Prostaglandin E₂ Mediates Cough via the EP₃ Receptor

Implications for Future Disease Therapy

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Rationale: A significant population of patients with severe asthma and chronic obstructive pulmonary disease is less responsive to β_2 -adrenoceptor agonists and corticosteroids, and there are possible safety issues concerning long-term use of these drugs. Inhaled prostaglandin E₂ (PGE₂) is antiinflammatory and a bronchodilator in patients with asthma, but it also causes cough.

Objectives: We aimed to identify the receptor involved in PGE₂-induced sensory nerve activation and cough using a range of *in vitro* and *in vivo* techniques.

Methods: Depolarization of vagal sensory nerves (human, mouse, and guinea pig) was assessed as an indicator of sensory nerve activity. Cough was measured in a conscious guinea pig model.

Measurements and Main Results: Using an extensive range of pharmacological tools, we identified that the EP₃ receptor mediates PGE₂-induced depolarization of sensory nerves in human, mouse, and guinea pig. Further supporting evidence comes from data showing that responses to PGE₂ are virtually abolished in isolated vagus nerves from EP₃-deficient mice (*Ptger3*^{-/-}). Finally, we demonstrated the role of the EP₃ receptor *in vivo* using a selective EP₃ antagonist to attenuate PGE₂-induced cough.

Conclusions: Identification of the receptor mediating PGE₂-induced cough represents a key step in developing a drug that is antiinflammatory and a bronchodilator but without unwanted side effects.

Keywords: airway sensory nerves; vagus nerve; cough; prostanoids; prostanoid receptors.

Asthma and chronic obstructive pulmonary disease (COPD) represent major problems for world public health as chronic respiratory diseases are currently the fourth leading cause of death (1). Asthma is characterized by reversible airflow obstruction caused by bronchoconstriction, inflammation, and airway hyperresponsiveness. In contrast, the airflow obstruction in COPD is not fully reversible and often worsens progressively (2). Currently, bronchodilators, such as long-acting β_2 -adrenergic receptor agonists, are the mainstay treatment but often fail to relieve symptoms of COPD and severe asthma (3) and some possible concerns have been raised over long-term use (4). Inhaled corticosteroids successfully control airway inflammation in most patients with asthma; however, they are of negligible benefit in patients with severe asthma (5) and are

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Prostaglandin E₂ (PGE₂) has been shown to be antiinflammatory and a bronchodilator in animal models and subjects with asthma. However PGE₂ causes cough as a side effect and the receptor mediating this effect is currently unknown.

What This Study Adds to the Field

We have identified the receptor involved in PGE₂-induced cough, which could lead to the development of a novel therapy for airway disease that is both a bronchodilator and antiinflammatory but devoid of tussive side effects.

often not effective in patients with COPD (6). Concerns exist over long-term side effects of high doses of inhaled corticosteroids (7). Collectively these factors represent a significant need for the development of new, safe therapies for airway inflammatory diseases.

Inhaled prostaglandin E₂ (PGE₂) is a bronchodilator in normal subjects (8–10) and in patients with asthma and chronic bronchitis (8, 11). In addition, PGE₂ has antiinflammatory properties in patients with asthma (12, 13), providing an ideal dual therapy for the treatment of these diseases. Despite the benefits of inhaled PGE₂, the development of prostanoid agonists for the treatment of airway inflammatory diseases has been hindered as prostanoids induce irritancy of the upper airway resulting in a reflex cough. Cough is initiated by stimulation of sensory afferent nerve endings in the airways. PGE₂ excites airway afferent nerves (14, 15), which concurs with the transient cough seen in both normal subjects and patients with asthma during studies with inhaled PGE₂ (8, 10, 13). However, it is not yet known how PGE₂ causes this tussive response.

There are currently nine known prostanoid receptors. PGE₂ has relatively low affinity for the FP, IP, TP, DP, and CRTh2 receptors (encoded by *Ptgrfr*, *Ptgir*, *Tbxa2r*, *Ptgdrr*, and *Gpr44* genes) and acts predominantly via the EP receptors. The EP receptors have been subclassified into EP₁, EP₂, EP₃, and EP₄ (encoded by *Ptger1*–*Ptger4* genes) (16–18) and distinct signaling pathways initiate diverse and opposing downstream effects in different tissues (17–19).

To date, no study has investigated the receptor responsible for the PGE₂-induced cough. The results of this study could provide key proof of concept data demonstrating the selectivity profile required for any candidate compound to elicit antiinflammatory/bronchodilator effects without the sensory irritant side effect liability. To identify the tussive receptor, we used models of sensory nerve activation and cough. Guinea pigs, as the only rodents that possess a cough reflex, are the most appropriate species for investigating cough *in vivo*. Although mice do not cough *per se*, comparing sensory nerve activation in nerves from prostanoid receptor-deficient mice is fundamental

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to this study. Thus, we established that responses to PGE₂ in guinea pig and mouse mimic those in human vagal sensory nerves. Using a pharmacological approach and prostanoid receptor-deficient mice, we provide substantial evidence that the EP₃ receptor mediates PGE₂-induced sensory nerve activation. Finally we provide *in vivo* proof of concept data demonstrating inhibition of PGE₂-induced cough by a selective EP₃ receptor antagonist.

METHODS

Animals

Male C57BL/6 mice (18–20 g) and Male Dunkin-Hartley guinea pigs (250–350 g) were purchased from Harlan (Bicester, Oxon, UK). Breeding pairs of mice devoid of one of the following genes: *Ptger1* (EP₁), *Ptger2* (EP₂), *Ptger3* (EP₃), *Ptgdr* (DP), *Ptgfr* (FP), *Ptgir* (IP), or *Tbxa2r* (TP), had been backcrossed at least eight times onto the C57BL/6 background. *Ptger4*^{−/−} mice do not survive on the C57BL/6 background due to patent ductus arteriosus (20), so they were backcrossed on a mixed background of 129/Ola X C57BL/6. Mice were kindly provided by Dr. Shuh Narumiya, Kyoto University, and breeding colonies maintained at Imperial College, London. Experiments were performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986.

Characterizing Responses to PGE₂ in Isolated Vagus Nerves

Sensory nerve depolarization was measured as previously described (21–23). Concentrations of vehicle (0.1% ethanol) or PGE₂ were applied to guinea pig, mouse, or human nerves in a random order for 2 minutes each, washing the tissue in between. No more than five stimulations were generated per section of nerve. Human vagus nerves were obtained from donor patients for heart or heart/lung transplants performed at The Royal Brompton or Harefield Hospital. Approval was obtained from the Royal Brompton and Harefield ethics committee after receiving the relevant consents from relatives.

Investigating PGE₂ Inhibition Using Selective Antagonists

A concentration of 10 μM PGE₂ was selected from the concentration response and the effect of a range of antagonists was investigated in the guinea pig and mouse: 0.1% dimethyl sulfoxide (DMSO) vehicle, 1 μM GW848687X (EP₁) (24), 0.2 μM L826266 (EP₃) (25), 1 μM GW627368X (EP₄) (26), 10 μM AL8810 (FP) (27), 10 μM AH6809 (EP_{1/2}DP) (28), 1 μM SQ29548 (TP) (29), and 1 μM RO3244794 (IP) (30). Concentrations of antagonists were selected that were approximately 100-fold the pA₂ value (defined as the negative logarithm of the molar concentration of an antagonist that would produce a twofold shift in the concentration–response curve for an agonist) as is common practice. Where available, agonists that act preferentially on one of the receptors (10 μM) were profiled in parallel to PGE₂:PGF_{2α} (FP), PGD₂ (DP), iloprost (IP), and U46619 (TP). Agonists were applied to the nerve for 2 minutes and washed. A second response was elicited to ensure the response was reproducible. The antagonist was then perfused onto the nerve for 10 minutes and immediately after, PGE₂ (or agonist) in the presence of antagonist was applied and the inhibition recorded. The nerve was then washed and agonist response repeated to ensure the nerve was still viable. Any antagonists that inhibited the PGE₂-induced depolarization were then investigated in the human vagus nerve.

Investigating the Effect of an EP₃ Antagonist on PGE₂-induced Cough *In Vivo*

The procedure for measuring coughs in conscious guinea pigs has previously been described (22, 23, 31). Male Dunkin-Hartley guinea pigs (250–350 g, Charles River, L'Arbresle, France) were exposed to a 10-minute aerosol of PGE₂ (30, 100, or 300 μg/ml) and the number of coughs recorded. Once the optimal concentration of PGE₂ had been established, a separate group of animals were dosed with vehicle (0.5% methylcellulose + 0.2% Tween 80 in saline, intraperitoneally) or the EP₃ antagonist L826266 (300 mg/kg, interperitoneally) 40 minutes before challenge with PGE₂. This high dose was chosen because the

pharmacokinetic profile demonstrated low plasma levels and a high degree of plasma protein binding after intraperitoneal dosing (report from Merck Frosst, Montreal, PQ, Canada). It was therefore predicted that this dose would be required to have some impact on the PGE₂ response. Indeed the data from a guinea pig pharmacokinetic study and assessment of the impact of plasma binding on antagonist potency suggested that even at this high dose there would only be approximately 0.1 μM of unbound (i.e., active) antagonist. Unfortunately, there was insufficient compound available to dose at a higher level and no other appropriate tool compounds available. Coughs were counted as described above.

Compounds and Materials

The EP₁ antagonist GW848687X (24) and the EP₄ antagonist GW627368X (26) were kind gifts from GlaxoSmithKline, and the EP₃ antagonist L826266 (25) and IP antagonist RO3244794 (30) were kind gifts from Merck Frosst and Roche Palo Alto (Palo Alto, CA), respectively. The FP antagonist AL8810 (27), EP_{1/2}DP antagonist AH6809 (28), and TP antagonist SQ29548 (29) were purchased from Cayman Europe. All antagonists were made in DMSO and stocks stored (1 mM or 10 mM) until required. PGE₂, PGF_{2α}, and PGD₂ were purchased from Sigma Aldrich (Poole, UK) and dissolved in ethanol to 10 mM. U46619 and Iloprost were purchased from Cayman Europe (Tallinn, Estonia) and dissolved in DMSO to 10 mM. Capsaicin was purchased from Sigma and stored in DMSO at 1 mM until required. A low pH solution was made to pH 5 as previously described (32). Krebs compounds were obtained from BDH (Dorset, UK) and all other chemicals and reagents were from Sigma Aldrich.

Data Analysis and Statistics

Antagonism of prostanoid agonists was analyzed using a 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 PGE₂ in prostanoid receptor-deficient mice were analyzed using Kruskal-Wallis test for multiple comparisons with Dunns *post hoc* test, comparing the responses in each prostanoid receptor-deficient group to the wild-type control. Inhibition of the PGE₂-induced cough was analyzed using Mann Whitney *U* test for nonparametric data. Data are presented as mean ± SEM and statistical significance was denoted as *P* less than 0.05.

RESULTS

PGE₂ Activates Isolated Vagus Nerves

Our model of sensory nerve activation has previously been characterized and is predictive of agents that cause cough *in vivo* (21, 22). Responses to PGE₂ in the guinea pig vagus nerve emulate responses in the human vagus nerve (23). In the present study we established a concentration-dependent increase in depolarization to PGE₂ (Figure 1) in mouse, guinea pig, and human isolated vagus nerves. With no disparity between the species in the response to PGE₂, we deduce that responses in guinea pig and mice are representative of those in human nerves.

Selective EP₃ Receptor Antagonist Inhibits PGE₂-induced Activation of Sensory Nerves

After confirming PGE₂-induced depolarization in all three species, we investigated the receptor responsible in guinea pig isolated nerves using an array of prostanoid receptor agonists and antagonists. Antagonists at FP (AL8810), EP_{1/2}DP (AH6809), TP (SQ29548), and IP (RO3244794) inhibited their corresponding receptor agonists (PGF_{2α}, PGD₂, U46619, and Iloprost, respectively) but did not influence PGE₂-induced depolarization (Figure 2A). Vehicle or antagonists at EP₁ (GW848687X) and EP₄ (GW627368X) had no effect on PGE₂; however, the EP₃ antagonist (L826266 [0.2 μM]) attenuated depolarization to PGE₂ in the guinea pig vagus nerve (Figure 2B). Example traces of the effect of the FP antagonist AL8810 on PGF_{2α} (Figure 2C) and PGE₂ (Figure 2D) are presented; AL8810 inhibited the responses to PGF_{2α} but did not

Figure 1. Depolarization (mV) of mouse, guinea pig, and human vagus nerves by vehicle (0.1% ethanol) or concentrations of PGE₂ (μM). Data are expressed as mean ± SEM of four to six experiments in guinea pig and mouse and two to four experiments in human isolated vagus nerves.

attenuate PGE₂. After the antagonist was washed out, the agonist responses were recovered after all antagonists. The same range of antagonists was investigated in wild-type mice and the effects were mirrored in both species. The EP₃ antagonist significantly inhibited PGE₂-induced (10 μM) depolarization of

Figure 2. Percentage inhibition of agonist-induced depolarization by selective prostanoid receptor antagonists in guinea pig vagus nerves. (A) The inhibition of PGE₂ (solid bars) or selective agonists (open bars; 10 μM PGF_{2α} [FP], PGD₂ [DP], U46619 [TP], and Iloprost [IP]) by antagonists 10 μM AL8810 (FP), 10 μM AH6809 (EP_{1/2}DP), 1 μM SQ29548 (TP), and 1 μM RO3244794 (IP). (B) Inhibition of PGE₂-induced depolarization (10 μM) by vehicle (0.1% dimethyl sulfoxide) and antagonists at EP₁ (1 μM GW848687X), EP₃ (0.2 μM L826266), and EP₄ (1 μM GW627368X). (A, B) data represent mean ± SEM, n = 4, * *P* < 0.05 comparing response in the same nerve before and after antagonist. Representative traces showing the effects of the FP antagonist AL8810 on (C) PGF_{2α} and (D) PGE₂.

Figure 3. Characterization of the EP₃ antagonist L826266. (A) Percentage inhibition of 10 μM PGE₂ by L826266 (0.1% dimethyl sulfoxide, 0.02, 0.2, 2, and 20 μM; n = 4). (B) Percentage inhibition of sulprostone (10 μM), capsaicin (1 μM), and low pH (pH 5) by L826266 (2 μM, n = 4) in guinea pig vagus nerves. (A, B) Data are expressed as mean ± SEM, * *P* < 0.05 comparing responses in the same nerve before and after antagonist. (C) A copy of an original trace in which a concentration of 0.2 μM EP₃ antagonist inhibited PGE₂-induced depolarization in the human vagus nerve by 80%.

the mouse vagus nerve by 64.8 ± 2.8% (n = 4; *P* < 0.05), whereas no inhibition by the other antagonists was observed (data not shown).

Further investigation of the EP₃ antagonist illustrated that L826266 concentration-dependently inhibited PGE₂ (Figure 3A). L826266 also completely abolished depolarization induced by sulprostone, an agonist at the EP₃ receptor, confirming that L826266 is indeed acting as an EP₃ antagonist in this system (Figure 3B). Furthermore, L826266 did not attenuate depolarization induced by 1 μM capsaicin or low pH solution (which both induce a tussive response *in vivo*) (Figure 3B) illustrating that the antagonist is inhibiting depolarization specifically induced by PGE₂. The EP₃ antagonist (at a submaximal concentration of 0.2 μM) had a remarkably similar effect in the human vagus nerve, inhibiting PGE₂ by 80% (Figure 3C), suggesting that the receptor responsible for PGE₂-induced sensory nerve activation is the same in guinea pigs, mice, and humans.

Presence of the EP₃ Receptor Is Essential for PGE₂-induced Sensory Nerve Activation

To confirm the role of the EP₃ receptor in PGE₂-induced sensory nerve activation, we used isolated vagus nerves from prostanoid receptor-deficient mice (Figure 4A). Isolated nerves from wild-type mice depolarized to the expected magnitude, whereas *Ptger3*^{-/-} mice had a significantly smaller, or completely abolished, response (Figure 4B). All of the mice were genotyped by a standard PCR technique. An example gel of amplification of DNA in the *Ptger3* gene in wild-type and *Ptger3*^{-/-} mouse tissue can be seen in Figure 4C. Primers for the wild type produced a band at 350 bp and *Ptger3*^{-/-} at 550 bp.

EP₃ Receptor Antagonist Attenuates PGE₂-induced Cough *In Vivo*

The role of the EP₃ receptor in PGE₂-induced sensory nerve activation and cough was then investigated *in vivo*. Guinea pigs

Figure 4. Investigating responses to PGE₂ in prostanoïd receptor-deficient mice. (A) Responses to 10 μM PGE₂ in isolated vagus nerves from prostanoïd receptor-deleted mice. The Ptger3^{-/-} mice had a significantly decreased response to PGE₂ compared with the C57BL/6 wild-type mice (n = 4–6; * P < 0.05). Data are expressed as mean ± SEM. (B) Representative trace showing the depolarization to 10 μM PGE₂ in the wild-type C57BL/6 mice (left) and no effect of PGE₂ in the Ptger3^{-/-} mice (right). (C) Example gel of genotyping for the Ptger3^{-/-} mice. Primers for the wild-type gene produced a band at 350 bp and Ptger3^{-/-} at 550 bp.

were exposed to an aerosol of 30, 100, or 300 μg/ml PGE₂ for 10 minutes to establish a suitable concentration (n = 8). The number of coughs were recorded: 30 μg/ml (0 coughs), 100 μg/ml (3.3 ± 3.3 coughs), and 300 μg/ml (12.6 ± 4 coughs). A concentration of 300 μg/ml was selected for use in subsequent experiments. In the study in which we tested the EP₃ receptor antagonist the numbers of coughs induced was higher (Figure 5). The reason for this is unknown but could be due to *in vivo* variation inherent in working with conscious models and/or the additional stress involved in dosing.

Investigation of L826266 on PGE₂-induced cough revealed that the antagonist significantly decreased the number of coughs in response to aerosolized PGE₂. Although one may expect that the high dose used would block the cough response completely, analysis of the pharmacokinetic profile and level of plasma protein binding (performed by Merck Frosst and detailed in METHODS) would suggest actual unbound/active levels to be around 0.1 μM. Data from the isolated vagus experiments (Figure 3) show that at this concentration PGE₂ responses are only attenuated by approximately 50%, which is remarkably reminiscent of the impact in the cough system. In addition we have data (not shown) in isolated guinea pig trachea showing that 2 μM (i.e., 20 times higher than predicted levels in the cough study) of this compound does not affect PGE₂-induced bronchodilation (reported to be through other EP receptors) (33) suggesting that this compound is unlikely to be acting on other EP receptors. Together these data confirm that the EP₃ receptor mediates PGE₂-induced cough (Figure 5).

DISCUSSION

Current treatments for airway inflammatory disease, such as inhaled glucocorticoids and long-acting β-agonists, have been associated with significant side effects (3, 4, 7) and furthermore, these treatments are often less effective in certain subpopulations of patients (5, 6). There is therefore an unmet need to develop new, safe drugs, free of adverse side effects, for the treatment of asthma and COPD. PGE₂ has been shown to be a bronchodilator and an antiinflammatory agent in several studies in patients with asthma (11–13). However, the development of prostanoïd agonists for the treatment of airway inflammatory diseases has been hindered due to airway irritancy and cough (8, 10, 13). Identifying the receptor triggering the reflex cough represents a major step in the development of a PGE₂ analog that is a bronchodilator and an antiinflammatory agent but devoid of tussive side effects.

To commence our investigation we measured vagal sensory nerve activation, because the vagus nerves supply the majority

of the sensory nerve fibers to the airway, using the isolated vagus nerve preparation. We have previously shown that responses to tussive stimuli in human vagus nerves are representative of those from guinea pig nerves (23). Here, we generated data comparing the responses to PGE₂ in mouse, guinea pig, and human species. Therefore we can conclude from these experiments that PGE₂ has a remarkably similar effect on human, mouse, and guinea pig vagus nerves. This would suggest that guinea pig and mouse vagi are representative of the effects of PGE₂ in human tissue. Moreover, because PGE₂ causes both sensory nerve activation and cough in humans and guinea pigs, we have evidence that our model of sensory nerve activity is representative of the human cough reflex.

Once we established that PGE₂ activates isolated vagal sensory nerves, we proceeded to investigate pharmacologically the receptor responsible for PGE₂-induced depolarization using a range of prostanoïd antagonists. The EP₃ antagonist L826266 was the only antagonist investigated that attenuated responses to PGE₂ in both guinea pig and mouse. Furthermore, we have provided evidence that this antagonism is mirrored in the isolated human nerve, illustrating that the EP₃-mediated sensory nerve activation, elicited by PGE₂, is an effect that is translated across species. Capsaicin and a low pH solution were not inhibited by the EP₃ antagonist, suggesting that the antagonist is specifically inhibiting PGE₂-induced sensory nerve activation rather than acting as a general inhibitor of nerve depolarization.

Although significant pharmacological evidence that the EP₃ receptor is responsible for PGE₂-induced sensory nerve activity was generated, we then corroborated these data by using isolated nerves from mice devoid of one of the prostanoïd receptors. The Ptger3^{-/-} mice, lacking EP₃ receptors, had a di-

Figure 5. Inhibition of PGE₂-induced cough with L826266. Guinea pigs were dosed (interperitoneally) with vehicle (n = 5) or 300 mg/kg L826266 (n = 6) 40 minutes before cough challenge. Data are represented as mean ± SEM, * P < 0.05 comparing vehicle to antagonist group.

inished response to PGE₂, whereas responses in nerves from other prostanoid receptor-deficient mice (*Ptger1*^{-/-}, *Ptger2*^{-/-}, *Ptger4*^{-/-}, *Ptgfr*^{-/-}, *Ptgdre*^{-/-}, *Ptgir*^{-/-}, *Tbxa2r*^{-/-}) depolarized to the same magnitude as wild types. This research culminated in the demonstration that L826266 inhibited PGE₂-induced cough in our *in vivo* guinea pig model. Taken together these studies confirm that cough induced by PGE₂ is caused mainly, if not solely, by activation of the EP₃ receptor.

PGE₂ is commonly presumed to be proinflammatory and has been implicated in several inflammatory disease conditions, including rheumatoid arthritis (34) and UVB-induced cutaneous inflammation (35). However, in humans, the lung appears to be unique in that PGE₂ has beneficial effects (8, 9, 11–13) as well as unwanted effects (8, 13). The receptor(s) responsible for the beneficial effects seem to differ from the EP₃ receptor causing the detrimental cough. Using human airway smooth muscle and rodent models, there is evidence that the EP₂ receptor is responsible for the PGE₂-induced bronchodilation (36–38). Antiinflammatory effects of PGE₂ have been identified in both human (12, 13) and rodent models (39). One isolated study has suggested that, in a mouse model, activation of the EP₃ receptor could suppress ovalbumin-induced inflammation (40). However, the wealth of information available, especially from human cell-based assays, would suggest that the receptor(s) responsible for the antiinflammatory actions of PGE₂ are the EP₂ and/or the EP₄ receptor subtype (41–45).

Rationale for the development of a PGE₂ analog that does not activate the EP₃ receptor is not just limited to cough, as the EP₃ receptor mediates other detrimental effects of PGE₂ in the airways. Although PGE₂-mediated bronchodilation is believed to be via the EP₂ receptor, bronchoconstrictor effects of PGE₂ have been shown to act through EP₁ and EP₃ (33). What is more, the EP₃ receptor has been implicated in the chemotaxis of mast cells to sites of inflammation (46).

In summary, we have established in this current study that PGE₂-induced sensory nerve activation and cough are mediated via the EP₃ receptor. Our research, and other published data, suggests that the unwanted effects of PGE₂ (proinflammatory effects, bronchoconstriction, and cough) are mediated by the EP₃ receptor and beneficial effects are likely to act via a different receptor. This research provides considerable support for the development of a PGE₂ analog that does not activate the EP₃ receptor, thereby providing a novel therapy for airway inflammatory diseases that is antiinflammatory and a bronchodilator but devoid of tussive side effects.

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