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Participation of thromboxane A_2 in the cough response in guinea-pigs: antitussive effect of ozagrel

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1 The purpose of this study was to investigate the involvement of thromboxane A_2 (TXA_2) in the cough response in a guinea-pig cough model. Here, we describe results obtained using a selective TXA_2 synthetase inhibitor, ozagrel, and a selective TXA_2 agonist, U-46619.

2 Guinea-pigs were anaesthetized and exposed to an aerosol of capsaicin (100 μM) to elicit coughing. The number of coughs was 20.0 ± 5.8 during capsaicin provocation (5 min), but only 2.8 ± 0.4 during a 5-min inhalation of phosphate-buffered saline (PBS) ($P < 0.05$).

3 TXB_2 levels in BAL were 101.4 ± 8.0 and 58.4 ± 8.7 pg ml^{-1} following capsaicin and PBS inhalation, respectively ($P < 0.01$), but there was no intergroup difference in the cell populations in BAL.

4 Inhalation of U-46619 did not induce a cough response by itself at concentrations of 100 ng ml^{-1} to 10 μg ml^{-1}. However, it caused a 2 fold increase in the number of capsaicin-induced coughs.

5 To explore the source of the TXA_2, BAL cells were stimulated with capsaicin and the supernatants collected for analysis. The TXB_2 concentration in BAL was increased dose-dependently, indicating that TXA_2 is released from BAL cells in response to capsaicin.

6 Ozagrel was administered orally 1 h before a 5 min capsaicin provocation and the number of coughs was counted during the capsaicin inhalation. Ozagrel decreased the number of coughs dose-dependently (ED_{50} value, 26.3 mg kg^{-1}).

7 These results show that TXA_2 modulates the capsaicin-induced cough response by increasing capsaicin-sensitivity.

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Keywords: Cough; antitussive; thromboxane A_2 synthetase inhibitor; ozagrel; U-46619

Abbreviations: ACE inhibitor, angiotensin-converting-enzyme inhibitor; BAL, bronchoalveolar lavage; CNS, central nervous system; PBS, phosphate-buffered saline; RAR, rapidly adapting receptor; TXA_2, thromboxane A_2

Introduction

Cough, a physiological defence mechanism for the clearance of foreign particles and excessive bronchial secretions from the airways, and occurs in healthy people but is also a common symptom of a variety of respiratory diseases, such as asthma (Irwin *et al*., 1981). The cough reflex is triggered by stimulation of myelinated rapidly adapting receptors (RARs) and unmyelinated C-fibres within the larynx, trachea and proximal bronchi (Karlsson *et al*., 1988; Widdicombe, 1995).

Although the benefical role of cough in the defence of the host is well known, persistent chronic cough can lead to physical exhaustion and is associated with significant morbidity (sleep loss, irritability and so on) (O'Connell *et al*., 1998). For that reason, a variety of agents have been used to try to suppress the cough response (Braman & Corrao, 1987). These agents are generally classified as central or peripheral antitussives (Braman & Corrao, 1987). Codeine and dextromethorphan, which are central antitussive agents, penetrate the central nervous system (CNS) following their systemic administration and reduce the responsiveness of the central neural elements that mediate cough (Chou & Wang, 1975; Bolser & DeGennaro, 1994; Bolser *et al*., 1994). Peripheral antitussive agents, such as benzonatate and BW443C, exhibit little or no penetration of the CNS after systemic administration but operate instead by reducing the responsiveness of the pulmonary vagal afferents that elicit cough (Korpas & Tomori, 1979; Adcock *et al*., 1988; Adcock, 1991; Bolser *et al*., 1994). However, there is still a need for new antitussive agents that, while exerting powerful effects against coughs caused by upper respiratory tract infection (URTI), the persistent coughing caused by angiotensin-converting-enzyme (ACE) inhibitors and cough-variant asthma, have no significant side effects (Widdicombe, 1999).

A few years ago, it was reported that ozagrel, which is a selective TXA_2 synthetase inhibitor, was effective in suppressing the cough response induced by capsaicin in asthmatics (Fujimura *et al*., 1995). Indeed, the threshold for the capsaicin response was increased by 400 mg day^{-1} following ozagrel administration for 4 days. Furthermore, Umemura *et al*. (1997) reported that ozagrel was effective against ACE-inhibitor-induced coughing in humans. In the present study, we used ozagrel to explore the mechanisms underlying the capsaicin-induced cough response in guinea-pigs.

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Methods

Animals

All experimental procedures conformed to international standards of animal welfare and were approved by the Laboratory Animal Committee of Kissei Pharmaceutical Co. Ltd.; they also conformed with current Japanese Law. Male Hartley guinea-pigs (SPF, 350–600 g) purchased from SLC Japan Inc. (Shizuoka, Japan) were maintained under a 12-h light-dark cycle with free access to water and standard laboratory food until the day of the experiment.

Capsaicin-induced cough in guinea-pigs

Hartley guinea-pigs (3–4 weeks old) were anaesthetized with urethane (1.5 mg kg^{-1}, s.c.) 1 h before a 5-min exposure to capsaicin. Each anaesthetized animal was placed in a body plethysmograph (Baxco, U.S.A.) and exposed to an aerosol of capsaicin (100 μM). The capsaicin aerosol was produced by a jet nebulizer (Pari GmbH, Starnberg, Germany; 67% of aerosol particles were less than 5 μm in diameter). The number of coughs occurring during this 5-min period was counted by visual inspection of the chart record and the animal's behaviour. No animal was exposed more than once to capsaicin.

Ozagrel (10, 30 and 100 mg kg^{-1}, p.o.) and codeine (1 and 3 mg kg^{-1}) were given 60 min before capsaicin provocation, while procaterol (3 and 10 mg kg^{-1}, i.v.) was given 5 min before capsaicin.

U-46619-induced cough

In our preliminary experiments, we could not count all the coughs induced by capsaicin in U-46619 pre-treated guinea-pigs because there were simply too many coughs. Therefore, we used 7 to 8-week-old guinea-pigs instead of the 3 to 4-week-old animals used for the other experiments (see Figures 1 and 4) because the former's sensitivity to cough was lower. Guinea-pigs were exposed to an aerosol of U-46619 delivered by jet nebulizer at a dose of 100 ng ml^{-1} for 5 min and the number of coughs was counted during this period. Five minutes after inhalation of U-46619 or PBS (vehicle control), the animals inhaled 100 μM capsaicin for 5 min; we then counted the number of coughs during the capsaicin inhalation.

Capsaicin-induced thromboxane release in BAL cells

BAL was performed by gentle washing of the lungs with 3×4 ml aliquots of PBS (from a syringe and *via* the tracheal cannula). The total number of cells present in the recovered fluid was counted using a haemocytometer, while differential cell counts were made using cytospin preparations stained with Diff-Quick. At least 200 cells were counted in each sample.

Collected BAL cells were resuspended in AIM medium (Gibco BRL, Rockville. MD, U.S.A.) and incubated with capsaicin at the desired concentration (0.3–100 μM) for 10 min at 37°C. Then, the cells were harvested by centrifugation and the supernatant collected for measurement of TXB_2. This was measured rather than TXA_2, which is highly unstable and is converted by nonenzymatic hydrolysis to TXB_2. The collected supernatant was stored at −80°C until measurement of the TXB_2 level could be performed.

Thromboxane B_2 (TXB_2) assay by ELISA

The TXB_2 concentration in the supernatant collected from capsaicin-stimulated BAL cells was measured using an EIA kit (Amersham, Bucks., U.K.). The sensitivity of the assay for TXB_2 was 3.6 pg ml^{-1} and no cross-reactivity was observed.

Drugs and chemicals

The following compounds were used: ozagrel ((E)-3-[p-(1H-imidazol-1-ylmethyl)phenyl]-2propenoic acid hydrochloride monohydrate), which is a selective thromboxane synthetase inhibitor. Its former name was OKY-046 HCl (Domenan®, Kissei Pharmaceutical Co. Ltd., Matsumoto, Japan) and it has been used for the treatment of asthma. It was dissolved in 0.5% carboxymethyl cellulose (CMC). U-46619 (5-heptenoic acid, 7-[6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-, [1R-1a,4a,5b(Z),6a(1E,3S*)]]-) (Cayman Chemicals, Ann Arbor, MI, U.S.A.), which was dissolved in methyl acetate, was diluted with PBS. Codeine phosphate (Takeda, Osaka, Japan) was dissolved in 0.5% CMC, while procaterol (Sigma Chemicals, St Louis, MO, U.S.A.) was dissolved in PBS. Capsaicin (Nakalai, Kyoto, Japan) was dissolved in 10% ethanol plus 10% Tween 80 and diluted with PBS.

Data analysis

Data are given as mean ± s.e.mean Pairs of groups were compared by use of a Student's *t*-test. The statistical analysis of the results was performed by an analysis of variance, using Dunnett's test for multiple comparisons. P values <0.05 were considered significant.

Results

Inhalation of 100 μM capsaicin for 5 min elicited 20.0 ± 5.8 coughs during the inhalation period ($n=5$, Figure 1A). On the other hand, only 2.8 ± 0.4 coughs were counted in animals that inhaled phosphate-buffered saline (PBS) ($n=5$, Figure 1A). The TXB_2 content of the BAL collected from these same animals was 101.4 ± 8.0 pg ml^{-1} in capsaicin-treated animals but only 58.4 ± 8.7 pg ml^{-1} in the PBS treated controls ($n=5$, Figure 1B). There were no significant differences in macrophage, eosinophil, neutrophil or lymphocyte numbers between the two groups (Figure 1C).

It is uncertain whether TXA_2 can induce (or enhance) the cough response. In this study, U-46619 was used instead of TXA_2, because TXA_2 is unstable and not suitable for *in vivo* experiments. When U-46619 was inhaled for 5 min at concentrations of 100 ng ml^{-1} to 10 μg ml^{-1}, it did not induce a cough response ($n=5$, Figure 2A). We therefore concluded that TXA_2 does not by itself induce a cough response. We next tested whether TXA_2 might enhance the cough response (again using U-46619). Five minutes after a 5-min inhalation of U-46619, capsaicin was inhaled to induce a cough response. Over the full 5-min measuring period, pre-treatment with 100 ng ml^{-1} of U-46619 increased the number of capsaicin-induced coughs by a factor of about two ($n=5$, Figure 2B), the number of coughs in U-46619 pre-treated animals being significantly greater than in PBS-pretreated controls at all time-points after 2 min into the capsaicin inhalation (Figure 2C). We therefore concluded that TXA_2 sensitizes the airway to capsaicin, even though it does not induce coughing by itself.

On this basis, the TXA_2 level in BAL would be expected to be an important determinant of the magnitude of the cough

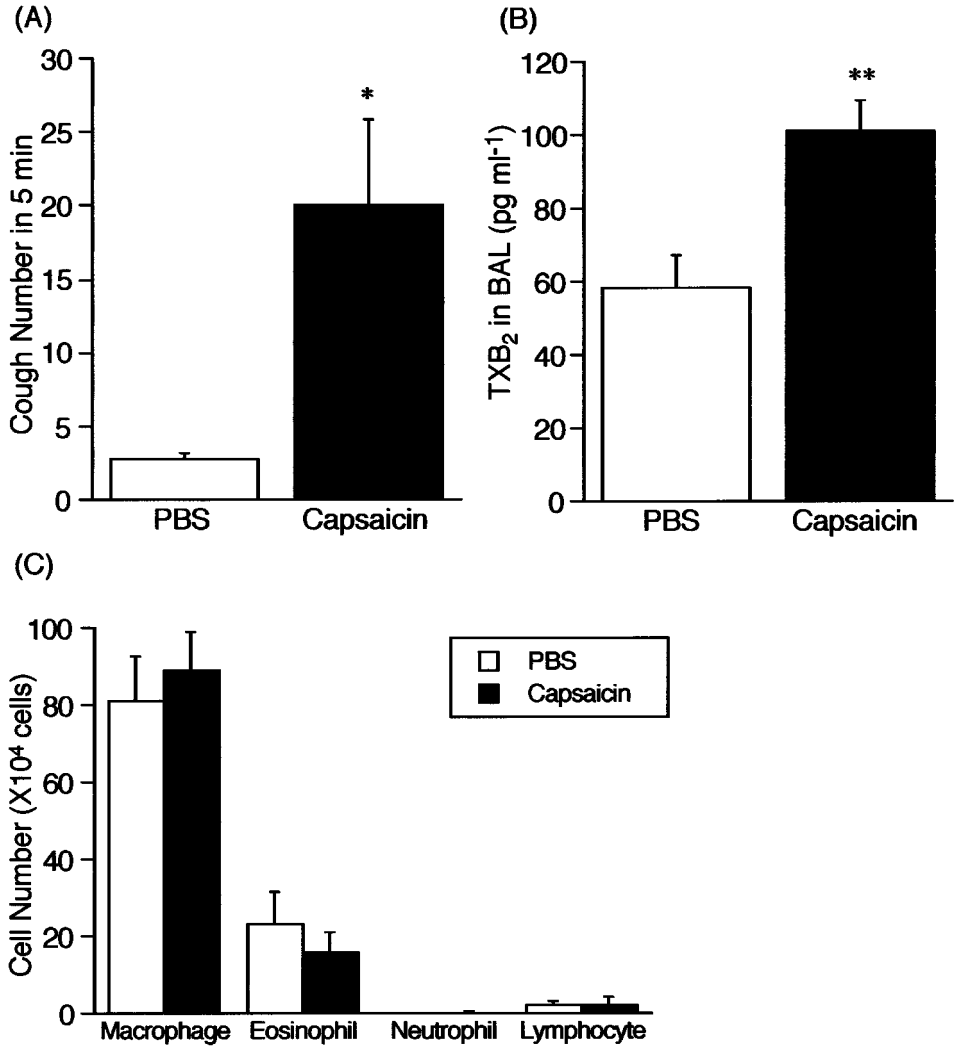

Figure 1 Effect of capsaicin on cough response and on TXB_2 concentration and cell population in BAL. Coughs were counted during a 5-min exposure to 100 μM capsaicin or vehicle (PBS) in 3- to 4-week-old guinea-pigs (A). TXB_2 concentration in BAL was measured by ELISA in the animals used for counting the number of coughs (B). Differential cell counts were obtained in BAL (C). Each column represents the mean$\pm$s.e.mean ($n=5$). $^*P<0.05$, $^{**}P<0.01$ vs PBS.

response. So, we next examined whether TXA_2 was indeed increased in BAL collected from capsaicin-treated animals. BAL cells collected from untreated guinea-pigs showed cell populations similar to those shown in Figure 1C. Using ELISA, we found that TXB_2 was increased dose-dependently by capsaicin, the increase being significant at 3 μM capsaicin or more ($n=5$, Figure 3).

Next, a TXA_2 synthetase inhibitor (ozagrel) was used to examine the effect of TXA_2 on capsaicin-induced cough. Guinea-pigs were given ozagrel or codeine orally 1 h before exposure to an aerosol of capsaicin. Ozagrel and codeine each inhibited the capsaicin-induced cough in a dose-dependent manner ($n=6$ for each drug, Figure 4), the ED_{50} values being 26.3 and 1.6 mg kg^{-1}, respectively ($n=6$). To determine whether bronchoconstriction was associated with the capsaicin-induced cough response, we administered the β-adrenoceptor agonist procaterol intravenously 5 min before capsaicin provocation. Procaterol inhibited capsaicin-induced cough in a dose-dependent manner, its ED_{50} value being 6.8 μg kg^{-1} ($n=6$). In contrast, only 0.1 μg kg^{-1} procaterol was needed completely to inhibit the histamine-induced bronchoconstriction in guinea-pigs (data not shown).

Discussion

The present study provides several pieces of evidence clearly indicating that the TXA_2 level in the lungs is strongly correlated with the magnitude of the cough response. Thus: (i) TXA_2 production increased in response to capsaicin, which also increased the cough number, (ii) the number of coughs was decreased by pre-treatment with the TXA_2 synthetase inhibitor ozagrel, (iii) U-46619, a TXA_2 analogue, increased the number of capsaicin-induced coughs, although it did not induce a cough response by itself and (iv) the U-46619 pre-treatment group showed an enhanced cough response within 2 min or so of the beginning of capsaicin provocation. These results suggest that TXA_2 may cause an enhanced cough response by heightening the airway sensitivity to capsaicin. However, the mechanism by which TXA_2 interacts with the cough reflex system is difficult to understand. We speculate that TXA_2 may stimulate sensory receptors such as C-fibres and/or RARs without eliciting a cough response when no other stimuli, such as capsaicin, are present and that TXA_2 may work by lowering the threshold of the relevant sensory receptors. Evidence in support of our speculation includes a

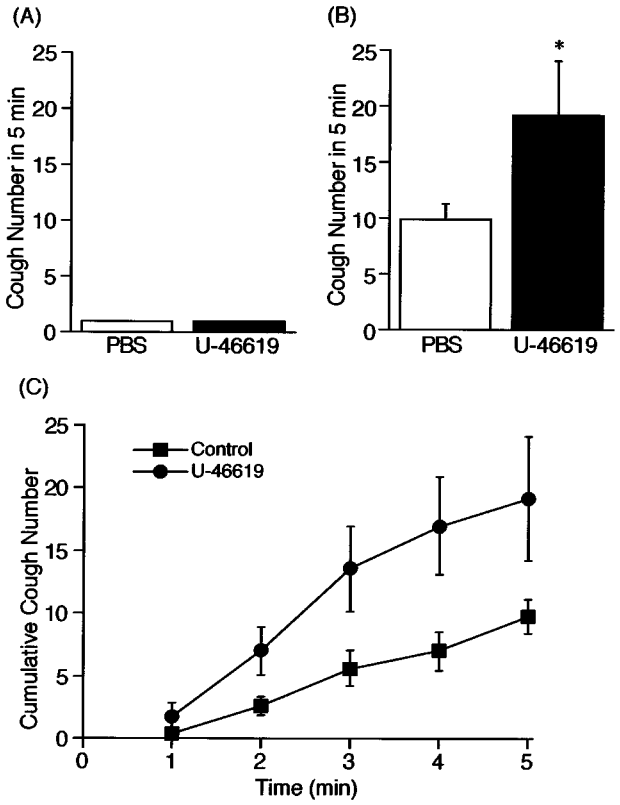

Figure 2 Effect of U-46619 on cough response. U-46619 (100 ng ml^{-1}) or vehicle (PBS) was inhaled for 5 min by guinea-pigs (7- to 8-week-old) and their cough response was monitored (A). Capsaicin was given for 5 min to guinea-pigs (7- to 8-week-old) that had pre-inhaled either U-46619 (100 ng ml^{-1}) or vehicle and the cough response was monitored during the capsaicin inhalation (B). Cumulative capsaicin-induced cough response of guinea-pigs (7- to 8-week-old) pretreated with either U-46619 (100 ng ml^{-1}) or vehicle (C). Each column represents the mean ± s.e.mean ($n=5$). *$P<0.05$ vs PBS.

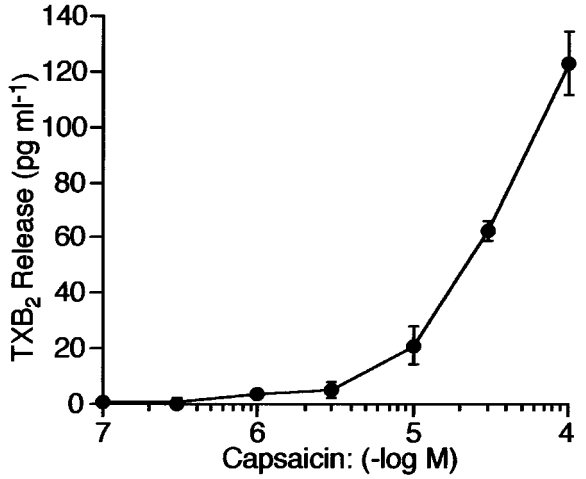

Figure 3 Dose-dependent effect of capsaicin on thromboxane release from BAL cells. BAL cells (2×10^5 cells) were stimulated with capsaicin for 5 min at 37°C. The supernatant was collected for measurement of TXB_2 concentration by ELISA. Data are expressed as the mean ± s.e.mean ($n=5$).

report that infusion of U-46619 stimulates C-fibres directly and slightly activates RARs (Karla *et al*., 1992) and another report showing that injection of the TXA_2 analogue STA_2 increases RARs activity (Matsumoto *et al*., 1994). Further, Widdicombe

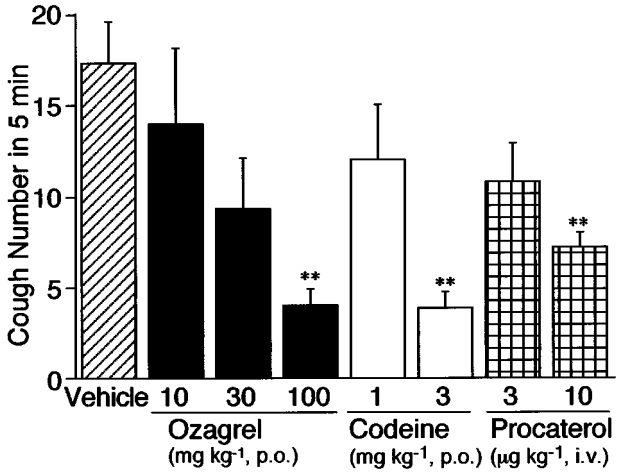

Figure 4 Effects of ozagrel, codeine and procaterol on capsaicin-induced cough. Ozagrel (10, 30 and 100 mg kg^{-1}, p.o.), codeine (1 and 3 mg kg^{-1}, p.o.) or vehicle (0.5% CMC, 1 ml kg^{-1}, p.o.) was administered to guinea-pigs (3- to 4-week-old) 1 h before capsaicin provocation. Procaterol (1 and 10 $\mu g\ kg^{-1}$, i.v.) was given 5 min before capsaicin provocation. Each column represents the mean ± s.e.mean ($n=6$) of the number of coughs produced during a 5-min exposure to capsaicin. **$P<0.01$ vs vehicle.

(1996) pointed out that sensitization and excitation are different mechanisms that can occur separately. Thus, we speculate (a) that TXA_2 stimulates C-fibres and RARs directly but not strongly enough to elicit a cough response by itself and (b) that enhanced coughing is seen early on in the period of capsaicin inhalation in U-46619 pre-treated animals because the sensitivity of these sensory receptors has already been heightened by TXA_2.

It is very difficult to assess whether the cough response is being affected by bronchoconstriction. TXA_2 is known to induce airway bronchoconstriction and, furthermore, an inhibition of bronchoconstriction by ozagrel has been reported (Komatsu *et al*., 1990). To explore the association between bronchoconstriction and the cough response, we administered a β-adrenoceptor agonist, procaterol, intravenously 5 min before capsaicin provocation. In our preliminary experiments, 0.1 $\mu g\ kg^{-1}$ procaterol was enough completely to inhibit histamine-induced bronchoconstriction and this value was of the same orders as one reported previously ($ED_{50}=0.053\ \mu g\ kg^{-1}$; Kikkawa *et al*., 1994). Therefore, we administered 3 and 10 $\mu g\ kg^{-1}$ of procaterol to be sure to block bronchoconstriction completely. However, the cough response was not completely blocked even when we used 10 $\mu g\ kg^{-1}$ of procaterol, (a dose 100 times higher than that needed for complete inhibition of bronchoconstriction). Furthermore, the TXA_2 analogues U-46619 and STA_2 have been found to stimulate sensory receptors with only a weak associated bronchoconstriction (Karla *et al*., 1992; Matsumoto *et al*., 1994). Thus, we speculate that the contribution of bronchoconstriction to the capsaicin-induced cough response was slight under our experimental conditions and that the effects of ozagrel were not due to any significant extent to an inhibition of bronchoconstriction.

Our results indicate the possibility that TXA_2 synthetase inhibitors might be useful drugs for inhibiting the cough response. Although dextromethorphan and codeine are clinically useful cough response suppressants, their use is limited because of their powerful side effects (such as sedation and addiction). Furthermore, it has been shown that codeine has no effect on allergic cough in passively sensitized guinea-

pigs (Winter & Flataker, 1955), raising concern about the effectiveness of codeine against allergic cough in humans. NK_1 and NK_2 tachykinin antagonists also inhibit the cough response but these agents are considered to stop mucus secretion in addition to their CNS actions. Therefore, the advent of antitussive agents with fewer side effects is awaited and ozagrel is a good candidate. In addition to our present results, there have been several studies on the clinical effectiveness of ozagrel. One report showed that ozagrel can suppress the cough response seen in some patients as a side effect of ACE inhibitors (Umemura *et al*., 1997), which are drugs used for heart failure or hypertension. Other reports have indicated that ozagrel is effective against chronic persistent cough (Nishi *et al*., 1996) as well as against post-infectious chronic cough (Fujimori *et al*., 1997). This latter report is a remarkably interesting observation, because the commonest cause of cough is upper respiratory tract infection (URTI) (Widdicombe, 1999). Furthermore, ozagrel increased the threshold for the capsaicin-induced cough response in asthmatics (Fujimura *et al*., 1995), raising the possibility that ozagrel may ameliorate the cough response in asthma. Thus, ozagrel shows great potential as a useful drug for inhibiting the cough response in a variety of disease states.

In conclusion, our results show that TXA_2 is involved in the capsaicin-induced cough response. Although TXA_2 does not itself induce a cough response, it enhances capsaicin-induced cough, possibly by sensitizing airway receptors. Thus, TXA_2 synthetase inhibitors show promise as useful drugs for inhibiting the cough response.

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