Altered expression and in vivo lung function of protease-activated receptors during influenza A virus infection in mice

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SECRETORY PROTEASES REGULATE multiple physiological functions by degrading acellular targets such as biological peptides and matrix proteins. However, proteases may also activate cells through their interaction with one of four members of the protease-activated receptor (PAR) family namely, PAR-1, PAR-2, PAR-3, or PAR-4 (40). PARs are seven-transmembrane domain, G protein-coupled receptors that act as sensors for extracellular proteolytic activity. Within human airways, the distribution of PAR mRNA and protein is widespread, with particularly high levels of immunoreactive PARs found on the bronchial epithelium and airway smooth muscle (20, 38). Activation of airway PARs can have profound influences on airway smooth muscle tone, although the effects produced can vary markedly depending on PAR subtype and the species. PAR-2 activation caused relaxation of isolated airway preparations from mice, rats, and guinea pigs (17, 41) and inhibited serotonin- and histamine-induced increases in airway resistance in anesthetized guinea pigs (15, 17). In contrast, PAR-2 activation caused contraction in human isolated bronchial rings (8, 59), although the effect of PAR-2-activating peptides on in vivo lung function in humans is not known.

In many instances, PAR-induced effects in the airways are mediated or modulated by the production of cyclooxygenase (COX) products, such as prostaglandin (PG) E₂. For example, the COX inhibitor indomethacin markedly inhibited PAR-2-mediated relaxation of airway preparations from mice, rats, and guinea pigs (12, 17, 39, 57) and modulated PAR-2-mediated contraction in human isolated bronchus (8, 59). Consistent with this, activation of PAR-2 in mouse isolated tracheal preparations (39) and airway epithelial cell cultures (3) induced PGE₂ production. Thus activation of epithelial PAR-2 stimulates the production and release of PGE₂, which induces airway smooth muscle relaxation.

Activation of airway PARs may also contribute to the recruitment, proliferation, and activation of various inflammatory cells (3, 25, 42, 44, 65, 73) and promote cellular growth and tissue remodeling (1, 5, 9, 51, 68, 74). The likelihood that PARs may play a role in the pathophysiology of chronic airway disease has been enhanced by the findings that biopsy specimens from patients with asthma had upregulated expression of PAR-2 in the respiratory epithelium (38) and that PAR-1 protein (but not PAR-2) is overexpressed in alveolar macrophages from smokers (58).

To further investigate the role of PARs in inflammatory airway disease, in the current study we examined the influence of influenza A viral infection on the distribution and function of PARs in murine airways. Several lines of circumstantial evidence indicate that influenza A viral infection may be associated with elevated expression of PARs and COX-2. For example, an early event in influenza A virus infection is the production of proinflammatory cytokines (33, 70), some of which have been reported to upregulate the expression of PARs (49) and COX-2 (4, 21, 46, 48, 52, 54, 72). Thus this study tested the hypothesis that influenza A virus infection elevates the expression of PARs and COX-2 within the airways, with concomitant increases in the bronchodilator activity of PAR agonists.

METHODS

Animals. Specified pathogen-free, male CBA/CaH mice at 8–10 wk of age (Animal Resource Centre, Murdoch, Australia) were used
throughout these studies. All experiments had approval from the University of Western Australia Animal Ethics Committee and adhered to guidelines established by the National Health and Medical Research Council of Australia.

**Influenza A/PR-8/34 virus.** Influenza A/PR-8/34 virus was propagated in the allantoic fluid of 10-day-old embryonated chicken eggs by procedures previously described (7, 77). Viral infectivity of the harvested allantoic fluid was 2.5 × 10^6 egg infectious doses (EID_{50}/ml) as previously described (41).

**Inoculation of mice with influenza A/PR-8/34 virus.** Mice were lightly anesthetized with methoxyflurane and intranasally inoculated with 15 μl of fluid containing either 1,000 EID_{50} influenza A/PR-8/34 virus (virus infected) or an equivalent dose of allantoic fluid from virus-free chicken eggs ( sham infected). Previous studies performed in our laboratory have shown that 1,000 EID_{50} influenza A/PR-8/34 virus induces epithelial disruption and inflammation in murine airways (7). Sham- and virus-infected mice were studied 48 h postinoculation. For in vitro studies, mice were killed (pentobarbitone sodium, 250 mg/kg), and the trachea and lungs were harvested.

**RT-PCR.** Total RNA was extracted from murine trachea and lung with Tri-Reagent (Molecular Research Center, Cincinnati, OH), as described by the manufacturer. cDNA was prepared with a Pro-Star First Strand RT-PCR kit. In brief, 1 μg of RNA was added to 3 μl of oligo(dT) to 39 μl with RNase-free water. The reaction mixture was incubated at 65°C for 5 min and allowed to slowly cool to room temperature. A master mix totaling 9 μl containing 5 μl 10× first-strand buffer, 40 units RNase block ribonuclease inhibitor, 4 mM dNTP, and 50 units StrataScript reverse transcriptase was prepared and gently mixed with the primer-annexed RNA template. The reaction mix was incubated at 42°C for 60 min, then at 90°C for 5 min. Forward and reverse PAR- and COX-specific primers were prepared commercially (Invitrogen): PAR-1, sense 5'-CTTGTCT-GATCGTGGCCCCCTG-3', antisense 5'-GCCATCCAAAACGGACAC-CAC-3' (PCR product 671 bp); PAR-2, sense 5'-CACCACCTGTCCATGATGTC-3', antisense 5'-CTCACTAGGAAGGTTTTAAAC-3' (PCR product 527 bp); PAR-3, sense 5'-CCCGAGGACAGTATT-TCAAC-3', antisense 5'-TGAGGCTGTGTAACGAAAG-3' (PCR product 646 bp); PAR-4, sense 5'-TAGAACCTCAAGAGCCGACGCGAAG-3', antisense 5'-CAGTGAACACATACTGTTGCG-3' (PCR product 395 bp); COX-1, sense 5'-AGGAGGATTTGCGTCTGGTGG-3', antisense 5'-AAATCTGACCTTTTCTAGGTGC-3' (PCR product 602 bp); COX-2, sense 5'-ACACCTTCTACGCGACTC-3', antisense 5'-TTCAGGAGAGCCTTGGC-3' (PCR product 274 bp). Each PCR was performed in a 12-μl reaction containing 1 μl cDNA, 1.2 μl 10× Taq DNA polymerase buffer, 0.2 mM dNTP, 2 mM MgCl₂, 100 μM selected primer, and 0.25 units Platinum Taq DNA polymerase. The conditions for amplification were: 94°C for 5 min for 1 cycle, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. PCR products were electrophoresed on 1.5% (wt/vol) agarose gel containing ethidium bromide and visualized by UV illumination.

**Tissue preparation for immunohistochemistry.** Mouse trachea and lungs were fixed (2% wt/vol paraformaldehyde, 30 min), rinsed (0.1 M phosphate buffer, 30 min), dehydrated in 70% ethanol, and embedded in paraffin. Tissue sections (5 μm) were cut and mounted onto tissue gelatin-chromalin-coated slides, dewaxed, cleared in xylene, and mounted with Depex (BDH Chemical) for viewing under a light microscope (Nikon Instruments, Melville, NY). Images were captured on a digital camera and processed with Adobe Photoshop software. Unless otherwise stated, all photomicrographs shown were representative of at least three independent studies. Negative controls included preincubating the PAR-2 antibody with a specific immunizing blocking peptide (SC-8207P; Santa Cruz Biotechnology, Santa Cruz, CA) and omission of the primary antibody.

**Immunohistochemistry by confocal microscopy.** Tissue sections were washed with 5% (wt/vol) BSA in PBS for 30 min at room temperature and then incubated with primary antibodies overnight at 4°C. After a brief rinse in PBS, sections were incubated with fluorescently labeled secondary antibodies for 60 min at room temperature, rinsed in PBS, mounted in a nonfluorescent mounting medium (Immu-mount; Shandon, Pittsburgh, PA), and kept in the dark until ready for viewing under a confocal microscope (MRC 1000/1024 UV laser scanning confocal microscope; Bio-Rad, Hemel Hempstead, UK) running COMOS software (Bio-Rad) at the Biomedical Confocal Microscopy Research Centre (Pharmacology Unit, University of Western Australia). We obtained three optical sections by scanning at 2-μm depths, and the image stacks obtained were digitally projected, colored, and superimposed with Adobe Photoshop. Unless otherwise stated, all photomicrographs shown were representative of at least three independent studies using three mice per study.

**In vivo lung function.** Individual mice were anesthetized (13 mg/kg xylazine and 130 mg/kg ketamine), and the jugular vein was cannulated with a saline-filled catheter for intravenous drug administration. The trachea was cannulated and connected to a Fleisch pneumotachograph (Fleisch, Lausanne, Switzerland). A saline-filled cannula connected to the pressure transducer was placed into the midtracheal esophagus to facilitate measurements of tranpsulmonary pressure. Spontaneous breathing was abolished with pancuronium bromide (450 μg/kg iv), and mice were mechanically ventilated with a SAR-830 ventilator (CWE, Ardmore, PA) at 150 breaths per min with a tidal volume of 3.5 ml/kg. Breath-to-breath measurements of airway resistance (cmH₂O·l⁻¹·s⁻¹) were calculated from flow and transpulmonary pressure recordings (PR800; Mumed Systems, London, UK) acquired using a modified method described previously (28). Preliminary experiments established that virus-infected mice were more sensitive to methacholine-induced increases in airway resistance than sham-infected mice. Thus a single submaximal dose of 250 μg/kg methacholine was administered to sham-infected mice and 180 μg/kg methacholine administered to virus-infected mice, to induce similar increases in airway resistance in each group of mice (typically 200 cmH₂O·l⁻¹·s⁻¹). In each mouse, the initial response was defined as 100% increase in airway resistance, and responses to subsequent methacholine challenges were expressed as a percentage of the initial response. In all mice, lungs were hyperinflated (delivering twice the tidal volume) to prevent atelectasis 2 min after every methacholine challenge. To examine the effect of PAR activation on airway function, we administered 5 mg/kg iv of PAR peptides 4 min after the initial methacholine challenge, and the responses induced by subsequent methacholine challenges were assessed 1, 6, and 11 min later. Thus these experiments examined the ability of PAR agonist peptides to inhibit or potentiate methacholine-induced increases in airway resistance. The 5-mg/kg dose of PAR agonist peptide was selected on the basis of results from preliminary experiments, which established that lower doses of 0.1, 0.5, and 1.0 mg/kg had no effect on methacholine-induced increases in airway resistance (data not shown).
Materials. Synthetic PAR agonist peptides and control peptides were synthesized with amidated carboxyl termini (>85% purity; Protein Facility, University of Western Australia). The sequences of the PAR agonist peptides and of the control peptides, respectively, were: PAR-1, SFFLRN-NH₂ and FSFLRN-NH₂; PAR-2, SLIGRL-NH₂ and LSIQLNL-NH₂; PAR-3, SFFNGP-NH₂ and FSNGGP-NH₂; and PAR-4, GYPGK-NH₂ and GYPGFK-NH₂. For immunohistochemistry, goat anti-mouse PAR-1 (SC-8204, Santa Cruz), PAR-2 (SC-8207), PAR-3 (SC-8209), and PAR-4 (SC-8462) antibodies (1:60 dilution), and rabbit anti-mouse COX-1 and COX-2 antibodies (1:200–1:1,000 dilution, Cayman) were used. In addition, biotinylated Griffonia simplicifolia-isolectin B₄ (GSI-B₄), which binds α-D-galactosyl moieties (24), was used to identify basal cells of the mouse trachea (22, 23, 29, 55, 61) (1:40 dilution, Sigma). Rabbit anti-goat (Vector) and swine anti-rabbit (Dako) biotinylated secondary antibodies were used for light microscopy immunohistochemistry, and donkey anti-goat (rhodamine-conjugated) and donkey anti-rabbit (FITC-conjugated) secondary antibodies (Santa Cruz) were used for confocal microscopy immunohistochemistry. 

Statistical analyses. Unless otherwise stated, data are expressed as means ± SE. Groups of data were compared by analysis of variance (SigmaStat software), and where appropriate a modified t-statistic was used to test for differences between means of specific pairs of groups (75).

RESULTS

Influenza A/PR-8/34 virus infection. Airway epithelium of tracheal preparations obtained from sham-infected mice was stratified and columnar, and its general integrity was well maintained (Fig. 1). However, the epithelium of tracheal preparations isolated from virus-infected mice 2 days post-inoculation appeared disorganized and swollen (Fig. 1), and isolated areas of epithelial sloughing were observed, which contributed to the presence of cellular debris in the tracheal lumen (not shown). In addition, mononuclear cells were also present in the underlying submucosa (Fig. 1). Peripheral lung sections isolated from virus-infected mice 2 days post-inoculation showed little distinctive morphological differences when compared with those obtained from sham-infected mice (Fig. 1). We assessed the coexpression of influenza antigen and α-D-galactosyl moieties by confocal microscopy, using anti-influenza antibodies and the basal cell marker GSI-B₄ isolectin, respectively (Fig. 2). In tracheal sections obtained from virus-infected mice, influenza antigens were detected on columnar cells of the epithelium, whereas α-D-galactosyl moieties were detected only on basal epithelial cells (Fig. 2). Superimposition of the images stained with the individual reagents showed no significant colocalization (Fig. 2). Staining was absent when the anti-influenza antibody and GSI-B₄ were omitted.

Expression of PARs in trachea and lungs of sham- and virus-infected mice. In sham-infected mice, expression of murine PAR-1, PAR-2, PAR-3, and PAR-4 mRNA was detected by RT-PCR in both the trachea and peripheral lung (Fig. 3). All PCR amplicons migrated to sites corresponding to their expected product size. Homogenous staining for PAR-1, PAR-2,
PAR-3, and PAR-4 was present in the epithelium and the airway smooth muscle in tracheal sections obtained from sham-infected mice (Fig. 4). In contrast, tracheal sections obtained from virus-infected mice revealed elevated PAR staining. Intense PAR-1 staining was present on the apical epithelium of virus-infected trachea and on some mononuclear cells (Fig. 4). Intense PAR-2 staining was present on the apical tracheal epithelium and a significant population of the infiltrating mononuclear cells (Fig. 4). Elevated staining for PAR-3 and PAR-4 was also observed, although the distribution of both PAR-3 and PAR-4 was present only in isolated regions of the epithelium, and the increased staining intensity was less striking than that for PAR-1 and PAR-2 (Fig. 4). Staining was absent when the primary antibodies were omitted or when the PAR-2 antibody was preabsorbed with its immunizing peptide (Fig. 4).

Expression of COX in trachea and lung of sham- and virus-infected mice. Expression of COX-1 and COX-2 mRNA was detected by RT-PCR in both the mouse trachea and peripheral lung (Fig. 3). COX-1 immunostaining was moderate and homogenous in both the airway epithelium and smooth muscle of sham-infected mouse trachea (Fig. 5). In contrast, in the airway, intense COX-2 staining was predominantly localized in the epithelium, with minimal smooth muscle staining (Fig. 5). Staining was absent when the primary antibody was omitted or when the COX-2 antibody was preabsorbed with its immunizing peptide, confirming immunostaining specificity (data not shown). Respiratory tract viral infection was not associated with any significant change in the levels of immunostaining for COX-1 and COX-2 (Fig. 5).

Colocalization of PAR-2, COX-2, and α-galactosyl moieties in mouse trachea. PAR-2 staining was present on both the airway epithelium and smooth muscle, whereas COX-2 staining was present in the airway epithelium only. GSI-B4 staining was present only in the basal layer of the tracheal epithelium. Superimposition of the images stained with the individual reagents showed that GSI-B4+ basal cells expressed both PAR-2 and COX-2 (Fig. 6).

Modulation by PAR agonist peptides of methacholine-induced bronchoconstriction in sham- and virus-infected mice. In vivo administration of PAR agonist peptides induced little change in baseline airway resistance in sham- or virus-infected mice (data not shown). However, PAR agonist peptides markedly influenced the increase in airway resistance induced by the bronchoconstrictor methacholine. In sham-infected mice, the PAR-1 agonist peptide transiently potentiated methacholine-induced increases in airway resistance (n = 5, Fig. 7). For example, 1 min after the injection of peptide, the methacholine-induced bronchoconstrictor response was increased by >50% (Fig. 7). In contrast, the peptide did not potentiate methacholine-induced increases in airway resistance in virus-infected mice (n = 5, Fig. 7). The effects of the PAR-2 agonist peptide were biphasic in sham-infected mice, characterized by a transient inhibition of methacholine-induced increases in airway resistance 1 min after the peptide was administered [82.4%, 95% confidence interval (CI), 79.7–85.2%, n = 16], and potentiation of methacholine challenges 11 min after peptide administration (114%, 95% CI, 107–121%, n = 16, Fig. 7). However, in virus-infected mice the peptide induced only inhibitory responses, significantly suppressing the effects of methacholine 1 and 6 min after the peptide was administered (n = 5, Fig. 7). The PAR-3 agonist peptide did not significantly modulate methacholine-induced increases in airway resistance at any time points assessed in sham- and virus-infected mice (n = 4–7, Fig. 7). The PAR-4 agonist peptide transiently potentiated methacholine-induced increases in airway resistance in both sham- and virus-infected mice (at 1 min, 144%, 95% CI, 133–155%, n = 6, Fig. 7). As shown in Fig. 7, the control peptides for PAR-1, PAR-2, PAR-3, and PAR-4 did not significantly modulate the effects of methacholine challenges 1, 6, or 11 min after the peptides were administered in sham- or virus-infected mice (n = 4–14).

Influence of the COX inhibitor indomethacin on in vivo effects induced by PAR agonist peptides in sham-infected mice. To examine the role of COX in the PAR-mediated changes in in vivo lung function described above, we pretreated selected mice with indomethacin. In sham-infected, indomethacin-pretreated mice, the magnitude of PAR-1 agonist peptide-induced potentiation of methacholine-induced increases in airway resistance was not significantly different from that in vehicle-pretreated mice (141%, 95% CI, 127–156%, n = 4) 1 min after the peptide was administered. However, PAR-1-pretreated potentiation was prolonged in indomethacin-pretreated mice and remained significantly elevated above baseline 11 min after the peptide was administered (130%, 95% CI, 114–149%, n = 4, Fig. 8). The transient inhibitory effects of the PAR-2 agonist peptide on methacholine-induced increases in airway resistance were abolished in indomethacin pretreated mice (n = 7–20, Fig. 8). The PAR-3 agonist peptide did not alter metha-
choline-induced increases in airway resistance 1, 6, or 11 min after the peptides were administered in vehicle- and indomethacin pretreated mice (n = 4–7, Fig. 8). In indomethacin-pretreated mice, the magnitude of PAR-4 agonist peptide-induced potentiation of methacholine-induced responses was not significantly different from that in vehicle-treated mice (150%, 95% CI, 130–174%, n = 8) 1 min after the peptide was administered. However, PAR-4-mediated potentiation was prolonged in indomethacin-pretreated mice and remained significantly elevated above baseline 6 min after the peptide was administered (128%, 95% CI, 118–139%, n = 7, Fig. 8).

Influence of indomethacin on in vivo effects induced by PAR-1 agonist peptide in virus-infected mice. As shown in Fig. 7, the PAR-1 agonist peptide potentiated methacholine-induced increases in airway resistance 1, 6, or 11 min after the peptides were administered in vehicle- and indomethacin pretreated mice (n = 4–7, Fig. 8). In indomethacin-pretreated mice, the magnitude of PAR-4 agonist peptide-induced potentiation of methacholine-induced responses was not significantly different from that in vehicle-treated mice (150%, 95% CI, 130–174%, n = 8) 1 min after the peptide was administered. However, PAR-4-mediated potentiation was prolonged in indomethacin-pretreated mice and remained significantly elevated above baseline 6 min after the peptide was administered (128%, 95% CI, 118–139%, n = 7, Fig. 8).
airway resistance in sham-infected mice but produced no such increase in virus-infected animals. Interestingly, the potentiating effect produced by the peptide was restored in virus-infected animals pretreated with indomethacin (Fig. 9). These findings suggest that in sham-infected mice the predominant effect of PAR-1 activation is potentiation of methacholine-induced bronchoconstriction, whereas in virus-infected mice this effect is counteracted by PAR-1-mediated generation of prostanoids.

![Fig. 5. Immunostaining of COX-1 and COX-2 in sham (left)- and virus-infected (right) tracheal sections. COX-1 was localized in both the airway epithelium and airway smooth muscle, whereas COX-2 was localized predominantly in the airway epithelium. All sections were counterstained with hematoxylin. Bar = 20 μm for all images. Images are representative examples of 3 separate studies, each using at least 1 pair of sham-infected and virus-infected mice.](image)

![Fig. 6. Immunostaining of PAR-2, COX-2, and α-D-galactosyl moieties in normal mouse tracheal sections, as determined by confocal microscopy. Photomicrographs of sham-infected mouse tracheal sections stained with rhodamine (red) conjugated to antibodies raised against PAR-2, FITC (green) conjugated to antibodies raised against COX-2, and Alexa 350 (purple) conjugated to the isolecitin GSI-B4. Superimposition of the 3 images (A–C) depicted cellular sites that stained for all 3 epitopes (arrowhead in D). The primary antibodies and isolecitins were omitted to assess background staining (E). Bar = 20 μm for all images.](image)
This study investigated the influence of respiratory tract viral infection on the distribution and function of PARs in CBA/CaH mice. mRNA and protein for each of the PARs were expressed on tracheal smooth muscle and tracheal epithelium, and immunostaining for PAR-2 and COX-2 was colocalized to the basal cells of the tracheal epithelium. In sham-infected mice, in vivo activation of PAR-2 induced a COX-dependent inhibition of methacholine-induced increases in airway resistance. In contrast, in vivo activation of PAR-1 and PAR-4 potentiated methacholine-induced effects. Of particular interest, respiratory tract viral infection with influenza A/PR-8/34 virus was associated with increased expression of immunoreactive PARs and with a concomitant shift in the in vivo effects of PAR activation toward suppression of methacholine-induced bronchoconstriction.

**DISCUSSION**

PARs are expressed in murine airways. Expression of PAR-1, PAR-2, PAR-3, and PAR-4 mRNA in the mouse trachea was confirmed by RT-PCR, and immunoreactive proteins of all four PARs were detected on the tracheal epithelium and airway smooth muscle. Consistent with this, immunoreactive PAR-1 and PAR-2 are present in the epithelium and airway smooth muscle of airway preparations obtained from various species, including mice (17, 31), rats (12, 30), guinea pigs (57), and humans (17, 20, 27, 38, 45). Furthermore, the epithelium of human bronchial tissue also expressed PAR-3 and PAR-4 (38).

Both the airway epithelium and airway smooth muscle of the murine trachea expressed all four PARs. Other cells in the...
Transient inhibition of methacholine-induced bronchoconstriction and PARs at the level of airway smooth muscle. The precise mechanism underlying PAR-mediated potentiation of methacholine-induced bronchoconstriction is not clear but the airway, including mast cells (PAR-1, PAR-2), human airway smooth muscle (PAR-1, PAR-2, and PAR-3), and type II pneumocytes (PAR-1, PAR-2, PAR-3, and PAR-4) have also been demonstrated to express more than one PAR subtype (3, 20, 27). Because different PARs are not only responsive to particular proteases but also vary in their intracellular signaling pathways (60, 76), the expression of multiple PARs on a single cell may permit biological tissues to discriminate and respond accordingly to the various types of proteases in the extracellular space.

Airway PARs differentially modulate in vivo bronchomotor tone. To characterize the effects of PAR activation on airway function, we administered PAR agonist peptides intravenously to anesthetized mice, and their effects on subsequent methacholine-induced increases in airway resistance in sham-infected (top) and virus-infected (bottom) mice pretreated with vehicle (left) or the COX inhibitor indomethacin (right). Methacholine challenges were assessed 1, 6, and 11 min after the PAR agonist peptide was administered. Results are expressed as a percentage of the first methacholine challenge, n = 4–7.

*Significant differences relative to basal (100%) responses, P < 0.05.

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PAR-mediated inhibition of methacholine-induced bronchoconstriction is mediated by COX. PAR-2-mediated inhibition of in vivo bronchoconstrictor challenges was abolished by the COX inhibitor indomethacin. The involvement of COX in PAR-mediated effects on bronchomotor tone has not previously been demonstrated in vivo, although several studies have shown that PAR agonist peptides, as well as trypsin and thrombin, induced indomethacin-sensitive relaxations in isolated airway preparations obtained from mice, rats, guinea pigs, and humans (12, 17, 41, 57). The most likely COX product involved is PGE2, since PAR-2 agonist peptides induce concentration-dependent PGE2 release and smooth muscle relaxation, and exogenously applied PGE2, but not PGD2, nor cigardost, stimulates marked relaxation responses in murine isolated tracheal preparations (39, and data not shown). Although the principal effect produced by PAR-1 and PAR-4 agonist peptides was augmentation of methacholine-induced bronchoconstriction, this effect was slightly prolonged in the presence of indomethacin. These findings are consistent with in vitro studies of murine trachea demonstrating that activation of PAR-1 and PAR-4 can also generate inhibitory COX products such as PGE2 (39).

Although indomethacin inhibited PAR-2-mediated bronchodilatory effects, the relative contributions of COX-1 and COX-2 to these effects was not evaluated in the current study. Nevertheless, previous studies in our laboratory have shown that a selective COX-2 inhibitor, nimesulide (6, 56), significantly attenuated relaxations induced by the agonist peptides to PAR-1, PAR-2, and PAR-4 in precontracted mouse isolated tracheal preparations (39). Consistent with this, COX-2 mRNA was present in the mouse trachea, and immunoreactive COX-2 was detected in the tracheal epithelium. These findings suggest that PAR-mediated bronchodiatory effects are mediated by constitutively expressed COX-2. The role of COX-1 is less clear, since although COX-1 mRNA and protein were detected in the mouse trachea (present study), a selective COX-1 inhibitor, valeryl salicylate (6, 32), did not inhibit relaxations mediated by PAR-1, PAR-2, or PAR-4 in mouse isolated tracheal preparations (39).

Fig. 9. Effects of the agonist peptide (5 mg/kg) to PAR-1 (SFFLRN) on methacholine-induced increases in airway resistance in sham-infected (top) and virus-infected (bottom) mice pretreated with vehicle (left) or the COX inhibitor indomethacin (right). Methacholine challenges were assessed 1, 6, and 11 min after the PAR agonist peptide was administered. Results are expressed as a percentage of the first methacholine challenge, n = 4–7.

*Significant differences relative to basal (100%) responses, P < 0.05.
PAR-2 and COX-2 protein is coexpressed in basal tracheal epithelial cells. Fluorescence imaging revealed colocalization of staining for COX-2, PAR-2, and GSI-B4 in mouse tracheal sections. Because GSI-B4 binds to basal epithelial cells of rodent and human conducting airways, these findings suggest that PAR-2 mediates airway smooth muscle relaxation through activation of COX-2 localized in the basal cells of the airway epithelium. These findings may well explain our earlier findings that mechanical disruption of the tracheal epithelium, which disrupts the columnar epithelial cells without removing the basal cells, had no significant effect on PAR-2-mediated relaxation in mouse isolated tracheal preparations (41). Thus PAR-2-mediated relaxation appears to occur independently of PAR-2+ /COX-2+ columnar epithelial cells. Although the in vivo functional roles of PAR-2 and COX-2 expressed on columnar epithelial cells in the mouse trachea remain unknown, PAR activation in isolated bronchial epithelial cells stimulates calcium signaling (19, 50, 69) and trachea remain unknown, PAR activation in isolated bronchial epithelial cells may also stimulate the release of various inflammatory mediators including eotaxin, granulocyte-monocyte colony-stimulating factor, IL-6, IL-8, and matrix metalloproteinases (3, 65, 73, 74), although the contribution of COX-2 in mediating these pathways has not yet been examined.

Influenza A virus infection is associated with increased PAR immunostaining. Tracheal preparations obtained from virus-infected mice showed increased staining for immunoreactive PAR-1 and PAR-2 on the tracheal epithelium, suggesting their upregulation during a respiratory virus infection. Previous studies have demonstrated that ex vivo virus infections in cultured epithelial cells stimulated the release of multiple inflammatory cytokines (2, 53, 67), some of which can modulate PAR expression (10, 16, 43, 47, 49, 62). Indeed, exogenous treatment of cultured cells with TNF-α, IL-1β, and IFN-γ increased PAR-1 and PAR-2 expression (43, 47, 49).

Increased PAR expression may contribute to increased sensitivity to PAR agonist peptides. For example, LPS and TNF-α upregulated PAR expression in isolated cells and tissues and, as a consequence, increased its sensitivity to PAR agonist peptides (14, 26, 34, 47). Consistent with this, PAR-stimulated release of IL-6 from human umbilical vascular endothelial cells was accentuated with LPS pretreatment (11).

PAR-mediated bronchodilator effects were augmented in influenza A virus-infected mice. In the current study, the effects induced by PAR agonist peptides on in vivo lung function were modulated by respiratory tract viral infection. For example, PAR-1 activation, which potentiated methacholine-induced bronchoconstriction in sham-infected mice, did not significantly alter methacholine-induced responses in virus-infected mice, and PAR-2-mediated inhibition of methacholine challenges were significantly prolonged in virus-infected mice. These findings indicate that increased expression of PAR-1 and PAR-2 observed in virus-infected mice is linked functionally to augmented bronchodilation. However, the precise mechanisms underlying these effects have not been fully resolved. In a previous study, we demonstrated that influenza A viral infection was not associated with any overt changes in the responsiveness of isolated tracheal smooth muscle preparations to PAR-stimulating peptides (5). Together, these findings suggest that other functions of epithelial PARs, such as regulation of ion channel function and transepithelial fluid transport (19), may contribute to in vivo responsiveness to PAR-stimulating peptides. Nevertheless, the findings that PAR-1-mediated potentiation of methacholine-induced bronchoconstriction was restored by pretreatment of virus-infected animals with indomethacin strongly indicate the involvement of relaxant prostanooids, perhaps PGE2, in the dampening of PAR-1-mediated potentiation of methacholine-induced bronchoconstriction in virus-infected mice.

Increased PAR expression has been reported in various animal models of inflammation (18, 63, 71). Indeed, PAR expression in alveolar cells of the peripheral lung was elevated in a rodent model of bleomycin-induced pulmonary fibrosis (30). In addition, biopsy specimens obtained from patients with asthma and bronchitis exhibit increased PAR expression in the epithelium and airway smooth muscle (38, 45). These findings suggest that proinflammatory stimuli may increase PAR expression in the airway. However, the significance of PAR upregulation on pulmonary function during airway inflammation had not previously been determined. The present study is the first to demonstrate that increased PAR expression on epithelial cells during influenza virus infection shifts the functional responses mediated by PARs toward bronchoprotection against spasmodogenic challenges in murine airways in vivo. Specifically, in virus-infected mice, PAR-1 agonist peptides induced markedly less bronchoconstriction, and PAR-2 agonist peptides induced prolonged bronchoprotection against spasmodogenic challenges. Although functional studies on PAR activation during airway inflammation have not yet been examined in detail, the current findings are consistent with the dual functional behavior observed with PGE2, and although PAR-2 is likely to mediate proinflammatory effects such as tissue genesis, vasodilation, and leukocyte trafficking during airway homeostasis, it may also act to preserve airway function by promoting bronchodilatation.

GRANTS
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