PAR-2 activation, PGE\textsubscript{2}, and COX-2 in human asthmatic and nonasthmatic airway smooth muscle cells

Linda S. Chambers, Judith L. Black, Qi Ge, Stephen M. Carlin, Wendy W. Au, Maree Poniris, Joanne Thompson, Peter R. Johnson, and Janette K. Burgess

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PAR-2 activation, PGE\textsubscript{2}, and COX-2 in human asthmatic and nonasthmatic airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 285: L619–L627, 2003. First published May 16, 2003; 10.1152/ajplung.00416.2002.—The protease-activated receptor-2 (PAR-2) is present on human airway smooth muscle (ASM) cells and can be activated by mast cell tryptase, trypsin, or an activating peptide (AP). Tryptase induced significant increases in PGE\textsubscript{2} release from human ASM cells after 6 and 24 h and also induced cyclooxygenase (COX)-2 mRNA expression and COX-2 protein. Tryptase and the PAR-2 AP did not alter PGE\textsubscript{2} release or COX-2 protein levels, suggesting a lack of PAR-2 involvement. When we compared results in asthmatic and nonasthmatic muscle cells, both trypsin and bradykinin induced less PGE\textsubscript{2} from asthmatic ASM cells, and bradykinin induced significantly less COX-2 mRNA in asthmatic cells. Significantly less PGE\textsubscript{2} was released from proliferating ASM cells from asthmatic patients. In conclusion, trypsin induces PGE\textsubscript{2} release and COX-2 in human ASM cells, which is unlikely to be via PAR-2 activation. In addition, ASM cells from asthmatic patients produce significantly less PGE\textsubscript{2} and COX-2 compared with nonasthmatic cells. These findings may contribute to the increase in muscar mass evident in asthmatic airways.

protease-activated receptor; tryptase; asthma; prostaglandin E\textsubscript{2}; cyclooxygenase-2

The protease-activated receptor-2 (PAR-2) belongs to a family of G protein-coupled receptors that are activated by specific proteases. Four PARs have been identified to date in which the extracellular amino terminal domain is cleaved to form a new amino terminus that functions as a tethered ligand to bind to and activate the receptor. PAR-2 is activated by trypsin, mast cell tryptase, and a synthetic peptide corresponding to the new tethered ligand domain [PAR-2-activating peptide (AP)-SLIGKV-NH\textsubscript{2} in humans and SLIGRL-NH\textsubscript{2} in mice]. Both the smooth muscle and epithelium of human airways express PAR-2 (12). Some studies have suggested that PAR-2 activation in the airways is associated with the release of endogenous prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) (10, 21, 22, 30).

PGE\textsubscript{2} is the predominant prostanoid produced by airway smooth muscle (ASM) cells (4, 24) and has a number of bronchoprotective effects in human airways including the inhibition of proliferation of ASM cells (18) and the inhibition of bronchoconstriction (26–28). PGE\textsubscript{2} generation is dependent on the rate-limiting enzyme prostaglandin hydroxendoperoxidase synthase, also known as cyclooxygenase (COX). Arachidonic acid generated from phospholipids is converted by COX to form PGH\textsubscript{2}, which is then further metabolized by specific synthases to produce various prostaglandins or thromboxanes. There are two isoforms of COX: COX-1, which is constitutively expressed in ASM cells, and COX-2, which is not normally detectable in ASM cells but is upregulated by inflammatory stimuli. COX-2 can be induced in ASM cells by IL-1β, bradykinin, and transforming growth factor-β (4, 14, 25, 35), leading to an increase in PGE\textsubscript{2} release.

It is possible that PAR-2 activation can also regulate PGE\textsubscript{2} release in the airways. We have previously shown that human bronchial segments contract in response to trypsin and the PAR-2 AP, and these contractions were increased in the presence of the COX inhibitor indomethacin (9). After 30 min in response to PAR-2 activation, there was no significant increase in immediate PGE\textsubscript{2} release from human ASM cells; however, longer incubation times have yet to be tested (9). While studying the proliferative effects of PAR-2 agonists on human ASM cells, we found that trypsin induced inhibition of proliferation of human ASM cells after 24 h and that this inhibition was decreased in the presence of indomethacin, suggesting the release of PGE\textsubscript{2} or another inhibitory prostanoid (9). Activating PAR-2 in mice and guinea pig airways induces a relaxation of isolated airways that is attenuated by inhibiting COX, suggesting release of PGE\textsubscript{2} (10, 22, 30). One study has shown that the PAR-2 AP induces release of PGE\textsubscript{2} from mice airways preferentially via COX-2 (21).

A recent study examining the levels of PAR-2 in asthmatic and nonasthmatic airway biopsies has shown that there is an increase in PAR-2 immunohistochemical staining in asthmatic epithelium compared with nonasthmatic epithelium (20). In addition, we have previously shown that ASM cells from asthmatic...
patients grow faster than those from nonasthmatic patients (19). The effects of PAR-2 activation and the release of PGE2 from asthmatic and nonasthmatic ASM cells have yet to be investigated.

The aims of this study were to determine the effect of PAR-2 activation on PGE2 release and COX-2 protein and mRNA levels of ASM cells as well as compare responses in ASM cells from asthmatic and nonasthmatic patients. The effects of Bradykinin, which is known to induce COX-2 and PGE2 release (25) but has yet to be investigated in asthmatic and nonasthmatic cells, were also examined. The effect of PAR-2 activation on the release of IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in asthmatic and nonasthmatic ASM cells was also included to see if the differences in responses we observed in asthmatic ASM cells were specific for PGE2 release.

METHODS

ASM cell isolation. Human lung tissue was obtained from patients undergoing lung transplantation, lobectomy, pneumonectomy, or endobronchial biopsies (16). Details for all asthmatic and nonasthmatic patients used in this study are presented in Table 1. The Human Ethics Committee of the University of Sydney and the Central Sydney Area Health Service provided approval for all experiments involving the use of human lung. Sensitization status was assessed for each patient from whom lung tissue was obtained as previously described (9). ASM cell cultures were established as previously described, from airways separate from those used for sensitization testing (9, 15, 18). Smooth muscle cell bundles were removed from surrounding airway tissue and were placed as explants in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20 U/ml penicillin, 20 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 10% fetal bovine serum (FBS). The smooth muscle cells were identified by their morphology (34) under a light microscope and by immunofluorescent staining for a specific α-smooth muscle actin antibody (18) and a calponin antibody (13). For all experiments, cells were plated in six-well plates at a density of 10^4 cells/cm^2 in DMEM supplemented with 10% FBS and antibiotics (20 U/ml penicillin, 20 μg/ml streptomycin, and 2.5 μg/ml amphotericin B).

PGE2 assay. After 7 days in DMEM with 10% FBS, the confluent cells were equilibrated for 24 h in 1% FBS. The cells were then treated with trypsin (50 U/ml), tryptase (1 nM), PAR-2 AP (100 nM), or diluent for controls. These concentrations of PAR-2 agonists were chosen since they have been shown to alter the proliferation of human ASM cells (9). On the basis of previous studies in smooth muscle, it is likely that the same concentration would also have an effect on the synthesis of mediators by ASM cells (6). All treatments were added in the presence of 1% FBS. Cells were also treated with trypsin in the presence of the

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M, male; F, female; N/A, not available. *Sensitization assessed by skin prick test.
irreversible serine protease inhibitor 4-aminophenylmethyl-
anesulfonyl fluoride (p-APMSF) to determine whether the 
actions of trypsin were dependent on its proteolytic activity. 
A concentrated solution of 2,500 U/ml of trypsin was 
incubated with 10 μM p-APMSF in 10 mM bis-Tris buffer (pH 6.1) 
at 4°C for 2 h (7). This solution was then diluted in DMEM 
supplemented with 1% FBS upon addition to the cells to 
achieve a final concentration of trypsin of 50 U/ml in the presence 
or absence of 0.2 μM p-APMSF.

The trypsin was prepared as 10−7 M aliquots made up in 
a buffer solution of 10 mM bis-Tris, pH 6.1, with 0.5 M NaCl 
and 60 μg/ml heparin. The controls for trypsin consisted of 
equivalent amounts of this heparin and buffer solution. After 
6- and 24-h incubation, the supernatant was collected and 
stored at −20°C until levels of PGE2 were assayed by an 
ELISA (Cayman Chemicals). A separate series of experi-
ments was also performed to examine the levels of PGE2 
produced as cells were proliferating. After 24 h in 10% FBS, 
the medium was changed to 1% FBS for 24 h to equilibrate 
the cells, which were then treated with 5% FBS to induce cell 
proliferation. Cell numbers were determined, and superna-
tants were collected on this day (day 0) and again after 3 and 
5 days of incubation with 5% FBS.

**IL-6 and GM-CSF assay.** Supernatants collected after 24-h 
incubation with trypsin (50 U/ml), PAR-2 AP (100 μM), 
tryptase (1 nM), and bradykinin (10 μM) for PGE2 analysis 
were also assayed for IL-6 and GM-CSF levels by the method 
previously described (32).

**Western immunoblots.** Cells were grown to confluence 
in 10% FBS for 7 days, equilibrated in 1% FBS for 24 h, and 
treated with trypsin (50 U/ml), trypsinate (1 mM), and PAR-2 
AP (100 μM) for 6 or 24 h. Bradykinin (10 μM) was tested 
over time points ranging from 0.5 to 24 h. Tryptase was also 
tested in the presence of 0.2 μM p-APMSF according to the 
same protocol as for the PGE2 release experiments. Plates 
were then kept on ice while the cells were rinsed with cold 
PBS. Lysis buffer (2 mM benzamidine, 500 μM PMSF, 0.5% 
Triton X-100, 30 mM Tris, and 1 mM EDTA, pH 7.4) was 
added to each well to produce whole cell extracts. Cells were 
harvested by cell scraping, and extracts were stored at 
−20°C. Extracts were centrifuged to remove cell debris, and 
protein concentration was determined by the Bio-Rad 
Bradford protein assay. Extracts were added to equal volumes of 
loading buffer (final concentration: 2% SDS, 7.5% glycerol, 
31.25 mM Tris·HCl, pH 6.8, 0.025% bromphenol blue, and 
200 mM DTT) and heated at 100°C for 5 min. Proteins were 
separated by SDS-polyacrylamide electrophoresis on 12% 
polyacrylamide gel. Proteins were then transferred to nitro-
cellulose membranes, which were incubated with blocking 
solution [1% BSA (wt/vol) in PBS] overnight at 4°C. The membranes 
were washed in 0.05% Tween 20 in PBS and then 
incubated with mouse monoclonal anti-human COX-1 or 
COX-2 antibody (5 or 2.5 μg/ml, respectively) at room 
temperature for 3 h. After further washes in 0.05% Tween 20 in 
PBS, the membranes were incubated for 1 h with horseradish 
peroxidase-conjugated goat anti-mouse IgG antibody 
(1:10,000). Immunoblot detection was then performed with a 
Supersignal West Dura Extended Duration Substrate kit, 
and bands were visualized with a Superscript one-step RT-PCR kit with Platinum Taq from Invitrogen. For each COX-1 or COX-2 reaction, RT-PCR was 
also carried out for 18S rRNA to allow the amounts of COX-1 
and COX-2 mRNA detected to be standardized. For each 
reaction, the master mix containing the RNA sample was 
split in half, with half used for the COX-1 or COX-2 reaction 
and the other half for the 18S rRNA reaction.

The COX-1 and COX-2 primers (1.6 μM) used were as 
described by Beasley (2). The 18S rRNA primers (0.5 μM) 
used were as follows: forward primer 5′-CTCAACCACG- 
GAAACCTCAC 3′ and reverse primer 5′ GACAATGCTC- 
CACCAACT 3′. For all reactions, the RT step was performed 
at 50°C for 30 min. The amplification reactions commenced 
with an initial denaturation step at 94°C for 2 min. For 
COX-1, this was followed by 24 cycles of the following: dena-
turation at 94°C for 30 s, annealing at 67°C for 30 s, and 
extension at 72°C for 1 min. For COX-2, 26 cycles of the following were performed: 94°C for 30 s, 55°C for 30 s, and 
72°C for 1 min. Twelve cycles of the following were used for 
the 18S rRNA: 94°C for 30 s, 56°C for 30 s, and 72°C for 1 
min. For all reactions, a final extension of 72°C for 10 min 
was then performed.

The reaction products were separated on Tris-acetate (40 
mM)-EDTA (1 mM)-polyacrylamide gels by electrophoresis 
and stained with 0.2% silver nitrate solution. We analyzed 
bands with a 440F Kodak imaging system and software. 
Amounts of COX-1 or COX-2 mRNA were expressed as a 
ratio of the amount of ribosomal 18S rRNA present in each sample.

**Assay of proteolytic activity.** The activity of trypsin in 
the presence and absence of the irreversible serine protease 
inhibitor p-APMSF was examined to assess the degree of 
inhibition induced. Proteolytic activity of trypsin was deter-
mined using the synthetic peptide substrate N-p-tosyl-Gly-
Pro-Lys-p-nitroanilide (9, 17). Two microliters of trypsin 
were added to 1 ml of the substrate solution [0.05 M Tris·HCl 
(pH 7.6), 0.12 M NaCl, and 0.1 mM N-P-tosyl-Gly-Pro-Lys-
p-nitroanilide] at 37°C. Tryptase was tested following incuba-
tion with p-APMSF or control diluent for 2 h as described 
for the PGE2 experiments. The rate of change of absorbance 
at 405 nm was recorded on a spectrophotometer and used to 
determine the activity as previously described (17).

**Analysis of results.** Changes in PGE2 release in response 
PAR-2 agonists were expressed as a percentage of the control 
(i.e., diluent) and were compared by Student’s paired t-test. 
The release of PGE2 from asthmatic and nonasthmatic ASM 
cells was compared by ANOVA and Fisher’s protected least 
squares difference (PLSD) tests. To compare asthmatic and 
nonasthmatic COX-2 mRNA levels, we expressed densitom-
etry readings of COX-2 mRNA bands as densitometry units 
normalized to the largest 18S band and compared by ANOVA 
with Fisher’s PLSD. For all statistical tests, significance was 
defined as a P value <0.05.

**Materials.** The following compounds were obtained from 
the sources given: tissue culture flasks and 96-well plates 
from Becton Dickinson; Hanks’ balanced salt solution, 
DMEM, penicillin, streptomycin, and amphotericin B from 
GIBCO-BRL, Life Technologies; FBS from CSL Biosciences; 
mouse monoclonal anti-human COX-1 antibody, mouse 
monoclonal anti-human COX-2 antibody, mouse monoclonal 
anti-human COX-2 antibody, and mouse monoclonal anti-human COX-2 antibody.
PGE₂ enzyme immunoassay kit from Cayman Chemical; Supersignal West Dura Extended Duration Substrate kit from Pierce; COX-1 and COX-2 primers from Geneworks; RNeasy mini kit from Qiagen; and Superscript one-step RT-PCR kit with Platinum Taq from Invitrogen.

RESULTS

Effect of PAR-2 activation on PGE₂ release from human ASM cells. Incubation with trypsin for 6 or 24 h produced large increases in PGE₂ release from ASM cells, 555 ± 193 and 588 ± 149%, respectively, of control release (Fig. 1A, n = 8, P < 0.05). Increases in PGE₂ release were also observed in response to the positive control bradykinin after 6 (371 ± 69%) and 24 (895 ± 518%) h (Fig. 1A, n = 8, P < 0.05). The PAR-2 AP and tryptase did not significantly alter PGE₂ release as shown in Fig. 1A (n = 6, P > 0.05). Examination of responses to trypsin and bradykinin in ASM cells from asthmatic and nonasthmatic patients reveals differences in the amount of PGE₂ released (Fig. 1, B and C; P < 0.05). Asthmatic ASM cells incubated with trypsin for 24 h released significantly less PGE₂ than nonasthmatic ASM cells [891 ± 160% (n = 4 nonasthmatic) and 285 ± 129% (n = 4 asthmatic), P < 0.05; Fig. 1B]. The trypsin-induced PGE₂ release in asthmatic and nonasthmatic ASM cells was dependent on the proteolytic activity of trypsin. After incubation with p-APMSF, trypsin had no detectable proteolytic activity. Trypsin-induced PGE₂ release was decreased in the presence of p-APMSF with responses reduced to 23 ± 6 and 25 ± 5% of responses to trypsin alone after 6 and 24 h, respectively (n = 6 patients, data not shown). These results were similar to the response in control cells treated with 1% FBS alone (18 ± 6 and 22 ± 4% of response to trypsin after 6 and 24 h,

Fig. 1. A: effect of protease-activated receptor (PAR)-2-activating peptide (AP, 100 μM), trypsin (50 U/ml), tryptase (1 nM), and bradykinin (10 μM) for 6 (open bars) and 24 h (solid bars) on PGE₂ release from human airway smooth muscle (ASM) cells. Graph shows means ± SE in n = 6 patients (3 asthmatic, 3 nonasthmatic) for PAR-2 AP and tryptase, and n = 8 patients (4 asthmatic, 4 nonasthmatic) for trypsin and bradykinin. Effect of trypsin (n = 4 asthmatic, 4 nonasthmatic; B) and bradykinin (10 μM, n = 7 asthmatic, n = 7 nonasthmatic; C) for 6 and 24 h on PGE₂ release from confluent ASM cells from asthmatic (solid bars) and nonasthmatic (open bars) patients. D: PGE₂ release from asthmatic (solid bars, n = 5) and nonasthmatic (open bars, n = 6) ASM cells after 0, 3, and 5 days in 5% fetal bovine serum. All values are means ± SE. *Significant increase compared with control. †Significant difference between responses in asthmatic and nonasthmatic patients; P < 0.05.
respectively). The decreases in PGE$_2$ release observed were similar in asthmatic (22 ± 11 and 21 ± 8% of response to trypsin alone after 6 and 24 h, n = 3 patients) and nonasthmatic patients (23 ± 5 and 29 ± 4% of response to trypsin after 6 and 24 h, n = 3 patients). Both 6- and 24-h incubation with bradykinin produced significantly less PGE$_2$ in asthmatic compared with nonasthmatic ASM cells [1,526 ± 596% (24 h, n = 7 nonasthmatic) and 366 ± 72% (24 h, n = 7 asthmatic), P < 0.05; Fig. 1C].

Levels of PGE$_2$ released from ASM cells during growth in 5% FBS were strikingly different in the two patient groups. After 24 h of quiescence in 1% FBS (day 0), the levels of PGE$_2$ released from asthmatic and nonasthmatic patients groups. After 24 h of incubation with bradykinin, the mean value for release from the nonasthmatic cells (36.9 ± 9.4 pg/10$^4$ cells, n = 6) was almost tenfold higher than the amount released from the asthmatic cells (3.9 ± 4 pg/10$^4$ cells, n = 5) (Fig. 1D, P < 0.001). This large difference between the PGE$_2$ released from ASM cells from the two patient groups was even more pronounced after 5 days of growth. The mean value for release from asthmatic ASM cells was only 1.2 ± 0.5 pg/10$^4$ cells (n = 5), whereas the mean value for the nonasthmatic cells was 20.1 ± 4.9 pg/10$^4$ cells (n = 6) after 5 days (P < 0.01).

IL-6 and GM-CSF release from ASM cells in response to PAR-2 agonists. Twenty-four hours of incubation with trypsin, tryptase, and bradykinin induced significant increases in IL-6 release from ASM cells of 266 ± 47, 147 ± 9, and 192 ± 24% of control responses, respectively (P < 0.05, n = 8 patients). Treatment with the PAR-2 AP for 24 h did not alter IL-6 levels (106 ± 3% of control response, P > 0.05, n = 8 patients). There were no significant differences in the levels of IL-6 released from asthmatic compared with nonasthmatic ASM cells (Fig. 2A, P > 0.05, n = 4 asthmatic and n = 4 nonasthmatic patients). Trypsin was the only agonist tested that significantly increased GM-CSF levels by 10.2 ± 2.2 pg/10$^4$ cells (n = 3) in both asthmatic and nonasthmatic patients. Trypsin and tryptase were not tested on nonasthmatic patients. As shown in Fig. 2B, there was no significant difference between responses in asthmatic and nonasthmatic ASM cells (P > 0.05, n = 4 asthmatic and n = 4 nonasthmatic patients).

COX protein levels in ASM cells following PAR-2 activation. Bradykinin (10 μM) induced time-dependent increases in COX-2 protein levels with responses maximal at 6 h (Fig. 3A). As represented in Fig. 3A, less COX-2 was consistently induced in ASM cells from asthmatic (n = 9) compared with nonasthmatic (n = 9) patients. However, due to problems regarding interpatient variability and reproducibility, it was not possible to accurately quantitate responses in asthmatic and nonasthmatic cells. Treatment for 6 h with trypsin induced large increases in COX-2 protein; however, tryptase and the PAR-2 AP had no detectable effect on COX-2 levels (Fig. 3B). None of the treatments was found to alter levels of COX-1 protein (Fig. 3B). The inhibition of all of the protease activity of trypsin abolished the induction of COX-2 protein by trypsin but did not alter COX-1 levels (n = 1 asthmatic, 3 nonasthmatic patients, Fig. 3C).

Effect of PAR-2 agonists on COX-2 mRNA levels of ASM cells. Small amounts of COX-2 mRNA were detected in untreated ASM cells from asthmatic and nonasthmatic patients. Trypsin (50 U/ml) induced increases in COX-2 mRNA in a time-dependent manner (Fig. 4A, n = 6, P < 0.05). There were no significant differences in responses to trypsin in asthmatic compared with nonasthmatic ASM cells (n = 3 asthmatic, n = 3 nonasthmatic patients, P > 0.05, data not shown). Bradykinin (10 μM) also induced time-dependent increases in COX-2 mRNA over 6 h with levels returning to baseline at 24 h (Fig. 4B). The level of mRNA for COX-2 in response to bradykinin in cells from asthmatic patients was less than in nonasthmatic patients with a significant difference between the responses after 1 and 2 h (n = 5 asthmatic, n = 6 nonasthmatic patients, P < 0.05; Fig. 4C). The responses after 1 h incubation with bradykinin were...
3.34 ± 0.78 × 10^5 (n = 5 asthmatic patients) and 5.57 ± 0.63 × 10^5 (n = 6 nonasthmatic patients) normalized densitometry units. After 2-h treatment with bradykinin, responses were 3.18 ± 0.67 × 10^5 (n = 5 asthmatic patients) and 5.70 ± 0.65 × 10^5 (n = 6 nonasthmatic patients) normalized densitometry units. By 24 h, COX-2 mRNA levels had returned close to baseline in both asthmatic and nonasthmatic ASM cells (1.45 ± 0.48 × 10^5 and 1.53 ± 0.38 × 10^5 normalized densitometry units, respectively). There were no significant differences in basal levels of COX-2 and COX-1 mRNA in asthmatic and nonasthmatic ASM cells (n = 3 asthmatic, n = 3 nonasthmatic patients, P > 0.05, data not shown).

**DISCUSSION**

In this study, we have shown for the first time that trypsin stimulates PGE_2 release from human ASM cells in culture as well as inducing COX-2 mRNA and protein. The other PAR-2 activators, PAR-2 AP and tryptase, had no significant effect on PGE_2 release or COX-2 protein levels. When asthmatic and nonasthmatic cells were compared, there was significantly less PGE_2 released from confluent asthmatic ASM cells in response to trypsin and bradykinin and from subconfluent, proliferating asthmatic ASM cells. Expression of COX-2 mRNA induced in asthmatic cells in response to 2-h treatment with bradykinin, responses were 3.18 ± 0.67 × 10^5 (n = 5 asthmatic patients) and 5.70 ± 0.65 × 10^5 (n = 6 nonasthmatic patients) normalized densitometry units. By 24 h, COX-2 mRNA levels had returned close to baseline in both asthmatic and nonasthmatic ASM cells (1.45 ± 0.48 × 10^5 and 1.53 ± 0.38 × 10^5 normalized densitometry units, respectively). There were no significant differences in basal levels of COX-2 and COX-1 mRNA in asthmatic and nonasthmatic ASM cells (n = 3 asthmatic, n = 3 nonasthmatic patients, P > 0.05, data not shown).
Trypsin was found to stimulate the release of PGE2, since levels of IL-6 and GM-CSF released in response to trypsin were similar in asthmatic and nonasthmatic smooth muscle. Tryptase and bradykinin induced IL-6 but not GM-CSF release from ASM cells with no differences between asthmatic and nonasthmatic cells.

Trypsin induced large increases in PGE2 release from ASM cells after 6- and 24-h incubation. These increases were comparable with those induced by bradykinin and were likely due to the induction of COX-2, as confirmed by an increase in both COX-2 mRNA expression and protein levels. Treatment with trypsin and the PAR-2 AP at concentrations that have previously been shown to increase the proliferation of human ASM cells (9) did not induce any significant changes in PGE2 release or COX-2 protein levels, which makes it unlikely that the effects of trypsin were mediated via activation of PAR-2. These results are consistent with our previous studies examining the effect of PAR-2 agonists on the proliferation of human ASM cells (9). We reported that tryptase and PAR-2 AP-induced proliferation of human ASM cells was not altered in the presence of indomethacin, suggesting that neither tryptase nor PAR-2 AP induced release of endogenous PGE2. Although not mediated via PAR-2 activation, the trypsin-induced PGE2 release and COX-2 induction in the current study were dependent on the proteolytic action of trypsin, since the irreversible proteolytic inhibitor APMSF abolished the increased PGE2 release and COX-2 protein levels in response to trypsin. This is consistent with the findings of Berger et al. (5), who reported that inhibition of the proteolytic activity of tryptase reduces the tryptase-induced proliferation of human ASM cells. However, in a recent study, APMSF did not alter the proliferation of human ASM cells induced by tryptase, suggesting a nonproteolytic mechanism of action (7).

PAR-2 activation in human airways appears to have the potential to contribute to the hyperplasia and hyperresponsiveness evident in the asthmatic airway (9); however, the findings that PGE2 is released in response to trypsin but not tryptase suggest that trypsin may also exhibit some non-PAR-2-mediated protective effects in the airways. Trypsin has been localized to human airway epithelial cells, but the stimulus for its release currently remains unknown (11).

To determine whether the lower levels of PGE2 released from asthmatic muscle cells was an indicator of a generalized defect in release of endogenous mediators, we examined release of two cytokines, IL-6 and GM-CSF, in response to PAR-2 stimulation. In contrast to our findings with PGE2, levels of these cytokines were not different in asthmatic and nonasthmatic cells. The decreased release of PGE2 seems therefore to be specific. Trypsin was found to stimulate the release of both IL-6 and GM-CSF. Neither trypsin nor the PAR-2 AP altered GM-CSF release, suggesting a lack of involvement of PAR-2. The PAR-2 AP also had no effect on IL-6 release, but both trypsin and tryptase did increase levels of this cytokine, which could suggest involvement of PAR-2 activation. It is possible that a concentration of AP higher than those that could be achieved in this system is required to detect an effect on IL-6 production. In a recent study published by Asokananthan et al. (1), 400 μM PAR-2 AP was used to increase IL-6 release from lung epithelial cells after 24 h. The trypsin-induced effects on PGE2, COX-2, and cytokine release described in this study may be mediated via a non-PAR mechanism or possibly via another PAR. Trypsin can cleave and activate PAR-4, which is known to be present on human ASM cells (20), and PAR-4 stimulation has been reported to increase IL-6, IL-8, and PGE2 release from human lung epithelial cells (1).

There were large differences in levels of PGE2 released from asthmatic and nonasthmatic ASM cells. Pronounced differences were evident in proliferating cells. Although there was no difference in basal PGE2 levels in quiesced, subconfluent cells, after 3 and 5 days of mitogenic stimulation, the levels of PGE2 released from asthmatic cells were ~10- and 17-fold lower, respectively, than those from nonasthmatic patients. Because PGE2 can inhibit proliferation of human ASM cells (3, 18), the reduced capacity for the asthmatic ASM cells to produce PGE2 during cell proliferation could contribute to the large increase in proliferation of asthmatic compared with nonasthmatic ASM cells as previously described (16).

In a separate series of experiments, both trypsin and bradykinin induced significantly less PGE2 release in confluent asthmatic ASM cells. There was also significantly less COX-2 mRNA induced by bradykinin in asthmatic ASM cells. The reduced release of prostaglandins such as PGE2 and possibly PGI2 resulting from less COX-2 induction could alter the properties of asthmatic airways. PGI2 also inhibits the proliferation of ASM cells and relaxes airways in vitro (3, 33). PGE2 has a number of anti-inflammatory and protective properties that, if impaired in asthmatic airways, could potentially amplify or contribute to the development of the hyperresponsiveness, airway remodeling, and inflammation evident in asthma.

This is the first study demonstrating significant differences in PGE2 levels and COX-2 mRNA expression in asthmatic and nonasthmatic ASM. However, similar results have previously been reported for another disease of human airways. Lung fibroblasts and macrophages in culture from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize PGE2, whereas the fibroblasts also exhibit reduced expression of COX-2 (23, 36). The results found in ASM cells are in contrast to a previous study investigating PGE2 release from asthmatic epithelium, which revealed increases in PGE2 release in response to calcium ionophore (8). The reduced expression of COX-2 in response to bradykinin in asthmatic ASM cells in the present study differs from results reported in asthmatic epithelium, where an increase in COX-2 immunostaining and mRNA levels in the epithelium and submucosa of asthmatic patients has been reported (29, 31). These contrasting findings in the ASM and...
epithelium may reflect the different role played by these two cell types in response to inflammatory events in the airway.

In summary, this study has shown for the first time that PAR-2 activation of human ASM cells does not appear to alter PGE\textsubscript{2} release or COX-2 induction. In contrast, prolonged stimulation with trypsin does increase both PGE\textsubscript{2} release and COX-2 mRNA and protein levels, probably via a PAR-2-independent mechanism. Profound reductions in PGE\textsubscript{2} release and COX-2 expression were observed in asthmatic ASM cells. If these in vitro findings were found to reflect in vivo events, then the diminished capacity for asthmatic ASM cells to synthesize PGE\textsubscript{2} could contribute to a number of the characteristic features of the asthmatic airway.

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DISCLOSURES

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