SEM of three experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with control cells (B).

mRNA, which was abolished by the transcription inhibitor actinomycin D. Subsequent studies using reporter gene assay also showed that tryptase activated the wild-type IL-8 promoter. These results suggest that tryptase induces IL-8 gene expression transcriptionally. Since the core IL-8 promoter contains binding sites for transcription factors AP-1, C/EBP, and NF- κ B, we then went on to determine whether these transcription factors were responsible for mediating tryptase-induced IL-8 gene transcription. We found that tryptase induction of wild-type IL-8 promoter activity was markedly reduced with the mutation at the binding sites for AP-1, C/EBP, or NF- κ B, respectively, suggesting that all three transcription factors are involved in the optimal IL-8 transcription by tryptase. Consistent with this, tryptase also stimulated the activity of reporter gene constructs with multiple repeat of the binding sequences for AP-1, C/EBP, and NF- κ B, respectively. To confirm that these transcription factors were binding to the IL-8 promoter in native chromatin environment, we performed chromatin immunoprecipitation (ChIP) assay as described in our previous studies (19, 23, 37), and we found that all three transcription factors bound to the IL-8 promoter after tryptase stimulation. The mechanisms involved in IL-8 gene transcription by cytokines, but not mast cell tryptase, have been investigated in other cell types. These studies have shown that the IL-8 gene is activated in a cell- and stimulus-specific manner, with different transcription factors being necessary for optimal IL-8 gene transcription in different cell types. Holtmann and colleagues have shown that NF- κ B is required for IL-8 transcription in all cell types studied, whereas AP-1 and C/EBP are dispensable for transcriptional activation in some cells, but contribute to activation in others (16). Our current study is the first to show that all three of these transcription factors contribute to optimal IL-8 transcription in response to tryptase in any cell system, which is also consistent with our previous findings that these transcription factors are all involved in bradykinin-induced IL-8 transcription in these cells (21). It is unlikely that the activation is mediated by prostanoids since tryptase, unlike bradykinin (21), does not induce prostanoid production in these cells (unpublished observations).

In addition to transcriptional regulation, many chemokine mRNAs undergo post-transcriptional modification. IL-8 mRNA is unstable and its rapid degradation is mediated by the adenosine- and uridine (AU)-rich elements (ARE) contained in its 3'-untranslated region (3'UTR) (16). It has been demonstrated that ARE forms stable complexes with either stabilizing or destabilizing ARE-binding proteins to regulate mRNA stability. By measuring IL-8 mRNA stability with transcription arrest experiment, we showed in this study that tryptase also stabilized IL-8 mRNA, suggesting that tryptase up-regulation of IL-8 gene expression requires not only transcriptional regulation but also additional regulation at the post-transcriptional level in human ASM cells. Recent studies have shown that stabilization of IL-8 mRNA is achieved through the activation of the p38 MAPK pathway by cytokines and direct cell stress (17, 38, 39) and that ultraviolet light induces interaction of the 3'UTR sequences of IL-8 with HuR (39), a known stabilizing ARE-binding protein (40). Further studies are required to determine whether the stabilization of IL-8 mRNA by tryptase requires the activation of the p38 MAPK pathway and HuR.

In conclusion, we have shown that tryptase up-regulates IL-8 expression in cultured human ASM cells through a PAR-2-independent proteolytic mechanism and coordinated actions of transcriptional regulation to induce mRNA transcription through the activation of transcription factors NF- κ B, AP-1, and C/EBP and the additional post-transcriptional regulation to suppress mRNA degradation. This study is the first to demonstrate that mast cell tryptase regulates chemokine gene expression through

both transcriptional and post-transcriptional mechanisms, and the findings will improve our understanding of the role and mechanisms of action of tryptase in mast cell-related diseases. It is also important to note that the molecular mechanisms that tryptase employs to regulate IL-8 gene expression described in this study are not restricted to IL-8 but may also be relevant for many other chemokines and growth factors regulated by tryptase.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Colin Clelland for providing us with specimens of human trachea, and Dr. Claire Seedhouse and Miss Lisa Corbett for helping with real-time PCR.

References

- Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135–142.
- Hallgren J, Pejler G. Biology of mast cell tryptase: an inflammatory mediator. *FEBS J* 2006;273:1871–1895.
- Caughey GH. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* 2007;217:141–154.
- Schwartz LB, Bradford TR. Regulation of tryptase from human lung mast cells by heparin: stabilization of the active tetramer. *J Biol Chem* 1986;261:7372–7379.
- Temkin V, Kantor B, Weg V, Hartman ML, Levi-Schaffer F. Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *J Immunol* 2002;169:2662–2669.
- Wang H, He S. Induction of lactoferrin and IL-8 release from human neutrophils by tryptic enzymes via proteinase activated receptor-2. *Cell Biol Int* 2006;30:688–697.
- Compton SJ, Cairns JA, Holgate ST, Walls AF. Human mast cell tryptase stimulates the release of an IL-8-dependent neutrophil chemotactic activity from human umbilical vein endothelial cells (HUVEC). *Clin Exp Immunol* 2000;121:31–36.
- Shpacovitch VM, Brzoska T, Buddenkotte J, Stroh C, Sommerhoff CP, Ansel JC, Schulze-Osthoff K, Bunnett NW, Luger TA, Steinhoff M. Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells. *J Invest Dermatol* 2002;118:380–385.
- Wang H, Zheng Y, He S. Induction of release and up-regulated gene expression of interleukin (IL)-8 in A549 cells by serine proteinases. *BMC Cell Biol* 2006;7:22.
- Howarth PH, Knox AJ, Amrani Y, Tliba O, Panettieri RA Jr, Johnson M. Synthetic responses of airway smooth muscle. *J Allergy Clin Immunol* 2004;114:S32–S50.
- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699–1703.
- Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R, Walls AF, Tunon de Lara JM. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J* 2003;17:2139–2141.
- Pang L, Nie M, Corbett L, Sutcliffe A, Knox AJ. Mast cell β -tryptase selectively cleaves eotaxin and RANTES and abrogate their eosinophil chemotactic activities. *J Immunol* 2006;176:3788–3795.
- Ossoskaya VS, Bunnett NW. Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 2004;84:579–621.
- Brown JK, Jones CA, Rooney LA, Caughey GH, Hall IP. Tryptase's potent mitogenic effects in human airway smooth muscle cells are via nonproteolytic actions. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L197–L206.
- Hoffmann E, Dittrich-Breiholz O, Holtmann H, Kracht M. Multiple control of interleukin-8 gene expression. *J Leukoc Biol* 2002;72:847–855.
- Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, Malinin NL, Cooper JA, Resch K, Kracht M. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol* 1999;19:6742–6753.
- Pang L, Knox AJ. Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: role of cyclooxygenase products. *J Immunol* 1998;161:2509–2515.
- Nie M, Corbett L, Knox AJ, Pang L. Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma

- agonists: interactions with glucocorticoids and β_2 -agonists. *J Biol Chem* 2005;280:2550–2561.
20. Pang L, Knox AJ. Synergistic inhibition by β_2 -agonists and corticosteroids on TNF α -induced IL-8 release from cultured human airway smooth-muscle cells. *Am J Respir Cell Mol Biol* 2000;23:79–85.
21. Zhu YM, Bradbury DA, Pang L, Knox AJ. Transcriptional regulation of interleukin (IL)-8 by bradykinin in human airway smooth muscle cells involves prostanoind-dependent activation of AP-1 and nuclear factor (NF)-IL-6 and prostanoind-independent activation of NF-kappaB. *J Biol Chem* 2003;278:29366–29375.
22. Pang L, Knox AJ. Effect of IL-1 β , TNF α and IFN γ on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells. *Br J Pharmacol* 1997;121:579–587.
23. Nie M, Knox AJ, Pang L. β_2 -adrenoceptor agonists, like glucocorticoids, repress eotaxin gene transcription by selective inhibition of histone H4 acetylation. *J Immunol* 2005;175:478–486.
24. Brasier AR, Jamaluddin M, Casola A, Duan W, Shen Q, Garofalo RP. A promoter recruitment mechanism for tumor necrosis factor- α -induced interleukin-8 transcription in type II pulmonary epithelial cells. Dependence on nuclear abundance of Rel A, NF-kappaB1, and c-Rel transcription factors. *J Biol Chem* 1998;273:3551–3561.
25. Casola A, Garofalo RP, Jamaluddin M, Vlahopoulos S, Brasier AR. Requirement of a novel upstream response element in respiratory syncytial virus-induced IL-8 gene expression. *J Immunol* 2000;164:5944–5951.
26. Bergmann M, Hart L, Lindsay M, Barnes PJ, Newton R. I κ B α degradation and nuclear factor- κ B DNA binding are insufficient for interleukin-1 β and tumor necrosis factor- α -induced κ B-dependent transcription. Requirement for an additional activation pathway. *J Biol Chem* 1998;273:6607–6610.
27. Kieser A, Kilger E, Gires O, Ueffing M, Kolch W, Hammerschmidt W. Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. *EMBO J* 1997;16:6478–6485.
28. Yang H, Mammen J, Wei W, Menconi M, Evenson A, Fareed M, Petkova V, Hasselgren PO. Expression and activity of C/EBP β and δ are upregulated by dexamethasone in skeletal muscle. *J Cell Physiol* 2005;204:219–226.
29. Pang L, Nie M, Corbett L, Knox AJ. Cyclooxygenase-2 expression by non-steroidal anti-inflammatory drugs in human airway smooth muscle cells: role of peroxisome proliferators-activated receptors. *J Immunol* 2003;170:1043–1051.
30. Mori S, Itoh Y, Shinohara R, Sendo T, Oishi R, Nishibori M. Nafamostat mesilate is an extremely potent inhibitor of human trypsin. *J Pharmacol Sci* 2003;92:420–423.
31. Schmidlin F, Amadesi S, Vidil R, Trevisani M, Martinet N, Caughey G, Tognetto M, Cavallero G, Mapp C, Geppetti P, et al. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *Am J Respir Crit Care Med* 2001;164:1276–1281.
32. Asano-Kato N, Fukagawa K, Okada N, Dogru M, Tsubota K, Fujishima H. Trypsin increases proliferative activity of human conjunctival fibroblasts through protease-activated receptor-2. *Invest Ophthalmol Vis Sci* 2005;46:4622–4626.
33. Ui H, Andoh T, Lee JB, Nojima H, Kuraishi Y. Potent pruritogenic action of trypsin mediated by PAR-2 receptor and its involvement in anti-pruritic effect of nafamostat mesilate in mice. *Eur J Pharmacol* 2006;530:172–178.
34. Brown JK, Hollenberg MD, Jones CA. Trypsin activates phosphatidylinositol 3-kinases proteolytically independently from proteinase-activated receptor-2 in cultured dog airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L259–L269.
35. Corteling R, Bonneau O, Ferretti S, Ferretti M, Trifilieff A. Differential DNA synthesis in response to activation of protease-activated receptors on cultured guinea-pig tracheal smooth muscle cells. *Naunyn Schmiedeberg Arch Pharmacol* 2003;368:10–16.
36. Akers IA, Parsons M, Hill MR, Hollenberg MD, Sanjar S, Laurent GJ, McNulty RJ. Mast cell trypsin stimulates human lung fibroblast proliferation via protease-activated receptor-2. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L193–L201.
37. Nie M, Pang L, Inoue H, Knox AJ. Transcriptional regulation of cyclooxygenase-2 by bradykinin and IL-1 β in human airway smooth muscle cells: involvement of different promoter elements, transcription factors and histone H4 acetylation. *Mol Cell Biol* 2003;23:9233–9244.
38. Winzen R, Kracht M, Ritter B, Wilhelm A, Chen CY, Shyu AB, Muller M, Gaestel M, Resch K, Holtmann H. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J* 1999;18:4969–4980.
39. Bollig F, Winzen R, Kracht M, Ghebremedhin B, Ritter B, Wilhelm A, Resch K, Holtmann H. Evidence for general stabilization of mRNAs in response to UV light. *Eur J Biochem* 2002;269:5830–5839.
40. Fan XC, Steitz JA. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J* 1998;17:3448–3460.