

Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells

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Background: Matrix metalloproteinases (MMPs) digest extracellular matrix components and might be important mediators of tissue remodeling. Proteinase activated receptor-2 (PAR-2) is expressed in a variety of cell types including epithelial cells. PAR-2 receptors are activated by serine proteases such as trypsin and mast cell tryptase and have been implicated in inflammation.

Objective: To study the effects of PAR-2-mediated airway epithelial cell activation on the production of MMP-9.

Methods: A specific PAR-2-activating peptide and trypsin were used to activate the human airway epithelial cell line A549 as well as primary cultures of small airway epithelial cells (SAEC). MMP-2 and MMP-9 messenger RNA and enzymatic activity were evaluated by RT-PCR and gelatin zymography, respectively.

Results: PAR-2-activating peptides upregulated MMP-9 mRNA expression and release of MMP-9 enzymatic activity from airway epithelial cells but had no effect on MMP-2 production. Dexamethasone and budesonide (10^{-6} to 10^{-10} mmol) inhibited PAR-2-mediated MMP-9 release. Pretreatment with indomethacin indicated that MMP-9 release was not prostaglandin dependent. Inhibitors of the MAP kinase MEK-1, and NF κ B showed that both pathways are important for PAR-2-mediated MMP-9 release. Trypsin, a physiologic PAR-2 activator, upregulated MMP-9 but also MMP-2 release from airway epithelial cells.

Conclusion: PAR-2 receptors appear to play an important role in the regulation of MMP-9 release from airway epithelial cells. As such, these receptors may be critical elements in tissue remodeling in asthma and other inflammatory conditions in the airways. (*J Allergy Clin Immunol* 2000;106:537-45.)

Key words: Inflammation, lung, matrix metalloproteinases, trypsin, corticosteroids

Tissue remodeling is an important feature of asthma. The latter is characterized by airway smooth muscle hypertrophy and a number of other changes, including subbasement membrane thickening, loss of epithelium, inflammatory cell infiltration, and goblet-cell hyperplasia. The exact mediators that cause these changes and their regulation are poorly understood.

Matrix metalloproteinases (MMPs) are zinc and calcium-dependent proteases that digest most extracellular matrix components. MMPs are produced by structural cells such as fibroblasts and endothelial and epithelial cells and by many inflammatory cells including macrophages,¹ eosinophils,² neutrophils,³ and mast cells.⁴ They are secreted as latent proenzymes and converted to the active form by proteolytic cleavage of an amino-terminal domain.⁵ MMPs might be important in tissue remodeling in the airways through their ability to affect the integrity of the basal lamina and the degree of infiltration by inflammatory cells.

MMP-2 and MMP-9 have been shown to be important mediators of inflammation in a murine model of asthma⁶ and in immune complex-mediated lung injury.⁷ Biopsy specimens from asthmatic patients show abundant expression of MMP-9 messenger RNA by in situ hybridization.² This mRNA localized mostly in eosinophils. MMP-9 in the bronchoalveolar lavage fluid is higher in asthmatic patients⁸ and in patients in status asthmaticus⁹ than in normal control subjects. Significant levels of MMP-9 are also found in the sputum of asthmatic patients.¹⁰ In addition, MMP-9 production by eosinophils is required for their transmigration through Matrigel monolayers in vitro.¹¹ Finally, gelatinases are also important for T-cell transmigration through the basal lamina.¹² These observations imply a significant role for MMPs in asthma, but the stimuli that induce their production during allergic inflammation are not known.

Airway epithelium plays an active role in inflammation through the production of a variety of lipid mediators, cytokines, chemokines, growth factors, and extracellular matrix components. Epithelial cells are also an important source of MMP-2 and MMP-9; the expression of the latter is upregulated by proinflammatory stimuli.^{13,14}

Protease-activated receptor-2 (PAR-2) has been detected in many tissues, but its exact physiologic and

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Abbreviations used

DMEM:	Dulbecco's modified Eagle's medium
MAP kinase:	Mitogen-activated protein kinase
MMP:	Matrix metalloproteinase
PAR:	Proteinase-activated receptor
SAEC:	Small airway epithelial cells
TIMP:	Tissue inhibitors of metalloproteinase

pathophysiologic role is not clear.¹⁵ Trypsin and mast-cell tryptase¹⁶ are the only proteases shown to activate PAR-2. There is evidence that PAR-2 activation is involved in inflammation. Inflammatory cytokines increase PAR-2 mRNA expression in human umbilical vein endothelial cells.¹⁷ PAR-2 activation increases vascular permeability¹⁸ and induces nitric oxide-mediated vasodilatation,^{19,20} hypotension,¹⁹ leukocyte rolling, adhesion and extravasation,²¹ and neurogenic inflammation.²² Finally, PAR-2 activation can induce chemokine secretion.²³ However, PAR-2 receptors on airway epithelial cells have been shown to offer protection from bronchoconstriction.²⁴

We hypothesized that PAR-2 receptor activation also has proinflammatory effects in the airways. We found that PAR-2-mediated activation of airway epithelial cells induces MMP-9 release but not MMP-2, tissue inhibitors of metalloproteinase (TIMP)-1, or TIMP-2 release. We also showed that MMP-9 upregulation involved mitogen-activated protein kinase (MAP-kinase) and NF κ B activation, was prostaglandin-independent, and was completely inhibited by corticosteroids.

MATERIAL AND METHODS

Material

Pancreatic trypsin, dexamethasone, and MG-132 (Cbz-Leu-Leu-leucinal; Sigma-Aldrich Canada LTD, Oakville, Ontario, Canada); recombinant human TNF (Cedarlane Laboratories Ltd, Ontario, Canada); Trizol, murine Moloney leukemia virus reverse transcriptase, Taq polymerase, oligo-dT primers, (Gibco BRL, Ontario, Canada); Fura-2 (Molecular Probes Inc, Eugene, Ore); Dulbecco's modified Eagle's medium (DMEM), FCS, penicillin, streptomycin, and L-glutamine (Biowhittaker, Walkersville, Md) were purchased as shown. Budesonide was generously provided by Astra Pharma Inc (Mississauga, Ontario, Canada).

The human pulmonary type II-like epithelial cell line A549 was acquired from the American Type Culture Collection (Rockville, Md); small airway epithelial cells (SAEC) that were isolated from human airways were purchased from Clonetics (Walkersville, Md).

PCR primers were synthesized by the DNA services laboratory, University of Alberta. PAR-1-, PAR-2-, and PAR-4-activating peptides (>95% pure by HPLC and mass spectrometry criteria) were synthesized by the Peptide Synthesis Facility of the Faculty of Medicine, University of Calgary. Two PAR-2-activating peptides (transcinnamoyl-LIGRLO-NH₂ or tc-LIGRLO-NH₂ and SLIGRL-NH₂) and 1 inactive peptide (LRGILS-NH₂) were used. The 2 peptides activate PAR-2 receptors with the same potency²⁵ and are thought not to activate other known PAR receptors.²⁶ The anti-PAR-2 rabbit polyclonal antiserum B5 was generated as previously described.²⁷

Airway epithelial cell culture and activation

A549 cells were cultured in 12-well plates with DMEM supplemented with 10% FBS, 4 mmol/L L-glutamine, and 100 μ g/mL penicillin/streptomycin until confluent. They were then maintained for 24 hours in DMEM without supplements and subsequently activated for 24 hours in DMEM with the indicated concentrations of PAR-2-activating peptides, trypsin, or TNF. Supernatants were collected, concentrated at $\times 10$, and kept at -70°C until analyzed. Cells were either lysed in Tris-buffered saline solution (supplemented with 1% Triton X-100, 10 μ g/mL aprotinin, 4 μ g/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 mmol/L EDTA, pH 7.4) for zymography and Western blotting or solubilized in Trizol for RNA extraction.

SAEC were cultured in 12-well plates in serum-free medium supplemented with growth factors (30 μ g/mL bovine pituitary extract, 0.5 μ g/mL hydrocortisone, 0.5 ng/mL human recombinant epidermal growth factor, 0.5 μ g/mL epinephrine, 10 μ g/mL transferrin, 5 μ g/mL insulin, 0.1 ng/mL retinoic acid, 6 ng/mL triiodothyronine, 50 μ g/mL gentamicin, 50 ng/mL amphotericin B, and 0.5 mg/mL human epithelial growth factor) until they were 80% to 90% confluent and were maintained in DMEM without supplements for a further 48 hours. Cells were then activated in DMEM for 24 hours.

Corticosteroids, indomethacin, NF κ B, and MAP-kinase inhibitors were added for 3 hours before A549 cell activation with PAR-2-activating peptides.

Ca⁺⁺ measurements

A549 cells were loaded with Fura-2 as described²⁸ and analyzed with a CAF-100 Ca⁺⁺ analyzer (Japan Spectroscopic Co LTD, Tokyo, Japan). Data were obtained with Virtual Bench (National Instrument Corporation, Austin, Texas) and analyzed with Excel (Microsoft Corporation, Redmond, Wash). The intracellular concentration of free Ca⁺⁺ was calculated as described.²⁹

RT-PCR analysis

Total RNA was isolated from A549 cells with Trizol according to the manufacturer's instructions. PCR was performed with the following primers (0.4 μ mol/L concentration) for MMP-9 (5'-CGTGGA-GAGTCGAAATCTCTG-3'; 5'-CCAAACTGGATGACGATGTCT-3'), and MMP-2 (5'-CCCAAAACGGACAAAGAGTT-3'; 5'-ACTGTCTCTGGGTCCAGATCA-3'). The amplification of β 2-microglobulin (5'-CTCGCGCTACTCTCTCTTTCTGG-3'; 5'-GCTTACATGTCTCGATCCGACTTAA-3') was used to verify that equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 54°C for MMP-9 and MMP-2 and 58°C for β 2-microglobulin. Amplified fragment sizes for β 2-microglobulin, MMP-9, and MMP-2 were 335 bp, 331 bp, and 293 bp, respectively.

Gelatin zymography

SDS-PAGE gelatin zymography was performed with 7% polyacrylamide gels containing 2% gelatin. Phorbol myristate acetate-treated (100 nmol; 48 hours at 37°C) HT 1080 human fibrosarcoma cell supernatant was used as a positive control to identify gelatinolytic activity of proenzymes and active forms of MMP-2 and MMP-9.³⁰ After electrophoresis at 4°C, the gels were washed 3 times in 2% Triton X-100 at room temperature, incubated in 50 mmol/L Tris-HCl buffer (pH 7.6, supplemented with 0.15 mol NaCl, 5 mmol/L CaCl₂, and 0.05% NaN₃) at 37°C for 24 hours and then stained with 0.05% Coomassie Brilliant Blue G-250. Proteolytic activity was identified as clear bands on a blue background.

Statistical analysis and optical density measurements

Optical densities of zymograms were measured with the use of

the Sigma Gel Analysis program (version 1.0.5.0, 1995; Jandel Scientific). Band intensities of zymograms were expressed as percentage of treated versus untreated cells (average $\pm$ SEM). For statistical analysis, the paired Student *t* test was used. A probability value of less than .05 was considered significant.

RESULTS

PAR-2 activation induces MMP-9 mRNA and enzymatic activity in A549 cells

Trypsin (10 U/mL) induced a sharp increase in the intracellular Ca^{++} concentration in A549 cells (Fig 1), as did the PAR-2-activating peptide tc-LIGRLO-NH₂ (70 $\mu\text{mmol/L}$; Fig 1). The inactive peptide LRGILS-NH₂ had no effect. Although treatment of the cells with trypsin desensitized the PAR-2 response to tc-LIGRLO-NH₂ (Fig 1), pretreatment of the cells with tc-LIGRLO-NH₂ did not completely desensitize the cell calcium response to trypsin (Fig 1) but abolished the response to the subsequent application of tc-LIGRLO-NH₂, which suggests that trypsin may also activate receptors other than PAR-2 on A549 cells. We verified the presence of PAR-2 receptors on A549 cells by flow cytometry using the rabbit polyclonal anti-PAR-2 antibody B5. These results document the presence of functional PAR-2 receptors on A549 cells.

A549 cells expressed low levels of MMP-9 mRNA. The PAR-2-activating peptide tc-LIGRLO-NH₂ (70 $\mu\text{mmol/L}$) upregulated MMP-9 mRNA expression within 2 hours, and the mRNA levels remained significantly higher than in unstimulated cells for up to 24 hours (Fig 2, A). TNF (100 ng/mL), known to induce MMP-9 in other systems,³¹ also upregulated MMP-9 mRNA expression.

The PAR-2-activating peptide tc-LIGRLO-NH₂, at concentrations between 7 and 70 $\mu\text{mmol/L}$, also induced a dose-dependent increase in pro-MMP-9 release from A549 cells (Fig 2, B). The increase ranged from 6.5 ± 0.6 -fold with 7 $\mu\text{mmol/L}$ tc-LIGRLO-NH₂ to 71.7 ± 18.7 -fold with 70 $\mu\text{mmol/L}$. The inactive peptide LRGILS-NH₂ had no effect on MMP-9 release. TNF (100 ng/mL) also upregulated pro-MMP-9 release (124.2 ± 53.8 -fold). No active form of MMP-9 was released after PAR-2 activation. A second PAR-2-activating peptide, SLIGRL-NH₂, had similar effects over the same concentration range (data not shown). Treatment with PAR-2-activating peptides or TNF had no effect on the level of PAR-2 expression on the surface of A549 or on the proliferation rate, under the culture conditions used. In all cases 2 to 2.5×10^5 cells (viability by trypan blue, >98%) were recovered from each well of the 12-well culture plates. Neither a highly specific PAR-1-activating peptide (AparafluoroFRCyclohexylACitY-NH₂ at 40 $\mu\text{mmol/L}$) nor a PAR-4-activating peptide (GYPGKF-NH₂ at 400 $\mu\text{mmol/L}$) induced MMP-9 release from A549 cells (data not shown).

A549 cells showed significant basal pro-MMP-2 release that did not change after activation with PAR-2-activating peptides or TNF (Fig 2, B). Similarly, no change in MMP-2 mRNA was detected by RT-PCR (data not shown). MMP-2 or MMP-9 enzymatic activity was

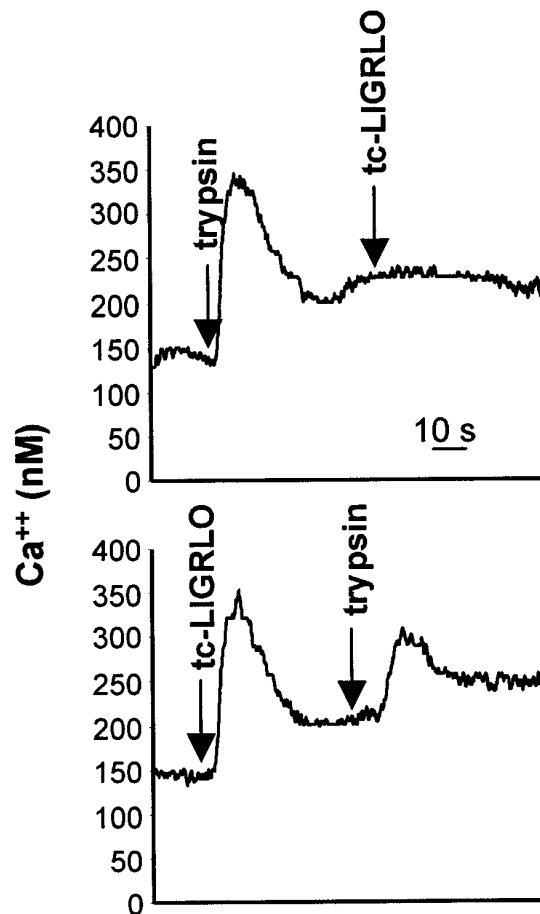


FIG 1. Free $[\text{Ca}^{++}]$ levels in A549 cells after activation with 10 U/mL of trypsin (top panel) or 70 $\mu\text{mmol/L}$ tc-LIGRLO-NH₂ (bottom panel).

not observed in A549 cell lysates after 24 hours of incubation with or without PAR-2-activating peptides, which indicates that most of the MMP-9 enzymatic activity released after stimulation is newly synthesized protein.

MMP-9 activity is partly regulated by TIMPs. PAR-2 activation did not affect TIMP-1 or TIMP-2 release from A549 cells (data not shown). These results pointed to a selective upregulation of synthesis and release of MMP-9 after PAR-2-mediated activation of A549 epithelial cells.

MMP production by human SAEC and effects of PAR-2 activation

SAEC cells released pro-MMP-9, pro-MMP-2, and the active form of MMP-2. SAEC produced higher basal amounts of MMP-9 than A549 cells. MMP-9 production by SAEC was upregulated after activation with PAR-2-activating peptides or TNF (Fig 2, C). The peptide tc-LIGRLO-NH₂ (18 $\mu\text{mmol/L}$) caused $56.3\% \pm 6.7\%$ and TNF (100 ng/mL) caused $55.7\% \pm 4.7\%$ increase of MMP-9 release compared with unstimulated cells. This effect was inhibited by approximately 30% after preincubation of SAEC with 10^{-6} mmol budesonide. The amount of MMP-9 mRNA was also increased by more than 2.5-

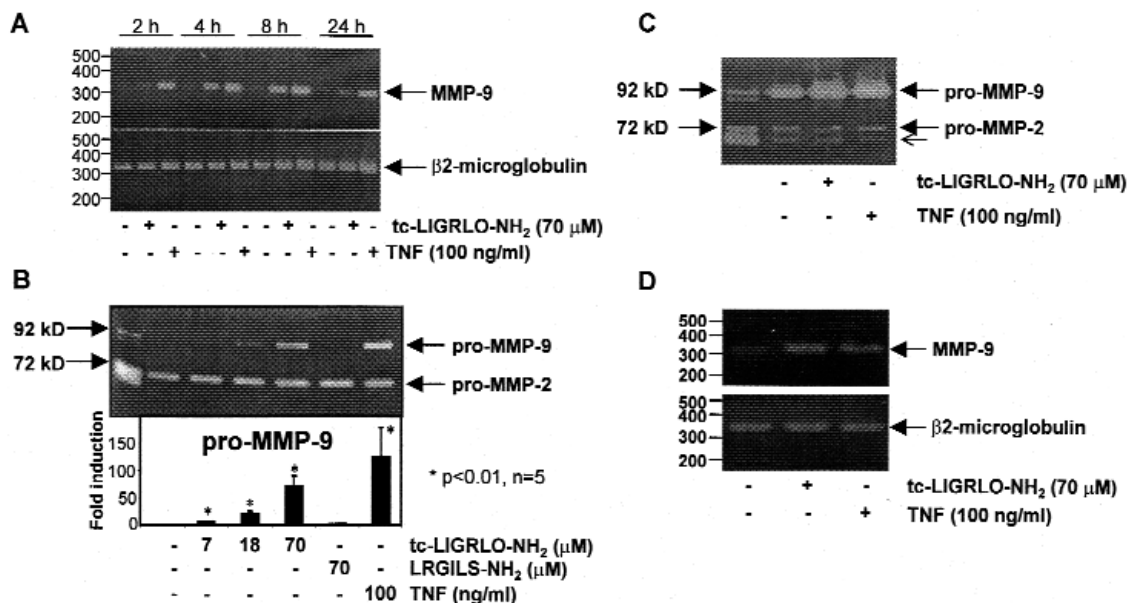


FIG 2. Effect of PAR-2 activation on MMP-9 production by A549 epithelial cells and SAEC. **A**, RT-PCR analysis of MMP-9 mRNA expression by A549 epithelial cells without activation or after stimulation with the PAR-2-activating peptide tc-LIGRLO-NH₂ (70 μmol/L) or TNF (100 ng/mL) for 2, 4, 8, or 24 hours (top panel, 35-cycle amplification of MMP-9 mRNA; lower panel, 22-cycle amplification of β2-microglobulin mRNA). The gel is representative of 4 similar experiments. **B**, Gelatin zymography of supernatants of A549 cells resting or after activation with various concentrations of tc-LIGRLO-NH₂, the inactive peptide LRGILS-NH₂, or TNF for 24 hours. A representative gelatin zymography gel (top panel) and the average (± SEM) of optical density measurements of MMP-9 bands from 5 separate experiments (lower panel) are shown. The left lane, as in all subsequent gels, shows a positive control for MMP-9 (92 kd) and MMP-2 (72 kd). **C**, Gelatin zymography of supernatants of SAEC cells with or without activation with tc-LIGRLO-NH₂ (70 μmol/L) or TNF (100 ng/mL). The thin arrow points to the activated form of MMP-2. Gel shown is representative of 4 similar experiments. **D**, RT-PCR of MMP-9 mRNA expression in SAEC cells with or without activation with tc-LIGRLO-NH₂ (70 μmol/L) or TNF (100 ng/mL). Representative of 3 similar experiments.

fold after 24 hours of incubation with PAR-2-activating peptides or TNF (Fig 2, D). A smaller increase (approximately 1.5-fold) was seen at 4 hours and 8 hours.

Effect of corticosteroids on MMP-9 induction

Corticosteroids have been shown to inhibit the production of MMP-9 in other systems. To study the effect of dexamethasone on pro-MMP-9 release, we preincubated A549 cells for 3 hours with 10⁻⁶ mmol dexamethasone before activating the cells as described earlier. Dexamethasone almost completely abolished the induction of MMP-9 release by the tc-LIGRLO-NH₂ or TNF (Fig 3, A). Budesonide (a corticosteroid used in its inhaled form in patients with asthma) inhibited PAR-2-mediated pro-MMP-9 release by more than 70% at concentrations between 10⁻⁶ and 10⁻⁹ mmol and by 33% ± 6.9% at 10⁻¹⁰ mmol (Fig 3, B). Budesonide had the same effect on SLIGRL-NH₂ and on TNF-mediated pro-MMP-9 release (data not shown). This effect of budesonide was mediated through the downregulation of MMP-9 mRNA (Fig 3, C). Dexamethasone and budesonide (10⁻⁶ mmol) also decreased basal MMP-2 release from A549 cells (Fig 3, A and B) by 65.3% ± 10.9% (*P*

< .02; *n* = 3 experiments) and 45.5% ± 7.8% (*P* < .001; *n* = 7 experiments), respectively.

Signal transduction pathways that induce MMP-9 release from A549 cells

PAR-2 activation induces prostaglandin production by airway epithelial cells,²⁴ and prostaglandins are known to mediate MMP-9 upregulation.^{31,32} The cyclooxygenase inhibitor indomethacin (10⁻⁵ mmol) did not affect PAR-2- or TNF-mediated MMP-9 release (Fig 4, A), which indicates that MMP-9 induction in our model is prostaglandin independent.

Because PAR-2 stimulation activates the MAP kinase pathway,³³ the inhibitor of MEK1, a kinase that is part of the MAP-kinase pathways, PD98059 was used. PD98059 (25 μmol/L) inhibited PAR-2-mediated MMP-9 release by 87% ± 8.2% (Fig 4, B). The same concentration of PD98059 had a lower (48.4% ± 2.62%) but still significant inhibitory effect on TNF-mediated MMP-9 release. The kinase p38 is also activated by TNF³⁴ and to a lesser extent by PAR-2 receptors.³³ The p38 inhibitor SB203580 (to 1 μmol/L) had no effect on MMP-9 release induced by PAR-2 or TNF (data not shown), which indicates that p38

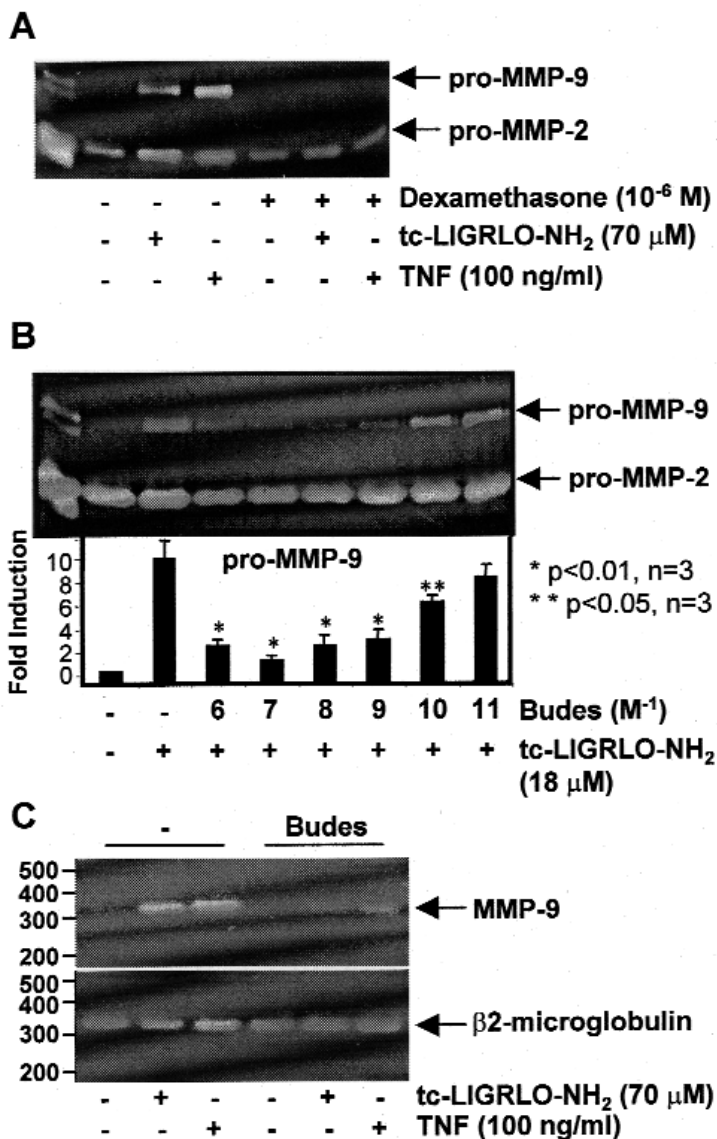


FIG 3. Effect of corticosteroids on PAR-2-mediated MMP-9 upregulation in A549 epithelial cells. **A**, Gelatin zymography of supernatants of A549 cells activated with tc-LIGRLO-NH₂ (70 μmol/L) or TNF (100 ng/mL) for 24 hours after 3 hours of preincubation with or without 10⁻⁶ mmol dexamethasone (left lane, positive control). Gel shown is representative of 4 similar experiments. **B**, Gelatin zymography of supernatants of A549 cells activated with tc-LIGRLO-NH₂ (18 μmol/L) for 24 hours after 3 hours of preincubation with various concentrations (10⁻⁶ to 10⁻¹¹ mmol) of budesonide. A representative experiment is shown in the top panel, and the average (± SEM) of densitometry measurements of the MMP-9 bands from 3 similar experiments is shown in the lower panel (left lane, positive control). **C**, RT-PCR of MMP-9 (top panel) and β2-microglobulin (bottom panel) mRNA expression, after activation with tc-LIGRLO-NH₂ (70 μmol/L) or TNF (100 ng/mL) for 4 hours with or without preincubation (3 hours) with budesonide 10⁻⁶ mmol. Gel shown is representative of 3 similar experiments.

does not participate in the MMP-9 release from A549 cells.

Corticosteroids have been shown to mediate some of their functions through NFκB inhibition.³⁵ MG-132, a proteasome inhibitor shown to inhibit NFκB in A549 cells,³⁶ was used to examine NFκB involvement in PAR-2-mediated MMP-9 release. MG-132 (after 3 hours of preincubation) significantly inhibited PAR-2-mediated

(70 μmol/L tc-LIGRLO-NH₂) MMP-9 release (67.8% ± 5.4% at 50 μmol/L) and to a lesser extent TNF-mediated (100 ng/mL) MMP-9 release (22.4% ± 3.4% at 50 μmol/L; Fig 4, B). PD98059 together with MG-132 (10 μmol/L each) caused over 90% inhibition of PAR-2-mediated MMP-9 release, which suggests that MAP-kinase and NFκB pathways work in parallel.

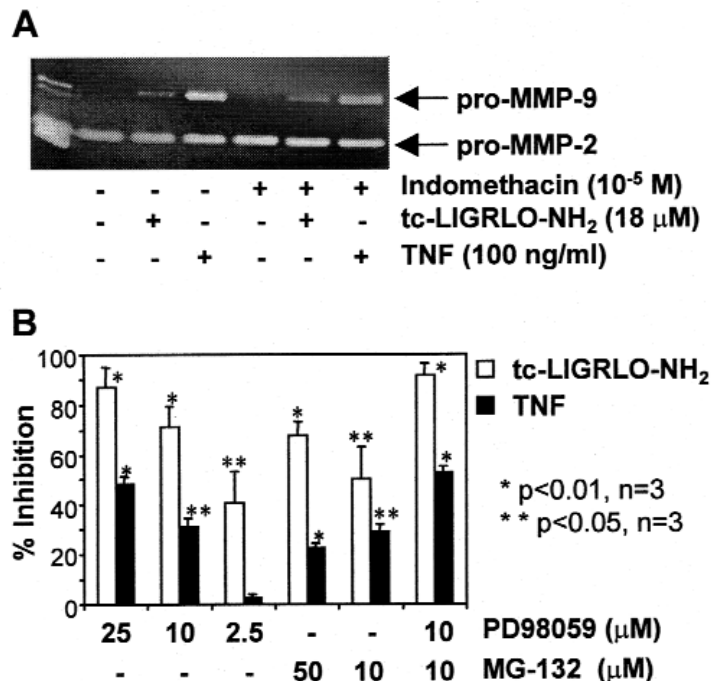


FIG 4. Signal transduction of PAR-2-mediated MMP-9 upregulation in A549 epithelial cells. **A**, Effect of indomethacin on MMP-9 release. Gelatin zymography of supernatants of A549 cells activated with tc-LIGRLO-NH₂ (70 μ mol/L) or TNF (100 ng/mL) for 24 hours after 3 hours of preincubation with 10^{-5} mol indomethacin. A representative gelatin zymography of 3 experiments performed, with the same results, is shown (left lane, positive control). **B**, Effect of different concentrations of the MAP kinase inhibitor PD98059 and the NF κ B inhibitor MG-132 on PAR-2- and TNF-mediated MMP-9 release from A549 cells. The cells were preincubated for 3 hours with the inhibitors before being activated with tc-LIGRLO-NH₂ (18 μ mol/L) or TNF (100 ng/mL) for 24 hours. The graph shows the percent inhibition of MMP-9 release compared with cells that were not preincubated with the inhibitors. TNF (100 ng/mL) caused 117.4 ± 38.3 and tc-LIGRLO-NH₂ (18 μ mol/L) 17.5 ± 1.2 -fold induction of MMP-9 over control.

Trypsin effect on MMP-9 production

Pancreatic trypsin in concentrations between 0.1 and 2 U/mL (higher concentrations of trypsin induced the detachment of the cells from the culture surface) caused significant upregulation of both pro-MMP-2 and pro-MMP-9 (Fig 5, A). Trypsin, at 0.5 U/mL, induced a 2.0 ± 0.4 -fold increase in pro-MMP-2 release and an 8.1 ± 1.0 -fold increase in pro-MMP-9 release (Fig 5, B). When concentrations of trypsin higher than 0.5 U/mL were used, the release of pro-MMP-2 was very high in concentrated supernatants, which rendered the gel unsuitable for densitometry. Using unconcentrated A549 supernatants, we found that the strongest upregulation of pro-MMP-2 release was a 30.2 ± 5.8 -fold with 2 U/mL of trypsin (data not shown). Trypsin also induced the release of at least 2 more proteins with gelatinolytic activity (Fig 5, A). These results indicate that, in epithelial cells, the effect of trypsin on MMP release might also involve the activation of receptors other than PAR-2.

DISCUSSION

In this study, we demonstrated that PAR-2-mediated activation of airway epithelial cells upregulated the synthesis and release of pro-MMP-9. MMP-9 was secreted in the proenzyme form from epithelial cells after PAR-2 activation. Recently, PAR-2 activation has been shown to cause relaxation of airways that was mediated by the release of cyclooxygenase products from airway epithelium,²⁴ which indicates a protective role for airway epithelial cell PAR-2. Our results indicate that the effect of PAR-2-receptor activation in the airways might be more complicated.

PAR-2 expression is abundant in the lung and the airways, but the physiologic functions and the physiologic ligands that activate PAR-2 are poorly understood. Trypsin immunoreactivity has been identified in airway epithelium,^{24,37} and a trypsin-like protease was purified from human airways.³⁸ It is likely, but yet to be verified, that this airway-derived trypsin-like enzyme may be able to activate PAR-2 receptors. Mast-cell tryptase might be

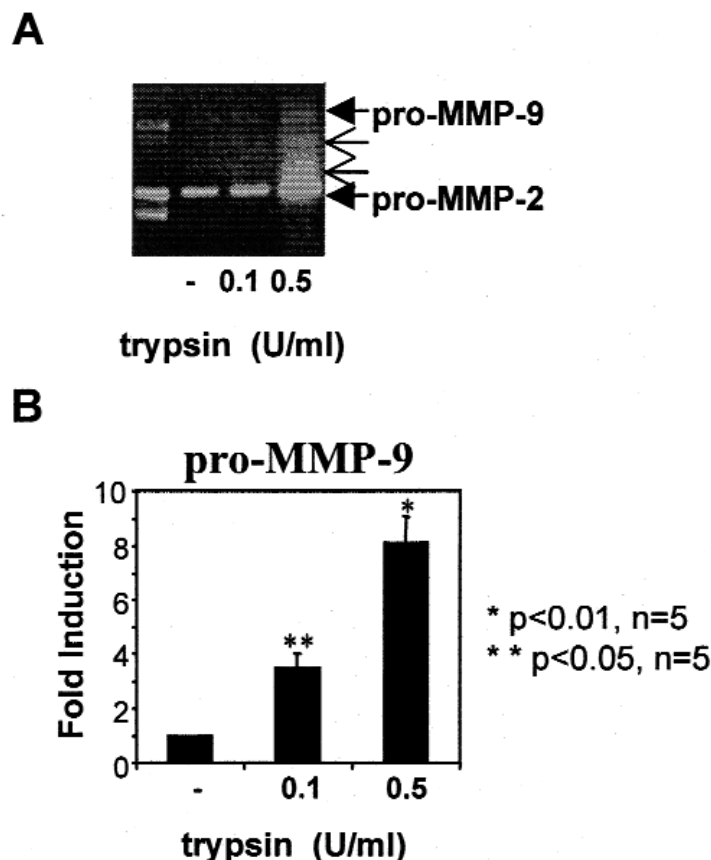


FIG 5. MMP-9 upregulation after A549 activation with trypsin. **A**, Gelatin zymography of supernatants of A549 cells activated for 24 hours with trypsin 0.1 to 0.5 U/mL. Gel shown is representative from 5 similar experiments. **B**, Results of optical density measurements (average $\pm$ SEM) of trypsin-mediated pro-MMP-9 release ($n = 5$ experiments).

another protease to activate PAR-2 receptors¹⁶ although there is evidence to suggest that it may activate only a subpopulation of PAR-2.³⁹ Mast cells, and in particular tryptase and TNF, have been implicated in the upregulation of MMPs.^{40,41} MMP release from airway epithelium, as a result of mast-cell activation, could facilitate inflammatory cell recruitment in the lung, particularly in the early stages of the inflammatory reaction when only a few inflammatory cells are present in these areas. Finally, many allergens are proteases, and at least 1 of them (Der p 9 from the house dust mite) can mediate some of its effects through trypsin-activated receptors.⁴² These molecules, together with other unidentified proteases, could regulate the release of MMP-9 by the respiratory epithelium during allergic inflammation.

Trypsin upregulated not only the release of pro-MMP-9 but also the release of pro-MMP-2 and induced the release of at least 2 other proteins with gelatinolytic activity. This effect could be due to trypsin's ability to activate proteinase-activated receptors (PARs) other than PAR-2. The identity and nature of these receptors are not

known, especially because PAR-1- and PAR-4-activating peptides had no effect on MMP release from A549 cells. The 2 other proteins with gelatinolytic activity could also represent proteolytic products of pro-MMP-9 after degradation by trypsin⁴³ or matrilysin,⁴⁴ which is also produced by airway epithelial cells.⁴⁵

Mast-cell tryptase appears to play an important role in respiratory disorders in which mast-cell activation is an important pathophysiologic feature, including asthma.^{46,47} Tryptase has also been implicated in the upregulation of MMP production.⁴⁰ We have evidence that tryptase induces MMP-9 release from airway epithelial cells, but its mode of action does not appear to be through PAR-2 activation (unpublished observations). This effect could be due to the inability of our tryptase preparation to activate PAR-2 receptors or to its ability to activate other receptors on epithelial cells with higher affinity. We are currently investigating in more detail the effects of tryptase on epithelial cell MMP release.

In addition to epithelial cells, PAR-2 is expressed in the airways by fibroblasts, smooth muscle cells,²⁴ and

inflammatory cells, such as neutrophils,⁴⁸ which produce significant amounts of MMP-9. Whether PAR-2 activation on these cells can have a similar effect on MMP-9 production remains to be elucidated. However, airway epithelial cells may be the first cells to respond to PAR-2-activating enzymes, especially when these enzymes are inhaled as airborne allergens.

PAR-2 activation has been shown to induce prostaglandin-mediated effects in epithelial cells,²⁴ and induction of MMP-9 in some cases is prostaglandin dependent.³¹ However, indomethacin, a prostaglandin synthase inhibitor, did not affect PAR-2- or TNF-mediated upregulation of MMP-9 release by epithelial cells. These results indicate that PAR-2 activation may have two independent effects on airway epithelial cells. It may induce prostaglandin release with the potential to mediate bronchodilation²⁴ or prostaglandin-independent pro-inflammatory mediator release.

Corticosteroids are used to modulate inflammation in asthma⁴⁹ and have the potential to inhibit the development of inflammatory changes in the lung.⁵⁰ Dexamethasone and budesonide inhibited PAR-2-mediated MMP-9 release from epithelial cells. Interestingly, both corticosteroids also inhibited the basal production of MMP-2 by airway epithelial cells. Corticosteroid treatment in asthmatic patients has been shown to decrease bronchoalveolar lavage MMP-9.⁸ The effects of corticosteroids on MMP-9 release indicate that NF κ B activation^{35,51} may be important for MMP-9 production. The proteasome inhibitor MG-132, which has been shown to inhibit NF κ B in A549 epithelial cells,³⁶ decreased MMP-9 release by up to 40%. Together, these observations indicate that part of the anti-inflammatory effects of corticosteroids may be mediated through the inhibition of MMP production in the airways.

PAR-2 receptors have been shown to activate the MAP-kinase pathway.³³ The MEK-1 inhibitor PD98059 caused more than 80% inhibition of PAR-2-mediated MMP-9 release from A549 cells. PD98059 has been shown to inhibit cyclooxygenase.⁵² However, our results with indomethacin indicate that the inhibitory effect of PD98059 must be mediated through MAP-kinase inhibition. PAR-2 also activates p38 kinase,³³ but the p38 inhibitor SB203580 did not affect the PAR-2-mediated upregulation of MMP-9 release.

The observed MMP-9 induction by PAR-2 activation in epithelial cells implicates serine proteases (such as trypsin-like enzymes, mast-cell tryptase and allergen proteases) in inflammatory responses in the airways. The release of MMP might be one of the pathways through which local production and release of serine proteases can affect the inflammatory response in the lung.

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