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Effects of inhaled thrombin receptor agonists in mice

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1 Active thrombin is found in the airways of patients with a variety of inflammatory lung diseases. However, whether thrombin contributes to the pathologies of these diseases is unknown, although thrombin is a potent inflammatory mediator in other organ systems. In the present study we have assessed the acute inflammatory effect of inhaled thrombin and investigated the possible receptors mediating any effects in mice.

2 Thrombin (200–2000 U kg^{-1} intranasally), induced the recruitment of a small, but significant, number of neutrophils into the airways as assessed by differential counts of cells retrieved by bronchoalveolar lavage (BAL). This small response was mimicked by peptide agonists of proteinase-activated receptor-4 (PAR_4; GYPGKF, AYPGKF; 2–20 mg kg^{-1}), but not PAR_1 (SFLLRN; 2–20 mg kg^{-1}). By contrast, trypsin (200–2000 U kg^{-1}) caused profound inflammation and lung damage.

3 Concentrations of tumour necrosis factor-α (TNF-α) were elevated in BAL fluid from thrombin-treated mice, and a TNF-α-neutralising antibody inhibited the influx of neutrophils in response to thrombin.

4 Although isolated alveolar macrophages appeared to express PAR_1- and PAR_4-immunoreactivity, these cells failed to release TNF-α above baseline levels in response to thrombin, trypsin or any of the peptide PAR agonists.

5 Neither thrombin (2000 U kg^{-1}) nor trypsin (200 U kg^{-1}) modified the airway neutrophilia in response to intranasal bacterial lipopolysaccharide (LPS; 100 μg kg^{-1}).

6 In conclusion, exogenous thrombin has only a modest acute inflammatory action in the lung that appears to be mediated by PAR_4 and involve release of TNF-α from an unknown source.
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Abbreviations: BAL, bronchoalveolar lavage; DFP, diisopropyl fluorophosphate; LPS, lipopolysaccharide; PAR, proteinase-activated receptor; PMNC, polymorphonuclear cell; TNF-α, tumour necrosis factor-α

Introduction

Most inflammatory lung diseases are characterised by episodic plasma leakage, during which thrombin may leave the circulation and become activated subsequent to other coagulation proteins coming into contact with tissue factor on extravascular cells such as the epithelium. For example, plasma proteins are found in bronchoalveolar lavage (BAL) fluids from patients with asthma promptly after inhalation of allergen (Persson *et al*., 1998) and levels of thrombin in BAL fluid correlate with disease severity (Terada *et al*., 2004). Indeed, thrombin is found in asthmatic sputum in sufficient quantities to exert proliferative effects on isolated airway smooth muscle cells (Gabazza *et al*., 1999). Thrombin levels are also elevated in BAL fluid from patients with acute respiratory distress syndrome, pneumonia and in patients with ventilator-associated pneumonia (Levi *et al*., 2003). Thus, the presence of thrombin in airway fluids appears to be a common feature of a variety of inflammatory diseases of the lung, but whether it might play a role in disease progression is currently uncertain.

Some of the cellular actions of thrombin are mediated by any of three proteinase-activated receptors (PAR_1, PAR_3, PAR_4), which are G-protein coupled receptors activated by cleavage of extracellular regions by serine and other proteases (Hollenberg & Compton, 2002). Other cellular effects of thrombin may be mediated by noncatalytic mechanisms *via* undefined receptors that bind at least two regions of thrombin (Hollenberg & Compton, 2002). In addition, thrombin may activate other zymogen forms of protease such as matrix metalloproteinases (e.g. Lafleur *et al*., 2001) and act *via* the production of biologically active fibrin fragments (Szaba & Smiley, 2002).

Although thrombin is known to exert an inflammatory effect in some animal models (Cirino *et al*., 1996; de Garavilla *et al*., 2001; Vergnolle *et al*., 2002; Chin *et al*., 2003; Copple *et al*., 2003), similar effects of thrombin in the lung have not been reported. In the present study we administered thrombin directly in order to examine any direct acute inflammatory effects in the airways.

Methods

Animals

Female specific-pathogen-free BALB/c mice (Charles River, Margate, Kent, U.K.), 6–8 weeks of age were used throughout

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the study. All procedures were performed according to The Animals (Scientific Procedures) Act (1986) and approved by the local ethics panel at King's College London.

In vivo *drug administration and BAL*

All drugs were dissolved in sterile saline and delivered intranasally under 5% isoflurane anaesthesia in a volume of 50 μl. At 24 h after administration, the animals were killed with an overdose of urethane (20 g kg^{-1}) and the trachea was cannulated for BAL with 3×0.5 ml volumes of PBS. A small aliquot of each BAL fluid sample was mixed with an equal volume of Turk's solution and total cell numbers were determined using a haemocytometer. Differential cell counts were performed on Diff-Quik (Gamidor, Abingdon, Oxon, U.K.)-stained cytospin (Cytospin II, Shandon Southern Instruments, Sewickley, PA, U.S.A.) preparations. Since polymorphonuclear cells (PMNC) are rarely found in BAL from normal pathogen-free mice, but are readily recruited into the airways following an inflammatory stimulus, we used the numbers of these cells in BAL as an indication of the inflammatory effect of thrombin receptor agonists. In all the experiments reported here, PMNC were >95% neutrophils and numbers of mononuclear cells in BAL were not significantly different between treatment groups.

Isolation and in vitro *stimulation of alveolar macrophages*

Alveolar macrophages were obtained under sterile conditions by repeated BAL (15 ml in total) with unsupplemented RPMI (Sigma, Poole, Dorset, U.K.) and washed three times. Cells were then plated onto 96-well plates at a density of 10–50×1000 cells/well (eight wells per animal) and allowed to adhere for 1 h before washing and the addition of agonists. One well from each animal was stimulated with lipopolysaccharide (LPS) (10 μg ml^{-1}) as a positive control. At 3 h after the addition of drugs the supernatants were collected, centrifuged to remove any cells and debris and assayed for tumour necrosis factor-α (TNF-α) content by ELISA as described below.

Isolation and stimulation of isolated tracheal epithelial cells

Tracheae were dissected free from animals killed with an overdose of urethane in a sterile field. Each trachea was then opened with a longitudinal cut and then sliced into eight flat sheets with a razor blade. The pieces of trachea were then incubated in DMEM containing 1 mg ml^{-1} pronase for 3 h at 37°C in a 5% CO_2 incubator. After incubation, the samples were triturated with a wide bore pipette 12 times before the pieces of trachea were removed and the remaining isolated cells centrifuged at $300 \times g$ for 5 min and washed three times before being plated onto 96-well plates at $20–50 \times 1000$ per well in DMEM supplemented with fetal calf serum (FCS) and antibiotics (penicillin 50 U ml^{-1}; streptomycin 50 μg ml^{-1}; Invitrogen, Paisley, U.K.). After 24 h the cells were washed to remove FCS and stimulated with thrombin and LPS exactly as described above for alveolar macrophages.

Measurement of TNF-α by ELISA

TNF-α concentrations were determined carried out using a commercially available kit (R&D Systems, Abingdon, Oxon, U.K.), following the manufacturer's instructions with minor modification. Thus, to prevent degradation of antibodies or antigens during incubation, 10% foetal calf serum was added to the samples before they were applied for the capture step. In preliminary experiments we found that this concentration of serum completely prevented any interference by proteases at the concentrations used (up to 10 U ml^{-1}).

Drugs and reagents used

PAR-activating peptides (SFLLRN, GYPGKF, AYPGKF; all C-terminally amidated) were purchased from Auspep (Parkville, Victoria, Australia). Bovine thrombin and diisopropyl fluorophosphate (DFP)-thrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT, U.S.A.), porcine trypsin from Worthington Biochemical Corp. (Lakewood, NJ, U.S.A.). Indomethacin was purchased from Sigma. The TNF-α neutralising antibody was purchased from R and D Systems.

Results

Effect of intranasal thrombin, trypsin and PAR-activating peptides

Intranasal thrombin (200–2000 U ml^{-1}) administration produced a small, dose-dependent influx of PMNC into the airways as assessed by differential cell counting of cells retrieved by BAL (Figure 1a). PARs have been shown to release anti-inflammatory prostaglandins from airway epithelial cells that might inhibit neutrophil influx (Cocks *et al*., 1999). However, in animals pretreated with the cyclooxygenase inhibitor indomethacin (10 mg kg^{-1}) 30 min before thrombin was administered, a significant reduction of the numbers of neutrophils found in BAL was observed (Figure 1b).

The mixed PAR_1/PAR_2 activating peptide SFLLRN (20 mg kg^{-1}) did not induce any cellular recruitment to the airways, whereas the PAR_4 activating peptide GYPGKF (2.0–20 mg kg^{-1}) induced a small PMNC influx, similar to that observed with thrombin (Figure 1c). The more potent synthetic PAR_4 peptide AYPGKV (2.0–20 mg kg^{-1}) also caused a PMNC influx that was significantly larger than that induced by the native peptide GYPGKV (Figure 1c).

Trypsin, which activates PAR_1, PAR_2 and PAR_4 (Hollenberg & Compton, 2002), induced a dose-dependent (200–2000 U ml^{-1}) inflammatory response that was approximately two orders of magnitude larger than that elicited by thrombin (Figure 2a). BAL from trypsin-treated animals was obviously contaminated by blood, but we are confident that most of the inflammatory cells that were found in BAL had migrated into the lungs since the number of PMNC greatly exceeded the number of mononuclear cells (data not shown) in contrast to the opposite ratio of these cell types found in murine blood (Sanderson & Phillips, 1981). Lungs from trypsin-treated animals had massive blood clots extending from the hilar regions of the lobes to the periphery, although some parts of the lobes appear less affected than others. Histological

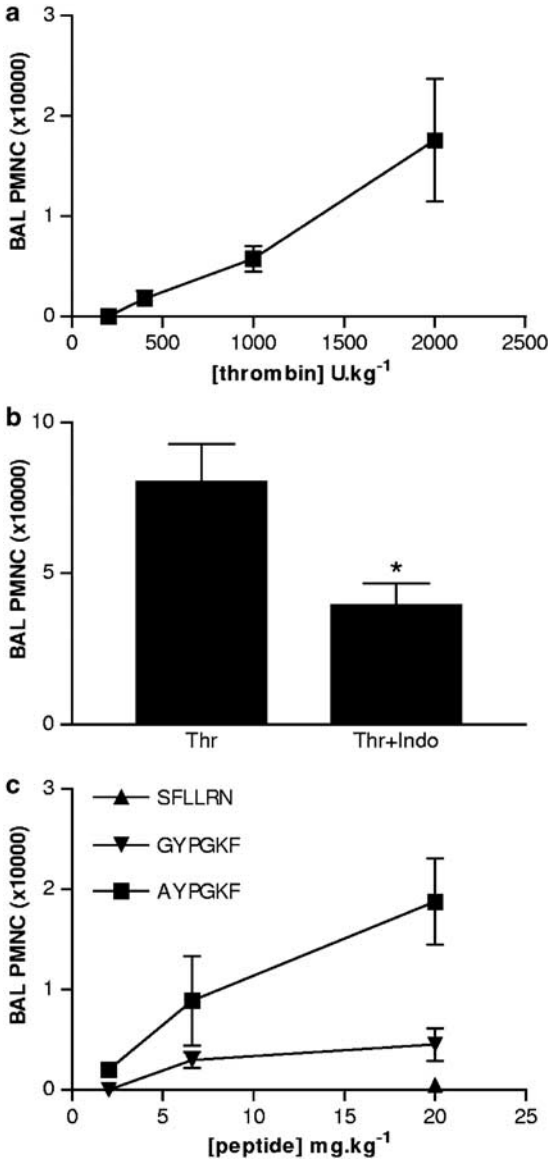

Figure 1 (a) Effect of intranasally administered thrombin on neutrophil numbers in BAL fluid in mice ($n=4$ for each dose). (b) Inhibition of thrombin (Thr; 2000 U kg^{-1})-induced neutrophil influx into the airways by indomethacin (Indo; 10 mg kg^{-1}; $n=5$). *Indicates significant difference between control and indomethacin-treated animals ($P<0.05$; unpaired *t*-test). (c) Effect of PAR-activating peptides administered *via* the same route ($n=4$–6 for each dose). The effect of doses of SFLLRN lower than 20 mg kg was not investigated.

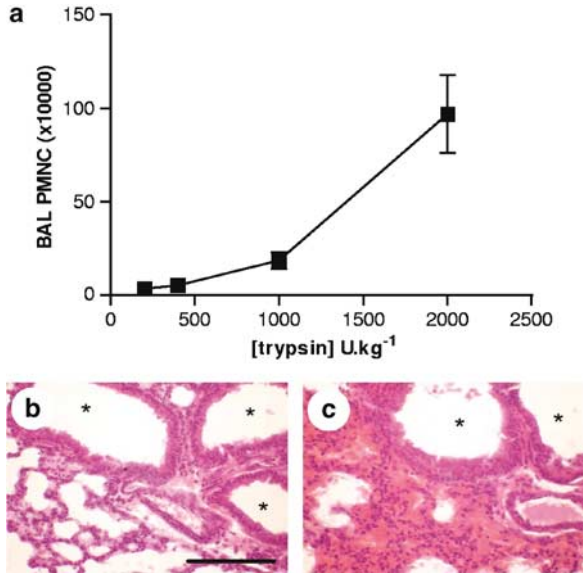

Figure 2 (a) Effect of intranasally administered trypsin (1000 U kg^{-1}) on neutrophil numbers found in BAL fluid ($n=4$). Photomicrographs of haematoxylin and eosin stained histological sections from control (b) and trypsin-treated (c) animals, showing the accumulation of red blood cells in the alveolar spaces surrounding bronchioles in trypsin treated animals, although the bronchial epithelial layer appears similar in both cases. *Indicates the lumen of bronchioles. Scale bar represents 100 μm and applies to both photomicrographs.

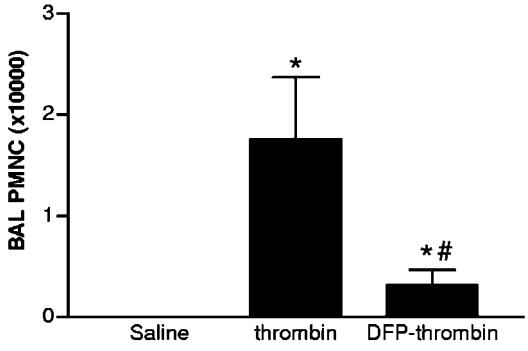

Figure 3 Comparison between thrombin and proteolytically inactive DFP-thrombin in inducing the influx of neutrophils into the airways, as assessed in BAL fluid. *Indicates significantly different to control ($n=4$; $P<0.05$), #Indicates significantly difference between thrombin and DFP-thrombin groups ($P<0.05$; one-way ANOVA with Bonferroni post-test).

assessment of these lungs (on which BAL had not been performed in order to preserve tissue integrity) showed that while the structure of the larger airways appeared to be intact in trypsin-treated animals, the alveolar spaces of the lungs contained high numbers of red blood cells (Figure 2b and c).

Effect of irreversibly inhibited thrombin

BAL retrieved from mice treated with DPF-inactivated thombin (equivalent to 2000 U kg^{-1} of active thrombin) contained significantly fewer PMNC than that from thrombin treated animals, implicating thrombin's proteolytic mechanism in its mild inflammatory action, although there was still a small, significant inflammatory response compared to control animals in this group (Figure 3).

Effect of a neutralising TNF-α antibody

The accumulation of neutrophils in the airways during an inflammatory response frequently involves macrophage-derived TNF-α (Mizgerd, 2002). Co-administration of thrombin (2000 U kg^{-1}) with a TNF-α neutralising antibody

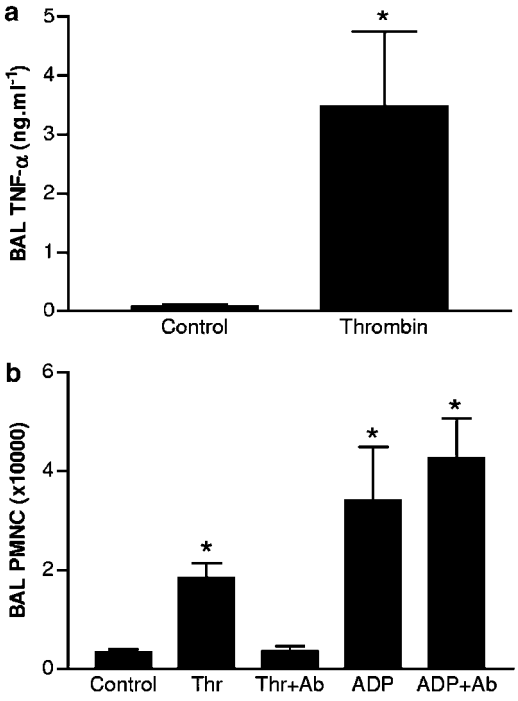

Figure 4 (a) TNF-α levels in BAL from animals administered thrombin or saline (control). *Indicates significant difference between control and thrombin-treated animals ($n=6$; $P<0.05$; unpaired t-test). (b) Comparison between thrombin and ADP in recruiting neutrophils into the airways and the effect of a neutralising TNF-α antibody. *Indicates significantly different to control ($n=6$; $P<0.05$; one-way ANOVA with Bonferroni post-test).

(5 μg/mouse) significantly reduced the number of neutrophils found in BAL fluid (Figure 4). However, while another platelet activator, ADP, also at a lethal intravenous dose (300 mg kg^{-1}; Hirsch *et al*., 2001), caused a mild inflammatory response, this was not altered by co-administration with the TNF-α neutralising antibody (Figure 4).

Effect of thrombin on TNF-α secretion by alveolar macrophages

Alveolar macrophages are considered the major source of TNF-α in the airways (Barnes *et al*., 1998). Both PAR_1 and PAR_4 were detected immunohistochemically on alveolar macrophages (Figure 5). Therefore, we isolated alveolar macrophages from mice in order to see if thrombin induced TNF-α release by these cells. Neither thrombin nor the PAR_1- and PAR_4-activating peptides induced detectable TNF-α secretion from alveolar macrophages that otherwise responded robustly to LPS (Figures 6 and 7). At the highest concentrations tested (1.0–10 U ml^{-1}), trypsin appeared to inhibit TNF-α secretion, but otherwise had no discernable effect (Figure 6).

TNF-α secretion by isolated tracheal epithelial cells

We could not detect TNF-α in the media from thrombin stimulated isolated tracheal epithelial cells, regardless of whether the cells were untreated or treated with LPS ($n=4$; data not shown).

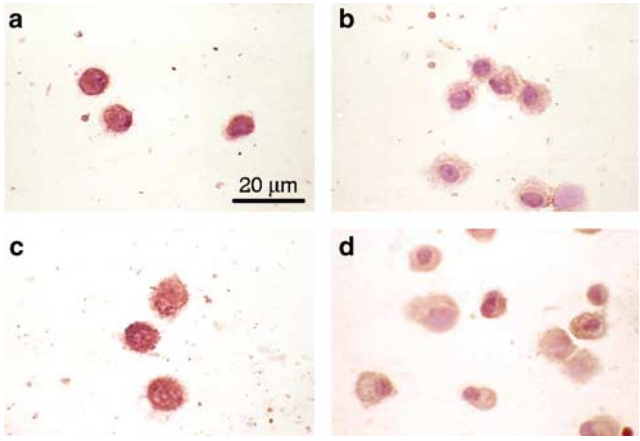

Figure 5 Immunohistochemical detection of PAR_1 (a) and PAR_4 (c) in isolated alveolar macrophages. Panels b and d show the control (primary antibody omitted). The preparations are counter-stained with haematoxylin and the scale bar in (a) applies to all panels. In the controls, only the haematoxylin-stained nuclei and faint cytoplasmic staining are apparent, whereas in (a) and (c) the whole cell is stained.

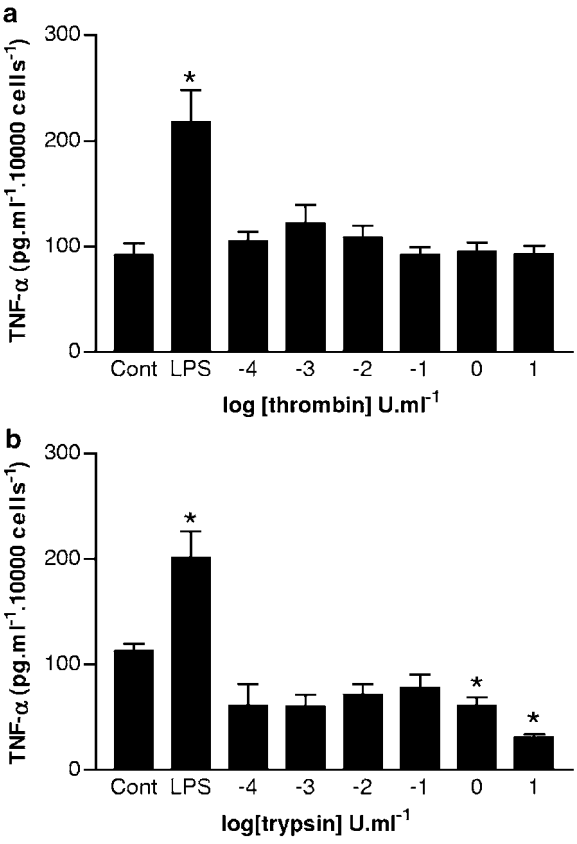

Figure 6 Effect of (a) thrombin or (b) trypsin on TNF-α secretion by isolated alveolar macrophages. LPS was used as a positive control to demonstrate cell responsiveness. *Indicates a significant difference compared with saline-treated controls (Cont; $n=5$; $P<0.05$; one-way ANOVA with Bonferroni post-test).

Effect of thrombin and trypsin in addition to LPS

Since some mediators have little inflammatory effect alone, but can powerfully amplify other inflammatory stimuli,

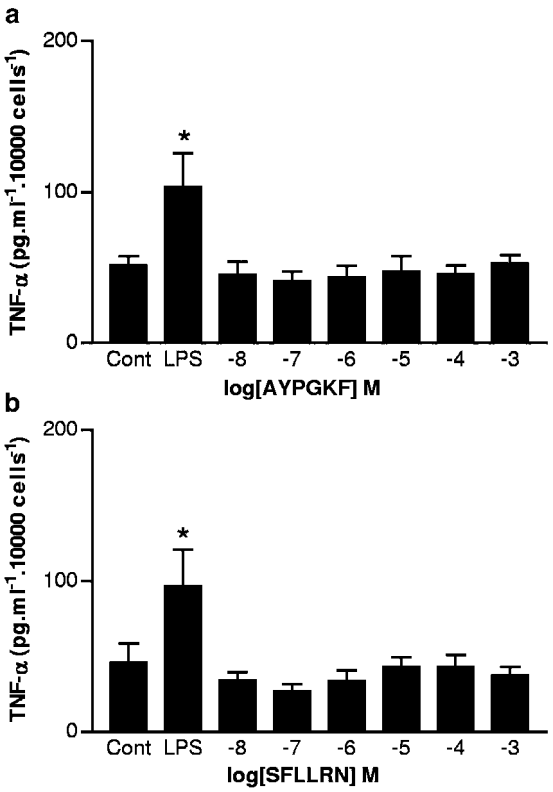

Figure 7 Lack of effect of PAR_4 (AYPGKF (a)) or PAR_1 (SFLLRN (b)) agonists on TNF-α secretion by isolated alveolar macrophages. *Indicates a significant difference compared with saline-treated controls ($n=4$–5); Cont; $P<0.05$; one-way ANOVA with Bonferroni post-test).

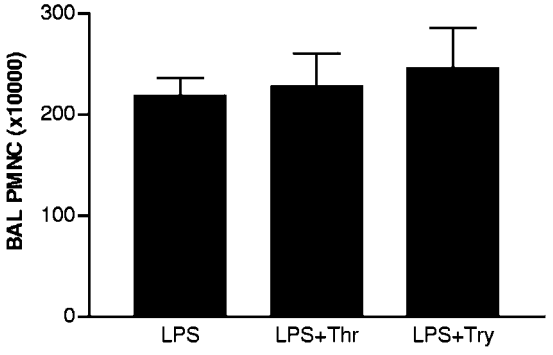

Figure 8 Trypsin and thrombin do not amplify the inflammatory response to LPS. Trypsin and thrombin were co-administered intranasally with LPS 10 $\mu g\,kg^{-1}$ at doses (100 and 1000 $U\,kg^{-1}$, respectively) that cause only mild inflammation individually ($n=4$–8).

we investigated the effect of thrombin and trypsin on the response to a submaximal dose of LPS. Thrombin and trypsin were administered at doses that cause minor, but significant, neutrophil accumulation in the airways (1000 and 100 $U\,kg^{-1}$, respectively) in combination with LPS (100 $\mu g\,kg^{-1}$). The numbers of neutrophils found in BAL fluid from both LPS + trypsin and LPS + thrombin were not different from those seen in animals treated with LPS only (Figure 8).

Discussion and conclusions

The present findings imply that thrombin is not a powerful inflammatory mediator in the airways of mice. Therefore, thrombin might not be a mediator of the acute symptoms of pulmonary conditions, even though it is readily detected in airway fluids from patients with a variety of inflammatory diseases (see Introduction). These findings contrast with previous findings in other organ systems, where thrombin is an effective inflammatory mediator, acting *via* multiple mechanisms to initiate an inflammatory response (Cirino *et al*., 1996; de Garavilla *et al*., 2001; Howell *et al*., 2001; Vergnolle *et al*., 2002; Chin *et al*., 2003; Copple *et al*., 2003).

The lack of efficacy of thrombin might be due to high levels of endogenous antiproteinases in the airways. However, the highest dose of thrombin administered in these experiments was twice the intravenous dose of thrombin required to cause fatal thromboembolism in mice (Momi *et al*., 2001). Therefore, we think that it is unlikely that the total airway antithrombin activity would exceed that of total plasma inhibitors. Furthermore, similar doses of trypsin had marked effects, indicating that any general antiprotease activity in the airways present can be overcome. Therefore, we are confident that a sufficient amount of proteolytically active thrombin reached the airways and that the minor inflammatory response that we observed is probably the maximal response that could ever occur *in vivo* in the mouse, in response to this mediator.

In the absence of widely available PAR antagonists, an analysis of the likely receptor that mediates the response to thrombin is necessarily based on comparison with peptide ligands for these receptors. Thrombin can activate PAR_1, PAR_3 and PAR_4 (Hollenberg & Compton, 2002). At present, there is no evidence that PAR_3 signals in mice, although it is a cofactor for activation of PAR_4 by thrombin. Any effect of thrombin at PAR_1 should be mimicked by the PAR_1/PAR_2 selective agonist SFLLRN and this was not the case in the present study, even though very high concentrations of the peptide were delivered both *in vivo* and *in vitro*. Thus, we are confident that PAR_1 did not mediate the mild proinflammatory action of thrombin in the airways. An agonist of the remaining candidate thrombin receptor, the PAR_4-activating peptide GYPGKF, mimicked the response to thrombin. Most compellingly, the synthetic activating peptide AYPGKF, which has been reported to be more potent than GYPGKF (Faruqi *et al*., 2000) was able to induce a larger inflammatory response in our experiments. Together, these findings suggest that the mild inflammatory effect of thrombin may be mediated by PAR_4.

Previous studies have found that all PARs are present on airway epithelial cells and activation of this receptor population initiates the release of anti-inflammatory prostanoids that might inhibit neutrophil influx (Lan *et al*., 2002). Therefore, we investigated whether inhibition of cyclooxygenase with indomethacin might potentiate the otherwise small response to inhaled thrombin. Surprisingly, indomethacin had the opposite effect and reduced the number of neutrophils found in BAL. This finding is also at odds with a report that indomethacin potentiates the neutrophil influx in response to LPS in mice (Goncalves de Moraes *et al*., 1996). Further studies would be required to determine the source and nature of the endogenous cyclooxygenase product that appears to be important to the proinflammatory action of thrombin in the airways.

In contrast to the effects of thrombin, trypsin elicited a massive inflammatory response, comparable to that seen in response to LPS. Trypsin is also likely to activate PAR_1 and PAR_4 (Hollenberg & Compton, 2002), although it is clear from the experiments with thrombin and PAR-activating peptides, that activation of these receptors could not account for the very different effects of trypsin. Similarly, although trypsin can activate PAR_2, inhalation of the PAR_2 activating peptide SLIGRL has been reported to cause very little, if any, proinflammatory reaction (Moffatt *et al*., 2002; Schmidlin *et al*., 2002). Based on the present experiments we are not in a position to ascribe the effects of trypsin to any particular mechanism, although the present findings have relevance in two areas of pathophysiology. Firstly, trypsin enters the circulation in pancreatitis and is thought to contribute to lung injury in this disease (Acioli *et al*., 1997). Indeed, activation of circulating trypsinogen is known to cause pulmonary inflammation in rats (Hartwig *et al*., 1999). Secondly, trypsin may mimic some of the PAR-independent effects of mast cell tryptase, another serine protease frequently found in the tissues in inflammatory disease states (Miller & Pemberton, 2002). We are currently investigating the possible mechanisms underlying trypsin-induced pulmonary inflammation.

PARs have been described on a variety of cells types within the airways (Lan *et al*., 2002). Most relevant to the present findings are that PAR_1 is found on human alveolar macrophages (Roche *et al*., 2003). Similarly, both PAR_1 and PAR_4 were identified on these cells by immunohistochemistry in the present study. Alveolar macrophages are known to be a major source of TNF-α in the airways (Barnes *et al*., 1998) that, in mice, recruits neutrophils after LPS administration (Goncalves de Moraes *et al*., 1996). Therefore, we were not surprised to find that a neutralising antibody against TNF-α was able to inhibit the small thrombin-induced neutrophil accumulation in the airways. However, isolated alveolar macrophages that clearly responded to LPS did not release significant TNF-α in response to thrombin or PAR_4-activating peptides. There are two simple explanations for these discrepant findings. Firstly, it is possible that the small amount of TNF-α released was below the detection threshold of our assay. This suggestion seems reasonable since the inflammatory effect of thrombin is quite limited and because, unstimulated, these cells secreted a significant amount of TNF-α *in vitro*. Secondly, it is possible that TNF-α is released from a source other than macrophages. One such potential source of TNF-α is the epithelium (Petterson & Adler, 2002), although in our hands neither thrombin nor LPS was able to elicit detectable TNF-α release from these cells in culture.

We considered the possibility that such a large dose of thrombin or PAR-activating peptides might not be acting on airway cells at all, but may elicit a response as a result of gaining access to the circulation. Thus, the small response in terms of neutrophil influx into the airways might be the result of minor platelet activation in the pulmonary vasculature. If this were the case, a smaller platelet-activating molecule, such as ADP, might be expected to mimic the effect of thrombin. ADP did cause a small neutrophil influx, but unlike the response to thrombin this process did not require TNF-α. Furthermore, ADP is more likely to gain access to the vascular compartment than thrombin, yet the response to ADP in terms of neutrophil influx was not considerably larger, as might be expected. While these data do not absolutely exclude the activation of platelets in the response to thrombin, we feel that it is highly suggestive that the effect of thrombin may be mediated by cells within the airway compartment.

The impetus for this study was the clinical observation that thrombin is found in airway fluids from patients with a variety of inflammatory airway diseases (Gabazza *et al*., 1999; Levi *et al*., 2003; Terada *et al*., 2004), combined with the reported extrapulmonary inflammatory actions of thrombin (Cirino *et al*., 1996; de Garavilla *et al*., 2001; Howell *et al*., 2001; Vergnolle *et al*., 2002; Chin *et al*., 2003; Copple *et al*., 2003). Clearly, intranasal administration of thrombin alone does not fully mimic the extravasation of thrombin that occurs during an acute or chronic inflammatory reaction. Yet, the present experiments do begin to define what effect thrombin within the airways might have. Given, for example, the profound response reported following equally unphysiological intraplantar injection of thrombin (Cirino *et al*., 1996; De Garavilla *et al*., 2001), we were surprised to observe no similar response in the airways. We therefore considered whether thrombin might potentiate another inflammatory response, that is, the innate immune response to LPS. However, even under these circumstances of independently evoked inflammation, thrombin appeared to have little pro-inflammatory activity. Therefore, the thrombin detected in clinical samples of airway fluids might not, on its own, substantially influence the ongoing inflammatory response. The interaction of thrombin with other plasma proteins, which extravasate simultaneously, might have a very different effect on the innate immune system. It is also possible that thrombin generated close to cell surfaces, as occurs during plasma extravasation, acts differently to exogenously administered thrombin. We are currently examining these possibilities.

From the present studies it does not seem that PAR_1 (present study) or PAR_2 (Moffatt *et al*., 2002; Schmidlin *et al*., 2002) cause any acute inflammatory response in the airways and that PAR_4 is able to mediate only a very mild response to supra-physiological concentrations of thrombin. However, thrombin is also known to mediate a variety of effects *in vitro* on other airway cells, such as smooth muscle and fibroblast proliferation, that could contribute to changes in airway morphology over a longer time scale (Lan *et al*., 2002). Indeed, there is emerging evidence that thrombin contributes to airway fibrosis *in vivo* (Howell *et al*., 2002).

In conclusion, despite the expression of thrombin receptors on a variety of cells in the airways and previous reports suggesting a pro-inflammatory role for thrombin *in vivo* in other tissues, intratracheal instillation of this coagulation protease induces a modest pro-inflammatory response in the airways of mice.

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