

# TRPA1 Agonists Evoke Coughing in Guinea Pig and Human Volunteers

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**Rationale:** Cough is the most frequent reason for consultation with a family doctor, or with a general or respiratory physician. Treatment options are limited and one meta-analysis concluded that over-the-counter remedies are ineffective. There is also increasing concern about their use in children. Environmental irritants such as air pollution and cigarette smoke are thought to evoke cough by stimulating airway sensory nerves; however, how this occurs is not fully understood.

**Objectives:** We hypothesized that the TRPA1 (transient receptor potential cation channel, subfamily A, member 1) receptor may have a role as a novel target for tussive agents given that many potential irritants have been shown to activate this channel.

**Methods:** We investigated the effect of TRPA1 ligands on vagal sensory nerve activity *in vitro* and in guinea pig and human tussive challenge models.

**Measurements and Main Results:** We demonstrated that TRPA1 agonists such as acrolein activate cloned human TRPA1 channels in HEK293 cells and also vagal sensory nerves in murine, guinea pig, and human tissues. A role for TRPA1 was confirmed, using specific inhibitors and tissue from *Trpa1*<sup>-/-</sup> gene-deleted animals. Finally, TRPA1 ligands evoked reproducible tussive responses in both a guinea pig model and normal volunteers.

**Conclusions:** This study identifies the TRPA1 receptor as a promiscuous receptor, activated by a wide range of stimuli, making it a perfect target for triggering cough and as such one of the most promising targets currently identified for the development of antitussive drugs.

**Keywords:** sensory nerves; cough; vagus

Cough is the most frequent reason for consultation with a family doctor (1), or with a general or respiratory physician. Patients with chronic cough probably account for 10–38% of respiratory outpatient practice in the United States (2). Chronic cough, of various etiologies, is a common presentation to specialist respiratory clinics and is reported as a troublesome symptom by 7% of the population (3). Treatment options are limited. A meta-analysis concluded that over-the-counter cough remedies are ineffective (4), and there is increasing concern about the use of

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

TRPA1 ligands are known to activate airway sensory nerves. However, there is little knowledge concerning the functional effects of these agents. No data currently exist profiling these ligands in clinical studies in the lung.

### What This Study Adds to the Field

TRPA1 ligands evoked reproducible tussive responses in an animal model and in humans.

over-the-counter therapies in children. Despite its importance our understanding of the mechanisms that provoke cough is poor.

Ion channels of the transient receptor potential (TRP) class have been implicated in the afferent sensory loop of the cough reflex (5–7) and in the heightened cough sensitivity seen in disease (8). Agonists of the TRPV1 (transient receptor potential cation channel, subfamily V, member 1) capsaicin receptor, such as vanilloids and protons, are among the most potent chemical stimuli that cause cough (5–7). Another TRP receptor, TRPA1 (transient receptor potential cation channel, subfamily A, member 1), which is not activated by capsaicin, has been shown to bind ligands such as acrolein, which is present in air pollution and the acrid smoke from organic material (9). We hypothesized that TRPA1 may have a role as a novel target for tussive agents.

TRPA1 is a Ca<sup>2+</sup>-permeant nonselective channel with 14 ankyrin repeats in its amino terminus, which belongs to the larger TRP family (10). TRPA1 has been characterized as a thermoreceptor that is activated by cold temperature (11). In addition, TRPA1 channels are activated by a range of natural products such as allyl isothiocyanate, allicin, and cannabidiol, found in mustard oil, garlic, and cannabis, respectively (12–14). The channel is also activated by a multitude of environmental irritants such as isothiocyanate, cinnamaldehyde, and acrolein. The latter is present in air pollution, vehicle exhaust, and cigarette smoke (9, 15–20). TRPA1 is expressed primarily in small-diameter, nociceptive neurons where its activation probably contributes to the perception of noxious stimuli and the phenomena known as inflammatory hyperalgesia and neurogenic inflammation (9, 17, 19).

The respiratory tract is innervated by sensory afferent nerves including the myelinated, rapidly adapting receptors and the nonmyelinated, chemosensitive C-fibers, which are activated by mechanical and chemical stimuli (21, 22). Activation of these vagal sensory afferents leads to central reflexes including cough. It has been demonstrated that stimulating TRPA1 channels activates vagal bronchopulmonary C-fibers in the guinea pig and rodent lung (23–25).

The aim of these experiments was to employ a series of preclinical and clinical studies to determine, for the first time, whether activation of the TRPA1 channel can evoke a tussive response. Evidence to this effect could result in a deeper understanding of the pathogenesis of cough and lead to the development of novel treatment modalities.

## METHODS

### Functional Characterization of Cloned TRPA1 Expressed in HEK293 Cells

TRPA1-expressing HEK293 cells and the method used to measure increases in intracellular calcium levels in this study have been described previously (26; and see the online supplement).

### Animals

Male Dunkin-Hartley guinea pigs (300–500 g) were housed in a temperature-controlled (21°C) room with food and water freely available for at least 1 week before commencing the experiment. The experiments were performed in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986.

### Effect of TRPA1 Ligands on Isolated Vagal Nerve Preparation

To demonstrate a functional response of TRPA1 channel activation in native tissue we used our fully characterized isolated vagal nerve preparation as described in previous publications (27, 28). Sensory nerve responses to the two TRPA1 agonists, acrolein and cinnamaldehyde, were determined. For these experiments only one response to one agonist was obtained in each segment of vagus. We also performed experiments with the selective TRPA1 inhibitor, HC-030031, or vehicle (dimethyl sulfoxide, 0.1% [vol/vol]) (17). To demonstrate antagonist selectivity HC-030031 was also tested against the TRPV1 agonist capsaicin, using a parallel protocol. To provide additional proof-of-concept data an alternative antagonist was also used, AP-18 (29).

To further confirm that the response observed was via activation of the TRPA1 channel we performed parallel agonist (acrolein) experiments using vagal tissue from wild-type and *Trpa1*<sup>-/-</sup> gene-deleted mice (Jackson Laboratory, Bar Harbor, ME). We confirmed the deletion of the *Trpa1*<sup>-/-</sup> gene in the knockout mice, and not the wild type, using standard genotyping techniques.

### Effect of TRPA1 Ligand Acrolein in Guinea Pig Conscious Cough Model

Cough was detected both by pressure change and by sound and recorded with a Buxco cough analyzer (Buxco, Wilmington, NC) as previously described (28). Guinea pigs received an aerosol of vehicle (0.9% sterile saline, *n* = 12) or acrolein (10, 30, 100, or 300 mM, *n* = 12, concentration derived from a preliminary study) for 5 minutes and coughs were counted during this period and for a further 5 minutes with the Buxco cough analyzer.

Having established a submaximal dose of acrolein, we confirmed the role of TRPA1 using the selective inhibitor HC-030031. Guinea pigs were dosed with vehicle (0.5% methyl cellulose in sterile saline at 1 ml/kg, administered intraperitoneally, *n* = 12) or HC-030031 (300 mg/kg, dose selected from data published by McNamara and colleagues [17]). One hour later the guinea pigs were exposed to a submaximal dose of TRPA1 agonist and cough was monitored as outlined previously.

### In Vitro Functional Characterization of TRPA1 in Isolated Human Vagal Tissue

Briefly, human trachea, with branches of the cervical vagus still attached, was obtained from three unused donor tissue samples surplus to clinical requirement (two males, 32–55 yr of age) collected for heart-lung transplantation. Relevant approvals were obtained from the next of kin and the Royal Brompton and Harefield Trust Ethics Committee. The isolated human vagus was exposed to a TRPA1 agonist and antagonist as described previously for the guinea pig experiments.

### Characterization of Tussive Response to Inhalation of TRPA1 Agonist in Human Volunteers

Healthy male and female, nonsmoking volunteers with normal lung function were entered into a randomized, crossover study of inhalational cough challenge of three tussive agents. Informed written consent was obtained from all the volunteers and the study was approved by the Hull and East Yorkshire Ethics Committee. Volunteers were randomized to inhalational cough challenge with the TRPA1 agonist diluent or cinnamaldehyde or capsaicin or citric acid. At Visit 1 a baseline cough challenge was performed and repeated 1 hour later with the same agent. The subjects were asked to return on two occasions 2–3 days apart when the protocol was repeated with an alternative cough stimulus. Each subject was recalled 2–3 days later for Visit 4 when a subthreshold dose (i.e., the dose below that evoking cough) of cinnamaldehyde or diluent was administered followed by a repeat cough challenge to capsaicin and citric acid, which was performed 1 hour later. This was repeated 2–3 days later at Visit 5 with the alternative subthreshold inhalation. Safety assessments were performed at all these visits.

Nebulizations were performed with a MEFAR MB3 dosimeter (Mefar Elletromedicale, Bovezzo, Italy) with a Respironics nebulizer chamber and mouthpiece according to our previously described methodology (30). The dosimeter was set to nebulize for 1 second and 2 ml of the agent was instilled into the nebulizer chamber. Coughs induced were assessed during the subsequent 15 seconds after the nebulization. Serial dilutions from stock (1 M citric acid, 1 mM capsaicin) were made with 0.9% saline whereas 50% ethanol was used as the diluent for cinnamaldehyde (800 mM).

### Compounds and Materials

Fluo-3 acetoxymethyl ester and LipofectAMINE 2000 were purchased from Invitrogen (Paisley, UK). Penicillin–streptomycin, fetal calf serum, Dulbecco's modified Eagle's medium, sodium pyruvate, and geneticin (G418) were all bought from GIBCO (Paisley, UK). The TRPA1 inhibitor HC-030031 was purchased from ChemBridge (San Diego, CA). All other agents were purchased from Sigma-Aldrich (Poole, Dorset, UK). All other details are in the online supplement.

### Data Analysis and Statistics

Antagonism of TRPA1 agonists was analyzed by two-tailed paired *t* test, comparing responses to agonist (in the same piece of vagus nerve) in the absence and presence of antagonist. Responses to TRPA1 ligands in gene-deleted mice were analyzed by Kruskal-Wallis test for multiple comparisons with Dunn's post-hoc test, comparing the responses in each TRPA1 receptor knockout with those of the wild-type control. Inhibition of acrolein-induced cough was analyzed by Mann-Whitney U test for nonparametric data. Data are presented as means ± SEM and statistical significance is denoted as \**P* < 0.05.

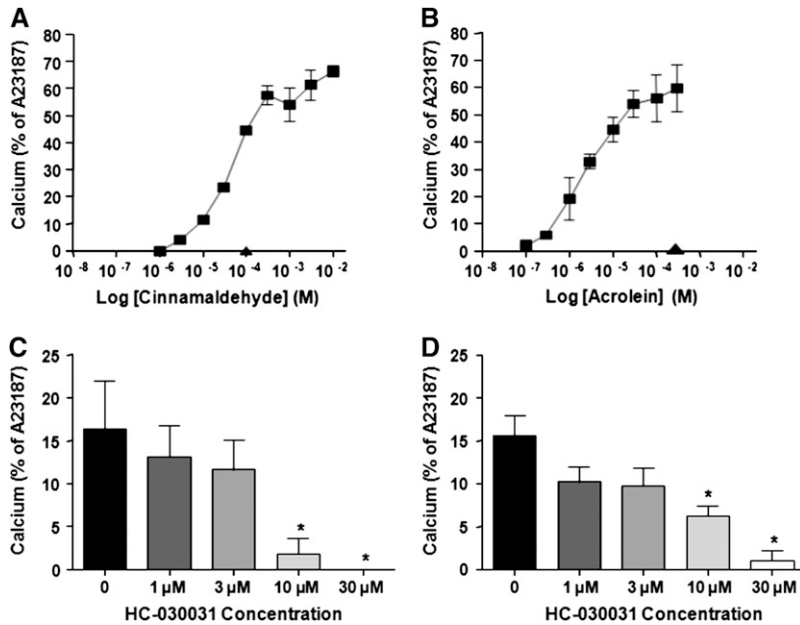
In the clinical study end points were taken as the concentration at which two or more coughs were induced (C2) and at which five or more coughs were induced (C5). For the purposes of statistical analysis the data were log transformed. SPSS version 13.0 was used for statistical analysis. Paired *t* tests or Wilcoxon analysis was performed when applicable. A *P* value of 0.05 or less was considered statistically significant.

## RESULTS

### In Vitro Functional Characterization of TRPA1 Ligands on Cloned Human TRPA1 Channels in HEK293 Cells

The TRPA1 receptor was successfully cloned from primary human fibroblasts and permanently expressed in HEK293 cells. Reverse transcription-polymerase chain reaction showed that HEK293 cells do not endogenously express TRPA1 mRNA (see Figure E1 in the online supplement).

Calcium signaling was used to assess agonist-induced activation of TRPA1-HEK cells (Figure 1). hTRPA1-HEK cells responded to both cinnamaldehyde and acrolein in a concentration-dependent manner (Figure 1). None of the agonists tested stimulated an observable increase in calcium in nontransfected



**Figure 1.** Calcium signaling was used to assess agonist induced activation of TRPA1-HEK cells and agonist-induced activation of TRPA1-HEK cells after preincubation with the antagonist HC-030031. (A) Cinnamaldehyde concentration-response curve (squares) in hTRPA1-HEK cells. hTRPV1-HEK cells failed to respond to 100 μM cinnamaldehyde (triangle). (B) Acrolein concentration-response curve (squares) in hTRPA1-HEK cells. hTRPV1-HEK cells failed to respond to 300 μM acrolein (triangle). (C) Effect of increasing HC-030031 concentrations on cinnamaldehyde (30 μM)-evoked calcium responses. (D) Effect of increasing HC-030031 concentrations on acrolein (3 μM)-evoked calcium responses. Results are expressed as means ± SEM of up to seven experiments, each performed in duplicate. \*Statistical significance ( $P < 0.05$ ) from vehicle-treated group.

or mock-transfected HEK cells or in hTRPV1-HEK cells (Figure 1). These results also demonstrated that HC-030031 caused inhibition of both cinnamaldehyde- and acrolein-evoked responses in a concentration-dependent manner.

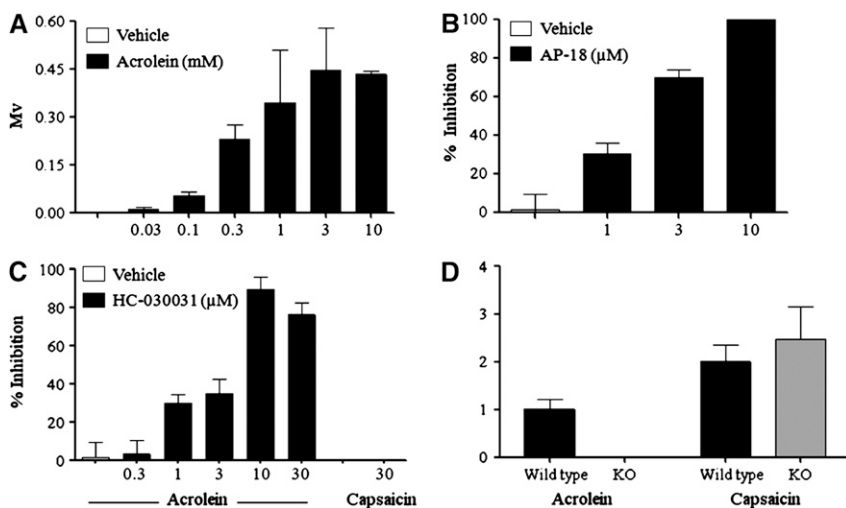
#### Effect of TRPA1 Ligands on Isolated Guinea Pig Vagal Nerve Preparation

Tussive agents such as capsaicin, low-pH solutions, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) are known vagal sensory nerve stimulants and as such isolated guinea pig, murine, and human vagus nerve preparations have been shown to elicit nerve depolarization responses to these stimulants (27, 28, 31). Furthermore, these agents are also known tussigenic agents in human and animal studies (5, 6, 32). These data suggest that the isolated vagus nerve is a useful and predictive preparation for conducting comprehensive pharmacological assessments of agents that may activate or inhibit sensory nerve function and thus the cough reflex. Previous data also suggest that the information generated is broadly predictive of data generated in single afferent nerve fiber recording studies, further validating the use of the isolated vagus preparation.

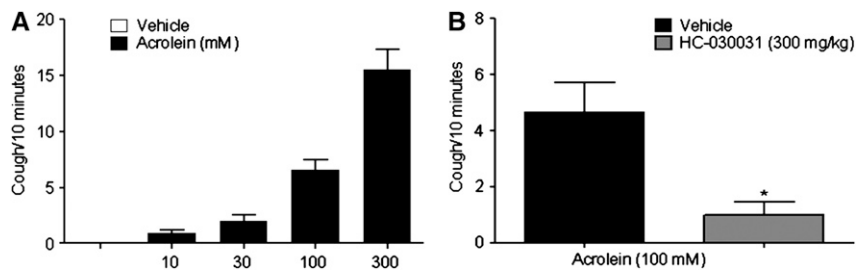
Therefore, to demonstrate a functional role for the TRPA1 channel in a native tissue, we demonstrated a concentration-related increase in the depolarization of the guinea pig isolated vagus (indicative of sensory nerve activation) with acrolein (Figure 2A). Furthermore, data obtained with cinnamaldehyde produced a similar pattern (see Figure E2 in the online supplement). To further confirm a role for TRPA1, we demonstrated that the selective TRPA1 inhibitors AP-18 and HC-030031 blocked depolarization in response to acrolein (Figures 2B and 2C). However, HC-030031 did not affect responses evoked by capsaicin (Figure 2C). In addition, we have shown that the vagus from *Trpa1*<sup>-/-</sup> mice failed to respond to the TRPA1 agonist acrolein, but depolarized in response to a TRPV1 agonist, capsaicin. The wild-type preparations responded appropriately to these stimuli (Figure 2D).

#### Effect of TRPA1 Ligand Acrolein in a Conscious Guinea Pig Cough Model

To demonstrate that the observations in the isolated tissue translate *in vivo* we exposed guinea pigs to aerosolized acrolein. As can be seen in Figure 3, the TRPA1 agonist caused a dose-



**Figure 2.** (A) Characterization of the depolarization (mV) responses elicited by isolated guinea pig vagus nerve preparations in response to the TRPA1 agonist acrolein. (B and C) Effect of TRPA1 channel blockers (AP-18, 1–10 μM; and HC-030031, 0.3–30 μM) on acrolein (300 μM)- or capsaicin (1 μM)-induced depolarization of isolated guinea pig vagus nerve preparations. Values are presented as means ± SEM of the percentage inhibition of depolarization before and after drug superfusion (n = 4). (D) Depolarization (mV) in response to TRPA1 (acrolein, 1 mM)/TRPV1 (capsaicin, 1 μM) agonists in vagal nerve tissue from wild-type and *Trpa1*<sup>-/-</sup> gene-deleted mice. Results are expressed as the mean ± SEM of four to six experiments. KO, knockout.



**Figure 3.** (A) Dose response to inhaled acrolein in conscious male guinea pigs (n = 12). (B) Blockade of the tussive response to acrolein with the TRPA1 ion channel blocker HC-030031 (300 mg/kg). Guinea pigs were dosed with vehicle or HC-030031 one hour before the tussive challenge (n = 12). Statistical significance was determined by Mann-Whitney U test for nonparametric data. Data are presented as means ± SEM and statistical significance is denoted as \*P < 0.05.

related increase in coughs. To confirm that the response was through the TRPA1 channel we performed experiments in which the guinea pigs were pretreated with the TRPA1 inhibitor before exposure to acrolein. In these experiments HC-030031 significantly inhibited the tussive response to acrolein (Figure 3).

**In Vitro Functional Characterization of Response to TRPA1 in Isolated Human Vagal Tissue**

To demonstrate that the observations in guinea pigs and mice translated to humans we paralleled the key vagal experiments with tissue collected from donor lung samples. Figure 4 shows that the human vagus is activated by acrolein (Figure 4A) and that this response is inhibited by a TRPA1 antagonist (Figure 4B).

**Demonstration of Tussive Response to TRPA1 Agonist in Human Volunteers**

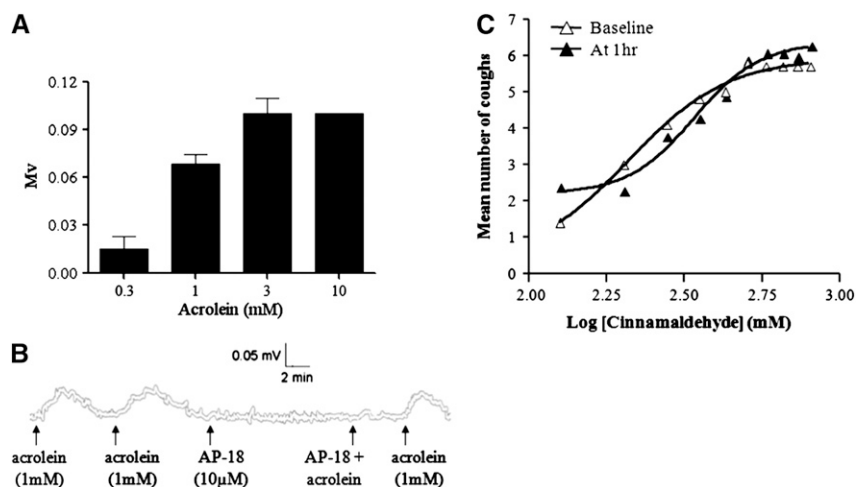
Ten subjects (6 males; mean age, 33.5 yr) were studied. Inhalation of nebulized cinnamaldehyde (but not diluent) induced cough in all subjects. One subject did not achieve C5 (the concentration inducing five or more coughs) at the highest concentration. One subject became nauseous at C2 (the concentration causing two or more coughs) and was withdrawn from further study. There was a distinct concentration–response relationship to inhaled cinnamaldehyde (Figure 4C). The median C2 for the baseline cinnamaldehyde challenge was 200 mM (range, 125–not achieved) with no significant tachyphylaxis seen with the second challenge (median C2, 275 mM [range 125–800]). The median C5 was 312.5 mM (range, 125–not achieved) compared with 387.5 mM (range, 125–not achieved) at 1 hour from baseline. These values are compared with citric acid and capsaicin challenge in the same subject in Table 1. Citric acid was the only agent that showed significant tachyphylaxis (P =

0.02) for C5. The prior inhalation of a subthreshold dose of cinnamaldehyde or of the diluent did not have any effect on subsequent cough challenge. Inhalation of cinnamaldehyde was well tolerated with no adverse effects other than mild nausea in one person.

**DISCUSSION**

Inhalational exposure to numerous irritating gases, fumes, dusts, vapors, chemicals, and endogenous mediators can lead to the development of cough. It has long been established that TRPV1 receptor activation can elicit a cough response in both animal models and in humans (5–7). This receptor is polyvalent, and is activated by vanilloids (e.g., capsaicin), noxious heat (≥42°C), extracellular protons (pH ≤ 5.9), and endogenous lipids (e.g., anandamide) and eicosanoids [e.g., leukotriene B<sub>4</sub>, 12-(S)-hydroperoxyeicosatetraenoic acid, and 15-(S)-hydroperoxyeicosatetraenoic acid]. However, it does not respond to many irritants known to initiate cough. The mechanism whereby a range of seemingly diverse irritants initiate acute and chronic cough has remained a mystery. These data have identified, for the first time, an alternative target for antitussive therapies that responds to known environmental irritants.

We began by cloning human TRPA1 and permanently expressing it in HEK293 cells and demonstrated that the reported TRPA1 agonists activated this channel, confirming previous data (19, 24). In house we have extensively characterized robust and clinically relevant preclinical systems in which to test potential sensory nerve modulators (27, 28). As the guinea pig is the only small animal that possesses a cough reflex that resembles the human response we used the isolated guinea pig vagus in conjunction with an *in vivo* conscious guinea pig cough model (28). Using the isolated guinea pig vagus preparation we demonstrated that two different TRPA1 agonists were able to



**Figure 4.** (A) Characterization of depolarization (mV) responses elicited by isolated human vagus nerve preparations in response to the TRPA1 agonist acrolein. Results are expressed as means ± SEM of three experiments. (B) A representative trace describing depolarization of isolated human vagus evoked by acrolein (1 mM) and inhibition in the presence of the TRPA1 antagonist AP-18 (10 μM). The response to acrolein is recovered after washout. (C) Concentration–response curve of cinnamaldehyde inhaled at baseline and repeated 1 hour later in human volunteers. Nebulizations were performed with the MEFAR MB3 dosimeter with a Respironics nebulizer chamber and mouthpiece. The dosimeter was set to nebulize for 1 second and 2 ml of the agent was instilled into the nebulizer chamber. Coughs induced were assessed during the subsequent 15 seconds after the nebulization.

TABLE 1. RESPONSE THRESHOLD FOR THE VARIOUS TUSSIVE CHALLENGES IN 10 NORMAL SUBJECTS

Tussive Agent	Response	Baseline	At 1 Hour	After Subthreshold Cinnamaldehyde	After Diluent
Cinnamaldehyde (mM)	C2	200	275	n/a	n/a
	C5	313	388	n/a	n/a
Citric acid (mM)	C2	125	125	125	94
	C5	200	250	250	250
Capsaicin ( $\mu$ M)	C2	4	4	8	4
	C5	12	16	16	16

Shown are median C2 and C5 values at baseline and 1 hour after baseline for cinnamaldehyde, citric acid, and capsaicin as well as median C2 and C5 values after inhalation of subthreshold TRPA1 agonist and diluent. C2 is the concentration inhaled at which two or more coughs were recorded and C5 is the concentration at which five or more coughs were recorded. In the citric acid group the concentration needed to induce cough was significantly greater at 1 hour ( $P = 0.02$ ).

activate sensory nerves and that this response could be attenuated with selective TRPA1 inhibitors. Similar data demonstrating inhibition of the TRPA1 channel with these inhibitors have been generated by other investigators (17–19, 29). To strengthen this pharmacological proof of concept we performed experiments with knockout mice. We demonstrated that TRPA1 and TRPV1 agonists both activate vagal sensory nerves in the wild-type mice; but that depolarization was achieved only by the TRPV1 agonist capsaicin, on the isolated vagal preparations from mice from which the *Trpa1* gene was deleted. Although the vagus is a useful and predictive preparation to use for a comprehensive pharmacological assessment of the activity of TRPA1 ligands on vagal sensory nerve activation the data should be viewed with some caution because pharmacological agents are applied to the axon of the isolated vagus nerve *in vitro* and not the nerve ending. The advantages and limitations of this preparation have been discussed at length in our previous publications (27, 28) however, evidence to date suggests that the isolated vagus preparation is predictive of data generated with single-fiber electrophysiological preparations and data generated in the conscious guinea pig cough mode (33). Furthermore, it is consistent with electrophysiological evidence suggesting that functional TRPA1 channels exist in respiratory nociceptive sensory neurons and their terminals (24). Next, we were able to show a dose–response relationship between increasing challenges with a TRPA1 agonist and the number of coughs in a guinea pig model. What is more, we were able to attenuate this tussive response with a selective TRPA1 inhibitor. To demonstrate that the preclinical data generated in animal *in vitro* and *in vivo* models actually translated to humans, we paralleled the previous experiments, using human vagal tissue.

With proof-of-concept data in an animal *in vivo* model and in human tissue *in vitro* we were able to move on with confidence to study normal human volunteers. The data from this study clearly show that a TRPA1 agonist causes a concentration-dependent cough in humans. The human cough response appears to be reproducible and without significant tachyphylaxis in this small data set. Agonizing TRPA1, using this protocol, does not appear to impact on cough responses to other tussive agents, suggesting a direct and specific response.

The TRPA1 receptor is activated by a number of irritant chemicals including mustard oil (i.e., isothiocyanate), acrolein, garlic, formalin, allicin, wasabi, cinnamon oil, diallyl disulfide, allyl isothiocyanate, 4-hydroxynonenal, and 4-oxononenal (12–14, 16–18, 20, 23, 25). The mechanism of action would appear to be through covalent modification of cysteine residues within the cytosolic N terminus of TRPA1 by reactive electrophilic molecules (16). This mode of action could be significant in pathological situations given that oxidant stress induced by either an inflammatory response or by exogenous irritants, such as cigarette smoke, can generate reactive electrophilic molecules including acrolein and 4-hydroxynonenal. This also suggests that

the TRPA1 receptor is a promiscuous receptor that can be activated by a wide range of stimuli, thus making it a perfect target for triggering protective cough reflexes. However, more work will be required to determine which of these putative agonists are sufficiently potent to stimulate cough *in vivo*.

Interestingly, inflammation can lead to the formation of endogenous electrophilic compounds *in vivo* (34). Examples of such compounds are the cyclopentenone ring-containing A and J series prostaglandins, which are formed as nonenzymatic dehydration products of PGE<sub>2</sub> and PGD<sub>2</sub>, respectively. Prostanoids that contain one or two electrophilic carbons such as 15PGJ<sub>2</sub>,  $\Delta^{12}$ -PGJ<sub>2</sub>, 8-iso-PGA<sub>2</sub>, and PGA<sub>2</sub> are therefore able to activate nociceptive neurons via direct interaction with TRPA1 (25). Furthermore, prostaglandins such as PGE<sub>2</sub> and the inducible form of cyclooxygenase-2 are elevated in respiratory disease states at the site of inflammation (35–37). Taken together, this information would suggest that reactive prostanoids and other endogenous TRPA1 ligands, which are produced in greater amounts during inflammation or oxidant stress, could evoke the cough seen in conditions such as asthma and chronic obstructive pulmonary disease.

It is still unclear whether there is cooperation between TRPV1 and TRPA1 channels. Both are activated by tussive agents and so it could be possible that these TRP channels act in concert to elicit functional responses. The dependence of TRPA1 on Ca<sup>2+</sup> may result in the activation of TRPA1 channels by an overflow of Ca<sup>2+</sup> in the locale of other activated channels without ever being modified by a reactive ligand. Furthermore, TRPA1 channels may also act as an amplifier of other Ca<sup>2+</sup>-mobilizing pathways, including activation of TRPV1 (38). However, whether this sort of cooperation exists in generating a cough reflex has yet to be determined.

In conclusion, we have shown for the first time that activation of the TRPA1 channel can evoke a tussive response in humans. This novel and exciting finding could have major implications for understanding the pathogenesis of respiratory diseases and for the treatment of cough, which presents as a significant unmet medical need. Because of their central role and activation by a wide range of irritant and chemical substances, either by exogenous agents, endogenously produced mediators during inflammation, or by oxidant stress, we suggest TRPA1 channels should be considered as one of the most promising targets currently identified for the development of novel antitussive drugs.

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