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The cysteinyl-leukotriene-1 receptor antagonist zafirlukast is a potent secretagogue in rat and human airways

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Abstract

Cysteinyl-leukotriene-1 receptor antagonists are important tools in the therapy of asthma. Although many studies have been performed concerning their effects on airway smooth muscle tone, there are no basic data on their effects on airway secretions. Therefore, we assessed the effects of zafirlukast and montelukast on rat tracheal secretion by quantification of secreted $^{35}S0_4$ labelled mucus macromolecules, and determined the influence of the arachidonic acid pathway using the modified Ussing chamber technique.

Zafirlukast (432±89.99%) and montelukast (167±16.74%) stimulated rat tracheal secretion. This was abolished by application of eicosatetraenoic acid, an inhibitor of the arachidonic acid metabolism. Whereas inhibition of cyclooxygenase did not show any significant effect on zafirlukast induced secretion, blockade of the 5-lipoxygenase pathway markedly reduced the secretagogue effects. Furthermore, inhibition of phosphatidylinositol-3-kinase completely inhibited the effects elicited by zafirlukast. Additional experiments revealed secretagogue effects of zafirlukast also in human bronchial tissue.

In conclusion, zafirlukast is a potent inducer of tracheal secretion. Obviously, these effects are induced by involvement of a phosphatidylinositol-3-kinase dependent pathway mediated by products of the arachidonic acid metabolism.

Keywords: Leukotriene receptor antagonist; Airway mucus; Secretion; Asthma; COPD (chronic obstructive pulmonary disease)

1. Introduction

Accumulating evidence suggests that products of the arachidonic acid pathway are involved in numerous homeostatic biological functions and inflammatory processes (Funk, 2001). Arachidonic acid, derived from hydrolysis of membrane phospholipids by phospholipase A_2 functions as a substrate of the eicosanoid family, comprising the prostaglandins, leukotrienes, and related compounds. Once released, a portion of the arachidonate is metabolized rapidly by several distinct enzyme systems to oxidated products, including cyclooxygenases, lipoxygenases or cytochrome *P*45Os. The leukotrienes are synthesized via activation of 5-lipoxygenase in cooperation with the 5-lipoxygenase-activating protein in a number of inflammatory cells (Devillier et al., 1999). The cysteinyl-leukotrienes leukotriene C_4, leukotriene D_4 and leukotriene E_4 have proven to be important biologically active compounds known to play a pivotal role in the pathogenesis of various diseases of the respiratory tract including bronchial asthma. The cysteinyl-leukotrienes are among the most potent bronchoconstrictors yet studied in human subjects, being up to 10,000-fold more potent than histamine or methacholine (Weiss et al., 1982; Adelroth et al., 1986; Griffin et al., 1983; Smith et al., 1985). They increase endothelial cell membrane permeability leading to edema formation (Wasserman et al., 1995), stimulate mucus secretion (Marom et al., 1982), and decrease mucus clearance (Ahmed et al., 1981). These lipid mediators exert their biological effects by binding to and activating specific receptors, known as cysteinyl-leukotriene-1 receptor and cysteinyl-leukotriene-2 receptor. Most of the actions of the cysteinyl-leukotrienes, including contraction of human airway smooth muscle,

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increasing mucus secretion or vascular permeability are mediated by the cysteinyl-leukotriene-1 receptor (Drazen et al., 1999). In humans the cysteinyl-leukotriene-2 receptor mediates contraction of pulmonary vascular smooth muscles while binding sites for the dihydroxy-leukotriene B_4, predominantly mediate chemotaxis (Brink et al., 2003). Specific cysteinyl-leukotriene-1 receptor antagonists have been developed and are already available for clinical use. Compounds such as zafirlukast, montelukast and pranlukast have shown efficacy in inhibiting leukotriene induced effects including bronchoconstriction, airway hyperreactivity and inflammatory processes and clearly demonstrate a clinical benefit in asthma patients (Drazen et al., 1999). It has been postulated that leukotriene receptor antagonists may have the potential to decrease mucus production and increase mucus clearance in patients with asthma (Hay, 1997). However, although many studies have been performed concerning the effects of leukotriene receptor antagonists on inflammatory cells and smooth muscle tone of the airways (Chan et al., 1990; Hui and Barnes, 1991; Reiss et al., 1997) there is only little knowledge about their effects on airway secretion (Liu et al., 1998). Therefore, it was the aim of the present study (i) to characterize the effects of zafirlukast and montelukast on airway secretory activity, (ii) to determine the influence of the arachidonic acid pathway and the related products in this context and, (iii) to clarify the role of phosphatidylinositol-3-kinase.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Harlan Winkelmann GmbH, Borchen, Germany) with an average body weight of 400±39 g were used for all experiments after approval of the protocol by the Regional Review Board for the care of animal subjects and in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985). The animals were kept in a light- and temperature-controlled room and had free access to water and a rat standard diet (Altromin, Lage, Germany). For each experiment $n=5–10$ animals were used.

2.2. Patients

For additional experiments with human tissue, bronchial explants were taken from patients suffering from bronchial carcinoma who underwent a pulmonary lobectomy. All human preparations used for the experiments were macroscopically tumor-free. Immediately after resection, human bronchial tissue was transferred to an organ bath filled with medium M199 in Earle's balanced salt solution (Gibco, Eggenstein, Germany), equilibrated with carbogen gas (95% oxygen, 5% carbon dioxide). The tissue preparation, radiolabelling of mucus macromolecules, and the study design were exactly the same as described for rat tissue in Sections 2.3, 2.4, and 2.5. All experiments were approved by the local ethics committee.

2.3. Reagents

Pentobarbital sodium (Nembutal®) for anesthesia was obtained from Sanofi (München, Germany). Sodium azide and acetylcholine was purchased from Merck (Darmstadt, Germany). 5,8,11,14-Eicosatetraynoic acid (ETYA), Ibuprofen and MK-886 were from Biomol (Hamburg, Germany). LY 294002 was from Calbiochem (Bad Soden, Germany). $Na_2^{35}SO_4$ for radiolabelling glycoproteins was from Amersham (Braunschweig, Germany). Montelukast (MK-476) was received as a gift from Merck Frosst (Quebeck, Canada) and Zafirlukast (ICI-204,219) from Zeneca (Cheshire, UK). All other reagents used were purchased from Sigma-Aldrich (Deisenhofen, Germany) if not specified otherwise. Stock solutions of MK-886 and ETYA were prepared in ethanol. The vehicle for Ibuprofen, LY-294002, montelukast and zafirlukast was dimethyl sulphoxide (DMSO). Maximum concentrations of ethanol or dimethyl sulphoxide during the experiments were 0.5%. None of the vehicles showed any significant effects on tracheal secretory activity (data not shown).

2.4. Tissue preparation

The modified Ussing chamber technique is a well established method for measurement of tracheal secretion and has been described in detail previously (Bredenbroker et al., 2001; Liu et al., 1998; Wagner et al., 1995a,c, 1996, 1998, 1999). Briefly, rats were anesthetized by an intraperitoneal injection of 70 mg/kg body weight pentobarbital sodium. The trachea was excised through a ventral collar midline incision and median sternotomy and immediately transferred to an organ bath filled with medium M199 in Earle's balanced salt solution, equilibrated with carbogen gas. After removing the connective tissue the trachea was opened along the paries membranaceus and mounted between the two halves of the modified Ussing chamber. According to the volume of the perfusion device, 7 ml of medium M199 was added to the luminal (i.e. mucosal) and submucosal sides, respectively. The pH was adjusted to 7.41 and a constant temperature of 37 °C was maintained during the whole experiment.

2.5. Radiolabelling and measurement of airway glycoprotein secretion

50 μCi $Na_2^{35}SO_4$ were added to the solution bathing the submucosal side and allowed to equilibrate with the tissues for the duration of the experiment. After 2 h the release of bound $^{35}SO_4$ to the mucosal side reaches steady state (Bredenbroker et al., 2001). Subsequently the luminal solution was collected every 15 min and replaced with fresh medium. The perfusate samples from the luminal side were collected in cellulose dialysis tubing (12,000–14,000 Da molecular mass cut-off, Serva, Heidelberg, Germany) and dialysed against distilled water containing unlabelled Na_2SO_4, to remove unincorporated $^{35}SO_4$, and sodium azide (10 mg/l) to prevent bacterial degradation. Dialysis was complete when the radioactive count of the dialysis fluid 3 h after the last change was the same as

that of tap water. The samples were transferred to plastic vials mixed with 10 ml of szintillant (Lumagel®, Baker, Deventer, Netherlands) and radioactivity was measured using a liquid szintillation counter (Rackbeta LKB 1219, LKB Instruments, Graefeling, Germany). The counts of labelled macromolecules represent the secretory activity. Former studies from our lab using high-performance liquid chromatography (HPLC) and autoradiography identified these labelled macromolecules as airway secretory glycoproteins from the submucosal glands, which were not digested by chondroitinase ABC. Thus, these macromolecules are true glycoproteins.

2.6. Study design

After 2 h of incubation, samples were collected every 15 min. The average of two samples before pharmacological intervention represented the basal secretion rate (=100%). Drugs were applied to the mucosal side and collections were taken 15 min later. Between each application, four fractions of 15 min were collected to allow the system to recover and reach a basal secretion again. Dose–response correlations for the substances tested were performed by luminal application in increasing steps of a factor of 10 according to the above mentioned design. In order to test the viability of the system, each experiment was finished with a stimulation of acetylcholine (1 μM), an established secretagogue for this system (Bredenbroker et al., 2001).

2.7. Data analysis

Data are expressed in percent of basal secretion±S.E.M. Statistical analyses were performed with Student's *t*-test. Experiments with at least five animals were performed for each experimental protocol. In additional experiments performed with human tissue, at least four bronchial explants per tested substance were used. Data were considered significant when $P<0.05$. Statistical analysis was performed using the Sigma Stat software package (Jandel Scientific, San Rafael, CA).

3. Results

3.1. Zafirlukast and montelukast stimulate rat tracheal secretory activity

The effects of the cysteinyl-leukotriene-1 receptor antagonists zafirlukast and montelukast on tracheal secretory activity in the rat were examined. As shown in Fig. 1, zafirlukast stimulated tracheal secretion in a concentration dependent manner. While zafirlukast at 0.01 μM and 0.1 μM had no effect on tracheal secretion, 1 μM zafirlukast showed a significant secretagogue activity (124.87±7.11%) compared to baseline levels and the same concentration of montelukast ($P<0.05$). Zafirlukast at 10 μM and 100 μM elicited secretion up to 260.33±16.57% and 432.30±89.99% of baseline levels ($P<0.01$), respectively. In contrary, montelukast had no secretagogue effects on tracheal secretion except at the highest concentration used (100 μM) where it shows a moderate, but significant increase of

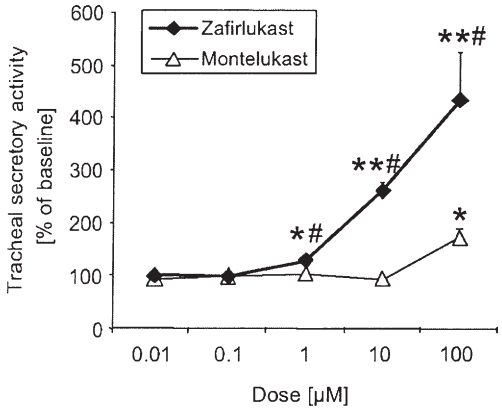

Fig. 1. Dose–response characteristics of zafirlukast (0.01–100 μM) and montelukast (0.01–100 μM) on tracheal secretory activity in the rat. Zafirlukast stimulated secretion dose dependently. Montelukast exerted secretagogue effects only at 100 μM. Data are expressed as mean±S.E.M. percent of baseline secretion for $n=5$ animals per group. *$P<0.05$ versus respective baseline values; **$P<0.01$ versus respective baseline values; #$P<0.05$ versus montelukast-induced secretion.

secretion up to 167.34±16.74% compared to the respective baseline value (Fig. 1; $P<0.05$).

3.2. Effects of ibuprofen, MK-886 and ETYA on zafirlukast induced tracheal secretory activity

The influence of the arachidonic acid pathway and its metabolites on zafirlukast induced tracheal secretory activity was examined. Zafirlukast was used at a submaximum concentration of 10 μM to possibly observe inhibitory or stimulating effects of the tested substances on zafirlukast induced secretion. Ibuprofen, a cyclooxygenase inhibitor, MK-886, a five-lipoxygenase-activating-protein inhibitor and ETYA, an inhibitor of the whole arachidonic acid cascade were used alone and in combination with zafirlukast to test the pathways related. In every single experiment zafirlukast itself profoundly stimulated tracheal secretion (Fig. 2A,B,C; $P<0.05$). Ibuprofen (100 μM; 125.32±40.87%) alone had no significant influence on secretion. In combination with zafirlukast, ibuprofen slightly but not significantly attenuated zafirlukast induced tracheal secretion (Zafirlukast: 278.13± 67.11%; Zafirlukast+ibuprofen: 195.83±50.05%; Fig. 2A). In contrast, MK-886 (10 μM; 134.54±11.91%) itself stimulated tracheal secretion ($P<0.05$) and markedly reduced zafirlukast induced secretions (Zafirlukast: 233.74±36.58%; Zafirlukast+MK-886: 148.28±9.25%; $P<0.05$; Fig. 2B). ETYA when given alone (100 μM; 106.33±17.88%) had no effect on secretion but it dramatically reduced zafirlukast induced tracheal secretion to baseline levels (Zafirlukast+ETYA: 107.12±15.27; $P<0.05$; Fig. 2C).

3.3. Effects of the phosphatidylinositol-3-kinase inhibitor LY-294002 on zafirlukast induced tracheal secretory activity

The effects of the phosphatidylinositol-3-kinase inhibitor LY-294002 on zafirlukast induced tracheal secretion are shown in Fig. 3. Zafirlukast (10 μM) induced secretion up to

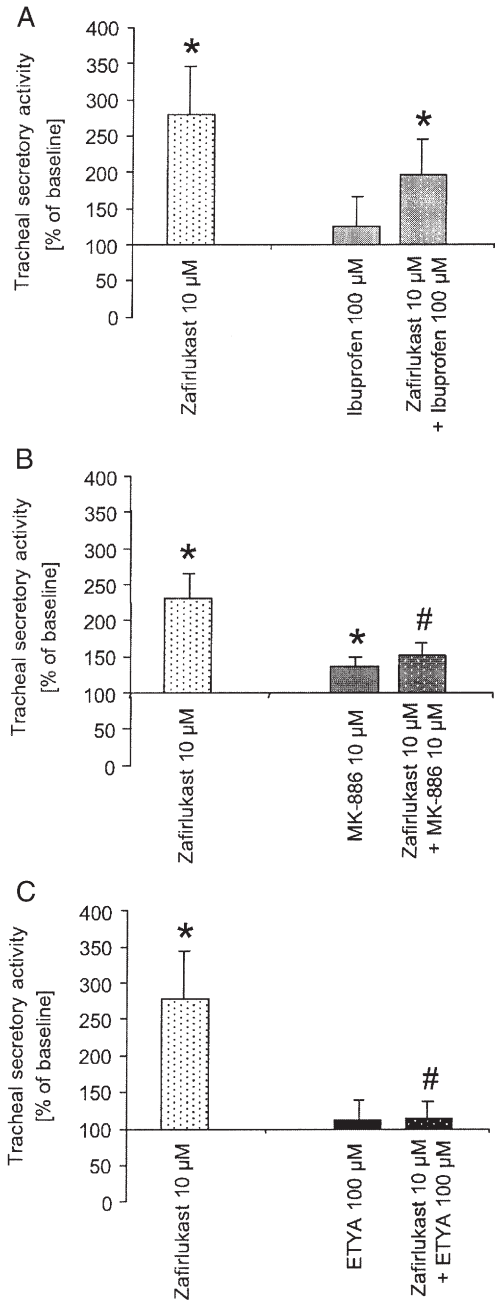

Fig. 2. Effects of ibuprofen (100 μM), MK-886 (10 μM) and eicosatetraynoic acid (ETYA; 100 μM) on zafirlukast (10 μM) induced tracheal secretory activity in the rat. Zafirlukast stimulated secretion. Since ibuprofen had no significant effect, MK-886 and ETYA inhibited zafirlukast induced secretions. MK-886 increased tracheal secretion by itself. Data are expressed as mean±S.E.M. percent of baseline secretion for $n=5$–10 animals per group. *$P<0.05$ versus respective baseline values; #$P<0.05$ versus zafirlukast-induced secretion.

228.82±32.63% of baseline level ($P<0.05$). LY-294002 (50 μM) alone stimulated tracheal secretion significantly (145.25±6.29%; $P<0.05$). To block phosphatidylinositol-3-kinase activity, LY-294002 was incubated four times 15 min within the culture medium before the combined zafirlukast and LY-294002 application. In sharp contrast to the application of zafirlukast alone, addition of zafirlukast into LY-294002 preincubated culture medium showed no further secretagogue effect. Thus, the secretagogue effect of zafirlukast was completely blocked (109.99±8.92%; $P<0.05$).

3.4. Effects of zafirlukast and montelukast on human bronchial secretory activity

The results of additional experiments with human bronchial explants are shown in Fig. 4. Zafirlukast (100 μM) induced secretion up to 146.59±14.58% of baseline level ($P<0.05$) while montelukast exerts no significant secretagogue effects (112.32±7.86% at 100 μM).

4. Discussion

The aim of the present study was to characterize the secretagogue activity of the cysteinyl-leukotriene-1 receptor antagonists zafirlukast and montelukast in tracheal explants of the rat. Our results demonstrate for the first time that zafirlukast profoundly stimulates tracheal secretion. Montelukast has a lower secretagogue potency. These effects showed a dose response relationship and were highly dependent on products of the arachidonic acid metabolism. Blockade of this pathway with ETYA completely inhibited the secretagogue activity of zafirlukast. Whereas Ibuprofen, a cyclooxygenase inhibitor, had no significant effect on zafirlukast induced airway secretion, MK-886, a five-lipoxygenase-activating-protein antagonist, inhibited zafirlukast induced tracheal secretion significantly. In addition to the involvement of the arachidonic acid pathway in stimulus secretion coupling we demonstrated the influence of phosphatidylinositol-3-kinase activity on zafirlukast induced tracheal secretion. Furthermore, MK-886 and LY-294002 stimulated tracheal secretory activity by themselves. Additional studies revealed secretagogue properties of zafirlukast also in human airway tissue.

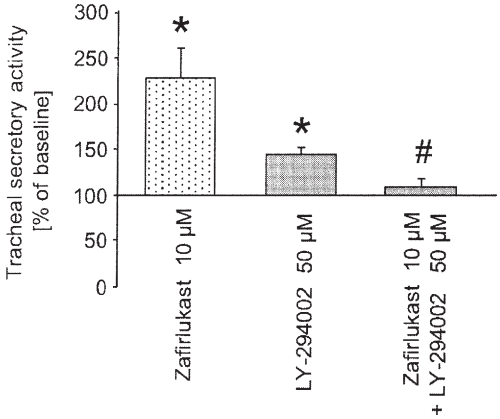

Fig. 3. Effects of LY-294002 (50 μM) on zafirlukast (10 μM) induced tracheal secretory activity in the rat. The phosphatidylinositol-3-kinase inhibitor LY-294002 completely blocked zafirlukast induced tracheal secretion, LY-294002 was incubated for four times 15 min within the culture medium before application of zafirlukast to guarantee inhibition of phosphatidylinositol-3-kinase. Data are expressed as mean±S.E.M. percent of baseline secretion for $n=5$ animals per group. *$P<0.05$ versus respective baseline values; #$P<0.01$ versus zafirlukast-induced secretion.

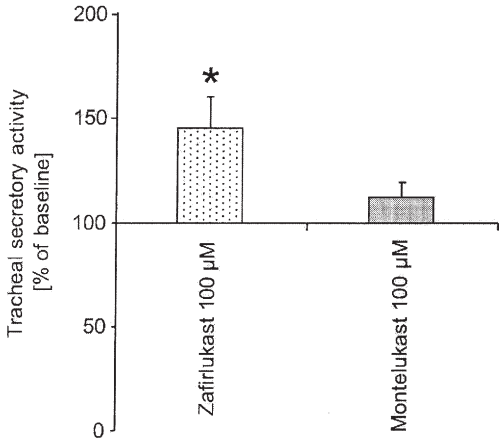

Fig. 4. Effects of zafirlukast (100 µM) and montelukast (100 µM) on tracheal secretory activity in human bronchial explants. Zafirlukast stimulated secretion significantly. Data are expressed as mean±S.E.M. percent of baseline secretion for $n=5$ (zafirlukast) and $n=4$ (montelukast) bronchial explants. *$P<0.05$ versus respective baseline values.

Our results show that zafirlukast and montelukast differed in their potency as secretagogues in tracheal explants. It is interesting that these two leukotriene receptor antagonists belong to different chemical classes which could be the reason for the differences observed. Whereas montelukast stimulated tracheal secretion only at the highest concentration used (100 µM), zafirlukast exerted its effects at a concentration of 1 µM with increasing action due to higher concentrations up to 432% of baseline values at 100 µM. In addition to these findings in the rat, we could also show secretagogue effects of zafirlukast tested in human bronchial explants. Zafirlukast was used in our experiments at concentrations in between 0.01 and 100 µM. Concentrations of zafirlukast measured in human plasma 2 h after oral administration of a single 20 mg dose led to a plasma concentration of 0.39 µM (Bui et al., 1997), or a 40 mg dose led to a plasma concentration of 0.50 µM (Smith et al., 1990), so that the concentrations used in our studies touch this range. Krell et al. (1990) evaluated the receptor selectivity of zafirlukast at 10 µM and demonstrated that the compound has virtually no affinity for most receptors: the single exception was the EP_1 receptor in guinea pig ileum where zafirlukast was 10,000-fold less potent than on guinea pig airway leukotriene receptors. However, additional non-selective effects of the cys-LT_1 antagonists in our experimental setting cannot be excluded. Recently, Liu et al. (1998) reported that the leukotriene receptor antagonists pranlukast and zafirlukast significantly suppressed leukotriene D_4 and ovalbumin induced secretion in tracheae from sensitized guinea-pigs (Liu et al., 1998). None of the antagonists stimulated secretion in their model. These data may not be contradictory to the present findings since the source of secretion in the guinea-pig model is predominantly from goblet cells and not from submucosal glands as in the present rat preparation (Jeffery, 1983; Jeffery and Reid, 1975; Liu et al., 1998). These two different observations therefore may indicate that zafirlukast- and montelukast induced tracheal secretion is primarily from submucosal glands. The specific pathogen free rats used in all of our experiments nearly completely lack goblet cells in their surface epithelium, so that the almost exclusive source of airway secretion are the submucosal glands (Jeffery and Reid, 1975; Jeffery, 1983).

Moreover, autoradiographic studies from our lab using ^{35}S-radiolabel demonstrate the accumulation of the label at the site of the submucosal glands, especially the acini. Nearly no label was found in the cells of the surface epithelium which furthermore suggests the submucosal origin of the glycoproteins. None of the other well established secretagogues including histamine, prostaglandines, leukotrienes or tachykinins had shown such high stimulatory activity like zafirlukast in inducing tracheal secretion (Wagner et al., 1995b,c; Marom et al., 1982; Liu et al., 1998; Kishioka et al., 1997, 2001).

No clinical data are available concerning zafirlukast induced mucus secretion so far. Thus, clinical trials would be necessary to assess the secretagogue effect of zafirlukast at usual doses, especially to answer the question whether the secretagogue effect of the leukotriene receptor antagonist observed in vitro is beneficial for example in asthma therapy. On the one side it may be useful to increase mucociliary clearance (Sabater et al., 2002) and take advantage of a higher amount of tracheobronchial glycoproteins, which are known to bind and trap inhaled particles to facilitate clearance out of the airways (Knowles and Boucher, 2002). On the other hand hypersecretion may contribute to a limitation of air flow like in acute asthma (Groneberg et al., 2002, 2004). Therefore, further studies are needed to evaluate the clinical significance of zafirlukast induced mucus secretion.

The arachidonic acid pathway and its related products are of critical importance in the pathogenesis of bronchial asthma. Our results demonstrate the involvement of the arachidonic acid metabolism in zafirlukast induced tracheal secretion. This pathway is essential for the generation of eicosanoids. Prostaglandins are generated by the action of cyclooxygenases. The leukotrienes are formed via activation of 5-lipoxygenase in connection with the five-lipoxygenase-activating-protein in a number of inflammatory cells including macrophages, mast cells, neutrophils, eosinophils, basophils, and B lymphocytes (Devillier et al., 1999). Application of ETYA, which completely blocked the arachidonic acid pathway through inhibition of phospholipase A_2, cyclooxygenases, lipoxygenases and cytochrome *P*45Os totally abolished the zafirlukast induced secretagogue effect. This phenomenon implies a direct involvement of the arachidonic acid pathway and suggests a zafirlukast triggered synthesis of arachidonic acid metabolites which in turn mediate the secretagogue effect demonstrated. Therefore, we tested the two major pathways of arachidonic acid metabolism catalysed by cyclooxygenase or 5-lipoxygenase. Inhibition of cyclooxygenase by application of ibuprofen slightly attenuated the zafirlukast induced effect without reaching significance. In contrast, MK-886, a five-lipoxygenase-activating-protein inhibitor significantly blocked zafirlukast induced tracheal secretion suggesting a role of 5-lipoxygenase metabolites in zafirlukast induced airway secretion. Furthermore, MK-886 stimulated secretion by itself up to 135% of baseline levels. This may be caused by an effect observed by Jan et al. who reported an arachidonic acid independent MK-886 induced

activation of cellular calcium mobilisation in Mardin–Darby canine kidney cells (Jan and Tseng, 2000). Intracellular calcium release or calcium influx is an important trigger leading to mucus secretion (Ishihara et al., 1990).

Recently, it has been shown that phosphatidylinositol-3-kinase is essential for the activation of group IV-phospholipase A_2, the enzyme that is of vital importance for the synthesis of inflammatory eicosanoids like prostaglandins or leukotrienes (Myou et al., 2003). Furthermore, Ito et al. (2002) showed that the phosphatidylinositol-3-kinase is necessary for leukotriene B_4 induced enzyme release, while Gibbs and Grabbe (1999) demonstrated the involvement of phosphatidylinositol-3-kinase in leukotriene C_4 synthesis. These facts prompted us to test whether phosphatidylinositol-3-kinase is also involved in zafirlukast induced airway secretion. Application of the phosphatidylinositol-3-kinase inhibitor LY-294002 led to a moderate increase of tracheal secretion in our setting. Likewise MK-886, LY-294002 has the ability to mobilize intracellular calcium stores, an effect described by Ethier and Madison in bovine and human smooth muscle cells that in turn could stimulate secretion (Ethier and Madison, 2002). Our results further indicate that the inhibition of phosphatidylinositol-3-kinase abolished zafirlukast induced tracheal secretion speaking in favour of the importance of this kinase as a crucial element involved in the secretagogue effects mediated by zafirlukast.

In summary, the present study demonstrates that zafirlukast is a potent inducer of experimental rat tracheal secretion. Montelukast has a lower secretagogue potency. These effects are likely induced by the involvement of a phosphatidylinositol-3-kinase dependent pathway mediated by products of the arachidonic acid metabolism. Additional experiments also demonstrated secretagogue effects of zafirlukast in human bronchial explants. Thus, further investigations are needed to clarify the clinical significance of these observations.

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