Title Page

Influence of influenza A infection on capsaicin-induced responses in murine airways

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Running title page

a) Influenza A and sensory nerve function in mouse airways

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d) List of nonstandard abbreviations:
   TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid type 1; PG, prostaglandin; SP, Substance P; EP, E-prostanoid; CDRC, cumulative dose response curve; NK, neurokinin; COX, cyclooxygenase; C_{max}, maximum contractile
response; EC₄₀, effective concentration of agonist inducing a 40% relaxation response to baseline; Eₘₐₓ, maximum response; BAL, bronchoalveolar lavage; ELISA, enzyme-linked immunosorbent assay; β₁AR, β₁-adrenoceptor; ASM, airway smooth muscle; CGRP, calcitonin-gene related peptide; cAMP, cyclic adenosine monophosphate; ANOVA, analysis of variance; EID, egg infectious dose; PBS, phosphate buffered saline.

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Abstract

The principal aim of the study was to determine the influence of influenza A virus infection on capsaicin-induced relaxation responses in mouse isolated tracheal segments, and to clarify underlying mechanisms. Anaesthetised mice were intranasally inoculated with influenza A/PR-8/34 virus (VIRUS) or vehicle (SHAM), and four days later tracheal segments were harvested for isometric tension recording, biochemical and histologic analyses. Capsaicin induced dose-dependent relaxation responses in carbachol-contracted SHAM trachea (e.g. 10 µM capsaicin produced 66±4% relaxation, n=11), which were significantly inhibited by capsazepine (TRPV1 antagonist), L-733,060 (NK₁ receptor antagonist), indomethacin (COX inhibitor) and the combination of AH6809 and AH23848 (EP₂ and EP₄ receptor antagonists) – indicating that capsaicin-induced relaxation involved the TRPV1-mediated release of SP, activation of epithelial NK₁ receptors and production of COX products capable of activating relaxant EP₂/EP₄ receptors. Consistent with this postulate, capsaicin-induced relaxation was associated with significant release of SP and PGE₂ from mouse tracheal segments. As expected, influenza A virus infection was associated with widespread disruption of the tracheal epithelium. Tracheal segments from VIRUS mice responded weakly to capsaicin (7±3% relaxation) and were 25-fold less responsive to SP than tracheas from SHAM mice. In contrast, relaxation responses to exogenous PGE₂ and to the β-adrenoceptor agonist isoprenaline were not inhibited in VIRUS trachea. Virus infection was associated with impaired capsaicin-induced release of PGE₂, but release of SP was not affected. In summary, influenza A virus infection profoundly inhibits capsaicin- and SP-induced relaxation responses, most likely by inhibiting the production of PGE₂.
Introduction

Sensory nerves innervate a range of key structures of the respiratory tract, including the airway epithelium, smooth muscle band and submucosal glands (Verhein et al., 2009). Afferent sensory nerves located within the airway epithelium serve to detect the presence of noxious stimuli and to initiate a reflex bronchoprotective response (Martling, 1987). Accumulating and compelling evidence indicates this sensory function is primarily mediated by members of the Transient Receptor Potential (TRP) family of ion channels, which are highly expressed on sensory nerve endings. For example, TRP vanilloid type 1 (TRPV1) is a calcium ion channel, activated by high temperature (>40°C), low pH and exogenous vanilloids such as the chilli plant extract, capsaicin (Manzini, 1992; Bevan & Geppetti, 1994; Caterina et al., 1997; Szallasi & Blumberg, 1999).

Upon TRPV1 activation by capsaicin, Ca\(^{2+}\) ions enter the sensory nerve ending and promote its depolarization. The newly-formed nerve impulse initiates a centrally-mediated, parasympathetic reflex that culminates in smooth muscle contraction and mucous gland secretion (Lee & Pisarri, 2001). However, of more relevance to this study, the nerve impulse also travels in an antidromic manner leading to local release of neuropeptides, such as Substance P (SP), from sensory nerve endings. SP is capable of exerting a wide range of biological effects within the airways, although these vary depending upon species and tissue type (Stewart et al., 2008). Application of SP (or capsaicin) to mouse isolated bronchial preparations induces airway smooth muscle relaxation (Manzini, 1992). In mouse and rat airways, SP activates Neurokinin-1 (NK\(_1\)) receptors on epithelial cells, leading to activation of cyclooxygenase (COX) and production of prostaglandin E\(_2\) (PGE\(_2\)) (Devillier et al.,
In turn, PGE$_2$ activates adenylate cyclase-linked EP$_{2/4}$ receptors on airway smooth muscle, increasing intracellular cAMP levels and promoting relaxation (Sheller et al., 2000; Narumiya et al., 1999). To date, no study has fully characterised the capsaicin-induced relaxation pathway. Hence a primary aim of this study was to apply pharmacological approaches to clarify and confirm the mechanisms that underpin capsaicin-mediated relaxation pathways in mouse isolated trachea.

An equally important aim of this study was to determine the effect of influenza A infection on airway sensory nerve function, and in particular on the capsaicin-induced relaxation pathway. It is currently unknown if an influenza A infection modulates sensory nerve function, although there exists circumstantial evidence that supports this postulate. Firstly, it is well established that influenza A infects and destroys the pseudo-stratified columnar epithelium, and as such may inhibit the epithelium-dependent, capsaicin-induced relaxation response. Secondly, certain neurotrophic influenza A strains can infect the CNS via the sensory nervous system (Shinya et al., 2000), and this may affect sensory nerve function. Finally, influenza A infection can alter the density, distribution and function of an array of receptors involved in controlling bronchomotor tone (Henry et al., 1991; Carr et al., 1996; Lan et al., 2004). This study tested the hypothesis that influenza A infection inhibits the capsaicin-induced relaxation pathway by altering sensory nerve, epithelial cell and smooth muscle function.
Methods

Animals and Viral inoculation. Groups of specified pathogen free male BALB/c mice aged 7-8 weeks (Animal Resources Centre, Murdoch, WA) were lightly anaesthetised with inhaled methoxyflurane, and administered a 20 µl intranasal inoculum containing either 1.43x10⁴ EID₅₀ doses of Influenza A/PR-8/34 virus (VIRUS) or diluted allantoic fluid (SHAM). Four days later mice were euthanised via lethal overdose of sodium pentobarbitone (160 mg kg⁻¹ i.p.) and exsanguinated by severing the right renal artery. All experiments were approved by the University of Western Australia Animal Ethics Committee and adhered to guidelines established by the National Health and Medical Research Council of Australia (2004).

Isometric tension recordings. On day four post-inoculation, the trachea was excised and cleaned of connective tissues. The trachea was cut into two segments, upper and lower, each approximately 2 mm in length. Each tracheal segment was loaded onto a pair of stainless steel hooks, suspended in an organ bath containing 2 ml Krebs solution (117 mM NaCl, 5.36 mM KCl, 25 mM NaHCO₃, 1.03 mM KH₂PO₄, 0.57 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, and 11.1 mM D-glucose), maintained at 37°C and bubbled continuously with 5% CO₂ in O₂. The tension of each preparation was maintained at ~230 mg and changes in tension were recorded via an FTO3 isometric force transducer (Grass instruments, Quincy, MA) connected to a Powerlab data acquisition and analysis system (AD Instruments, Castle Hill, Australia). Following a 30 min equilibration period, the viability of each segment was determined by sequential exposure to a sub-maximal dose of carbachol (0.2 µM) and a maximal dose of carbachol (10 µM). Following a 15 min washout and rest period,
segments were re-exposed to sequential doses of carbachol (0.2 µM and 10 µM). Segments that responded weakly to 10 µM carbachol (<600 mg tension) were deemed unviable and discarded. Viable segments were washed and left to rest for a further 15 mins before a carbachol cumulative dose response curve (CDRC, 0.01 µM to 10 µM using half log dose increments) was performed. The maximum response obtained to the 10 µM dose of carbachol was deemed the maximum contractile response (100% C\text{\text{max}}). Mouse isolated tracheal segments do not exhibit intrinsic airway tone, hence precontraction with 1 µM carbachol was required to reveal relaxation responses to agents such as capsaicin. Contractile responses to 1 µM carbachol plateaued at approximately 70-80% C\text{\text{max}}. Upon reaching a plateau, a single bolus dose of capsaicin (0.01, 0.1, 1 or 10 µM) was added to the carbachol-contracted segment. Capsaicin rapidly depletes the stores of sensory neuropeptides in isolated trachea, therefore only a single dose of capsaicin was administered to each preparation (Manzini, 1992). Once the peak relaxation response to capsaicin was observed (within 10 mins), preparations were washed and left to equilibrate for 15 mins with fresh Krebs solution. This process of precontraction, testing of an active agent and washing out then resting was repeated several times per preparation using an array of agents: SP (0.01 nM to 100 nM - bolus dosing, whole log increments), PGE\text{\text{2}} (3 nM to 3000 nM CDRC in half log increments or 30 nM bolus) and isoprenaline (1 nM to 1000 nM CDRC in half log increments). In separate mouse isolated tracheal segments, this protocol was repeated in the presence of various antagonists/inhibitors (or vehicles) including: capsazepine (TRPV1 antagonist – 10 µM; Ellis & Undem, 1994), indomethacin (non-selective COX inhibitor – 5 µM; Manzini, 1992), AH6809 (EP\text{\text{2}} receptor antagonist – 3 µM; Lan et al.,
2001), AH23848 (EP4 receptor antagonist – 10 µM; Davis et al., 2004) and L-733,060 (NK₁ receptor antagonist – 10 µM; Kubo et al., 2007). The antagonists/inhibitors were added to the baths after each rest period and allowed to equilibrate for at least 10 mins before each precontraction.

**Bronchoalveolar lavage and inflammatory cell influx.** Following exsanguination, the trachea was cannulated and the airways washed out six times with 0.5 mL of sterile phosphate-buffered saline (PBS). The extracted fluid was pooled for each mouse and centrifuged at 4°C for 5 mins at 400 g (Rotina 35R centrifuge). The pellet was resuspended (1% bovine serum albumin in PBS) and total inflammatory cell number determined using a haemocytometer. Each sample was then cytocentrifuged (Shandon cytospin 4) onto a microscope slide and stained with a modified Wright’s stain (aqueous stain Rapid I and Rapid II, Amber Scientific, Perth, WA). Cells were differentially counted under a light microscope, counting 400 leukocytes per slide.

**Histology.** For morphological examination, mouse tracheas obtained on day four post-inoculation (SHAM and VIRUS) were immersion fixed (2% paraformaldehyde / 0.2% wt/vol saturated picric acid in PBS) for 48 hr at 4°C. Following fixation, the tracheas were dehydrated through graded alcohols and embedded in paraffin wax. Five micron transverse sections of trachea were cut and stained with haematoxylin and eosin.

**PGE₂ and SP ELISA studies.** As described above (isometric tension recordings), viable VIRUS and SHAM tracheal segments were washed with Krebs solution and precontracted with 1 µM carbachol for 15 mins. Segments were then exposed to either 10 µM Capsaicin or vehicle (final bath concentration of ethanol <0.1%) for 10
mins and the bathing fluid collected and frozen (-80°C) for subsequent analysis of PGE₂ and SP content. Tissue SP was then extracted from these tracheal segments using an amended protocol from Erin & Ulusoy (2009). Capsaicin- or vehicle-exposed tracheal segments were washed repeatedly (>20 times) with Krebs solution, and transferred to tubes containing 1 mL of 2% acetic acid at 95°C for 5 mins. The supernatant was collected and concentrated (Savant Speedvac SPD121P) at 50°C for 3 hr. The dry remnants were solubilised in 130 µL of EIA buffer (Cayman Chemicals, Ann Arbor, MI) and stored at -80°C for subsequent analysis of SP content. Levels of SP and PGE₂ were determined utilising commercially available ELISA kits (Cayman Chemicals, Ann Arbor, MI) in accordance with the manufacturer’s instructions.

**Materials.** Capsaicin, carbachol, PGE₂, isoprenaline-hydrochloride, indomethacin, capsazepine, AH6809 and AH23848 were obtained from Sigma-Aldrich (St Louis, MO). SP and L-733,060 were purchased from TOCRIS Biosciences (Ellisville, MO). SP and PGE₂ ELISA plates were obtained from Cayman Chemicals (Ann Arbor, MI). Sodium pentobarbitone was obtained from Virbac Animal Health (Peakhurst, Australia) and methoxyflurane from Medical Developments International Ltd (Springvale, Australia).

**Solutions.** Capsaicin and capsazepine stock solutions were made up in 100% ethanol and serially diluted in sterile saline. The resulting bath concentrations of ethanol were ≤0.1% which is sufficiently low to minimise ethanol-induced effects upon TRPV1 (Trevisani et al., 2002). Isoprenaline stock solution and serial dilutions were made in 0.1 mM ascorbic acid. Indomethacin and AH6809 stock solutions were
made up in 0.1 mM Na₂CO₃ and serially diluted in sterile saline. All other agents (carbachol, PGE₂, SP and L-733,060) were dissolved and diluted in sterile saline.

**Statistical analysis.** The two main parameters used in statistical analysis of the isometric tension data were the –logEC₄₀ (the logarithm of the concentration of agent required to produce 40% relaxation of the carbachol-induced contraction) and the E₉₅ (the maximal relaxation response reached in percent relaxation of the carbachol-induced contraction). Parametric tests (1- and 2-way ANOVA) with post-hoc comparisons (Holm-Sidak method) were the prioritised statistical tests used to analyse the isometric tension recording data, BALF cell counts and ELISA data. Differences between groups were considered statistically significant if p<0.05.
Results

Characterization of capsaicin-induced relaxation responses in SHAM trachea.
Capsaicin elicited dose-dependent relaxation responses in SHAM mouse isolated trachea, and responses were similar in both upper and lower segments (Fig. 1A). In lower tracheal segments, the response to 10 µM capsaicin (66 ± 4%, n=11) was abolished by 10 µM capsazepine (TRPV1 antagonist; -1 ±2%, p<0.001, n=4-5; Fig. 1B) and by 5 µM indomethacin (COX inhibitor; 0 ± 1%, p<0.001, n=4; Fig. 1D). Relaxation responses to capsaicin were also significantly inhibited by 10 µM L-733,060 (NK1 receptor antagonist; p<0.001, n=5; Fig. 1C) and by 3 µM AH6809 and 10 µM AH23848 (EP2 and EP4 receptor antagonists respectively, n=6; Fig. 1E). Capsaicin-induced relaxation responses were also blocked by capsazepine, indomethacin, L-733,060, or the combination of AH6809 and AH23848 in upper tracheal segments (data not shown).

SP elicited dose-dependent relaxation responses in SHAM tracheal segments (Fig. 2A), although it was almost 10-fold more potent in lower segments (-logEC40: 10.0 ± 0.1, n=29) than upper segments (-logEC40: 9.1 ± 0.1, n=21, P<0.01). As expected, the potency of SP was not affected by 10 µM capsazepine (Fig. 2B) but was significantly reduced by 10 µM L-733,060 (Fig. 2C). SP-induced relaxation responses were also suppressed by 5 µM indomethacin (Fig. 2D) and the combination of 3 µM AH6809 and 10 µM AH23848 (p<0.001, n=7; Fig. 2E).

Cumulative addition of PGE2 elicited dose-dependent relaxation in upper and lower segments of SHAM trachea (-logEC40: 6.6 ± 0.1, n=16, lower segment; Fig. 3A). Relaxation responses to PGE2 in lower segments were not inhibited by
capsazepine, L-733,060 or indomethacin (Fig. 3B-D), but were significantly inhibited by the combination of AH6809 and AH23848 (Fig. 3E).

Cumulative addition of isoprenaline elicited dose-dependent relaxation in SHAM trachea (Fig. 4A). The maximum response ($E_{\text{max}}$: 79 ± 2%, n=12, lower segment) and potency (-logEC$_{40}$: 7.6 ± 0.1, n=12) were not significantly inhibited by 5 µM indomethacin (Fig. 4B) or the combination of 3 µM AH6809 and 10 µM AH23848 (Fig. 4C).

**Influenza A infection.** At day 4 post-inoculation, prominent pathological changes were observed in tracheal sections from VIRUS mice (Fig. 5B) compared to SHAM mice (Fig. 5A). Examination of haematoxylin and eosin-stained transverse sections of VIRUS mouse trachea revealed extensive denudation of the epithelium (Fig 5B). Furthermore, a number of inflammatory cell infiltrates, particularly neutrophils, were evident within the lumen, blood vessels and interstitial space of VIRUS mice but not SHAM mice. The inflammatory response was further investigated using BAL fluid cell counts, which revealed an eight-fold increase in the total number of cells infiltrating the airways (p<0.001, n=9-10; Fig. 5C). The inflammatory cells in the BAL fluid from SHAM mice consisted primarily of macrophages (0.7 ± 0.1 million cells/BAL) and a minor population of lymphocytes. The infiltrates from VIRUS mice consisted of significantly elevated numbers of macrophages (3.5 ± 0.3 million cells/BAL, p<0.001, n=10), and neutrophils (2.2 ± 0.1 million cells/BAL, p<0.001, n=10).

**Characterization of capsaicin-induced relaxation responses in VIRUS trachea.** In stark contrast to segments from SHAM mice, both upper and lower tracheal
segments from VIRUS mice responded very weakly to capsaicin (e.g. 10 µM capsaicin produced 7 ± 3% relaxation in lower tracheal segments, n=11, Fig. 6A). Furthermore, VIRUS trachea were 25-fold less responsive to exogenous SP (p<0.001, n=29, Fig 6B). On the other hand, the potencies of exogenous PGE_2 (Fig. 6C) and isoprenaline (Fig. 6D) were not reduced in VIRUS trachea. Indeed, PGE_2 was 2.5-fold more potent in VIRUS trachea compared to SHAM trachea (p<0.001, n=16, lower segment; Fig. 6C).

Carbachol-induced contractions were marginally reduced in tracheal segments from VIRUS mice compared to SHAM mice (e.g. 1µM carbachol produced 71 ± 1% contraction in lower tracheal segments from VIRUS mice and 78 ± 1% contraction in SHAM mice, p<0.001, n=30). Similarly small, but statistically significant, differences were observed in upper tracheal segments (data not shown).

As shown in Table 1, the effects of antagonists and inhibitors on responses to SP, PGE_2 and isoprenaline in upper and lower segments of VIRUS trachea were qualitatively similar to those obtained in SHAM preparations.

**Capsaicin-induced changes in PGE_2 and SP levels in SHAM and VIRUS trachea.** Exposure of SHAM tracheal segments to 10 µM capsaicin for 10 mins caused a significant release of PGE_2 into the surrounding media (Fig. 7A). In stark contrast, capsaicin caused no significant release of PGE_2 from VIRUS tracheal segments (Fig. 7A). Basal levels of PGE_2 release were also lower in VIRUS mice than SHAM mice (Fig. 7A). Levels of SP in media surrounding VIRUS or SHAM trachea following exposure to capsaicin or vehicle were below the level of detection of the ELISA kit (<3.9 pg/ml). However, capsaicin caused a significant reduction in the tissue levels of SP recovered from mouse tracheal segments (Fig. 7B).
Importantly, there was no significant difference in the magnitude of the capsaicin-induced reduction in tracheal SP levels between SHAM and VIRUS mice (Fig. 7B).
Discussion

Our pharmacological studies indicate that capsaicin relaxes mouse isolated tracheal segments through the sequential activation of sensory nerve TRPV1, epithelial NK₁ receptors and COX, and smooth muscle EP₂/₄ receptors (Figure 8). Consistent with this schema, complementary biochemical assays show that capsaicin induces the rapid release of SP and PGE₂ from mouse tracheal segments. Of particular interest, both capsaicin-induced relaxation and PGE₂ generation was dramatically reduced in airways obtained from influenza A virus-infected mice.

Capsaicin induced concentration-dependent relaxation responses in mouse isolated trachea, consistent with previous studies in rodent bronchial preparations (Manzini, 1992; Szarek et al., 1995, 1998). Capsaicin-induced relaxation responses in mouse trachea are most likely mediated by TRPV1 as they were abolished by the TRPV1 antagonist capsazepine. Immunohistochemical studies indicate the predominant cellular location of TRPV1 in mouse lung is upon sensory nerves endings (Dinh et al., 2004), which co-localise with the neuropeptide SP. Indeed, previous studies suggest that capsaicin-induced relaxation responses are mediated by SP-induced activation of epithelial NK₁ receptors (Manzini, 1992; Ichikawa et al., 1995). This postulate is supported by our data showing relaxation responses induced by capsaicin and exogenous SP were inhibited by an NK₁ receptor antagonist, L-733,060, and moreover by the finding that exposure to capsaicin caused a significant reduction in residual tissue levels of SP in mouse tracheal segments.

Relaxation responses to both capsaicin and SP were also abolished by indomethacin, indicating that a relaxant COX product mediates these responses. Consistent with this, capsaicin-induced relaxation responses were associated with
elevated production of the COX-derived prostanoid PGE$_2$. This agrees with studies in rat trachea showing that PGE$_2$ is the primary COX product generated as a result of SP-induced NK$_1$ receptor stimulation (Devillier et al., 1992).

PGE$_2$-induced relaxation responses in airway smooth muscle are typically mediated by the EP$_2$ or EP$_4$ receptor subtype, via signalling through $G_{\alpha s}$, adenylate cyclase and intracellular cAMP. In the current study, the combined application of an EP$_2$ receptor antagonist, AH6809, and an EP$_4$ receptor antagonist, AH23848, produced a more effective inhibition of relaxation responses to capsaicin, SP and PGE$_2$, than the use of either EP receptor antagonist alone – indicating that both EP receptor subtypes may be mediating the relaxation response. Previous studies have confirmed a role for the EP$_2$ receptor in SP and PGE$_2$-induced relaxations (Sheller et al., 2000; Fortner et al., 2001), and further experiments are warranted to more definitively establish the role of EP$_4$ receptors. In summary, capsaicin-induced relaxation in the mouse isolated trachea appears to involve activation of TRPV1 on sensory nerve endings, NK$_1$ receptors on the epithelium and subsequent generation of relaxant COX products such as PGE$_2$, which promote relaxation via smooth muscle EP$_{2/4}$ receptors (Figure 8).

A unique finding of this study was the observation that tracheal segments from influenza A-infected mice were markedly hyporesponsive to the relaxant actions of capsaicin and SP. Given the likely sequential involvement of sensory nerves, epithelial cells and smooth muscle cells in capsaicin-induced relaxation responses, the attenuated responses to capsaicin (and SP) may well be explained by virus-induced changes in the function of these key cellular components.
One possibility worthy of consideration is that influenza A virus infection damages sensory nerves, thereby reducing their capacity to respond to capsaicin. For example, certain neurotrophic strains of influenza A virus have been reported to infect the CNS through the sensory nerves in murine airways (Shinya et al., 2000) and to cause neuronal damage (Jang et al., 2009). However, H1N1 strains of influenza A virus (such as A/PR-8/34) do not appear to be neurotrophic (Kobasa et al., 2007), which reduces the likelihood that the current findings can be explained by virus-induced damage of the nerves leading to dysfunction. Consistent with this, the capacity of capsaicin to cause release of SP from tracheal tissue was not affected by influenza virus infection. These key findings indicate that the virus-induced attenuation of the capsaicin relaxation response is unlikely to be caused by an impaired ability of the sensory nerves to release SP in response to capsaicin. Rather, it indicates the locus of the lesion lies downstream from sensory nerve activation.

Histological examination of tracheal segments from influenza A-infected mice revealed widespread and extensive damage to the epithelium, which may reduce its capacity to generate relaxant factors such as PGE₂ in response to capsaicin and SP. Previously published studies have used epithelium denudation techniques to establish the importance of the epithelium in relaxation responses to capsaicin (Szarek et al., 1995, 1998) and SP (Szarek et al., 1995, 1998; Kao et al., 1999; Liu et al., 2006) in rat and mouse isolated airway preparations. Moreover, Szarek and coworkers (1998) demonstrated that epithelium removal by mechanical disruption blocked both capsaicin- and SP-induced release of PGE₂. Consistent with this postulate, capsaicin did not induce the release PGE₂ from tracheal segments of
VIRUS mice. Thus, the reduced sensitivity of VIRUS trachea to exogenously administered capsaicin and SP may be at least partly explained by a reduced capacity of VIRUS trachea to generate epithelial PGE$_2$.

Influenza A virus infection has been associated with reduced relaxation responses in airway smooth muscle (Henry et al., 1991; Ashraf et al., 2001). However, virus-induced reductions in relaxation responses were only observed for capsaicin and SP, which cause relaxation indirectly via the release of COX products such as PGE$_2$. In contrast, responses to exogenous PGE$_2$ and isoprenaline – which cause relaxation by directly acting on EP receptors and β-adrenoceptors located on smooth muscle cells – were not reduced in tracheal preparations from influenza A-infected mice. Thus, virus-induced loss of responsiveness to capsaicin and SP cannot be explained by hypofunction of relaxation pathways within tracheal smooth muscle cells, and indicate that the locus of the dysfunction lies upstream, principally at the level of the epithelium.

In addition to attenuating capsaicin-induced generation of PGE$_2$, influenza A viral infection also significantly reduced the basal release of PGE$_2$ from mouse tracheal segments. A potential homeostatic response to reducing basal PGE$_2$ levels would be an increase in the sensitivity of the EP receptors signalling pathway (perhaps by promoting EP receptor expression). The development of such a compensatory response may well explain the heightened sensitivity of tracheal segments from VIRUS mice to exogenous PGE$_2$ – tracheal segments from VIRUS mice were less responsive to capsaicin and SP, but were hyperresponsive to exogenous PGE$_2$ (significant 2.5-fold leftward displacement of concentration-response curve in VIRUS mice compared to SHAM mice, Fig. 6C). Although the
underlying mechanism for virus-induced hypersensitivity to PGE$_2$ has not been
determined, a driving factor may be the reduced capacity of tracheal epithelial cells
to release basal PGE$_2$ at the levels observed prior to influenza infection.

In conclusion, this study has characterised capsaicin-induced relaxation in
mouse isolated trachea, and demonstrated that these responses are greatly
attenuated by influenza A/PR-8/34 virus infection (Figure 8). The mechanism through
which influenza A attenuates this response is likely to involve a reduced ability to
generate PGE$_2$ in response to SP. Although this phenomenon has not yet been
described in humans and requires further characterisation, it is tempting to speculate
that the loss of this sensory nerve-mediated bronchoprotective response may be
responsible for compounding viral-induced asthma exacerbations.
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Authorship Contributions

Participated in research design: Henry and Taylor

Conducted experiments: Taylor and Mann

Performed data analysis: Taylor, Mann and Henry

Wrote or contributed to the writing of the manuscript: Taylor, Mann and Henry
References


Footnotes

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d)
Legends for Figures

**Figure 1:** Characterisation of capsaicin-induced relaxation responses in SHAM trachea. (A) Bolus dose-response curves to capsaicin in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on capsaicin-induced relaxations are only presented graphically for the lower tracheal segments. Effects of (B) 10 µM capsazepine, (C) 10 µM L-733,060, (D) 5 µM indomethacin and (E) 3 µM AH6809 plus 10 µM AH23848 on capsaicin-induced relaxation responses in lower tracheal segments. Black bars indicate capsaicin-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 5-15 (regional dose response curves) or 3-7 (antagonist studies) lower tracheal segments. ***p<0.001 compared to the lower segment (vehicle). Comparable data was obtained for upper tracheal segments (data not shown).

**Figure 2:** Characterisation of Substance P (SP)-induced relaxation responses in SHAM trachea. (A) Bolus dose-response curves to SP in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on SP-induced relaxations are only presented graphically for the lower tracheal segments. Effects of (B) 10 µM capsazepine, (C) 10 µM L-733,060, (D) 5 µM indomethacin and (E) 3 µM AH6809 plus 10 µM AH23848 on SP-induced relaxation responses in lower tracheal segments. Black bars or symbols indicate SP-induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 29-32 (regional dose response curves) or 4-10 (antagonist studies) lower tracheal segments. **p<0.01, ***p<0.001, SP Emax compared to lower segment (vehicle). †††p<0.001, SP -
logEC40 compared to lower segment (vehicle). Comparable data was obtained for upper segment in the presence of the antagonists (Table 1).

**Figure 3:** Characterisation of PGE2-induced relaxation responses in SHAM trachea. (A) Cumulative dose-response curves to PGE2 in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on PGE2-induced relaxations are only presented graphically for the lower tracheal segments. Effects of (B) 10 µM capsazepine, (C) 10 µM L-733,060, (D) 5 µM indomethacin and (E) 3 µM AH6809 plus 10 µM AH23848 on PGE2-induced relaxation responses in lower tracheal segments. Black bars or symbols indicate PGE2–induced responses in the presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 15-16 (regional dose response curve) lower tracheal segments or 3-10 (antagonist studies) lower tracheal segments. *p<0.05, PGE2 -logEC40 compared to lower segment (vehicle), ***p<0.001, PGE2 -logEC40 compared to lower segment (vehicle).

Comparable data was obtained for upper segment in the presence of the antagonists (Table 1).

**Figure 4:** Characterisation of the isoprenaline-induced relaxation responses in SHAM trachea. (A) Cumulative dose-response curves to isoprenaline in upper (●) and lower (○) tracheal segments. For clarity the effects of the antagonists/inhibitors on isoprenaline-induced relaxations are only presented graphically for the lower tracheal segments. Effects of (B) 5 µM indomethacin and (C) 3 µM AH6809 plus 10 µM AH23848 on isoprenaline-induced relaxation responses in lower tracheal segments. Black bars or symbols indicate isoprenaline–induced responses in the
presence of antagonist/inhibitor. Shown are mean responses ± S.E.M from 12 (regional dose response curve) lower tracheal segments or 3-4 (antagonist studies) lower tracheal segments. ***p<0.001, isoprenaline -logEC₄₀ compared to lower segment. Comparable data was obtained for upper segment in the presence of the antagonists (Table 1).

**Figure 5:** Haematoxylin and eosin-stained photomicrographs of SHAM (A) and VIRUS (B) mouse trachea at 4 days post-inoculation, illustrating marked disruption of airway epithelium during influenza A virus infection. Scale bar = 20μm. (C) Levels of inflammatory cell infiltrates found in bronchoalveolar lavage fluid from SHAM (white bars) and VIRUS (black bars) mice at 4 days post-inoculation. Shown are mean levels of cells ± S.E.M from 9-10 BAL samples. ***p<0.001 compared to SHAM infected cell count.

**Figure 6:** Relaxation responses to (A) capsaicin, (B) substance P, (C) PGE₂ and (D) isoprenaline in SHAM (white symbols) and VIRUS (black symbols) lower tracheal segments. Shown are mean responses ± S.E.M from 5-15 (Capsaicin), 29-32 (Substance P), 16 (PGE₂) and 12 (isoprenaline) lower tracheal segments. ***p<0.001, maximal response in virus-infected compared to sham infected tracheal segments. †††p<0.001, -logEC₄₀ in virus infected compared to sham infected tracheal segments. Comparable data was obtained for upper tracheal segments (Table 1).

**Figure 7:** (A) Levels of PGE₂ in media surrounding SHAM and VIRUS mouse
isolated whole tracheas following 10 mins exposure to 10 μM capsaicin (black bars) and vehicle (white bars).  (B) Levels of residual SP in SHAM and VIRUS trachea following exposure to 10 μM capsaicin (black bars) and vehicle (white bars). Shown are mean responses ± S.E.M from 9 (VIRUS) and 9 (SHAM) whole tracheas. *P<0.01 (2-way ANOVA, Holm-Sidak post-hoc comparison).

**Figure 8: Influenza A-induced modulation of capsaicin-induced smooth muscle relaxation pathway in mouse airways.** In trachea from uninfected mice, capsaicin activates TRPV1 ion channels on sensory nerve endings evoking a ‘local axonal reflex’ resulting in the release of substance P, which acts upon epithelial NK1 receptors to activate COX leading to the generation of PGE2. In turn, PGE2 activates EP2/4 receptors on airway smooth muscle leading to an increase in cAMP and subsequent relaxation. Similarly, isoprenaline mediates a cAMP-dependent airway smooth muscle relaxation through its actions on β1-adrenoceptors. In contrast, tracheal segments from influenza A-infected mice did not respond to capsaicin (neither PGE2 release nor relaxation response) and were hyporesponsive to substance P. Influenza A infection was not associated with impaired relaxation responses to either PGE2 or isoprenaline. Thus, influenza A-induced modulation of the function of epithelial cells, rather than sensory nerves or smooth muscle cells appears to be primarily responsible for suppressed responsiveness to capsaicin and substance P.
Table 1  Effect of selected antagonists and inhibitors on the relaxant activity of Substance P, PGE₂ and Isoprenaline in upper and lower tracheal segments from SHAM and VIRUS mice

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Treatment</th>
<th>Region (n)</th>
<th>-logEC₄₀</th>
<th>E_max</th>
<th>-logEC₄₀</th>
<th>E_max</th>
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<tr>
<td>Substance P</td>
<td>None (vehicle)</td>
<td>Upper (21-31)</td>
<td>9.1±0.1</td>
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<td>8.2±0.1</td>
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<td></td>
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<td>Lower (29-32)</td>
<td>10.0±0.1</td>
<td>81±1</td>
<td>8.6±0.1</td>
<td>67±2</td>
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<tr>
<td></td>
<td>L-733,060 (10uM)</td>
<td>Upper (5)</td>
<td>8.4±0.2</td>
<td>69±3</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Lower (4-5)</td>
<td>9.3±0.2</td>
<td>78±3</td>
<td>ND</td>
<td>22±5</td>
</tr>
<tr>
<td></td>
<td>Indomethacin (5uM)</td>
<td>Upper (5)</td>
<td>ND</td>
<td>-2±1</td>
<td>ND</td>
<td>-2±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lower (5)</td>
<td>ND</td>
<td>-2±1</td>
<td>ND</td>
<td>-2±1</td>
</tr>
<tr>
<td></td>
<td>Capsazepine Vehicle</td>
<td>Upper (7-10)</td>
<td>9.1±0.1</td>
<td>71±3</td>
<td>8.2±0.2</td>
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<tr>
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<td></td>
<td>Lower (8-10)</td>
<td>9.9±0.1</td>
<td>82±3</td>
<td>8.4±0.1</td>
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</tr>
<tr>
<td></td>
<td>Capsazepine (10uM)</td>
<td>Upper (5-7)</td>
<td>9.4±0.2</td>
<td>78±5</td>
<td>8.2±0.1</td>
<td>46±8</td>
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<td></td>
<td>Lower (5-7)</td>
<td>9.8±0.2</td>
<td>85±3</td>
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<td>55±10</td>
</tr>
<tr>
<td>PGE₂</td>
<td>None (vehicle)</td>
<td>Upper (14-15)</td>
<td>6.3±0.1</td>
<td>75±3</td>
<td>6.7±0.1</td>
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<td>Lower (16)</td>
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<td>86±2</td>
<td>6.7±0.1</td>
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<td>6.8±0.1</td>
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<td>Indomethacin (5uM)</td>
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<td>90±6</td>
</tr>
<tr>
<td></td>
<td>Capsazepine Vehicle</td>
<td>Upper (10)</td>
<td>6.5±0.1</td>
<td>81±3</td>
<td>6.7±0.1</td>
<td>87±3</td>
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<td>Lower (9-10)</td>
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<td>88±2</td>
<td>7.2±0.1</td>
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<tr>
<td></td>
<td>Capsazepine (10uM)</td>
<td>Upper (5)</td>
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<td>88±4</td>
<td>7.1±0.2</td>
<td>94±2</td>
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<tr>
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<td></td>
<td>Lower (5)</td>
<td>7.1±0.2</td>
<td>94±1</td>
<td>6.5±0.2</td>
<td>96±1</td>
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<td>7.2±0.1</td>
<td>77±2</td>
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<tr>
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<td></td>
<td>Lower (12)</td>
<td>7.6±0.1</td>
<td>79±2</td>
<td>7.4±0.1</td>
<td>76±3</td>
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<tr>
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<td>7.1±0.2</td>
<td>76±4</td>
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<td>Lower (4)</td>
<td>7.4±0.2</td>
<td>79±5</td>
<td>7.4±0.2</td>
<td>78±5</td>
</tr>
</tbody>
</table>

ND, -logEC₄₀ not determined because 40% Emax value not achieved.

* p<0.05 in comparison to respective SHAM segment

b p<0.05 in comparison to respective vehicle

c p<0.05 in comparison to respective upper segment.
Figure 3

A  

Relaxation (%)  

100  

80  

60  

40  

20  

0  

PGE\textsubscript{2} (nM)  

1  

10  

100  

1000  

***

B  

C  

D  

E  

Relaxation (%)  

100  

80  

60  

40  

20  

0  

PGE\textsubscript{2} (nM)  

1  

10  

100  

1000  

Relaxation (%)  

100  

80  

60  

40  

20  

0  

PGE\textsubscript{2} (nM)  

1  

10  

100  

1000  

30 nM PGE\textsubscript{2}  

+AH6809  

+AH23848  

+AH6809+AH23848  

***
Figure 4

(A) Relaxation (%) as a function of Isoprenaline (nM).

(B) Additional curve details.

(C) Bar graph showing relaxation percentages with different conditions.
Figure 5

A  
B

C

Number of leucocytes (million cells BAL)

Total cell count  Macrophages  Neutrophils  Lymphocytes

***  ***  ***
Figure 7

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Figure 8

Sensory nerve

Capsaicin → TRPV1

Substance P → NK₁

Prostaglandin E₂ → EP₂/₄

Isoprenaline → β₁-AR

Epithelial cell

COX

Relaxation

Smooth muscle cell

cAMP

Influenza virus infection