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Delayed and persistent suppression of bronchoconstriction by trypsin in the airway lumen

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ABSTRACT: Mucosal trypsin, a protease-activated receptor (PAR) stimulant, may have an endogenous bronchoprotective role on airway smooth muscle. To test this possibility the effects of lumenal trypsin on airway tone in segments of pig bronchus were tested.

Bronchial segments from pigs were mounted in an organ chamber containing Kreb's solution. Contractions were assessed from isovolumetric lumen pressure induced by acetylcholine (ACh) or carbachol added to the adventitia.

Trypsin, added to the airway lumen (300 $\mu g \cdot mL^{-1}$), had no immediate effect on smooth muscle tone but suppressed ACh-induced contractions after 60 min, for at least 3 h. Synthetic activating peptides (AP) for PAR_1, PAR_2 or PAR_3 were without effect, but PAR_4 AP caused rapid, weak suppression of contractions. Lumenal thrombin was without effect and did not prevent the effects of trypsin. Effects of trypsin were reduced by N_ω-nitro-L-arginine methyl ester but not indomethacin. Trypsin, thrombin and PAR_4 AP released prostaglandin E2. Adventitially, trypsin, thrombin and PAR_4 AP (but not PAR_2 AP) relaxed carbachol-toned airways after $<$3 min.

The findings of this study show that trypsin causes delayed and persistent bronchoprotection by interacting with airway cells accessible from the lumen. The signalling mechanism may involve nitric oxide synthase but not prostanoids or protease-activated receptors.

KEYWORDS: Airway smooth muscle, protease-activated receptors, trypsin

Endogenous proteases, such as thrombin, trypsin and tryptase, are now thought to regulate a range of physiological and pathophysiological events. For example, endogenous proteases regulate respiratory and cardiovascular tissue including smooth muscle, epithelium and endothelium, and are implicated in disease [1–3]. Many of the regulatory effects of endogenous proteases, in particular trypsin and thrombin, are mediated by interactions with a family of G protein-coupled receptors called protease-activated receptors (PARs) [4]. Currently four PARs (PAR_1, PAR_2, PAR_3 and PAR_4) have been cloned and characterised and are activated by cleavage of the extracellular N-terminus, revealing a tethered ligand which then self-activates the receptor domain.

In the respiratory system, a protective role for trypsin in the airway lumen has been proposed [1, 2, 5, 6]. Functionally, trypsin can produce rapid and short-lived relaxation of airway smooth muscle (ASM) strip preparations, although findings from different species and/or studies are not uniform [5, 7–10]; for example, in human ASM, trypsin may cause excitation rather than relaxation [11]. Trypsin activates PAR_2 and PAR_4 [1, 2, 4] and, since synthetic peptides (PAR-activating peptides, PAR AP) to these receptors also relax ASM preparations, the relaxant effects of trypsin have been attributed to activation of PAR-associated signalling pathways. Several studies using ASM strips or cell culture suggest that trypsin could act indirectly to produce relaxation, possibly *via* release of the prostanoid prostaglandin E2 (PGE_2) [7–10, 12] or nitric oxide (NO) [10] from the epithelium. The epithelium is a rich source of paracrine-type mediators including eicosanoids, NO and other substances, with either bronchoprotective or contractile effects on ASM (reviewed in [13, 14]).

Both PAR_2 and PAR_4 are expressed by airway epithelium [12], and trypsin, or its precursor trypsinogen, co-localises with PARs in the epithelium providing a structural framework needed for a physiological role of trypsin at that site [5]. Evidence for a lumenal role of trypsin

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from *in vivo* studies is unclear, however. Tracheal instillation of trypsin produces bronchoconstriction in guinea-pigs and aerosol PAR_2 AP produces little or no effect [10]. These results run counter to the bronchoprotection concept derived from data generated from some *in vitro* studies. Intravenous PAR_2 activators provide contrasting findings (bronchoconstriction or bronchoprotection), possibly because of differing contributions from lung neural pathways [10, 15].

Some of the above studies implicate trypsin as a regulator of ASM through an interaction with mucosal cells [5, 7–10, 12]. It was reasoned that if trypsin has a physiological role at the mucosa then this could be shown by studying its effects at the mucosal surface of an intact airway. An isolated bronchial preparation was used where agents could be selectively added to the lumen because the normal three-dimensional airway structure is retained. Trypsin or other agents could also be added to the adventitial surface of the airway, where they would act directly on ASM. As the experiments showed some unexpected actions of trypsin delivered to the airway lumen, the actions of trypsin were also compared with some synthetic PAR AP to assess the contribution of PARs to the trypsin-mediated responses observed.

METHODS

Pigs (25–30 kg) were euthanised with sodium pentabarbital (*i.v.*). A stem bronchus was dissected from the left and right lung, the parenchyma was removed by gentle dissection and the side branches were ligated [16]. An airway segment some 25 mm in length and 2–3 mm internal diameter was removed. Segments were classified as small or medium–sized based on their diameter, location (spanning generations 9–14), and the presence of cartilage [17]. Each segment was cannulated and mounted horizontally in an organ bath containing Kreb's solution (mM: NaCl 121, KCl 5.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaMOPS (sodium morpholinopropane sulphonic acid, pH 7.3) 5.0, glucose 11.5, $CaCl_2$ 2.5, gassed with 95% O_2 and 5% CO_2 and warmed to 37°C). The segment adventitia was bathed in Kreb's solution and the segment lumen was filled with Kreb's solution from a separate reservoir. The volume of solution in the lumen was ~0.15 mL and the bath volume was 30 mL. Stopcocks at either end of the segment allowed the airway lumen to be sealed so that intralumenal pressure could be monitored using a calibrated pressure transducer.

After a 30-min equilibration period, bronchi were electrically field stimulated (EFS, 60 V, 20 Hz and 3-ms pulses) using platinum ring electrodes and a Grass S44 stimulator (Grass Instruments, MA, USA). EFS-induced responses were subsequently used to assess recovery of tissue after treatment with contractile spasmogens. When a repeatable EFS response was obtained, a submaximal concentration of acetylcholine (ACh), 10^{-4} M, was then added to the bath to provide a stable contraction history. The optimum passive transmural pressure (5 cmH_2O) was established by adjusting the height of the reservoir used to perfuse the segment lumen and recording responses to EFS.

Prostaglandin E2 assay

Kreb's solution was withdrawn from the airway lumen and assayed for PGE_2 using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions.

Inactivation of trypsin

Trypsin was dissolved in phosphate-buffered saline and incubated with an equimolar concentration of Pefabloc (Boehringer-Mannheim, Mannheim, Germany) for 3 h at 37°C. The above solution was then gel filtered through a PD 10 column (Amersham Biosciences, Uppsala, Sweden) to remove unbound Pefabloc. The eluant was freeze dried and trypsin activity determined using *N*-benzoyl-DL-arginine *p*-nitroanilide as substrate [18].

Protocol and data

Two protocols were used to examine the effects of lumenal trypsin and other PAR AP on airway contractile responses. In one, the effects of lumenal trypsin and PAR AP were determined on ACh concentration-response curves (10^{-7}–10^{-2} M) where ACh was added to the solution bathing the airway adventitial surface. Three concentration-response curves were recorded. The first was a control; the second was started 15 min after introduction of trypsin or PAR AP to the lumen; and the third was recorded ~60 min after washout of agents from the lumen. Effects of trypsin or PAR AP on the maximum pressure developed in response to ACh (E_{max}) and the ACh concentration that produced half the maximum pressure (EC_{50}) were determined. To further explore possible mechanisms of action of trypsin, and its time course, a second protocol used repeated challenges with an EC_{50} concentration of ACh (3×10^{-5} M). ACh was added adventitially at 25-min intervals before and after exposure of the lumen to trypsin. When trypsin was used it was added to the lumen solution for 45 min, which was the period of time that trypsin was present in the lumen in the first protocol described above where full concentration-response curves to ACh were recorded. As ACh contractions were recorded with a 25-min time cycle, the lumen fluid had to be removed when the bath solutions were washed. Trypsin was replaced in the lumen after such washout periods. Control experiments were run (see Results section) to show that contractile responses in the absence of trypsin were consistent over the study period. In some of the experiments, the Kreb's solution contained in the airway lumen was withdrawn for assay for PGE_2.

In a separate group of experiments, the effects of adventitial trypsin, thrombin and PAR AP were tested on airways that were pre-toned with carbachol (10^{-6} M). Control runs with carbachol alone were carried out in each bronchial segment to ensure that airway pressure was fully sustained during the recording period (fig. 1). Tone in control and trypsin-, thrombin- or PAR AP-treated airways was compared over a 15-min period.

Means were compared by ANOVA or t-test for paired or unpaired data. Data presented in figure 2 were analysed by Chi-squared test for trends (see Results). Data presented are mean ± SEM, with n=number of airway segments.

Drugs and materials

Porcine pancreatic trypsin (Type IX-S, 13,000–20,000 BAEE $U \cdot mg^{-1}$ protein), indomethacin and L-NAME (N_ω-nitro-L-arginine methyl ester) were obtained from Sigma Chemical

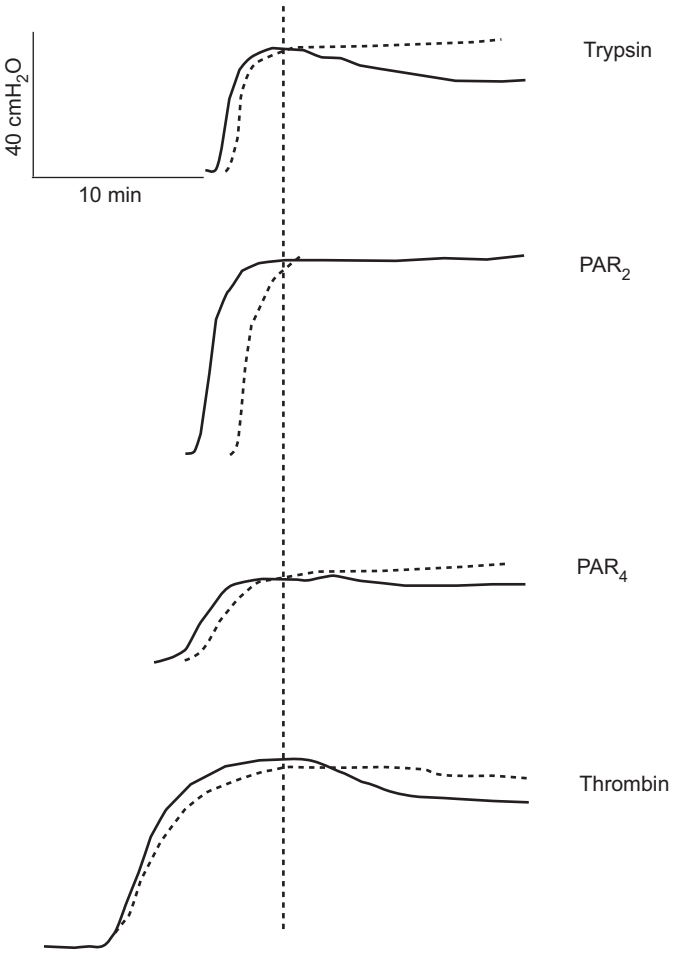

FIGURE 1. Representative tracings from bronchial segments showing the effects of trypsin, protease-activated receptor activating peptides (PAR AP) and thrombin added to the airway adventitia of pre-toned airways (carbachol, 10^{-6} M). ------: control responses to carbachol, *i.e.* without addition of agonists (trypsin or PAR AP), to show maintenance of pressure over the study period (15 min). ——: responses in the same airway, showing the effects of adventitial addition of trypsin (300 μg·mL^{-1}), PAR_2 and PAR_4 AP (10^{-4} M) or thrombin (10 U·mL^{-1}). The dashed vertical line denotes the time point where agonists were introduced to the bath. Scale bar: 40 cmH_2O and 10 min.

Co. Ltd. (St Louis, MS, USA). Human thrombin was obtained from CSL (Parkville, Australia). The following PAR AP were synthesised by Proteomics International (Perth, Australia); PAR_1 (SFLLRN-NH_2), PAR_2 (SLIGKV-NH_2), PAR_3 (TFRGAP-NH_2) and PAR_4 (GYPGQV-NH_2). A negative control for PAR_4 was GYPGVQ-NH_2.

RESULTS

Lumenal trypsin, thrombin and PAR AP

Trypsin (1-300 μg·mL^{-1}) had no direct effect on airway pressure (*i.e.* contraction or relaxation) when it was added to the airway lumen. However, lumenal trypsin modified contractile responses to ACh added to the adventitial side (fig. 3). As described in the Protocols section, concentration-response curves to ACh were repeated: 1) before addition of trypsin; 2) in the presence of lumenal trypsin; and 3) after

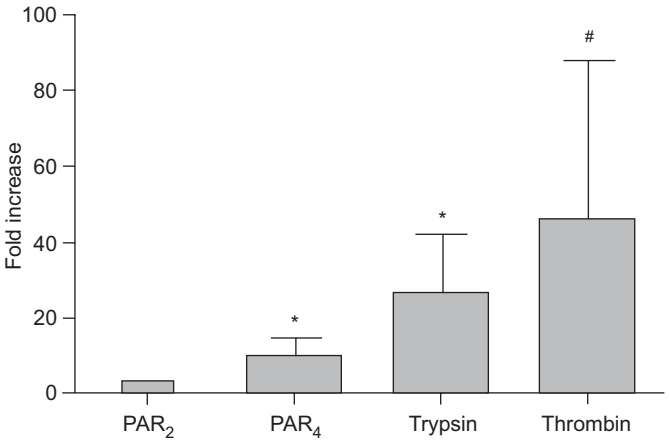

FIGURE 2. Prostaglandin E2 accumulation in the lumen of isolated bronchial segments after exposure to protease-activated receptor activating peptides (PAR AP; 4×10^{-4} M), trypsin (300 μg·mL^{-1}) and thrombin (10 U·mL^{-1}). Acetylcholine was present in the bath when control or test samples were taken. Data are fold increases in prostaglandin E2 concentrations compared with respective control samples in the absence of the test agent. *: $p<0.05$; #: $p=0.057$ (Chi-squared test, see Results). n=5–8.

washout of trypsin. The concentration-response curve to ACh recorded in the presence of 300 μg·mL^{-1} trypsin (*i.e.* curve indicated by ■), did not differ from that seen in the control curve (*i.e.* curve indicated by ○). However, the third concentration-response curve (*i.e.* curve indicated by ▲), recorded some 60 min after washout of trypsin, was suppressed with a 39.3±9.6% reduction in E_{max} compared with the control ($p<0.001$, n=4), although the EC_{50} value of ACh was unchanged. Indomethacin did not alter the suppressive effect of trypsin pre-treatment (E_{max} of third ACh concentration-response curve was suppressed 39.1±13.1%, n=4, $p<0.05$, fig. 3).

The time course of the trypsin-mediated effect is shown in figure 4. The inhibitory response to trypsin became apparent ~1 h after trypsin was initially introduced to the lumen for 45 min (*i.e.* 15 min after its washout). The effect of trypsin was long lasting, remaining for at least 3 h from the initial introduction of trypsin. Inactivated trypsin did not cause prolonged suppression of ACh-induced contraction (fig. 4), although a small transient inhibition was still present at 85 min.

The inhibitory effect normally seen with trypsin was almost abolished by L-NAME, except at 85 min when there was a small but significant reduction in contraction (fig. 4). Thrombin treatment (10 U·mL^{-1}) for 60 min had no effect on ACh-induced contractions (fig. 5). The effect of trypsin was still present after tissues had been exposed to thrombin (fig. 5), indicating that the tissues were not desensitised to trypsin.

K^+ depolarising solution

To assess the integrity of the epithelial barrier in airways exposed to lumenal trypsin for 45 min, the Kreb's solution in the airway lumen was replaced with high K^+ Kreb's solution (NaCl replaced with 80 mM K_2SO_4). Previous studies have shown that high K^+ does not cause contraction of bronchial

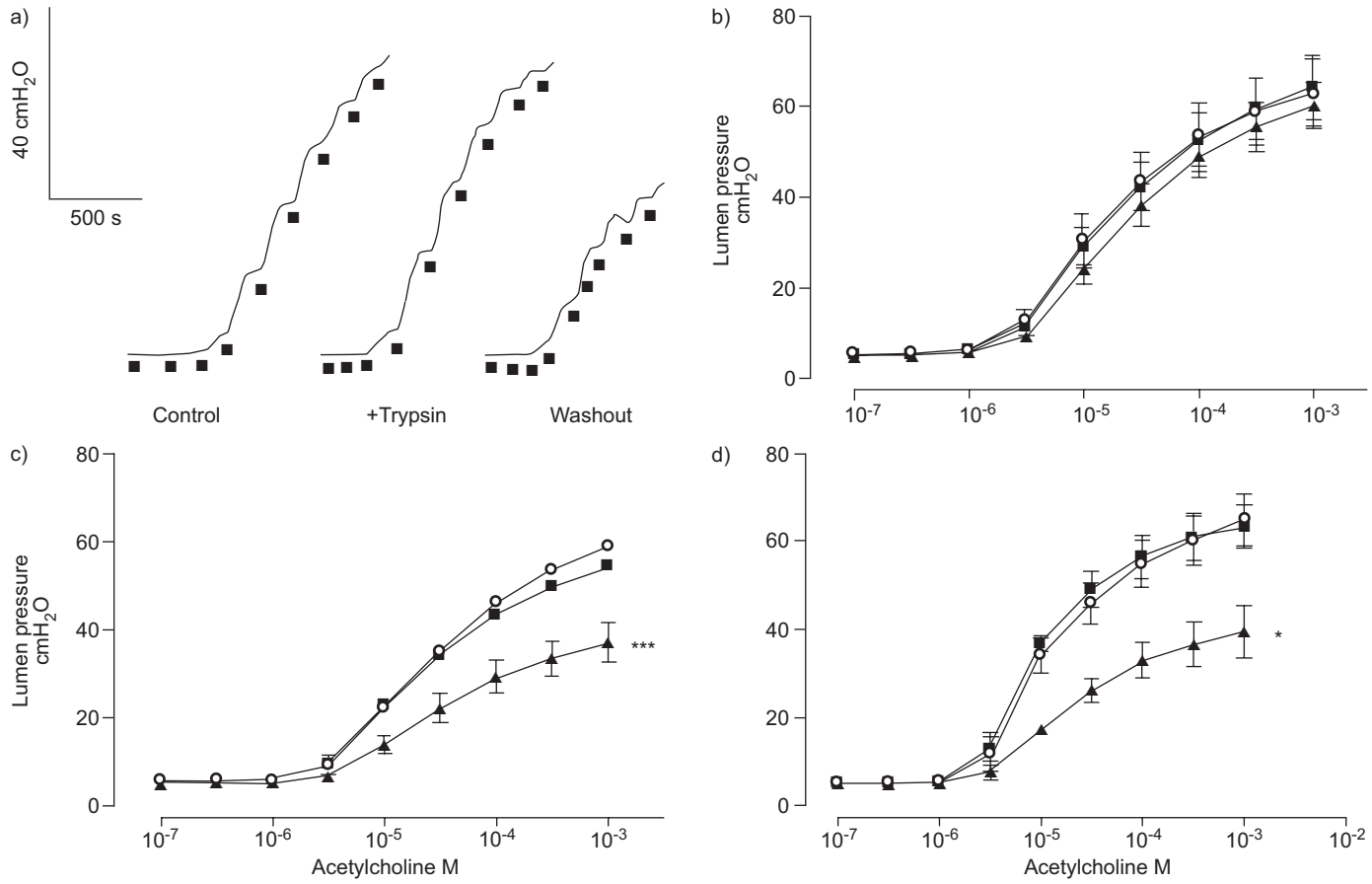

FIGURE 3. Effects of lumenal trypsin (300 μg·mL^{-1}) on concentration-response curves to acetylcholine (ACh) in isolated bronchial segments. ACh was added to the airway adventitia cumulatively. a) Representative traces showing the experimental protocol. Three ACh concentration-response curves were recorded (addition of drugs is shown by ■). The first was a control, the second was recorded with trypsin in the airway lumen, and the third was recorded after washout of trypsin. b) Control experiment showing repeatability of triplicate concentration-response curves to ACh, without trypsin. c) Concentration-response curves to ACh before trypsin, with trypsin and after washout of trypsin. d) Concentration-response curves with same conditions as c) but recorded in the presence of indomethacin 10^{-5} M. In c) and d) ○: before trypsin; ■: +300 μg·mL^{-1} trypsin; ▲: after washout of trypsin. *: $p<0.05$; ***: $p<0.001$ compared with E_{max} of concentration-response curve without trypsin. n=4.

segments with normal intact epithelium, but does so when the epithelium is mechanically or chemically damaged (fig. 6) [19–21]. In six of six experiments, high lumenal K^+ did not cause contraction either after washout of trypsin or for up to 120 min afterwards (fig. 6).

Lumenal PAR AP

PAR_1, PAR_2, PAR_3 and PAR_4 AP (up to 4×10^{-4} M) had no effect on the resting tone of airways when placed in the lumen (n=3–6). Lumenal PAR_1, PAR_2 and PAR_3 AP (4×10^{-4} M) also had no effect on ACh concentration-response curves. By contrast, PAR_4 AP (4×10^{-4} M) rapidly suppressed the ACh concentration-response curve, reducing the E_{max} by 13.1±3.0% ($p<0.05$, n=6), but without change to the EC_{50}. After washout of PAR_4 AP, ACh responses remained suppressed ($p<0.001$). The negative control peptide for PAR_4 AP had no effect on the ACh concentration-response curve (n=4). Indomethacin (10^{-5} M) abolished the effects of PAR_4 AP on ACh concentration-response curves (n=7). The effects of PAR_4 AP, and of indomethacin, are shown in figure 7.

Adventitial trypsin, thrombin and PAR AP

Trypsin (1–300 μg·mL^{-1}) added to the adventitia side had no effect on airway pressure. However, in pre-toned airways (10^{-6} M carbachol to the adventitia), trypsin (300 μg·mL^{-1}) produced a short latency (<3 min) relaxation which, over 15 min, amounted to 19.3±3.6% reduction of tone ($p<0.05$, n=9, fig. 1). Lower concentrations of trypsin (1–100 μg·mL^{-1}) had little or no effect on tone. Trypsin-induced relaxation was not affected by indomethacin (18.6±2.7% reduction in tone, n=9, $p>0.05$ *versus* trypsin alone).

To assess whether the relaxation produced by the exposure of the airway adventitia to trypsin was reversible, and to look for possible nonspecific changes to ASM contraction by trypsin, carbachol-induced contractions that were evoked before adding trypsin (control) and contractions ~60 min after washout of trypsin were studied. Contractile responses to carbachol were fully recovered after washout of trypsin and there was no evidence for any long-lasting change in ASM function. The size of the second carbachol-induced contraction

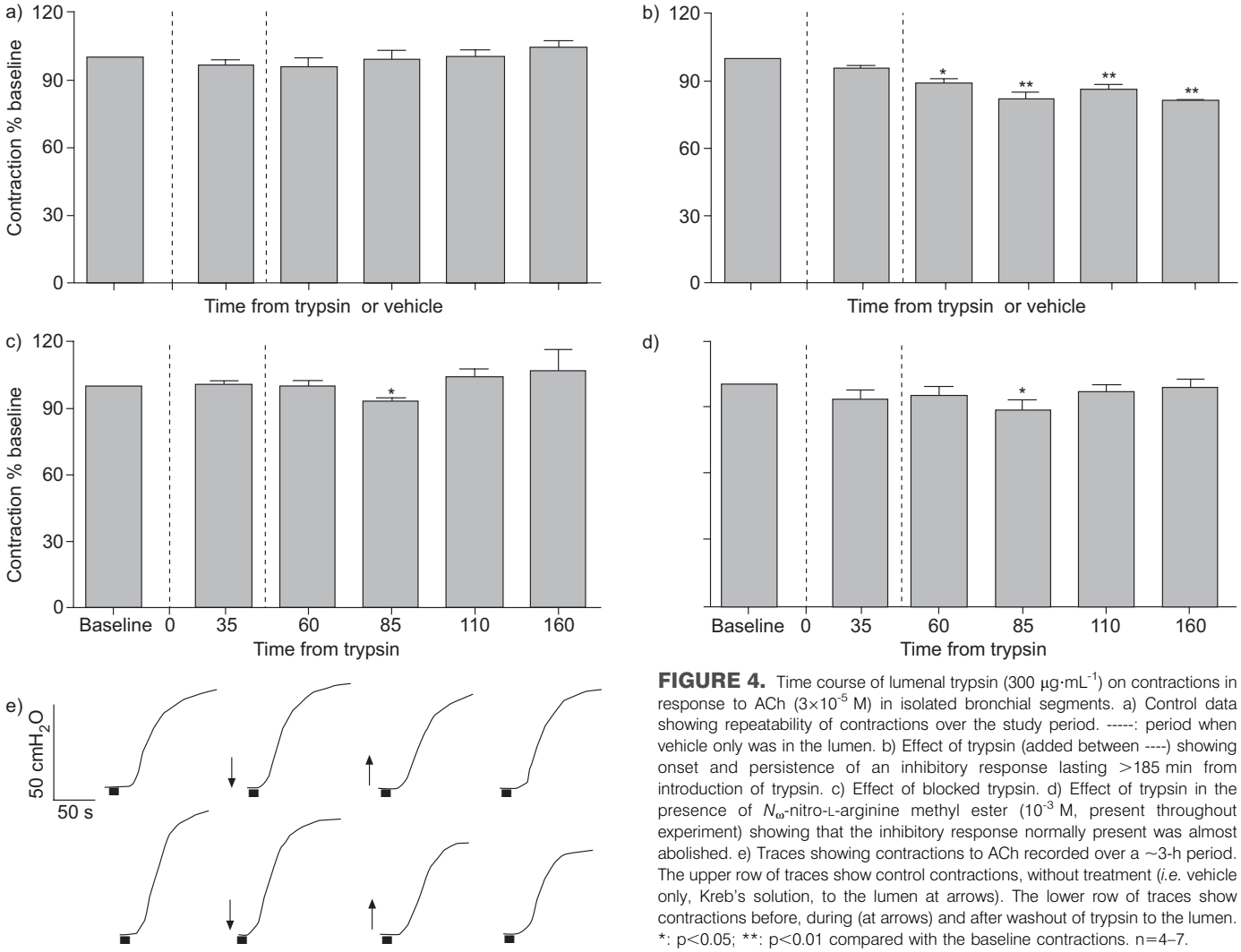

FIGURE 4. Time course of lumenal trypsin (300 μg·mL^{-1}) on contractions in response to ACh (3×10^{-5} M) in isolated bronchial segments. a) Control data showing repeatability of contractions over the study period. -----: period when vehicle only was in the lumen. b) Effect of trypsin (added between ----) showing onset and persistence of an inhibitory response lasting >185 min from introduction of trypsin. c) Effect of blocked trypsin. d) Effect of trypsin in the presence of N_ω-nitro-L-arginine methyl ester (10^{-3} M, present throughout experiment) showing that the inhibitory response normally present was almost abolished. e) Traces showing contractions to ACh recorded over a ~3-h period. The upper row of traces show control contractions, without treatment (*i.e.* vehicle only, Kreb's solution, to the lumen at arrows). The lower row of traces show contractions before, during (at arrows) and after washout of trypsin to the lumen. *: $p<0.05$; **: $p<0.01$ compared with the baseline contractions. n=4–7.

after washout of trypsin was not significantly different (48.4±5.6 cmH_2O) from controls (49.7±4.7 cmH_2O, n=5).

PAR_2 AP (10^{-4} M) had no effect in carbachol-toned airways (n=4) but PAR_4 AP (10^{-4} M) produced relaxation (12.0±3.4% reduction of tone, $p<0.05$, n=4, fig. 1). Thrombin (10 U·mL^{-1}) also relaxed airways (37.0±6.5% reduction of tone, n=4).

Trypsin, thrombin and PAR AP on prostaglandin E2 accumulation

PGE_2 accumulation in the airway lumen was recorded before and 45 min after administration of lumenal trypsin, thrombin and PAR AP treatment (fig. 2). PGE_2 concentrations either in control samples or in the presence of test agents were variable. However, trend analyses indicated trypsin, PAR_4 AP and thrombin-increased PGE_2 accumulation in the airway (Chi-squared test). Lumenal PAR_2 AP had no effect on PGE_2 accumulation and indomethacin abolished trypsin-induced PGE_2 accumulation (n=4). A negative control for PAR_4 AP (n=3) had no effect on PGE_2 accumulation.

DISCUSSION

The findings of this study provide clear evidence that trypsin interacts with airway cells accessible from the lumen and reduces bronchoconstriction. The previous finding that trypsin produces direct relaxation of pre-contracted ASM [5, 7–10] was also confirmed; this was shown by adding trypsin to the adventitia of the airway preparation so that ASM was activated directly. Thus, trypsin exerts a dual effect, reducing contractions when it is added lumenally and causing relaxation when added adventitially.

The mechanisms concerned with the two effects of trypsin were different, however, as judged by the widely different kinetics of the response. Lumenally, trypsin produced a delayed response, detectable ~60 min after it was first introduced then removed, compared with the short-latency, reversible relaxation to adventitial trypsin that was characteristic of the rapid responses to trypsin and PAR agonists reported in ASM strip studies [5, 7–10]. The suppression of ACh-induced contractions by lumenally added trypsin persisted for at least 3 h, which was more than 2 h after trypsin was washed from the bath. As discussed below, the persistent effects of trypsin were independent of PAR and appeared to involve a paracrine mechanism, possibly involving NO. Trypsin has also been shown to cause a delayed expression of the inducible form of cyclooxygenase (COX-2) in human

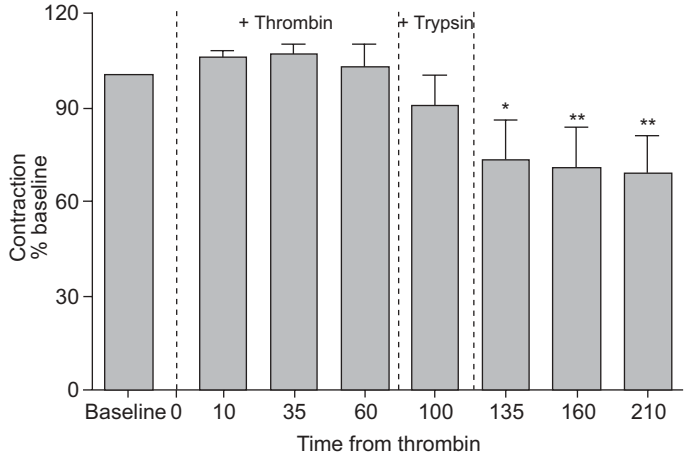

FIGURE 5. Effects of lumenal thrombin (10 U·mL^{-1}) on contractions in response to acetylcholine (3×10^{-5} M) in isolated bronchial segments. Thrombin was present in the lumen for 1 h (between -----), then replaced with trypsin (300 μg·mL^{-1}). Thrombin had no effect on contractions and it did not prevent the inhibitory response to trypsin. *: $p<0.05$; **: $p<0.01$ compared with the baseline contractions. n=6.

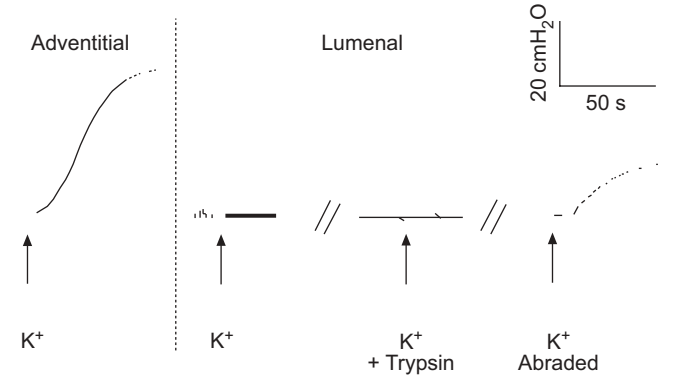

FIGURE 6. Traces showing contractions of isolated bronchial segments to high K^+ placed adventitally and lumenally in a normal airway preparation, and lumenally in a trypsin-exposed airway and an airway in which the lumen had been gently abraded with a stainless steel metal rod.

cultured ASM, taking hours rather than minutes, which also appears to be independent of PAR [22]. These findings suggest a more sustained regulation of airway tone by trypsin, distinct from the rapid, short-lived effect more commonly associated with this enzyme [5, 7–11]. Trypsin is expressed by normal airway epithelium [5] and elevated levels of trypsin and/or tryptase are present in airways presenting with asthma and chronic inflammatory disease [23–25]. If lumenal trypsin exerts a similar persistent bronchoprotective effect in humans *via* a paracrine mechanism, as suggested here in an animal model, then an interrelationship between airway trypsin, the release of one or more mediators including NO, and ASM could represent an important long-term control mechanism involved in airway narrowing in health and lung disease.

Different protocols were used to show that trypsin reduced the size of maximal contractions to in response to ACh without

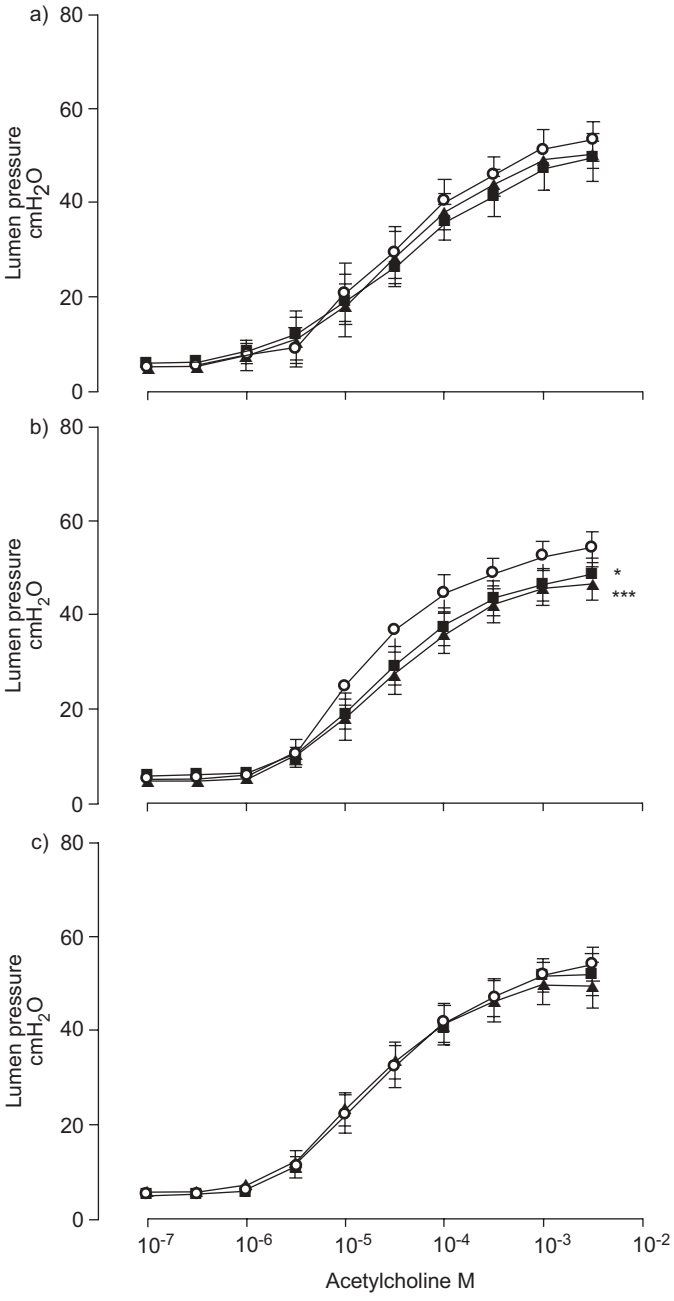

FIGURE 7. Effects of lumenal protease-activated receptor activating peptides (PAR AP; 4×10^{-4} M) on concentration-response curves to acetylcholine (ACh) in isolated bronchial segments. Responses to ACh were recorded before PAR AP (○), in the presence of PAR AP in the airway lumen (■) and after washout of PAR AP (▲). a) PAR_2 AP; b) PAR_4 AP; and c) PAR_4 AP in the presence of indomethacin (10^{-5} M). *: $p<0.05$; ***: $p<0.001$ compared with E_{max} of first concentration-response curve. n=4–7.

altering the sensitivity of the tissue to ACh, and to show the delayed and long-lasting effects of trypsin. In the former protocol, which involved recording the effect of trypsin on complete concentration-response curves to ACh (fig. 3), there was more suppression of maximum contractions than in the latter protocol, which recorded the effects of trypsin on repeated submaximal contractions in response to ACh

(fig. 4). The reason for the different levels of suppression was not established but may relate to the sustained presence of trypsin, and possibly to mediators released into the airway lumen during the measurement of the ACh concentration-response curve.

The concentrations of trypsin in this study were higher than those reported in other airway strip or cell studies, possibly because of the expression of endogenous antiproteases by airways [6]. However, the concentration of trypsin caused no nonspecific damage or loss of functionality of ASM, or change in effect of muscarinic receptors on ASM, since there were no long-term changes to the contractile responses to carbachol after exposure of the airway to trypsin when it was added to the adventitia. Furthermore, the long-term suppression of contraction by trypsin was largely abolished in the presence of a pharmacological blocker for NO (L-NAME), which would not occur if either the ASM or its receptors were damaged. Finally, the lumenal application of trypsin did not alter lumenal responses to K^+ depolarising solution, indicating that structural properties of the epithelium (*e.g.* tight junctions) were not compromised by exposure to the protease, as discussed below.

The response to lumenal trypsin appears to result from an interaction with the airway mucosa, rather than from direct inhibition of ASM, whereas the response to adventitial trypsin is likely to result from direct relaxation of ASM. The observation that a delayed suppressive effect of trypsin was only seen after lumenal trypsin, and not after adventitial trypsin, supports an indirect affect of lumenal trypsin. The possibility that a delayed effect of lumenal trypsin might be caused by the slow passage of trypsin across epithelial–intercellular junctions before reaching the underlying ASM was considered. However, the epithelium is highly impermeant to ions and small molecules [19, 20] which would prevent diffusion of this enzyme. For diffusion to occur, loss of epithelial tight junction proteins would be required before trypsin could reach ASM. To test this possibility, responses to lumenal K^+ depolarising solution after trypsin administration and for a further 2 h after washout were monitored. At no time were there any contractions in response to K^+, indicating that the epithelium had retained its impermeant property. This study and others have shown that while ASM contracts to high K^+ depolarising solution adventitially, K^+ is without effect when it is placed in the lumen of intact airways, unless the epithelium is first breached as illustrated in this study and elsewhere [19–21]. Further evidence that trypsin was largely restricted to the lumenal surface of the airway was: 1) the observation that trypsin produced a persistent effect after it had been washed from the bath; 2) the observation that lumenal application of another enzyme, thrombin, although strongly relaxing ASM, did not produce an effect lumenally; and 3) that low concentrations of trypsin would be achieved at the level of ASM; even if there was no diffusion barrier between lumenal and adventitial solutions the equilibrium concentration of trypsin in the solution bathing the adventitia would be some 200-fold lower than the concentration added to the airway lumen because the bath volume was ~200-fold greater than the lumen volume.

The effects of lumenal trypsin were not mimicked by synthetic activators of PAR_1, PAR_2, PAR_3, or by thrombin, all of which were without effect when given lumenally. In contrast, lumenal PAR_4 AP did produce a small suppression of ACh-induced contraction, raising a possibility that some effect of lumenal trypsin might be due to activation of PAR_4. However, the findings of the current study show that the effect of lumenal trypsin was not due to PAR_4 because the PAR_4 AP response was blocked by indomethacin whereas the trypsin response was not, indicating that these two molecules act by different mechanisms. Furthermore, thrombin did not block the effect of trypsin, as seen with other PAR-mediated effects [5, 12]. Lastly, the relaxant effect induced by PAR_4 was more rapid than with trypsin, consistent with different modes of action. Although not mimicked by synthetic ligands to PARs, the response to trypsin in pig airways required catalytically active enzyme, since inactivated trypsin produced little or no suppression of airway contraction. Although not excluded, it is unlikely that the inability to detect PAR-mediated effects (other than PAR_4) on airway contraction was due to a lack of effectiveness of the synthetic agonists or trypsin on PARs. The activating peptides were shown in numerous studies, including the current study, to be effective activators of PARs [5, 8–12]. The concentrations of PAR AP used were higher than those reported in other airway strip studies, but the same as those used by us to obtain PGE_2 and cytokine release *via* PAR_1, PAR_2 and PAR_4 activation in cultured epithelium [12]. PGE_2 accumulation was measured as a marker of PAR activation [12], which suggested that trypsin, thrombin and PAR_4 AP activated one or more PARs in the airway preparation, since they caused PGE_2 release. The findings of the current study, that PAR_4 AP and thrombin reduced tone in the intact airway when they were given adventitially, are also consistent with activation of PAR_4 in this system. Other studies in porcine vascular tissue also show that PARs are expressed in this species and that PAR AP produce functional responses [26, 27]. A negative control to PAR_4 AP (QYPGVQ-NH_2) was without effect either on PGE_2 release or on ACh concentration-response curves suggesting that responses to PAR_4 AP were receptor specific.

The results of the current study support a paradigm in which mucosal trypsin produces long-lasting regulation of ASM tone by a PAR-independent mechanism. The findings of this study further indicate that the signalling pathway does not involve COX, as previously shown in other airway studies [7–10, 12], but instead show a major requirement for nitric oxide synthase (NOS). The contribution of COX to the effects of lumenal trypsin in this study was investigated using indomethacin and measurements of PGE_2 as a marker of COX activity. Trypsin produced marked PGE_2 accumulation in agreement with studies in other species [7, 12]. Despite demonstrating a release of PGE_2 in pig bronchi, trypsin-induced bronchoprotection appeared independent of COX metabolites, since after abolition of PGE_2 by indomethacin, trypsin still produced its characteristic inhibitory responses. The role of NOS in airway responses to lumenal trypsin was investigated, as there is evidence for its involvement in PAR-mediated responses in airways [10] and blood vessels [27–29]. In bronchial segments, the effects of trypsin were largely abolished by L-NAME, suggesting that the persistent suppression of airway contraction was mediated by NO. There was a small residual effect of trypsin in the presence of L-NAME, some 85 min after trypsin

was first added, which may have been a result of incomplete blockade of NOS by L-NAME, or could represent some additional nonspecific suppressive action of trypsin on ASM. A source of NOS was not established, but airway epithelium expresses constitutive and inducible isoforms of NOS and is a known source of NO [13, 30, 31]. Many endogenous mediators activate NOS isoforms, including cytokines, thrombin and other biologically active substances [13], which could potentially act as intermediaries in a delayed, nonspecific effect of trypsin (*i.e.* PAR-independent) *via* NO release. Additionally, NOS activation leads to the production of *S*-nitrosothiols, which themselves exert biological activity on ASM but with a longer time course than NO [13]. Further studies to elucidate a role of NOS in the actions of trypsin reported here are needed to investigate these possibilities.

In summary, the findings reported here provide evidence for a delayed and persistent bronchoprotective action of mucosal trypsin, that under physiological conditions may involve signalling *via* nitric oxide synthase and, unlike previously described actions of trypsin, is independent of classical protease-activated receptors. The duration of the bronchoprotective effect of trypsin lasts as long (~3 h), or longer than the study period, suggesting that it has the capacity to provide long-term regulation of tone in conditions where there is chronic airflow limitation.

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