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Protective effect of a PAR2-activating peptide on histamine-induced bronchoconstriction in guinea-pig

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1 Protease activated receptor-2 (PAR2) is a seven transmembrane domain G protein coupled receptor proteolytically activated. PAR2, together with other PARs, can be also activated by peptides mimicking the sequence of the receptor tethered ligand. We have evaluated the effect of systemic administration of a peptide activating PAR2 (PAR2-AP, SLIGRL) on histamine-induced increase in lung resistances in the guinea-pig.

2 Intravenous administration of PAR2-AP (1 mg kg^{-1}) significantly inhibited histamine-induced increase in lung resistance in a time-dependent fashion that was not abolished by indomethacin or vagotomy.

3 Bronchoprotective effect of PAR2-AP was not reversed by the cyclo-oxygenase inhibitor, indomethacin, the nitric oxide synthetase inhibitor, L-NAME, nor by the non-selective beta-antagonist, propranolol.

4 Indomethacin augmented the bronchoconstriction to histamine which was inhibited by PAR2-AP. Furthermore, in vagotomized animals, the bronchial hyper-responsiveness to histamine was significantly reduced, and in these circumstances, PAR2-AP still retained the capacity to provide bronchoprotection against histamine.

5 PAR2-AP also produced a modest reduction in histamine-induced protein leakage in trachea and upper bronchi.

6 Our results indicated that PAR2 might have a bronchoprotective role in the guinea-pig *in vivo* independent of prostaglandin or nitric oxide release.
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Abbreviations: DMF, dimethylformamide; DPCDI, 1,3-diisopropylcarbodiimide; Fmoc, fluorenylmetoxycarbonyl; HOBt, 1-hydroxybenzotriazole; IL-1α, interleukin 1α; MBHA, 4-methylbenzhydrilamine; PAR, protease activated receptor; PAR-AP, protease activated receptor-activating peptide; PGE_2, prostaglandinE_2; R_L, airway resistance; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; TNFα, Tumour Necrosis Factor α

Introduction

Protease activated receptor-2 (PAR2) is a seven transmembrane domain G protein coupled receptor activated by proteolytic cleavage which, together with other PARs, shows a unique mechanism of autoactivation. Indeed, following proteolytic cleavage of the receptor, a new N-terminus peptide is exposed that functions as a tethered ligand. In the absence of proteolytic cleavage, PAR2 can be activated by synthetic peptides mimicking the sequence of the tethered ligand (Dery *et al*., 1998). The physiological ligand(s) for PAR2 is still unknown although trypsin (Nystedt *et al*., 1994), tryptase (Molino *et al*., 1997) and factor Xa (Fox *et al*., 1997) are all able to activate this receptor.

Immunohistochemistry studies have shown that PAR2 is expressed on several human tissues, under physiological conditions (D'Andrea *et al*., 1998), including the airways of subjects with asthma (Knight *et al*., 2000). There is also evidence for an up-regulation of PAR2 by inflammatory stimuli, such as tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) (Nystedt *et al*., 1996), suggesting that the activation of PAR2 may be regulated by inflammatory mediators. Furthermore, local injection of a small peptide activating PAR2 (PAR2-AP) in the rat hind paw causes oedema (Kawabata *et al*., 1998; Vergnolle *et al*., 1999) and, more recently, a neurogenic mechanism has been proposed as the basis of the pro-inflammatory effect of PAR2-AP (Steinhoff *et al*., 2000).

We have shown that, *in vivo*, inflammatory stimuli, such as endotoxemia induced by bacterial lipopolysaccharide, leads to an increased expression of PAR2 on vascular endothelium and smooth muscle cells that correlates to an increase in the hypotensive effect of the synthetic peptide

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activating PAR2 receptor (Cicala *et al*., 1999a). All these data would point to a pro-inflammatory effect following activation of PAR2. In contrast, there is also evidence for a protective anti-inflammatory effect following activation of PAR2. PAR2 is expressed in human lung (D'Andrea *et al*., 1998; Hauck *et al*., 1999) and, in the airways, activation of PAR2 causes an epithelium dependent relaxation of mouse isolated bronchi, that correlates with PAR2 immunoreactivity in cytoplasmic regions of airway epithelial cells (Cocks *et al*., 1999), and of mouse tracheal ring (Lan *et al*., 2000). *In vivo*, it has been demonstrated that activation of PAR2 produces a protective effect against 5HT-induced bronchoconstriction in rats (Cocks *et al*., 1999), while a recent study suggests that PAR2 activation leads to a sensory neuropeptide dependent bronchoconstrictor response (Ricciardolo *et al*., 2000).

In the present study we have investigated the role of PAR2 on histamine-induced bronchoconstriction in the guinea-pig and whether the bronchoprotective effect of PAR2 receptor activation *in vivo* was dependent upon prostaglandin release.

Methods

Drugs

PAR2-AP (SLIGRL) and control peptide with a scrambled sequence (LSIGRL) were synthesized by standard solid phase method as C-terminal amides on a 4-methylbenzhydrilamine polystyrene resin (MBHA resin) functionalized with the linker 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl) phenoxyacetamidonorleucine, using dimethylformamide (DMF) as solvent. The stepwise synthesis, by a fully automated continuous-flow peptide synthesizer, was carried out by fluorenylmetoxycarbonyl (Fmoc) chemistry, and no special efforts were made to optimize the repetitive steps. The Nα-Fmoc aminoacids carrying standard side chain protective groups were converted to benzotriazolyl esters with 1-hydroxybenzotriazole (HOBt) and 1,3-diisopropylcarbodiimide (DIPCDI) in the synthesizer. The Fmoc group was cleaved with 20% piperidine-DMF solution. After completion of the synthesis, the protected peptides were cleaved from the resin and the aminoacid side chains were simultaneously deprotected by treatment with trifluoroacetic acid (TFA)/H_2O_2/Et_3SiH (88 : 5 : 7) mixture. The resulting peptides were purified by preparative reverse phase high performance liquid chromatography (RP–HPLC) and homogeneity of the purified products was assessed by analytical RP–HPLC. Aminoacid analysis, mass spectrometry and NMR spectroscopy achieved structure verification.

Urethane, heparin, histamine, indomethacin, PEG 400, L-NAME, propranolol, Evan's Blue, formamide and bradykinin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Measurement of bronchoconstriction

Guinea-pigs (250–300 g) were anaesthetized with urethane (2.5 g kg^{-1} i.p.). The jugular vein and carotid artery were cannulated for drug administration and blood pressure monitoring, respectively. Animals were artificially ventilated (60 breaths min^{-1}; 1 ml 100 g^{-1} tidal volume) and airway resistance (R_L) evaluated by an on-line respiratory analyser (PMS, Mumed, U.K.). Initially, increasing doses of histamine (1.25, 2.5, 5 μg kg^{-1} i.v.) were administered to check airway responsiveness. Subsequently, the submaximal dose of 5 μg kg^{-1} of histamine was chosen for evaluating the effects of PAR2-AP. Five minutes thereafter, animals were treated with PAR2-AP (SLIGRL, 1 mg kg^{-1} i.v.) or the scrambled peptide (LSIGRL, 1 mg kg^{-1} i.v.) and histamine was re-administered 1, 6 and 11 min later and the response compared to that obtained prior to administration of the peptides.

Drug treatment

To investigate the role of endogenous prostaglandins on the effect of PAR2-AP, different groups of animals were pre-treated with indomethacin (5 mg kg^{-1} i.p.) 30 min before the administration of histamine and the effect of PAR2-AP or of the control peptide was then evaluated. A control group was pre-treated with vehicle alone (PEG 400). The dose of indomethacin was chosen on the basis of its ability to completely block bradykinin (2 μg kg^{-1} i.v.)-induced increase in lung resistance. To investigate whether nitric was involved in PAR2-AP effect, a different group of animals was pre-treated with L-NAME (30 mg kg^{-1} i.v.) 15 min before the administration of histamine and the effect of PAR2-AP was then evaluated. To rule out the possibility that the effect of PAR2-AP was due to adrenergic system activation, a group of animals was pre-treated with propranolol (1 mg kg^{-1} i.v.) 15 min before the administration of histamine and the effect of PAR2-AP was then evaluated.

Effect of vagotomy

Different groups of animals were vagotomized by excision of both vagi nerves in the neck region. The effect of PAR2-AP on histamine-induced bronchoconstriction was then investigated following the protocol described above. The effect of indomethacin (5 mg kg^{-1} i.v. 30 min pre-treatment) was also evaluated in other groups of vagotomized animals.

Measurement of airway microvascular leakage

To determine whether the protective effect of PAR2-AP on bronchial responsiveness to histamine was associated with a reduction of histamine induced microvascular leakage, we examined lung tissues isolated from treated animals, using the Evan's Blue dye extravasation technique (Udaka *et al*., 1970). Different groups of animals were anaesthetized with urethane (2.5 g kg^{-1} i.p.) and jugular vein and trachea were cannulated. Evan's Blue dye (2.5% w v^{-1}; 20 mg kg^{-1}) was injected intravenously. One minute before the administration of histamine (15 μg kg^{-1} i.v.), animals were treated with PAR2-AP (1 mg kg^{-1} i.v.) or with the scrambled peptide. After 15 min, animals were sacrificed, the thorax was opened and animals were exsanguinated by cutting the inferior vena cava below an occlusion with thread. Lungs were perfused with saline through the jugular vein to eliminate dye present

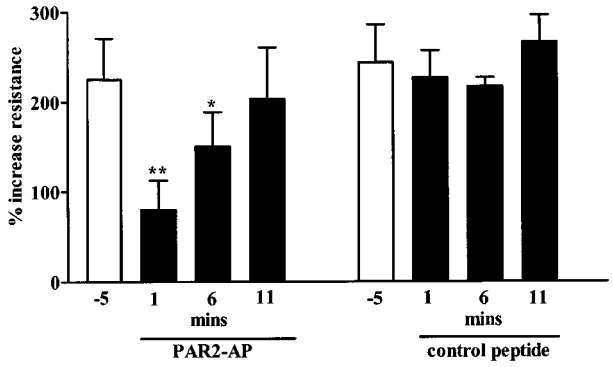

Figure 1 Effect of PAR2-AP and of the control peptide (1 mg kg^{-1} i.v.) on increase in airway resistance induced by histamine (5 μg kg^{-1} i.v.). Response to histamine was evaluated 1, 6 and 11 min after administration of peptides and compared to that obtained 5 min prior to PAR2-AP (open bar). *$P<0.05$ and **$P<0.01$ ($n=4$).

in the pulmonary circulation. Lungs were removed and trachea and main bronchi were gently excised. Wet tissue weight was determined. Evans Blue dye was completely extracted by keeping tissues in formamide (3 ml) at room temperature for 72 h. The absorption of these samples was then measured at 630 nm using a microplate reader. The amount of Evans Blue dye that was extravasated was quantified by interpolation on a standard curve of dye concentrations ranging from 0.98–31.25 μg ml^{-1} as was expressed as μg dye g^{-1} wet tissue.

Statistical analysis

Data are expressed as mean ± s.e.mean and analysed with one way analysis of variance (ANOVA), followed by Dunnett's test, or by two-tailed Student's *t*-test when appropriate. A value of $P<0.05$ was taken as significant.

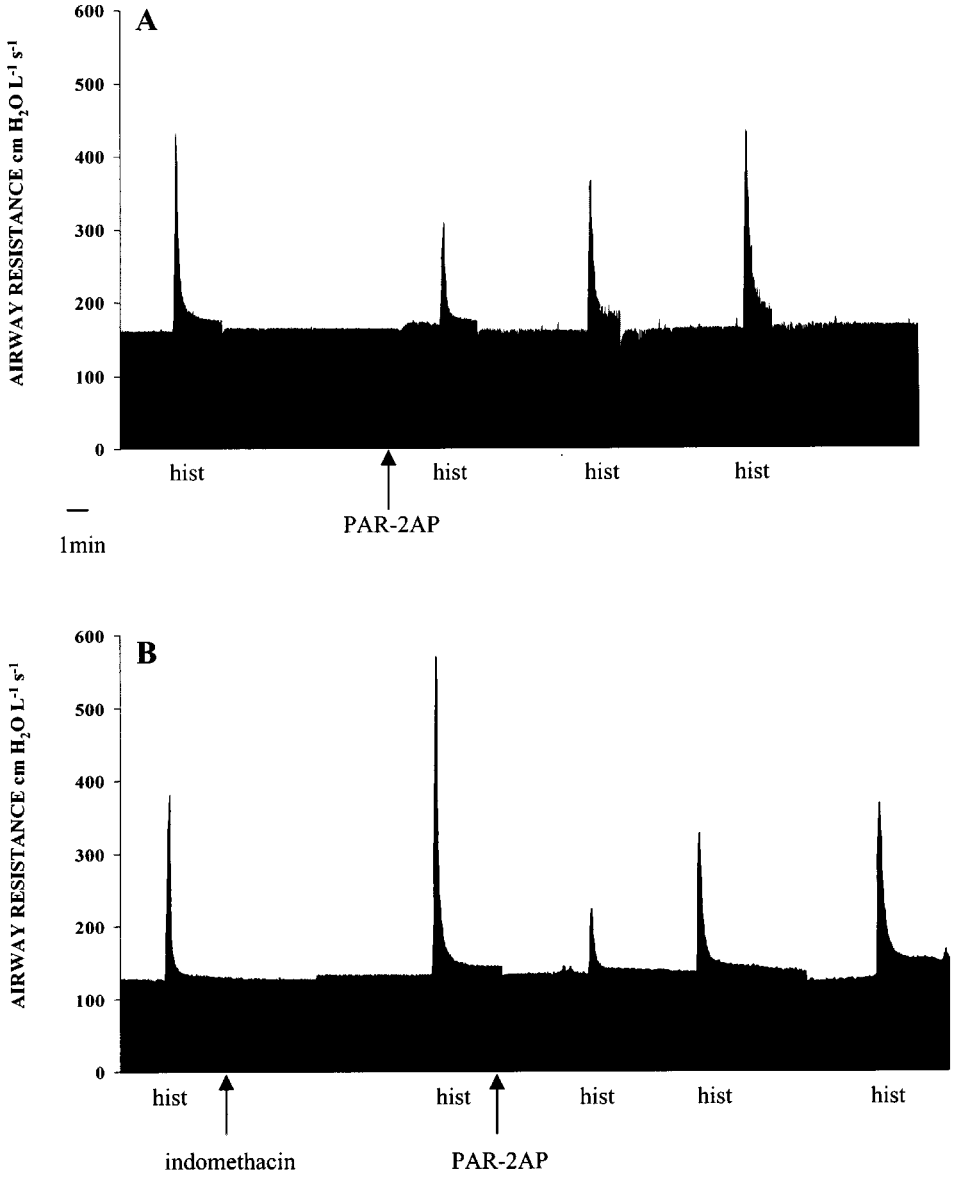

Figure 2 Typical traces representing the effect of PAR2-AP (1 mg kg^{-1} i.v.) on histamine (5 μg kg^{-1} i.v.)-induced increase in airway resistance in guinea-pig. Response to histamine was evaluated 1, 6 and 11 min after peptide administration and compared to that obtained before. (A) control guinea-pig and (B) guinea-pig treated with indomethacin (5 mg kg^{-1} i.p.) 30 min before.

Results

Effect of PAR2-AP on histamine-induced bronchoconstriction

Baseline airway resistance (R_L) in guinea-pigs was 140 ± 10 cm H_2O l^{-1} s^{-1}. Histamine 5 μg kg^{-1} i.v. caused an increase in airway resistance of $225 \pm 46\%$ above baseline ($n=4$). When animals were pre-treated 1 min before with PAR2-AP (1 mg kg^{-1}, i.v.), this increase was significantly reduced after 1 ($n=4$; $P<0.01$) and 6 ($n=4$; $P<0.05$) min respectively. The response to histamine returned to control values 10 min after PAR2-AP administration. The control peptide did not have any effect on histamine-induced increase in baseline resistance (Figures 1 and 2A). In animals pre-treated with indomethacin (5 mg kg^{-1} i.p., 30 min pre-treatment), the response to histamine was significantly increased compared to pre-treatment values (Figure 2B and 3; $n=4$, $P<0.05$). However, indomethacin did not affect the inhibitory response to PAR2-AP on histamine-induced increase in airway resistance. Rather, the effect of PAR2-AP appeared to be prolonged after indomethacin pre-treatment, since the airway response to histamine was still reduced after 10 min following PAR2-AP administration (Figures 2B and 3). Conversely, in animals injected with the control peptide, the response to histamine was still increased 10 min thereafter (Figure 3). Neither pre-treatment with L-NAME nor propranolol altered the inhibitory effect of PAR2-AP on histamine-induced bronchoconstriction (Figure 4).

Effect of PAR2-AP in vagotomized animals

In vagotomized guinea-pigs, the protective effect of PAR2-AP against histamine induced bronchoconstriction was still present and was not different from that obtained in intact animals (Figure 5A). After vagotomy, indomethacin did not cause significant airway hyper-responsiveness to histamine. The inhibitory effect of PAR2-AP was not modified by pre-treatment with indomethacin in vagotomized animals (Figure 5B).

Effect of PAR2-AP on histamine-induced increase in airway permeability

Histamine induced an increase in tracheal and main bronchial permeability, measured as Evan's Blue dye leakage was modestly, but significantly, reduced by PAR2-AP pre-treatment (control, 17.27 ± 0.89 μg ml^{-1} $n=6$; vs PAR2-AP, 14.62 ± 67 μg ml^{-1}, $n=5$, $P<0.05$).

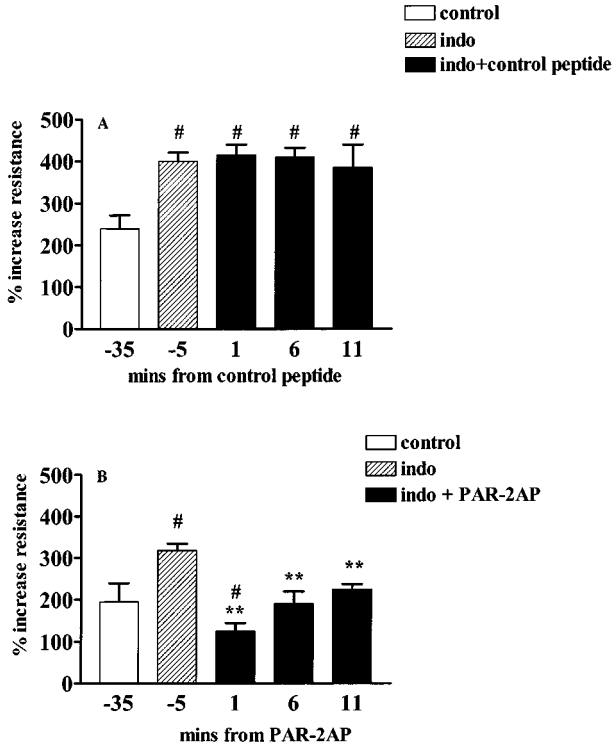

Figure 3 Bar graph representing the effect of indomethacin on bronchoprotection induced by PAR2-AP. Response to histamine (5 μg kg^{-1} i.v.) was evaluated 1, 6 and 11 min after administration of the control peptide (A) or PAR2-AP (B) at the dose of 1 mg kg^{-1} i.v., in guinea-pigs previously treated with indomethacin (5 mg kg^{-1} i.p., 30 min before). #$P<0.05$ vs control; **$P<0.01$ vs indo ($n=4$).

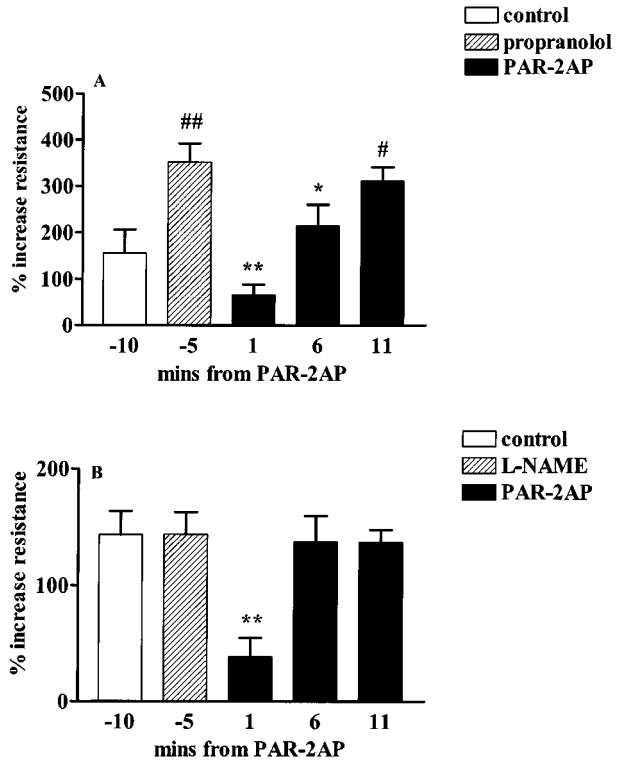

Figure 4 Bar graph representing the effect of propranolol (A) and L-NAME (B) on bronchoprotection induced by PAR2-AP. Response to histamine (5 μg kg^{-1} i.v.) was evaluated 1, 6 and 11 min after administration of PAR2-AP at the dose of 1 mg kg^{-1} i.v., in guinea-pigs previously treated with propranolol (1 mg kg^{-1} i.p., 15 min before; (A) or L-NAME (30 mg kg^{-1}, 15 min before; (B), ## $P<0.01$ and # $P<0.05$ vs control; *$P<0.05$ and **$P<0.01$ vs propranolol ($n=5$) or L-NAME ($n=5$).

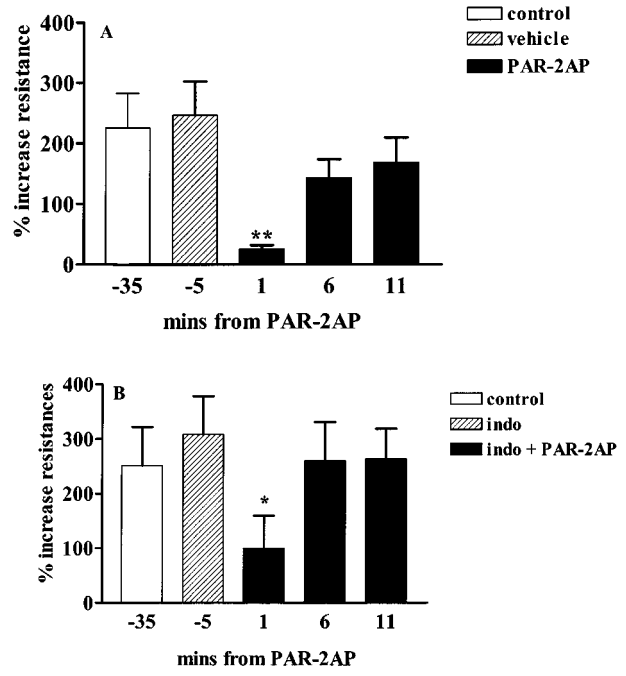

Figure 5 Bar graph representing the effect of PAR2-AP (1 mg kg^{-1} i.v.) in vagotomized guinea-pigs. Response to histamine was evaluated 1, 6 and 11 min after administration of PAR2-AP in guinea-pigs treated with vehicle (A) or indomethacin (5 mg kg^{-1} i.p., 30 min before, (B). $^{*}P<0.05$ and $^{**}P<0.01$ vs time -5 min ($n=5$).

Discussion

Our results have demonstrated that intravenous administration of PAR2-AP to guinea-pigs inhibits histamine-induced increase in lung resistance by a mechanism independent of the release of prostaglandin, nitric oxide nor to the effect of circulating adrenaline. *In vitro* it has been proposed that the protective effect of PAR2-AP on airway reactivity is dependent on the involvement of epithelial PGE_2 (Cocks *et al*., 1999). The discrepancy between our data and those obtained by others on *in vitro* airway preparations is likely due to the complex interactions present in an *in vivo* setting. Likewise, a similar discrepancy between data obtained *in vitro* and *in vivo* has also been observed for haemodynamic changes mediated by PAR2 activation. Indeed, while PAR2-AP-induced relaxation of isolated vascular tissues is clearly dependent upon nitric oxide release from endothelial cells *in vitro* (Emilsson *et al*., 1997; Moffatt & Cocks, 1998), the hypotension induced by PAR2 activation is not dependent upon NO release *in vivo* (Cicala *et al*., 1999b).

We demonstrated that prostanoids do not appear to play a role in the bronchoprotection attributed to PAR2-AP. Moreover, indomethacin treatment *per se*, significantly augmented histamine-induced increase in baseline resistance. The mechanism by which indomethacin induces an increase in airway responsiveness to histamine is still uncertain. It has been attributed to a vagal stimulation, as the effect is abolished in vagotomized animals (Ito & Tajima, 1981), but also to the inhibition of airway derived PGE_2, which is known to have a bronchoprotective effect (Pavord & Tattersfield, 1995; Spina, 2000). A further possibility is that NSAIDs attenuate adrenergic and non-adrenergic inhibitory activity thereby leading to an increase in airway responsiveness (McCullock *et al*., 1967; Coburn & Tomita, 1973). Our data confirm the increase in histamine-induced bronchoconstriction following pre-treatment with indomethacin, which was evident in intact but not in vagotomized animals, in agreement with previous data (Ito & Tajima 1981; Mitchell & Adcock, 1988). Interestingly, we observed that in indomethacin treated guinea-pigs, the effect of PAR2-AP was prolonged. Indeed, suppression of bronchial hyperresponsiveness was observed 10 min following administration of PAR2-AP, at a time when, under normal conditions, the inhibitory response to PAR2-AP had returned to pre-dosing levels. This finding suggests that activation by PAR2-AP *in vivo* leads to a bronchoprotective effect independent of PGE_2 synthesis and more pronounced when hyper-responsiveness is established. Recently, PAR2 has been shown to be expressed on primary spinal afferent neurones and to be involved in inflammation through the release of neuropeptides (CGRP and Substance P) (Steinhoff *et al*., 2000) and PAR2 activation has been linked to a sensory nerve-dependent bronchoconstriction (Ricciardolo *et al*., 2000). However, it is clear from our experiments that the predominant action of PAR2-AP is bronchoprotection.

Vagal stimulation is thought to be involved in both histamine response and in indomethacin-induced increase in airway responsiveness to histamine. Furthermore there is evidence that parasympathetic nervous system mediates both cholinergic contractions and non-adrenergic non-cholinergic (NANC) relaxation of airway smooth muscle (Canning & Undem, 1997). In order to establish whether the effect of PAR2-AP was modulated by vagal tone we also performed experiments in vagotomized animals. Our data show that in vagotomized animals the bronchoprotective action of PAR2-AP was still present and that it was not affected by indomethacin. Under these circumstances, indomethacin did not prolong the PAR2-AP protective effect, demonstrating that the prolongation of PAR2-AP effect induced by indomethacin in intact animals is likely due to indomethacin-induced bronchial hyper-responsiveness *per se*. In this respect, activation of PAR2 in the airways might have an important regulatory role in the nervous control of airways. We also rule out the possibility that PAR2-AP-induced bronchoprotection is mediated secondary *via* sympathetic nerve activation, since this response was not modified by propranolol. Moreover, it remains to be established whether PAR2-AP activates iNANC nerves directly since the response was not abolished by vagotomy, although it is unlikely to be due to the release of nitric oxide, as L-NAME failed to reverse the bronchoprotection observed with PAR2-AP. Our results have also demonstrated that PAR2-AP showed a modest, but significant, protection against histamine-induced increase in airway permeability in guinea-pigs. This finding further supports the concept that PAR2 may have a protective role in the airways and that this protective effect extends beyond the control of airway smooth muscle and airway nerves.

In conclusion, our *in vivo* data confirm previous *in vitro* evidence of a protective role for PAR2 in airways, however, it appears that PAR2-AP mediates this effect independently of prostanoids, nitric oxide or circulating adrenaline.

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