Acrolein relaxes mouse isolated trachea via a TRPA1-dependent mechanism

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ABSTRACT

Airway sensory C-fibres express TRPA1 channels which have recently been identified as a key chemosensory receptor for acrolein, a toxic and highly prevalent component of smoke. TRPA1 likely plays an intermediary role in eliciting a range of effects induced by acrolein including cough and neurogenic inflammation. Currently, it is not known whether acrolein-induced activation of TRPA1 produces other airway effects including relaxation of mouse airway smooth muscle. The aims of this study were to examine the effects of acrolein on airway smooth muscle tone in mouse isolated trachea, and to characterise the cellular and molecular mechanisms underpinning the effects of acrolein. Isometric tension recording studies were conducted on mouse isolated tracheal segments to characterise acrolein-induced relaxation responses. Release of the relaxant PGE₂ was measured by EIA to examine its role in the response. Use of selective antagonists/inhibitors permitted pharmacological characterisation of the molecular and cellular mechanisms underlying this relaxation response. Acrolein induced dose-dependent relaxation responses in mouse isolated tracheal segments. Importantly, these relaxation responses were significantly inhibited by the TRPA1 antagonists AP-18 and HC-030031, an NK₁ receptor antagonist RP-67580, and the EP₂ receptor antagonist PF-04418948, whilst completely abolished by the non-selective COX inhibitor indomethacin. Acrolein also caused rapid PGE₂ release which was suppressed by HC-030031. In summary, acrolein induced a novel bronchodilator response in mouse airways. Pharmacologic studies indicate that acrolein-induced relaxation likely involves interplay between TRPA1-expressing airway sensory C-fibres, NK₁ receptor-expressing epithelial cells, and EP₂-receptor expressing airway smooth muscle cells.

Keywords. Acrolein, mouse tracheal smooth muscle, relaxation, TRPA1, NK₁ receptors, EP receptors

Abbreviations. AITC, allyl isothiocyanate; CGRP, calcitonin gene related peptide; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay; EtOH, ethanol; PGE₂, prostaglandin E₂; NK₁, neurokinin 1; SP, substance P; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor vanilloid 1

1. INTRODUCTION

The respiratory tract is innervated by a complex network of sensory nerves. These nerves function as a line of defence against airborne insults by initiating powerful and co-ordinated reflex responses to protect the lung from injury [1]. A large number of sensory nerves belong to the unmyelinated C-fibre family, a group of nerve fibres characterised by their distinct sensitivity to an array of natural pungent products and chemical irritants, including acrolein [2]. There is evidence that activation of sensory C-fibres by these irritants is mediated principally by members of the transient receptor potential (TRP) superfamily of ion channels. For example, the TRP vanilloid 1 (TRPV1) channel is readily activated by capsaicin, the major pungent ingredient of hot chilli peppers [3, 4], and TRPV1 activation likely plays an important role in mediating the effects of capsaicin.

Although readily activated by capsaicin, the TRPV1 channel is not thought to play a chemosensory role in detecting other airborne irritants, particularly those of an electrophilic nature. Rather, this role has recently been attributed to the TRP ankyrin 1 (TRPA1) channel, a tetrameric, non-selective cation channel co-expressed in 30-40% of TRPV1-expressing C-fibres [2]. Acrolein, a highly electrophilic and abundant α , β -unsaturated aldehyde found in smoke derived from combustion of a range of organic materials including wood and tobacco, is a robust activator of TRPA1 [5]. As a highly reactive electrophile and amongst the most noxious substances known in toxicology, acrolein elicits a raft of effects on the airway [6], some of which may be mediated via activation of the

TRPA1 channel. For example, activation of TRPA1 plays a major intermediary role in acrolein-induced cough and neurogenic inflammation [7, 8]. In addition to acrolein, several other volatile irritants also readily activate TRPA1, including the electrophilic smoke component crotonaldehyde, [5, 7], the pungent component of mustard and wasabi allyl isothiocyanate (AITC), and the major pungent component of cinnamon cinnamaldehyde [8, 9].

The activation of TRPV1 by capsaicin likely mediates a range of profound airway responses including bronchorelaxation in mouse isolated airways via local axonal release of neuropeptides such as Substance P (SP) [4, 10]. However, it is not currently known whether activators of TRPA1 produce similar responses in mouse airways. Thus, the first aim of the current study was to examine the effects of known activators of TRPA1 including acrolein, crotonaldehyde, AITC, and cinnamaldehyde, on airway smooth muscle tone in mouse isolated trachea. Upon observing robust relaxation responses, the second aim of the study was to characterise the cellular and molecular mechanisms underpinning the novel relaxation response to acrolein. Using a combination of isometric tension recording studies and complementary biochemical assays, we demonstrated that acrolein induces a novel relaxation response in mouse isolated trachea which most likely involves interplay between multiple components of the airways including TRPA1-expressing sensory C-fibres, neighbouring epithelial cells and the airway smooth muscle.

2. MATERIALS AND METHODS

2.1. Mouse isolated tracheal preparations

Male BALB/c mice aged 7-8 weeks (Animal Resources Centre, Murdoch, WA) were kept on a 12 h light/dark cycle and given access to autoclaved food and water ad libitum until killed with an overdose of sodium pentobarbitone (160 mg kg^{-1} , i.p.) and exsanguinated by severing the right renal artery. Tracheae were then excised from the airways, cleared from surrounding tissue, and, for functional studies, bisected to yield two equal segments (upper and lower). All studies were conducted with the approval of the University of Western Australia Animal Ethics Committee and adhered to the guidelines of the National Health and Medical Research Council of Australia.

2.2. Isometric tension recordings

Tracheal segments were mounted onto two L-shaped stainless steel hooks and suspended in a mini tissue organ bath system (miniTOBs) (Danish, Myotecnology, Denmark) filled with 20 ml (for functional studies) or 2 ml (for PGE₂ studies) of Krebs solution (25 mM NaHCO₃, 1.03 mM KH₂PO₄, 0.57 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, 11.1 mM D-glucose). The organ bath was maintained at 37°C and bubbled continuously with 5% CO₂ in O₂. Changes in tension were recorded via an isometric force transducer connected to a PowerLab[®] data acquisition program (ADInstruments Pty Ltd, Castle Hill, Australia). Following a 30 min equilibration period in which tissues were washed with fresh Krebs every 10 min and the resting tension periodically re-adjusted to ~0.23 g, tracheal segments were sequentially exposed to submaximal (0.2 μM) and supramaximal (10 μM) doses of carbachol in a viability test [11]. After a 15 min washout and recovery period, the viability test was repeated and preparations that responded weakly (<500 mg tension increase) were considered unsuitable for subsequent testing. For viable preparations, the response to the supramaximal dose of carbachol (10 μM) in the second viability test was deemed C_{max}.

2.3. Dose-response curves to TRPA1 activators

Because mouse tracheal smooth muscle does not exhibit inherent airway tone, it was necessary to pre-contract tracheal segments with a spasmogen in order to examine subsequent relaxation responses of the airways [10, 12]. Tracheal preparations were therefore pre-contracted using 0.3 μM carbachol to 60-80% C_{max} and upon reaching a plateau, preparations were exposed to a single bolus dose of one of four TRPA1 activators; acrolein (30 μM), crotonaldehyde (100 μM), cinnamaldehyde (500 μM) or AITC (100 μM), and the maximum relaxation response recorded. The preparation was then washed and rested for 15 min. For acrolein studies, the process was repeated with a second, higher concentration of acrolein. A maximum of two sequential doses of acrolein were administered to any single tracheal preparation. By administering different combinations of acrolein doses to individual tracheal preparations, bolus dose-response curves to acrolein were generated (1-100 μM , half-log increments). All relaxation responses were expressed as % R_{max} , where 100% R_{max} represented a full reversal of the carbachol pre-contraction.

2.4. Antagonist studies to characterise acrolein-induced relaxation responses

To elucidate the mechanisms underlying acrolein-induced relaxation of mouse tracheae, separate tracheal segments were isolated and mounted in the miniTOBs. Preparations were then exposed to an array of inhibitors and antagonists including AP-18 (30 μM , TRPA1 channel inhibitor [13]), HC-030031 (20 μM , selective TRPA1 channel inhibitor [7]), capsazepine (3 μM , TRPV1 channel inhibitor [14]), RP-67580 (20 nM, Neurokinin 1 (NK_1) receptor antagonist [15]), CGRP (8-37) (human) (1 μM , CGRP₁ receptor antagonist [16]), indomethacin (5 μM , non-selective cyclooxygenase (COX)-inhibitor [10]), and PF-04418948 (100 nM, novel EP₂ receptor antagonist [17]), or their respective vehicles (0.02% ethanol (EtOH) or 0.02% dimethyl sulfoxide (DMSO)) for 20 min prior to pre-contraction with 0.3 μM carbachol and exposure to a single bolus dose of 30 μM acrolein. The process of incubation of inhibitors/antagonists for 20 min, pre-contraction with 0.3 μM carbachol, and exposure to an active agent was repeated in single tracheal preparations to

examine responses to SP (single bolus dose of 1 nM for upper segments, 0.1 nM for lower segments), calcitonin gene related peptide (CGRP, multiple bolus doses, 0.01–1 μ M, whole-log increments), or Prostaglandin E₂ (PGE₂, cumulative dose response curve, 0.03–1 μ M, half-log increments) in the absence and presence of the inhibitors/antagonists.

2.5. Quantitation of PGE₂ release

To measure acrolein-induced changes in PGE₂ release, whole tracheal segments were mounted in 2 ml organ baths filled with Krebs solution (as earlier described). Viable tracheal segments were then washed with fresh Krebs to remove any existing PGE₂ in solution, before pre-contraction with 0.3 μ M carbachol (5 min) and exposure to a single bolus dose of acrolein (30 μ M) or a saline control (0.9% NaCl) for 10 min. In some preparations, segments were first exposed to HC-030031 (20 μ M) or vehicle (0.02% DMSO) for 20 min prior to pre-contraction and exposure to acrolein. The entire bathing fluid was then collected and stored at -80°C until analysed using a commercial PGE₂ enzyme immunoassay (EIA) kit according to the manufacturer's instructions (Cayman Chemical Co, no. 514010).

2.6. Chemicals

Acrolein, carbachol (carbamylcholine chloride), crotonaldehyde, HC-030031 (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide), PGE₂, indomethacin, and *trans*-cinnamaldehyde were purchased from Sigma-Aldrich (St Louis, MO), whilst AP-18 (4-(4-Chlorophenyl)-3-methyl-3-buten-2-one oxime), capsazepine (N-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide), PF-04418948 (1-(4-Fluorobenzoyl)-3-[[[(6-methoxy-2-naphthalenyl)oxy]methyl]-3-azetidinecarboxylic acid), RP-67580 ((3aR,7aR)-Octahydro-2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7-diphenyl-4H-isoindol), SP and α -CGRP were obtained from Tocris Bioscience (Ellisville, MO). CGRP 8-37 (human) was purchased

from Auspep (Tullamarine, VIC). Sodium pentobarbitone was supplied by Virbac Australia (Peakhurst, NSW) and the PGE₂ EIA kit purchased from Cayman Chemicals (Ann Arbor MI).

2.7. Solutions

Acrolein, carbachol, PGE₂ and SP were dissolved and serially diluted in sterile saline whilst indomethacin was dissolved in 100mM Na₂CO₃ and α -CGRP in sterile water. *Trans*-cinnamaldehyde, AP-18, capsazepine and RP-67580 were diluted in 100% EtOH, and HC-030031 and PF-04418948 diluted in 100% DMSO such that final bath concentrations of both vehicles (EtOH and DMSO) were ~0.02%.

2.8. Data and statistical analyses

Relaxation (% R_{max}) data and PGE₂ quantitation data are presented as mean $\pm$ SEM, and were analysed by one or two-way ANOVA with post-hoc comparisons (Holm-Sidak method) and Student's t-test, respectively, using SigmaPlot 12.5 (Systat Software, San Jose, CA). Differences were considered significant if $p < 0.05$

3. RESULTS

3.1. TRPA1 activators evoke robust relaxation responses in mouse isolated airways

The TRPA1 activators acrolein (30 μ M), crotonaldehyde (100 μ M), *trans*-cinnamaldehyde (500 μ M) and AITC (100 μ M) all induced rapid relaxation responses in mouse isolated tracheal preparations pre-contracted with carbachol (0.3 μ M), with maximum responses observed within 1 min of administration (Fig.1). Interestingly, the relaxant response produced by 30 μ M acrolein was similar to those produced by the other TRPA1 activators administered at higher concentrations

(100–500 μ M) (Fig.1). Responses to the four TRPA1 activators were significantly greater in lower tracheal segments than in the upper tracheal segments ($p<0.01$, Table 1).

3.2. Acrolein-induced relaxation responses exhibit a dose-dependent relationship

To further characterise acrolein-induced relaxation responses, a bolus dose-response curve to acrolein was generated (0.5 log increments, 1–100 μ M) from which a dose-dependent relationship to acrolein in both upper and lower tracheal segments was established (Fig.2). There were no regional differences between the maximal relaxation responses induced by the highest concentration of acrolein tested (100 μ M) in upper ($58\pm 8\%$, $n=5$) and lower tracheal ($71\pm 6\%$, $n=5$) segments ($p>0.05$). In contrast, responses evoked by a mid-range concentration of acrolein (10 μ M) were significantly greater in lower segments ($53\pm 12\%$, $n=5$) than in upper segments ($24\pm 11\%$, $n=7$, $p<0.05$), indicative of regional differences with respect to acrolein potency.

3.3. Pharmacological characterisation of acrolein-induced relaxation responses

Relaxation responses elicited by 30 μ M acrolein were inhibited by a number of selective TRPA1 inhibitors. In lower tracheal segments, pre-treatment with the selective TRPA1 channel inhibitor AP-18 (30 μ M) attenuated relaxation responses by greater than 90% relative to the EtOH vehicle control ($p<0.001$) (Fig.3A) Also, HC-030031 (20 μ M) significantly inhibited acrolein-induced relaxant responses relative to the DMSO vehicle control ($p<0.001$) (Fig.3B). In contrast, acrolein-evoked relaxation responses were unaffected by the selective TRPV1 channel inhibitor capsazepine (Table 2). The effects of AP-18, HC-030031 and capsazepine on acrolein-induced relaxation responses observed in upper tracheal segments were similar to those observed in lower tracheal segments (Table 2).

Acrolein-elicited relaxations were also suppressed by modulation of effectors which act downstream of the TRPA1 channel. Responses to 30 μ M acrolein were suppressed by the potent

NK₁ receptor antagonist RP-67580 (20 nM) and the novel EP₂ receptor antagonist PF-04419848 (100 nM) in both upper and lower tracheal segments (Fig.3, Table 2). In addition, the non-selective COX inhibitor indomethacin (5 μ M) completely abolished relaxation responses elicited by the highest concentration of acrolein examined (100 μ M) in both upper ($-3\pm 1\%$ vs $58\pm\%$, $n=4$, $p<0.001$) and lower ($-0.5\%\pm 2$ vs $69\pm 6\%$, $n=4$, $p<0.001$) tracheal segments (Fig. 4, Table 2). In contrast, responses to 30 μ M acrolein were unaffected by the CGRP₁ receptor antagonist CGRP 8-37 (Table 2).

3.4. Substance P and PGE₂-induced relaxation responses

The neuropeptide SP elicited relaxation responses in tracheal segments, with 1 nM SP inducing responses in upper segments of a magnitude similar to that produced by 0.1 nM SP in lower segments (Fig. 5, Table 2, 3), reflective of regional differences in sensitivities to SP. In contrast, the neuropeptide CGRP (0.01–1 μ M) failed to evoke consistent relaxation responses in either upper or lower segments ($<5\%$ relaxation, Table 3). As expected, SP-elicited responses were significantly blocked by RP-67580 (20 nM) and inhibited by PF-04418948 (100 nM), but were unaffected by HC-030031 (20 μ M) and AP-18 (30 μ M) (Fig.5, Table 2).

The prostanoid PGE₂ elicited relaxation responses in tracheal segments, with cumulative addition of exogenous PGE₂ inducing responses which were significantly suppressed by PF-04418948 (100 nM) in both upper (78% inhibition, $p<0.001$) and lower (81% inhibition, $p<0.001$) segments such that 50% relaxation was not achieved (Fig. 6B, Table 4). In contrast, maximal PGE₂-induced relaxations were unaffected by pre-treatment with HC-030031 (20 μ M), AP-18 (30 μ M) and RP-67580 (20 nM) (Fig.6, Table 4).

3.5. Acrolein-induced changes in levels of PGE₂ released by tracheal segments

Exposure of tracheal segments to 30 μ M acrolein for 10 min significantly elevated the level of PGE₂ released into the surrounding bathing fluid above basal levels of PGE₂ release, an increase which was attenuated by 20 μ M HC-030031 (Fig. 7A). In addition, a clear correlation was established between acrolein-induced elevations in PGE₂ release and the relaxation responses observed in the same tracheal preparations from which the bathing fluid was collected for EIA analysis (Fig. 7B).

4. DISCUSSION

In the present study, we demonstrated that the TRPA1 channel activator acrolein is capable of evoking robust relaxation responses in mouse isolated airways. Chemically-related electrophilic and volatile compounds known to activate TRPA1 including crotonaldehyde, cinnamaldehyde and AITC also produced relaxation responses in mouse isolated tracheal preparations. Further pharmacologic studies indicated that the novel relaxation response induced by acrolein likely occurs via sequential (1) activation of the TRPA1 channel expressed on sensory C-fibres, (2) release of neuropeptides by activated sensory C-fibres, (3) activation of NK₁ receptors expressed on neighbouring epithelial cells, (4) generation and release of a PGE₂ by epithelial cells, and (5) activation of EP₂ receptors expressed on neighbouring airway smooth muscle cells.

We first examined the ability of acrolein and additional volatile TRPA1 channel activators, including crotonaldehyde, cinnamaldehyde, and AITC to evoke relaxation of mouse isolated airways. This relaxation response has been previously reported to be induced by the TRPV1 activator capsaicin in mouse airways [4, 10]. The four TRPA1 activators were examined at biologically-relevant concentrations and which are thought to activate the TRPA1 channel [5, 8, 9],

Common to the four TRPA1 activators evaluated in the present study were a volatile nature and high electrophilicity, the latter feature permitting covalent modification of nucleophilic cysteine residues within the ankyrin repeat sequences in the N-terminus, a likely prerequisite to channel activation [18, 19]. Our studies have shown, for the first time, that an array of electrophilic compounds and activators of the TRPA1 channel produce relaxation responses in both upper and lower regions of mouse isolated trachea.

The findings that acrolein-induced relaxation responses were attenuated by two selective TRPA1 channel inhibitors HC-030031 and AP-18 whilst unaffected by the selective TRPV1 channel inhibitor capsazepine suggest that activation of TRPA1, but not TRPV1, plays a key role in mediating this response. This concurs with prior observations that the TRPA1 channel is not only robustly activated by acrolein but a key chemosensory receptor for acrolein, with sensory neurons from TRPA1-deficient mice shown to lack any responsiveness to acrolein [5, 7]. Whilst complete abolition of acrolein-induced relaxation responses was not achieved by HC-030031 or AP-18 in either upper and lower segments in our study, the marked suppression of relaxation responses by these TRPA1 inhibitors suggests that TRPA1 channel activation plays a major role in mediating this response. Future use of TRPA1-deficient mice may yield further insights into whether the TRPA1 channel is the sole chemosensory receptor involved in relaxation responses evoked by acrolein.

Downstream of TRPA1 channel activation, neuropeptides may be released peripherally by activated C-fibre endings via a local axonal reflex. Indeed, high concentrations of acrolein exposure have been reported to deplete the neuropeptide content of airway sensory C-fibres, resulting in the release of tachykinins such as SP [20], and CGRP [21, 22]. SP possesses well-documented relaxant effects, producing relaxation of airway smooth muscle in several rodent species including mouse and rat [4, 10, 16, 23-25], consistent with the findings of our study. In contrast, the relaxant effects of CGRP are less clear, with some reports of small and erratic relaxations responses evoked in

mouse, rat, and pig airways [4, 16, 26]. In the current study, CGRP failed to evoke consistent relaxation responses in mouse tracheae. Furthermore, blockade of the CGRP₁ receptor by CGRP 8-37 did not affect relaxations produced by acrolein. Taken together, these findings suggest that CGRP is unlikely to play a major mediatory role in acrolein-induced relaxation. The well-established relaxation elicited by SP has been shown to be an epithelium-dependent response [24] and the complete absence of responses in NK₁ -/- mice has provided definitive evidence for a role of the NK₁ receptor in SP- evoked relaxation responses [16]. In accordance with previous studies [15, 16], the NK₁ receptor inhibitor RP-67580 abolished exogenous SP-induced relaxation responses in the current study thereby confirming its pharmacologic site of action. Importantly, RP-67580 significantly suppressed acrolein-evoked relaxation, providing evidence for a role of SP and activation of its cognate receptor, the NK₁ receptor, in this novel response.

SP-induced relaxation responses are thought to involve the release of epithelial cell relaxant products, and in mouse and rat airways there is compelling evidence that the COX product PGE₂ is a key relaxant product mediating this response [10, 15, 24, 27]. Acrolein-evoked relaxation responses observed in our study were completely abolished by the non-selective COX inhibitor indomethacin, indicating that a COX-derived epithelial cell product is essential to mediating this response. Consistent with this, acrolein was found to elevate the levels of PGE₂ released into the surrounding bathing fluid. Furthermore, there was a clear correlation between changes in PGE₂ release and relaxations produced; the relaxation responses were associated with a significant increase in PGE₂ release and the inhibition of the relaxation response by HC-030031 (20μM) associated with a reduction in PGE₂ release. These findings are congruent with reports that PGE₂ is released in response to relaxant agents, such as capsaicin, which elicit C-fibre nerve activation via the TRPV1 channel [10]. Thus, it appears that C-fibre-mediated relaxation of mouse airways by different irritant stimuli is produced via a common pathway involving the release of epithelial-derived PGE₂.

Following generation and release from epithelial cells, PGE₂ induces relaxation responses via activation of target receptors on airway smooth muscle cells. Although four EP receptor subtypes exist (EP₁₋₄), the EP₂ receptor, which is preferentially coupled to the G_s protein to activate adenylate cyclase activity and increase cyclic AMP levels, is the major subtype mediating PGE₂-induced relaxation of smooth muscle in mouse airways [27-29]. Recent discovery of PF-0441948, a selective EP₂ receptor antagonist shown to exhibit a markedly improved selectivity for the EP₂ receptor compared to the commonly-used EP₂ receptor antagonist AH6809 [30], has consolidated these findings [31]. In the current study, marked suppression of exogenous PGE₂-induced relaxation responses by PF-04418948 provides further evidence that the EP₂ receptor is a key mediator in the relaxation of murine tracheal smooth muscle by PGE₂. Importantly, suppression of acrolein-induced relaxation responses by PF-04418948 firmly implicates involvement of the EP₂ receptor activation in this response, and is consistent with the target receptor of the relaxant mediator proposed to be released by neuropeptide-activated epithelial cells, namely PGE₂. In summary, results of the present study provide strong evidence implicating prostanoid involvement in acrolein-induced relaxation of mouse airway smooth muscle. Firstly, responses were completely abolished by the (non-selective) COX inhibitor indomethacin. Secondly, increases in PGE₂ released into the surrounding bathing fluid were measured and correlated strongly with the extent of the relaxation responses observed, and thirdly, inhibition of the target prostanoid receptor EP₂ with PF-04418948 attenuated the relaxation response.

In keeping with reported potencies of the four TRPA1 channel activators examined in our study [2], acrolein induced similar relaxation responses at concentrations lower than those of the other three activators (crotonaldehyde, cinnamaldehyde, AITC). Though difficult to measure, concentrations of acrolein reached in the lung during smoking have been reported to be as high as 80 µM [32], which may be exceeded during acute intoxication with larger volumes of smoke. Thus, the concentrations of acrolein examined in the present study are likely to represent a biologically-

relevant range that may be encountered *in vivo*. Closer examination of acrolein-induced relaxation responses revealed regional differences, with acrolein shown to be more potent in lower segments than in upper segments. Similar regional differences were observed for the other TRPA1 activators examined in the current study and have also been reported for other relaxant agents including the PAR₂ activator SLIGRL [33] and the neuropeptide Substance P [10] which exhibit a greater potency in lower mouse airways than in upper airways. The reasons for these regional differences to acrolein remain to be fully elucidated but might arise from differences in the quantity of, or sensitivity to, mediators released downstream of TRPA1 channel activation, for reasons yet to be fully elucidated. For example, it is conceivable that in lower tracheal segments, increases in the quantity of PGE₂ released (which accounts for potency differences seen for SLIGRL [33]) or sensitivity to Substance P (as observed in our study and in previous studies [10]) might underpin observations of increased potency of acrolein in lower tracheal segments compared to upper segments. Parallel regional differences may not have been observed at higher concentrations of acrolein because the maximum response to PGE₂ and/or SP may have been reached.

The physiological significance of the relaxation response induced by acrolein may lie in its potential modulatory role and bronchoprotective function, representing an additional protective response initiated by the network of sensory nerves widely thought to play a crucial protective role in the airways. The acute release of neuropeptides is known to confer protection against acrolein, with prior depletion of neuropeptides and desensitisation of C-fibres exacerbating acrolein-induced lung damage [20]. Just how might the bronchodilation response observed in the current study confer a bronchoprotective outcome? Upon inhalation of an irritant, many of the other well-documented effects evoked by neuropeptides, including bronchoconstriction, mucous hypersecretion, vasodilatation and increased vascular permeability, collude to promote narrowing of the airway lumen [34-36]. Uncontrolled or unmodulated activation of target receptors by protective neuropeptides may lead, somewhat paradoxically, to the production of inappropriate reflexes that

may shift from being protective to deleterious in nature [35]. Therefore, it is possible that a co-existing but opposing bronchodilatory effect of SP may help protect the airways against excessive neurogenic activity and uncontrolled airway lumen narrowing. The modulatory role of this relaxation response has indeed been characterised, and proposed to form an important component of the “sensory nerve inhibitory system”; a system first described by Szarek and co-workers to exist in rodent airways which may be crucial for maintaining airway patency [16, 23]. Negative regulation of smooth muscle contraction by this inhibitory system may therefore represent an additional protective effect of sensory nerve activation by virtue of its role in maintaining open and unobstructed airways.

In summary, our study has demonstrated that acrolein causes relaxation of mouse isolated trachea. This novel response appears to involve activation of cellular targets on several major components of the airways. Pharmacologic studies indicated that acrolein-induced relaxation likely involves the activation of TRPA1 channels on sensory C-fibres. It is postulated that acrolein-activated sensory C-fibres release neuropeptides that stimulate NK₁ receptors on neighbouring epithelial cells. These neuropeptide-activated epithelial cells release relaxant prostanoid products, including PGE₂, which stimulate EP₂ receptors on airway smooth muscle to induce relaxation (Fig.8). This relaxation response provides intriguing insight into a potential protective role of the airway sensory nerves by way of their modulatory function in maintaining the balance between bronchoconstriction and airway patency.

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CONFLICT OF INTEREST

None.

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FIGURES and TABLES

Fig. 1. Representative isometric tension recordings of relaxation responses evoked by the TRPA1 activators acrolein (ACR, 30 μM), crotonaldehyde (CRA, 100 μM), cinnamaldehyde (CNA, 500 μM), and allyl isothiocyanate (AITC, 100 μM) in mouse isolated lower tracheal smooth muscle preparations pre-contracted with 0.3 μM carbachol.

Fig. 2. Bolus dose-response curve to acrolein in upper and lower mouse tracheal segments. Data are presented as mean $\pm$ SEM (n=4–7). *p<0.05 compared to response evoked by acrolein at corresponding concentrations in upper segments.

Fig. 3. Effect of 30 μ M AP-18, 20 nM RP-67580 (A), 20 μ M HC-030031 and 100 nM PF-04418948 (B) on acrolein-induced relaxation responses in lower mouse tracheal segments. Data are presented as mean $\pm$ SEM (n=3–9). ***p< 0.001 compared to respective vehicle.

Fig. 4. (A) Effect of 5 μ M indomethacin (indo) on acrolein-induced relaxation responses of carbachol pre-contracted upper and lower mouse tracheal segments. Data are presented as mean $\pm$ SEM (n=4). ***p< 0.001 compared to respective control. (B) Representative isometric tension recordings demonstrating full abolition of acrolein-induced relaxation responses by 5 μ M indomethacin.

Fig. 5. Effect of 30 μ M AP-18, 20 nM RP-67580 (A), 20 μ M HC-030031 and 100 nM PF-04418948 (B) on Substance P-induced relaxation responses in lower mouse tracheal segments. Data are presented as mean $\pm$ SEM (n=4–5). *p<0.05, ***p< 0.001 compared to respective vehicle.

Fig. 6. Cumulative dose-response curves to PGE₂ in lower mouse tracheal segments. Effects of 30 μM AP-18, 10 μM capsazepine and 20 nM RP-67580 on PGE₂ induced relaxation responses (A). Effects of 20 μM HC-030031 and 100 nM PF-04418948 on PGE₂ –induced relaxation responses (B). Data are presented as mean ± SEM (n=3–9). ***p< 0.001, maximal relaxation response produced in the presence of 20 μM HC-030031 compared to DMSO vehicle.

Fig. 7. Quantitation of PGE₂ released by tracheal segments into surrounding bathing fluid. (A) Effect of acrolein (ACR, 30 μM) on PGE₂ release from carbachol pre-contracted mouse tracheal preparations. The effect of HC-030031 (20 μM) on acrolein-induced PGE₂ release was also assessed. (B) Correlation between acrolein-induced PGE₂ release and tracheal smooth muscle relaxation (n=7-9). Data are presented as mean ± SEM (n= 7–9). ***p<0.001, *p<0.05 compared to respective control/vehicle.

Fig. 8. Schematic outlining the postulated paradigm of acrolein-induced relaxation of mouse airways involving the interplay between three major structures of the airways – the airway sensory nerves, epithelium and smooth muscle.

Table 1. Relaxation responses induced by volatile TRPA1 channel activators in upper and lower mouse tracheal segments

TRPA1 activator (concentration)	Chemical structure	Properties & source(s)	Region (n)	Relaxation (%)
Acrolein (30 μ M)		Highly electrophilic α,β -unsaturated aldehyde, volatile and prevalent component of organic smoke	Upper (7)	37 $\pm$ 7
			Lower (4)	68 $\pm$ 11
Crotonaldehyde (100 μ M)		Electrophilic α,β -unsaturated aldehyde, volatile component of smoke	Upper (5)	30 $\pm$ 7
			Lower (4)	47 $\pm$ 8
<i>Trans</i> -Cinnamaldehyde (500 μ M)		Electrophilic derivative of acrolein, major pungent ingredient in cinnamon	Upper (6)	15 $\pm$ 4
			Lower (5)	24 $\pm$ 7
Allyl isothiocyanate (100 μ M)		Electrophilic, major pungent ingredient in mustard and wasabi	Upper (5)	32 $\pm$ 8
			Lower (4)	52 $\pm$ 11

Table 2. Effect of selected inhibitors and antagonists on relaxation responses induced by acrolein and Substance P in mouse tracheal segments

Agonist (concentration)	Treatment	Notes	Region (n)	Relaxation (%)
Acrolein (100 μ M)	None	–	Upper (4)	58 $\pm$ 9
			Lower (4)	69 $\pm$ 6
	Indomethacin (5 μ M)	Non-selective cyclooxygenase inhibitor	Upper (4)	-3 $\pm$ 1 ^a
			Lower (4)	-0.5 $\pm$ 2 ^a
Acrolein (30 μ M)	None	–	Upper (3)	18 $\pm$ 0
			Lower (3)	33 $\pm$ 1
	CGRP (8-37) (human) (1 μ M)	Peptide CGRP ₁ antagonist	Upper (3)	13 $\pm$ 3
			Lower (4)	33 $\pm$ 7
	EtOH (0.02%, vehicle)	Vehicle for AP-18, RP-67580,	Upper (8)	37 $\pm$ 5
			Lower (8)	36 $\pm$ 5
	AP-18 (20 μ M)	Selective TRPA1 channel blocker	Upper (3)	10 $\pm$ 7 ^b
			Lower (3)	3 $\pm$ 1 ^d
	RP-67580 (20 nM)	Selective NK ₁ receptor antagonist	Upper (8)	17 $\pm$ 6 ^b
			Lower (9)	9 $\pm$ 4 ^d
	Capsazepine vehicle (0.02% EtOH)	–	Upper (5)	18 $\pm$ 3
			Lower (3)	53 $\pm$ 10
	Capsazepine (3 μ M)	Selective TRPV1 channel blocker	Upper (9)	17 $\pm$ 3
			Lower (5)	34 $\pm$ 3
Substance P (0.1 nM, lower segments; 1 nM, upper segments)	DMSO (0.02%, vehicle)	Vehicle for HC-030031, capsazepine, PF-04419848,	Upper (4)	33 $\pm$ 5
			Lower (4)	50 $\pm$ 3
	HC-030031 (20 μ M)	Selective TRPA1 channel blocker	Upper (3)	3 $\pm$ 2 ^c
			Lower (3)	25 $\pm$ 3 ^d
	PF-04418948 (100 nM)	Novel EP ₂ receptor antagonist	Upper (3)	15 $\pm$ 3 ^b
			Lower (3)	17 $\pm$ 4 ^d
	EtOH (0.02%, vehicle)	–	Upper (8)	52 $\pm$ 7
			Lower (5)	71 $\pm$ 8
	AP-18 (20 μ M)	<i>See above</i>	Upper (4)	51 $\pm$ 11
			Lower (4)	56 $\pm$ 9
	RP-67580 (20 nM)	<i>See above</i>	Upper (4)	2 $\pm$ 1 ^c
			Lower (5)	3 $\pm$ 2 ^d
	Capsazepine vehicle (0.02% EtOH)	–	Upper (3)	37 $\pm$ 3
			Lower (5)	59 $\pm$ 12
	Capsazepine (3 μ M)	<i>See above</i>	Upper (6)	43 $\pm$ 9
			Lower (12)	42 $\pm$ 7
	DMSO (0.02%, vehicle)	–	Upper (6)	44 $\pm$ 5
			Lower (6)	70 $\pm$ 7
	HC-030031 (20 μ M)	<i>See above</i>	Upper (6)	37 $\pm$ 6
			Lower (5)	47 $\pm$ 15
	PF-04418948 (100 nM)	<i>See above</i>	Upper (4)	17 $\pm$ 3 ^b
			Lower (5)	26 $\pm$ 4 ^b

^a p<0.001 compared to respective control

^b p< 0.05 compared to respective vehicle

^c p< 0.01 compared to respective vehicle

^d p< 0.001 compared to respective vehicle

Table 3. Relaxation responses evoked by neuropeptides Substance P and CGRP in upper and lower mouse tracheal segments

Agonist	Concentration (nM)	Region (n)	Relaxation (%)
Substance P	1	Upper (13)	49±5
	0.1	Lower (14)	68±4
CGRP	10	Upper (6)	-4±2 ^a
		Lower (4)	-4±2 ^a
	100	Upper (5)	0±2
		Lower (4)	-4±3 ^a
	1000	Upper (5)	-7±4 ^a
		Lower (4)	-9±3 ^a

^a Negative relaxations reflect a contractile response ("negative" reversal of pre-contraction with 0.3 µM carbachol)

Table 4. Effects of selected antagonists on relaxation responses evoked by PGE₂ in mouse tracheal segments

Agonist	Treatment	Region (n)	Relaxation parameters	
			-log EC ₅₀	R _{max} (%)
PGE ₂	EtOH vehicle (0.02%)	Upper (8-9)	6.7 ± 0.3	83 ± 6
		Lower (7)	7.0 ± 0.2	86 ± 4
	RP-67580 (20nM)	Upper (4)	7.0 ± 0.1	94 ± 4
		Lower (5-9)	7.3 ± 0.1	91 ± 5
	AP-18 (20μM)	Upper (6)	6.8 ± 0.2	94 ± 3
		Lower (5)	6.8 ± 0.2	96 ± 3
	Capsazepine (3μM)	Upper (3)	7.1 ± 0.1	99 ± 1
		Lower (3)	7.3 ± 0.1	98 ± 1
	DMSO vehicle (0.02%)	Upper (4)	5.8 ± 0.1	90 ± 5
		Lower (3)	7.3 ± 0.1	86 ± 4
	PF-04418948 (100nM)	Upper (3)	N.D.	20 ± 6 ^a
		Lower (3)	N.D.	16 ± 4 ^a
	HC-030031 (20μM)	Upper (5)	6.6 ± 0.2	99 ± 1
		Lower (3)	6.5 ± 0.3	95 ± 2

N.D., -log EC₅₀ not determined because 50% relaxation not reached

^a p < 0.001 compared to DMSO vehicle