Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways

Cory Ebeling, BSc,[a] Paul Forsythe, PhD,[a]* Jason Ng,[a] John R. Gordon, PhD,[b] Morley Hollenberg, MD, PhD,[c] and Harissios Vliagoftis, MD[a] *Edmonton and Calgary, Alberta, and Saskatoon, Saskatchewan, Canada*

Background: Serine proteinases such as mast cell tryptase, trypsin-like enzymes, and certain allergens are important in the pathogenesis of asthma. These proteinases can activate the proteinase-activated receptor (PAR)–2, which has been shown to be upregulated in the airways of patients with asthma.
Objective: The purpose of this study was to investigate PAR-2 activation in the airways during allergen challenge and its effects on the 2 principle features of asthma, airway inflammation and airway hyperresponsiveness (AHR).
Methods: Proteinase-activated receptor 2 activating peptide SLIGRL-NH_2 (PAR-2 activating peptide [ap]) or control peptide LSIGRL-NH_2 (PAR-2 control peptide [cp]) was administered alone or in conjunction with ovalbumin intranasally to mice, and AHR and airway inflammation were evaluated.
Results: PAR2ap did not induce AHR or airway inflammation in ovalbumin-sensitized mice that had not been challenged with ovalbumin. When administered with ovalbumin, PAR-2ap enhanced AHR and airway inflammation compared with ovalbumin administered alone or with PAR-2cp. The enhanced AHR persisted for 5 days, whereas the enhancement to airway inflammation dissipated. Mice administered PAR-2ap alone during the 5 days after the final antigen challenge demonstrated an additional enhancement to airway inflammation compared with the control animals. PAR-2ap administered with allergen increased TNF and IL-5 mRNA in lung tissue and IL-13 and TNF in bronchoalveolar lavage fluid.
Conclusion: Exogenous PAR-2 activation in parallel with allergen challenge enhances allergen-mediated AHR and airway inflammation through distinct mechanisms. PAR-2 activation can also enhance established airway inflammation even when dissociated from exposure to allergen. Therefore, PAR-2 activation may play a pathogenic role in the development of AHR and airway inflammation. (J Allergy Clin Immunol 2005;115:623-30.)

Key words: *Proteinase-activated receptor 2, allergy, rodent, airway inflammation, eosinophils, IL-13*

Allergic asthma is characterized by eosinophilic airway inflammation and airway hyperresponsiveness (AHR). There is mounting evidence that proteinases are involved in the pathogenesis of asthma. Mast cell tryptase, a serine proteinase released by mast cells, is increased in bronchoalveolar lavage (BAL) fluid of patients with asthma after allergen challenge[1] and can induce smooth muscle hyperreactivity.[2,3] Furthermore, many aeroallergens associated with asthma, such as house dust mite allergens and various fungal allergens, are proteinases.[4-6]

Serine proteinases are capable of mediating some of their effects through the G-coupled proteinase-activated receptors (PARs). This family of receptors has 4 members (PAR-1 to PAR-4). PAR-1 and PAR-3 are activated primarily by thrombinlike proteinases, whereas PAR-2 and PAR-4 are activated primarily by trypsinlike proteinases.[7]

Proteinase-activated receptor 2 is of particular interest in asthma because it can be activated by mast cell tryptase,[8] and aeroallergens[9,10] and has been implicated in inflammation. PAR-2 activation induces the release of GM-CSF, eotaxin,[11] and matrix metalloproteinase 9[12] from airway epithelial cells. These mediators could be involved in eosinophil recruitment into the lung. PAR-2 agonist injected into rat hindpaws induces edema and granulocyte infiltration.[13] PAR-2 mediates microvascular inflammation by inducing P-selectin–mediated leukocyte rolling[14] and participates in the induction of intestinal inflammation.[15]

From [a]the Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada; [b]the Immunology Research Group, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; and [c]the Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, Canada.
*Current address: Brain-Body Institute, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada.
Supported by the Canadian Institute of Health Research and the Alberta Heritage Foundation of Medical Research. Dr Vliagoftis is an Alberta Heritage Foundation of Medical Research Clinical Investigator and a Canadian Institute of Health Research Scholar.
Received for publication June 7, 2004; revised November 15, 2004; accepted for publication November 22, 2004.
Available online January 31, 2005.
Reprint requests: Harissios Vliagoftis, MD, Pulmonary Research Group, Department of Medicine, 550 HMRC, University of Alberta, Edmonton, AB, T6G 2S2, Canada. E-mail: harissios.vliagoftis@ualberta.ca.
0091-6749/$30.00
© 2005 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2004.11.042

Abbreviations used
AHR: Airway hyperresponsiveness
ap: Activating peptide
BAL: Bronchoalveolar lavage
cp: Control peptide
MCP-1: Monocyte chemoattractant protein 1
MIP: Macrophage inflammatory protein
PAR: Proteinase-activated receptor

Little is known about the pathophysiological importance of PAR-2 in the airways. PAR-2 is upregulated on the airway epithelium of patients with asthma.[16] Transgenic mice overexpressing PAR-2 develop increased levels of ovalbumin-mediated eosinophilic airway inflammation and AHR compared with wild-type mice,[17] whereas PAR-2 knockout mice show the opposite effect. Others have shown a possible protective role for PAR-2 in the airways. PAR-2 activation relaxes isolated rat tracheas[18] and has a protective effect against bronchoconstriction *in vivo*.[19] It is therefore not clear whether PAR-2 activated in the airway is protective, ie, induces bronchodilation, or detrimental by inducing inflammation, bronchoconstriction, and/or hyperresponsiveness.

We hypothesized that PAR-2 activation in the airways has proinflammatory effects. Here we report that PAR-2 activation in the respiratory tract increases allergen-mediated accumulation of eosinophils and mononuclear cells in the airways and increases AHR, and does so by different mechanisms. We also report that PAR-2 activating peptide (ap) administered with allergen increased TNF and IL-5 mRNA in lung tissue and IL-13 and TNF protein levels in BAL fluid.

METHODS

Animals

Male Balb/c mice (18-20 g) were purchased from Charles River Laboratories (St Constant, Quebec, Canada). The mice were housed and experimented in virus-free and antibody-free conditions and maintained on a 12-hour light-dark schedule. The University of Alberta Health Sciences Laboratory Animal Ethics Board (Edmonton, Alberta, Canada) approved all experiments described in this study.

Sensitization and challenge with ovalbumin

Male Balb/c mice were sensitized on days 1 and 6 via intraperitoneal injection of 0.9% sterile saline (0.5 mL) containing 10 μg ovalbumin and 2 mg $Al(OH)_3$. After light anesthesia with ketamine (360 mg/kg) and acepromazine (12 mg/kg), groups of mice were challenged intranasally with 25 μL saline, PAR-2ap (SLIGRL-NH_2; 100 μmol/L) or PAR-2 control peptide (cp; LSIGRL-NH_2; 100 μmol/L) alone (Fig 1, *A*) or with ovalbumin (50 μg) on days 12 and 14. Concentrations of peptides chosen were similar to those used in other *in vivo* animal studies.[19,20] Mice were then assessed for airway inflammation and AHR on days 15 or 19 (short-term and follow-up protocols, respectively; Fig 1, *B and C*). Other mice were further challenged with saline, PAR-2ap, or PAR-2cp on days 16 and 18 and analyzed on day 19 (long-term protocol; Fig 1, *D*).

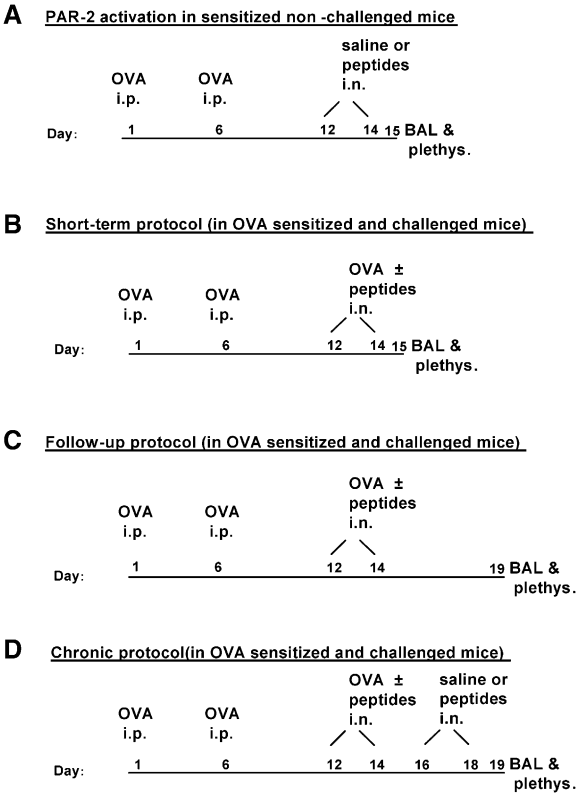

FIG 1. Ovalbumin sensitization/challenge protocols used in this study to explore the role of PAR-2 on airway inflammation and AHR. *i.n.,* Intranasal; *i.p.,* intraperitoneal; *OVA,* ovalbumin; *plethys,* plethysmography.

AHR

Enhanced pause was determined in mice by using whole-body plethysmography (Buxco Electronics, Inc, Sharon, Conn) to determine AHR in response to methacholine challenge (2-64 mg/mL) as described.[21,22]

BAL

Airway inflammation was assessed by inflammatory cell counts in BAL fluid as described.[21]

PAR-2 localization in tissue sections by immunohistochemistry

Lungs were placed in Histo Prep (Fisher Scientific, Ottawa, Ontario, Canada) and quick-frozen in supercooled methanol. Frozen sections (5 μm) were prepared and placed in cold acetone (20°C) for 10 minutes, washed in PBS (5 minutes), placed in methanol containing 0.3% H_2O_2 for 35 minutes to block endogenous peroxidases, and incubated with the anti–PAR-2 B5 Ab[23] (1/250 dilution in 100 μL PBS) or anti–PAR-2 B5 Ab preabsorbed with immunizing peptide (65 μg/mL in 100 μL PBS for 1 hour at 4°C) for 4 hours at room temperature. Sections were then washed in PBS (5 minutes), incubated with horseradish peroxidase–labeled $F(ab')_2$ goat antirabbit IgG (1/2000 dilution in 100 μL PBS; Jackson ImmunoResearch Inc, West Grove, Pa) for 1 hour at room temperature, washed in PBS (5 minutes), then stained with diaminobenzidine substrate (Vector Laboratories, Burlingame, Calif). Sections were then rinsed in water (5 minutes), counterstained with Harris' hematoxylin (Sigma

Aldrich, Oakville, Ontario, Canada), and dehydrated in increasing ethanol/xylene gradient. Slides were photographed by using a Nikon DXM 1200 digital camera mounted on a Nikon E 800 microscope (Nikon Corp, Tokyo, Japan).

PAR-2 localization in BAL cells by immunohistochemistry

Smears of BAL cells on slides were subject to the same staining protocol as the tissue sections with the exception that slides were incubated with the primary B5 Ab (or preabsorbed B5 Ab) at 1/100 dilution for 2.5 hours at room temperature, whereas the secondary $F(ab')_2$ fragment was incubated at 1/1000 dilution for 1.5 hours at room temperature.

Inflammatory cytokine mRNA analysis

Lungs from ovalbumin-sensitized mice were excised at various time points after intranasal administration of PAR-2cp or PAR-2ap with or without ovalbumin, and RNA was isolated. RNA (20 μg) was lyophilized and kept at −70°C until analyzed by Ribonuclease (RNase) protection assay. Multi-Probe RNase Protection Assay was performed according to the manufacturer's instructions by using RNA probes synthesized from RiboQuant (BD Pharmingen, Mississauga, Ontario, Canada) template plasmids (TNF, IL-4, IL-5, IL-10, IL-13, macrophage inflammatory protein (MIP)–1α, MIP-2, monocyte chemoattractant protein 1 (MCP-1), IL-6, IFN-γ, and the 2 housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase). Densitometry was performed on all genes, and quantification was determined against the L32 housekeeping gene by using AlphaImager software (Alpha Innotech Corporation, San Leandro, Calif).

Cytokine assays

IL-4, IL-5, IL-9, IL-10, IL-13, TNF-α, and IFN-γ were measured in BAL samples by ELISA as previously described.[24]

Ovalbumin specific IgE in blood serum

Mice were sacrificed, and total circulating blood volume was obtained by direct cardiac puncture. Serum was collected, and ovalbumin specific IgE was determined by ELISA as previously described.[25]

Statistical analysis

Statistical differences in the means of inflammatory cell counts between treatment groups were determined by using 1-way ANOVA. The paired Student *t* test was used to compare the means of 2 groups. Differences in AHR were determined by *F* test analysis that compared the entire curves of each treatment group with one another. From this *F* test, a *P* value was generated. In all cases, a *P* value of less than .05 was considered statistically significant.

RESULTS

Localization of PAR-2 in the airways/lungs of Balb/c mice

Proteinase-activated receptor 2 was localized to the airway epithelium, endothelium, and airway smooth muscle of ovalbumin-sensitized mice (Fig 2, *A*). PAR-2 was also abundantly expressed on the epithelium of the nasal cavity (Fig 2, *B*). Preabsorbtion of the B5 Ab with the immunizing peptide abolished staining in all cases, verifying the specificity of the results (Fig 2, *C and D*).

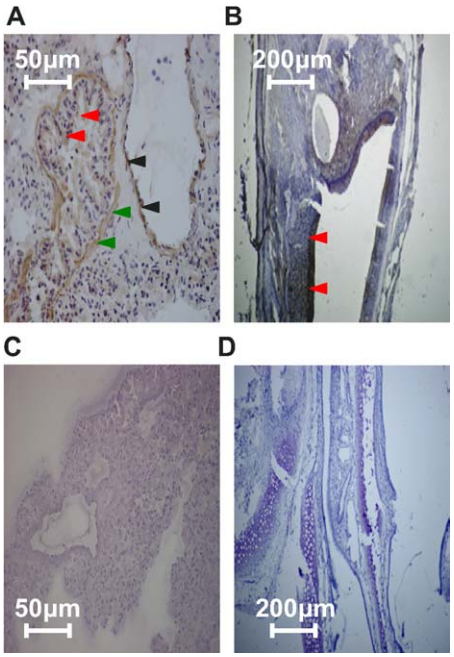

FIG 2. PAR-2 localization in the lung tissue **(A)** and nasal tissue **(B)** of mice that were sensitized but not challenged with ovalbumin. PAR-2 was localized to the airway epithelium *(red arrows)*, smooth muscle *(green arrows)*, and vascular endothelium *(black arrows)*. As a negative control, B5 Ab was preabsorbed with immunizing peptide **(C and D)**.

Effects of PAR-2 activation on allergen-mediated airway inflammation and AHR

To study whether PAR-2 activation in the airways induces airway inflammation or AHR, ovalbumin-sensitized mice were challenged intranasally with PAR-2ap, PAR-2cp, or saline on days 12 and 14 (ovalbumin and peptides were found to be endotoxin-free as determined by E-Toxate LAL (limulus amebocyte lysate) assay (Sigma Aldrich). Assessment of airway inflammation by BAL and AHR by plethysmography occurred on day 15 (protocol in Fig 1, *A*). For both airway inflammation and AHR, mice receiving PAR-2cp showed no differences compared with mice receiving saline (data not shown). In both the PAR-2cp and PAR-2ap groups, the cells seen in the BAL were exclusively alveolar macrophages. There were no differences in total cell numbers between the 2 groups (n = 5; Fig 3, *A, upper panel*). AHR was also not induced in any of the treatment groups (Fig 3, *A, lower panel*). Therefore, PAR-2 activation by PAR-2ap was not capable of initiating airway inflammation or AHR in ovalbumin-sensitized mice.

We then studied the effects of PAR-2 activation on ovalbumin-induced airway inflammation and AHR. Ovalbumin-sensitized mice were challenged twice with ovalbumin alone or in combination with PAR-2ap or PAR-2cp on days 12 and 14. Inflammation and AHR were assessed the day after the last challenge (short-term protocol in Fig 1, *B*). Mice that received PAR-2cp with

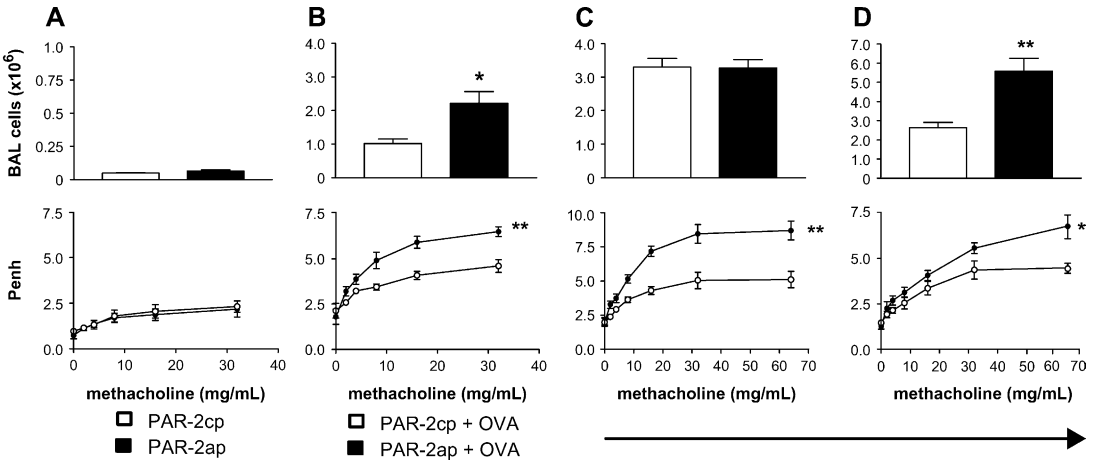

FIG 3. BAL total cell numbers *(upper panels)* and enhanced pause values from increasing doses of methacholine *(lower panels)* in ovalbumin-sensitized mice administered PAR-2 peptides alone (n = 5) **(A)** or mice subject to the short-term (n = 11) **(B)**, follow-up (n = 9) **(C)**, and chronic (n = 9) **(D)** activation protocols as outlined in Fig 1. *Open columns/circles* represent PAR-2cp treatment, whereas *closed columns/circles* represent PAR-2ap treatment. Results are shown as means ± SEMs. *$P < .05$; †$P < .01$ compared with PAR-2cp treatments. *OVA,* Ovalbumin.

ovalbumin showed no differences in airway inflammation or AHR compared with mice that received ovalbumin alone (data not shown). Coadministration of PAR-2ap with ovalbumin increased total inflammatory cell numbers in the BAL ($2.2 \pm 0.4 \times 10^6$) compared with ovalbumin with PAR-2cp ($1.0 \pm 0.1 \times 10^6$; $P < .05$; n = 11; Fig 3, *B, upper panel*). PAR-2ap–treated mice had increased numbers of eosinophils ($1.8 \pm 0.3 \times 10^6$) and mononuclear cells ($2.8 \pm 0.5 \times 10^5$) compared with PAR-2cp ($0.9 \pm 0.1 \times 10^6$; $P < .05$; and $0.8 \pm 0.2 \times 10^5$; $P < .01$, respectively). Ovalbumin plus PAR-2ap–treated mice exhibited a significantly higher methacholine responsiveness when compared with ovalbumin plus PAR-2cp–treated mice ($P < .01$; Fig 3, *B, lower panel*). Thus, PAR-2 activation after intranasal administration of PAR-2ap enhanced ovalbumin-mediated allergic airway inflammation and AHR.

A hallmark of murine models of allergic diseases is the production of allergen specific IgE. To determine whether the enhancing effects of the PAR-2ap were IgE-dependent, serum levels of ovalbumin-specific IgE were assessed in animals receiving PAR-2ap or PAR-2cp with ovalbumin and in control nonsensitized mice (Table I). We detected ovalbumin-specific IgE in the serum of mice in both treatment groups. PAR-2ap administered with ovalbumin did not increase the levels of ovalbumin-specific IgE when compared with PAR-2cp with ovalbumin.

To explore whether the PAR-2–induced enhancement to airway inflammation and AHR was long-lasting, we evaluated animals 5 days after the final challenge (day 19; follow-up protocol seen in Fig 1, *C*). Again, mice that received PAR-2cp with ovalbumin showed no differences in airway inflammation or AHR compared with mice that received ovalbumin alone (data not shown). Furthermore, there were no differences in total BAL cell numbers between the PAR-2cp and PAR-2ap treatment groups (Fig 3, *C, upper panel*), although inflammation as a whole was increased compared with animals examined on day 15. Mice that received ovalbumin with PAR-2ap had $3.3 \pm 0.2 \times 10^6$ cells in their BAL, whereas mice receiving PAR-2cp with ovalbumin had $3.4 \pm 0.3 \times 10^6$ cells (n = 9). There were also no differences in the differential counts of BAL cells between treatment groups. Therefore, the enhanced inflammation seen after PAR-2ap administration dissipated 5 days after the last challenge. Although no differences were seen in inflammation, AHR was still enhanced in the PAR-2ap–treated mice. Assessment on day 19 showed that ovalbumin plus PAR-2ap–treated mice exhibited a significantly higher methacholine responsiveness compared with ovalbumin plus PAR-2cp ($P < .01$; Fig 3, *C, lower panel*). Therefore, the effect of PAR-2 activation on allergen-induced AHR was long-lived (≥5 days).

Since the inflammation seen in the other groups caught up to the PAR-2ap–treated group, this experiment showed that the enhancing effect of the PAR-2ap on airway inflammation was lost with time. However, asthmatic airways are constantly exposed to proteinases whether they are exogenous or endogenous in nature. To mimic this chronic PAR-2 activation, we administered on days 16 and 18 PAR-2ap, PAR-2cp, or saline to mice that had been challenged on days 12 and 14 with ovalbumin plus PAR-2ap, ovalbumin plus PAR-2cp, or ovalbumin alone, respectively. Assessment of inflammation by BAL and AHR by plethysmography followed on day 19 (chronic protocol in Fig 1, *D*). No differences were seen in airway inflammation or AHR between mice given saline or PAR-2cp (data not shown). PAR-2ap administration in this

TABLE I. Serum levels of OVA-specific IgE in the short-term protocol

Sensitization	Challenge	OVA-specific IgE (OD_{405})
None	Saline solution	0.12 ± 0.05
OVA + $Al(OH_3)$	OVA + PAR-2cp	0.40 ± 0.04**
OVA + $Al(OH_3)$	OVA + PAR-2ap	0.44 ± 0.03**

Groups of mice were subjected to the short-term protocol, and 24 hours after the final challenge, the total circulating volume of blood was taken, serum was collected, and OVA-specific IgE was measured. Each result is shown as mean ± SEM.
**$P < .01$ in comparison with nonsensitized animals challenged with saline solution (n = 4).

situation increased total inflammatory cell numbers in the BAL ($5.6 \pm 0.7 \times 10^6$) compared with PAR-2cp ($2.6 \pm 0.3 \times 10^6$; $P < .05$; n = 9; Fig 3, *D, upper panel*). PAR-2ap treatment increased the numbers of eosinophils ($4.1 \pm 0.5 \times 10^6$) and mononuclear cells ($1.3 \pm 0.2 \times 10^6$) compared with PAR-2cp ($2.2 \pm 0.3 \times 10^6$; $P < .05$; and $3.2 \pm 0.2 \times 10^5$; $P < .01$, respectively). Thus, sustained PAR-2ap administration further enhanced ovalbumin-induced airway inflammation. The difference in AHR seen in the follow-up protocol remained, because methacholine responsiveness in PAR-2ap–treated mice was still significantly enhanced compared with the PAR-2cp ($P < .01$) curve (Fig 3, *D, lower panel*).

PAR-2 expression by BAL cells

The PAR-2ap administered intranasally could also affect alveolar macrophages and inflammatory cells recruited during the development of airway inflammation. To explore whether PAR-2ap could affect these cells directly, we studied the expression of PAR-2 by these cells. Immunohistochemistry using the B5 Ab showed that the alveolar macrophages and inflammatory cells, consisting of eosinophils and mononuclear cells, stained positive for PAR-2 (Fig 4, *A and B,* respectively). Alveolar macrophages exhibited a more intense staining than did the other inflammatory cells. Preabsorbtion with immunizing peptide abolished all staining (Fig 4, *C and D*).

Cytokine and chemokine mRNA expression in the lung

To define further the mechanism of enhanced airway inflammation and AHR, we studied cytokine/chemokine expression in the lung after 1 administration of PAR-2ap alone or with ovalbumin, or after the first administration of peptides alone in the chronic protocol. PAR-2ap alone upregulated TNF and MIP-2 and, to a smaller extent, IL-13 mRNA levels in the lungs of naive mice 4 hours after administration (Fig 5, *A*). PAR-2ap administered with ovalbumin increased cytokine/chemokine expression in the lung 4 hours after challenge. TNF and IL-5 were the most noticeable of these (Fig 5, *B*). All differences resolved by 8 hours. No differences were seen in any of the cytokines/chemokines after the first administration of PAR-2ap or PAR-2cp in the chronic protocol (data not shown).

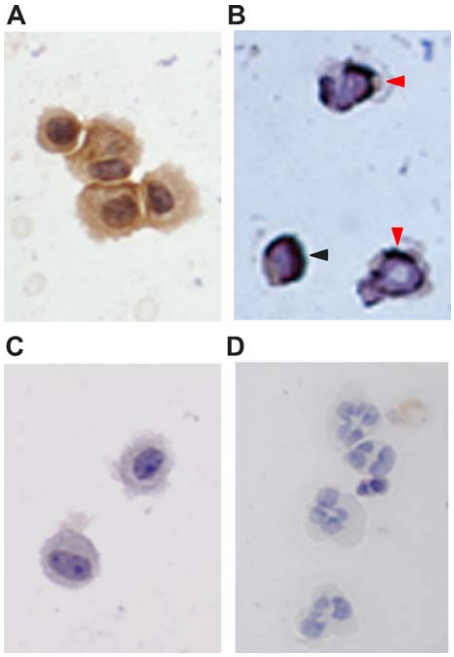

FIG 4. PAR-2 localization on alveolar macrophages **(A)** and inflammatory cells **(B)**. Of the inflammatory cells, PAR-2 was localized to eosinophils *(red arrows)* and mononuclear cells *(black arrows)*. As a negative control, B5 Ab was preabsorbed with immunizing peptide **(C and D)**.

Cytokine levels in BAL fluid

We also looked at cytokine levels in BAL fluid of mice in our protocols. Administration of PAR-2ap alone increased the levels of IL-13 in BAL fluid compared with PAR-2cp (53.57 ± 13.27 pg/mL and undetectable levels, respectively; n = 3; $P < .05$), and the levels of TNF (72.19 ± 28.16 pg/mL vs 19.45 ± 19.45 pg/mL; n = 3; $P < .05$; (Fig 6, *A*). The same was true in the short-term protocol, in which levels of IL-13 (112.20 ± 92.53 pg/mL vs undetectable levels; n = 3; $P < .05$) and TNF (81.85 ± 19.21 pg/mL vs 9.28 ± 5.82 pg/mL; n = 3; $P < .01$; Fig 6, *B*) were increased when PAR-2ap was administered with ovalbumin compared with PAR-2cp with ovalbumin. This increase in IL-13 (32.81 ± 26.58 pg/mL vs undetectable levels; n = 3; $P < .05$) and TNF (99.95 ± 27.12 pg/mL vs 18.79 ± 11.89 pg/mL; n = 3; $P < .05$) carried over into the follow-up protocol (Fig 6, *C*). In the chronic protocol, only IL-13 was increased with PAR-2ap treatment (244.71 ± 119.60 pg/mL vs 66.32 ± 22.38 pg/mL; n = 3; $P < .05$), whereas no difference was seen in TNF levels (58.45 ± 27.36 pg/mL vs 49.75 ± 24.91 pg/mL; n = 3; Fig 6, *D*). In all cases IL-4, IL-9, IL-10, and IFN-γ were not significantly altered, whereas IL-5 showed an upward trend but did not reach statistical significance.

DISCUSSION

The current literature indicates that PAR-2 activation in the airways may have both protective and pathogenic roles

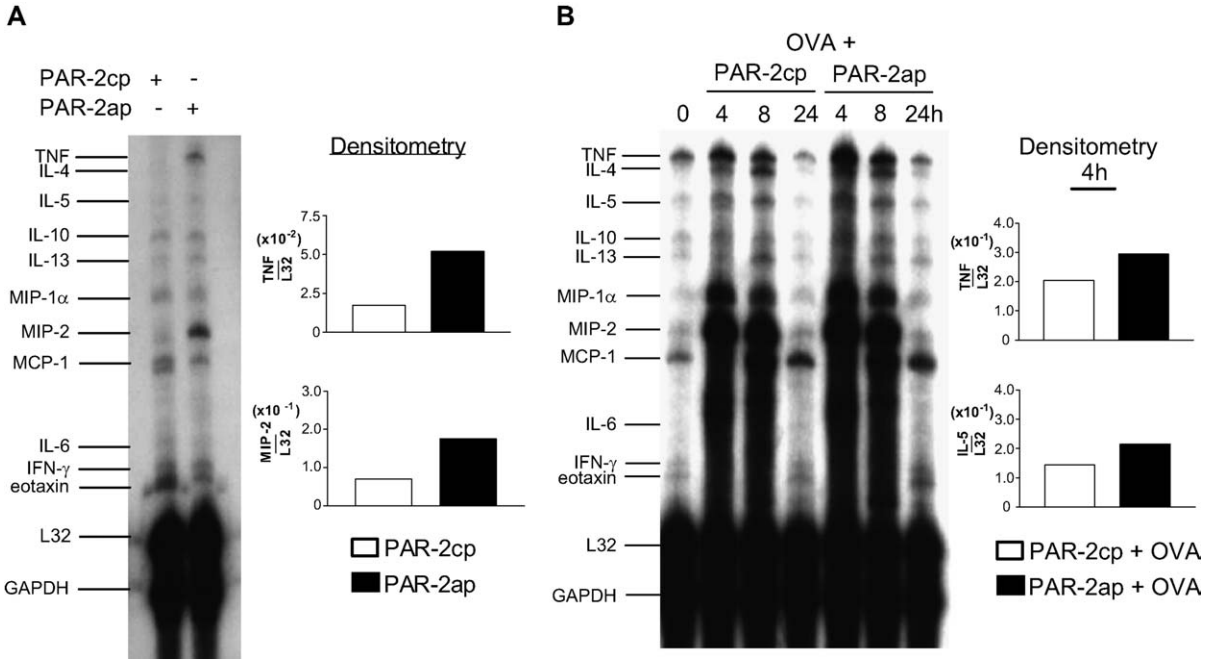

FIG 5. A, Ribonuclease (RNase) protection assay for inflammatory cytokines present in the lung 4 hours after administration of PAR-2cp or PAR-2ap to naive mice and densitometry graphs of upregulated genes (1 representative experiment of 2 is shown). **B,** RNase protection assay for inflammatory cytokines present in the lung 0, 4, 8, and 24 hours after one challenge of ovalbumin with PAR-2cp or PAR-2ap in sensitized mice and densitometry graphs of upregulated genes (1 representative experiment of 2 is shown). *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *OVA,* ovalbumin.

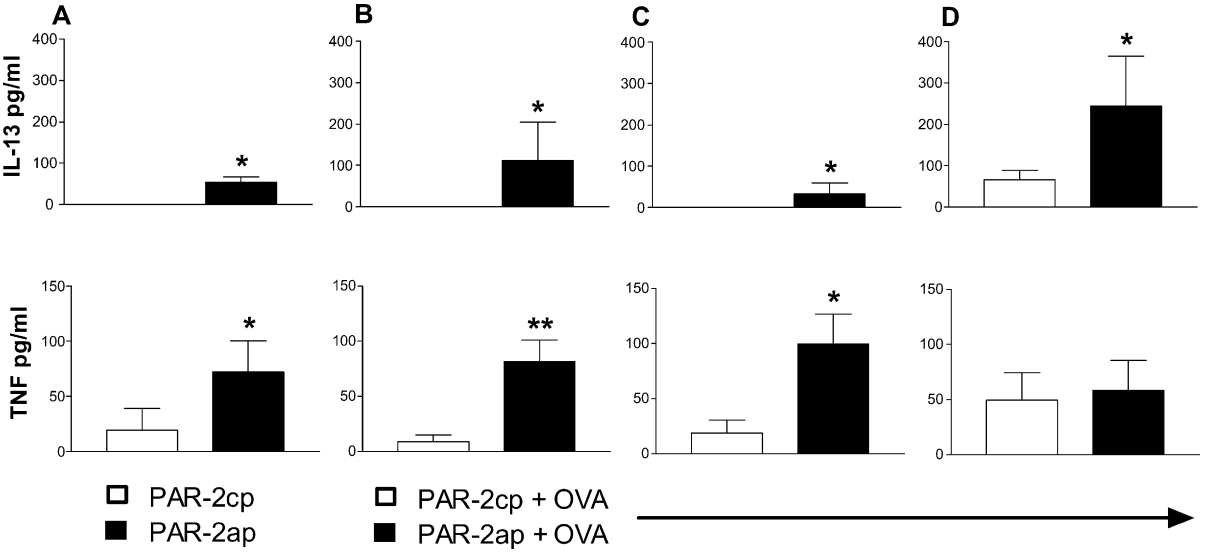

FIG 6. IL-13 *(upper panels)* and TNF *(lower panels)* levels in BAL fluid of mice treated with PAR-2cp *(open columns)* or PAR-2ap *(closed columns)* without **(A)** or with ovalbumin challenge in the short-term **(B)**, follow-up **(C)**, or chronic **(D)** activation protocols as outlined in Fig 1. Results are shown as means ± SEMs (n = 3). *$P < .05$; †$P < .01$ compared with PAR-2cp treatments. *OVA,* Ovalbumin.

in inflammatory airway diseases, depending on the model used and the route of PAR-2 activation.[19,26,27] We have shown that administration of a PAR-2ap through the nasal cavity was incapable of initiating inflammatory cell influx in the airways or AHR in ovalbumin-sensitized mice, but enhanced ovalbumin-induced airway inflammation and AHR. These results indicate that PAR-2 activation in the airways may have detrimental effects in asthma.

A role for PAR-2 in allergic airway inflammation has been previously shown.[17] In that study, the authors used PAR-2 knockout mice to study the role of endogenous proteinases, released from inflammatory cells, in allergic inflammation and AHR. In contrast, in our study, we challenged normal mice with ovalbumin and PAR-2ap to mimic the effects of inhaled allergens that have the potential to activate PAR-2. In this article, we have shown for the first time that although PAR-2 may be involved in both airway inflammation and AHR, it regulates these 2 principle features of asthma through different pathways. We have demonstrated that AHR remains elevated for more than 5 days after exogenous PAR-2 activation, whereas the enhancement to airway inflammation was short-lived. We have also shown, for the first time, that PAR-2 activation in inflamed airways increases airway inflammation even in the absence of continued allergen exposure. This observation indicates that, in cases of established allergic airway inflammation, nonspecific stimuli that activate PAR-2 are sufficient to perpetuate allergic inflammation. These nonspecific stimuli may include both exogenous and/or endogenous serine proteinases. This observation may explain why allergen avoidance is not very effective as the sole therapeutic measure as long as airway inflammation persists.

The demonstrated changes in AHR correlated with the increased IL-13 seen in the BAL fluid of PAR-2ap–treated mice. This increase in IL-13, although not strong enough to induce AHR, could potentiate the responsiveness of airway smooth muscle to methacholine after allergen challenge. Such an effect could then be translated into elevated AHR once inflammation begins. IL-13 has shown to be critical in the development of allergen induced AHR in Balb/c mice, and its function cannot be compensated for by IL-4, nor does it correlate with eosinophil airway inflammation.[28] Indeed, it has been demonstrated that administering recombinant IL-13 to the airway of Balb/c mice induces a rapid and potent AHR response before airway inflammation is seen.[29] TNF seen in the BAL fluid may be responsible for the enhanced inflammation through increased adhesion molecule expression on the endothelium.[30]

Because we administered the PAR-2ap intranasally, it is very likely that its effects were mediated through PAR-2 activation on the epithelium of the upper or lower airways. PAR-2 activation on human airway epithelial cells induces the release of proinflammatory mediators such as eotaxin, GM-CSF,[11] and matrix metalloproteinases,[12] which are capable of promoting eosinophil recruitment and survival in the lung. Furthermore, if the effects seen in this study are the result of PAR-2 activation of the epithelium in the nose, it would be consistent with the 1 airway/1 disease concept[31,32] in which inflammatory events in the nose can promote similar events in the lower airway and vice versa. Release of proinflammatory mediators from the airway epithelium after PAR-2 activation could be the cause of increased eosinophilic inflammation in our model.

If given in the absence of allergen challenge, PAR-2ap was not capable of initiating airway inflammation of AHR. This could mean that the PAR-2 agonist is working synergistically with the proinflammatory effects mediated by allergen challenge. For example, it may activate PAR-2–expressing inflammatory cells that accumulate in the tissues. We have shown that eosinophils, which predominated in the BAL of ovalbumin–challenged mice, express PAR-2. PAR-2–mediated activation of eosinophils induces degranulation and superoxide production.[33] Alternatively, it could also mean that the increased epithelial permeability or epithelial desquamation induced by allergen challenge allows the PAR-2ap to penetrate deeper into the lung.

Endothelium throughout the lung also stained positive for PAR-2. PAR-2–mediated endothelial cell activation releases proinflammatory cytokines that could affect airway inflammation[34] and increase endothelial permeability,[13,35] allowing more inflammatory cell infiltration. Similarly, peripheral nerves have been implicated in PAR-2–mediated neurogenic inflammation[36] and may be of greater access for the PAR-2ap after allergen challenge. Thus, PAR-2 activation of any or all of these cell types may explain further increases in inflammation when PAR-2ap is administered alone after initial challenges.

We propose that allergens possessing intrinsic serine protease activity may be more potent in inducing airway inflammation and AHR because of their PAR-2–mediated effects. Our model also indicates that PAR-2 activation in the airways by inhaled particles or contaminants with serine protease activity may exacerbate established allergen-induced airway inflammation and AHR. The effects of PAR-2 activation occur independently of continuing allergen exposure. Therefore, further exploration of the role of PAR-2 in allergic airway inflammation may offer new targets for therapeutic intervention.

We thank Sorin Musat-Marcu, MD (HistoBest Inc, Edmonton, Alberta, Canada), for preparing nasal cavity tissue sections. We also thank Dean Befus, PhD, for his critical reading of the manuscript, and Melanie Abel for her help with animal handling.

REFERENCES

1. Schwartz LB. Tryptase, a mediator of human mast cells. J Allergy Clin Immunol 1990;86:594-8.
2. Sekizawa K, Caughey GH, Lazarus SC, Gold WM, Nadel JA. Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. J Clin Invest 1989;83:175-9.
3. Berger P, Compton SJ, Molimard M, Walls AF, N'Guyen C, Marthan R, et al. Mast cell tryptase as a mediator of hyperresponsiveness in human isolated bronchi. Clin Exp Allergy 1999;29:804-12.
4. Shen HD, Tam MF, Chou H, Han SH. The importance of serine proteinases as aeroallergens associated with asthma. Int Arch Allergy Immunol 1999;119:259-64.
5. Hewitt CR, Foster S, Phillips C, Horton H, Jones RM, Brown AP, et al. Mite allergens: significance of enzymatic activity. Allergy 1998;53:60-3.
6. Kauffman HF, Van Der HS. Exposure, sensitization, and mechanisms of fungus-induced asthma. Curr Allergy Asthma Rep 2003;3:430-7.
7. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev 2001;53:245-82.
8. Compton SJ, Renaux B, Wijesuriya SJ, Hollenberg MD. Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. Br J Pharmacol 2001;134:705-18.

9. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. J Immunol 2001;167:1014-21.
10. Asokananthan N, Graham PT, Stewart DJ, Bakker AJ, Eidne KA, Thompson PJ, et al. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. J Immunol 2002;169:4572-8.
11. Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. J Allergy Clin Immunol 2001;107:679-85.
12. Vliagoftis H, Schwingshackl A, Milne CD, Duszyk M, Hollenberg MD, Wallace JL, et al. Proteinase-activated receptor-2-mediated matrix metalloproteinase-9 release from airway epithelial cells. J Allergy Clin Immunol 2000;106:537-45.
13. Vergnolle N, Hollenberg MD, Sharkey KA, Wallace JL. Characterization of the inflammatory response to proteinase-activated receptor-2 (PAR2)-activating peptides in the rat paw. Br J Pharmacol 1999;127:1083-90.
14. Lindner JR, Kahn ML, Coughlin SR, Sambrano GR, Schauble E, Bernstein D, et al. Delayed onset of inflammation in protease-activated receptor-2- deficient mice. J Immunol 2000;165:6504-10.
15. Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, et al. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am J Pathol 2002;161: 1903-15.
16. Knight DA, Lim S, Scaffidi AK, Roche N, Chung KF, Stewart GA, et al. Protease-activated receptors in human airways: upregulation of PAR-2 in respiratory epithelium from patients with asthma. J Allergy Clin Immunol 2001;108:797-803.
17. Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J Immunol 2002; 169:5315-21.
18. Chow JM, Moffatt JD, Cocks TM. Effect of protease-activated receptor (PAR)-1, -2 and -4-activating peptides, thrombin and trypsin in rat isolated airways. Br J Pharmacol 2000;131:1584-91.
19. Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, et al. A protective role for protease-activated receptors in the airways. Nature 1999;398:156-60.
20. Vergnolle N, Bunnett NW, Sharkey KA, Brussee V, Compton SJ, Grady EF, et al. Proteinase-activated receptor-2 and hyperalgesia: a novel pain pathway. Nat Med 2001;7:821-6.
21. Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H. Opposing effects of short- and long-term stress on airway inflammation. Am J Respir Crit Care Med 2004;169:220-6.
22. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med 1997;156:766-75.
23. Al Ani B, Saifeddine M, Kawabata A, Renaux B, Mokashi S, Hollenberg MD. Proteinase-activated receptor 2 (PAR(2)): development of a ligand-binding assay correlating with activation of PAR(2) by PAR(1)- and PAR(2)-derived peptide ligands. J Pharmacol Exp Ther 1999;290: 753-60.
24. Gordon JR, Zhang X, Stevenson K, Cosford K. Thrombin induces IL-6 but not TNFalpha secretion by mouse mast cells: threshold-level thrombin receptor and very low level FcepsilonRI signaling synergistically enhance IL-6 secretion. Cell Immunol 2000;205:128-35.
25. Schneider AM, Li F, Zhang X, Gordon JR. Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease after sensitization with limiting doses of ovalbumin-alum. Cell Immunol 2001;212:101-9.
26. Ricciardolo FL, Steinhoff M, Amadesi S, Guerrini R, Tognetto M, Trevisani M, et al. Presence and bronchomotor activity of protease-activated receptor-2 in guinea pig airways. Am J Respir Crit Care Med 2000;161:1672-80.
27. Barrios VE, Jarosinski MA, Wright CD. Proteinase-activated receptor-2 mediates hyperresponsiveness in isolated guinea pig bronchi. Biochem Pharmacol 2003;66:519-25.
28. Walter DM, McIntire JJ, Berry G, McKenzie AN, Donaldson DD, DeKruyff RH, et al. Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. J Immunol 2001;167:4668-75.
29. Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, et al. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. Am J Respir Cell Mol Biol 2001;25:522-30.
30. Haraldsen G, Kvale D, Lien B, Farstad IN, Brandtzaeg P. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human microvascular endothelial cells. J Immunol 1996;156:2558-65.
31. Leynaert B, Bousquet J, Neukirch C, Liard R, Neukirch F. Perennial rhinitis: an independent risk factor for asthma in nonatopic subjects: results from the European Community Respiratory Health Survey. J Allergy Clin Immunol 1999;104:301-4.
32. Yawn BP, Yunginger JW, Wollan PC, Reed CE, Silverstein MD, Harris AG. Allergic rhinitis in Rochester, Minnesota residents with asthma: frequency and impact on health care charges. J Allergy Clin Immunol 1999;103:54-9.
33. Miike S, McWilliam AS, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. J Immunol 2001;167:6615-22.
34. Shpacovitch VM, Brzoska T, Buddenkotte J, Stroh C, Sommerhoff CP, Ansel JC, et al. 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; 118:380-5.
35. Klarenbach SW, Chipiuk A, Nelson RC, Hollenberg MD, Murray AG. Differential actions of PAR2 and PAR1 in stimulating human endothelial cell exocytosis and permeability: the role of Rho-GTPases. Circ Res 2003;92:272-8.
36. Steinhoff M, Vergnolle N, Young SH, Tognetto M, Amadesi S, Ennes HS, et al. Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. Nat Med 2000;6:151-8.