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SHORT COMMUNICATION

Erlotinib: Lacking of Cholinergic Effects on Tracheal Smooth Muscle

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Erlotinib (Tarceva) is an oral epidermal growth factor receptor-tyrosine kinase inhibitor that is mainly used for patients with advanced or metastatic non-small-cell lung cancer. Tyrosine kinase signaling cascades also play a critical role in the pathogenesis of allergic airway inflammation and airway remodeling. However, cholinergic effects caused by erlotinib on tracheal smooth muscle remain unclear. The objective of this study was to determine the effects of erlotinib on the isolated rat tracheal smooth muscle *in vitro*. To examine the cholinergic effects of erlotinib, *in vitro* rat tracheal smooth muscle was used to assess alterations in methacholine-induced contraction (served as a parasympathetic mimetic) and electrically induced contraction. The results demonstrated that the addition of erlotinib (from 1×10^{-8}M to 1×10^{-4}M) induced no significant effects on tracheal tension after methacholine treatment. Furthermore, erlotinib did not affect electrical field stimulation-induced spike contraction. This study demonstrated that erlotinib had no cholinergic effects *in vitro*, suggesting it may be safe for asthmatic patients with non-small-cell lung cancer after further investigation.

1. Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide, and its incidence has been increasing. Non-small-cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers. Patients with NSCLC harboring mutations in the epidermal growth factor receptor (EGFR) gene have a dramatic response to EGFR-tyrosine kinase inhibitor (EGFR-TKI).[1] Therapeutic modalities used in thoracic oncology include molecularly targeted therapy using low-molecular-weight TKIs that block the activation of the EGFR cascade. Erlotinib (Tarceva) is an oral EGFR-TKI that is mainly used for patients with advanced or metastatic NSCLC who have failed at least one prior chemotherapy regimen. First-line treatment with EGFR-TKIs such as erlotinib showed higher efficiency than standard chemotherapy regimens in patients harboring EGFR mutations.[2,3]

Asthma is a very common chronic disease that occurs in all age groups. Association between asthma and lung cancer has been reported,[4] suggesting that asthma is a risk factor of lung cancer development. However, there is a paucity of studies evaluating the risk of lung cancer treatment in patients with asthma. Comparisons of the efficacy and safety of erlotinib with standard chemotherapy regimens for second-line therapy confirmed that erlotinib has comparable efficiency and a better toxicity profile.[5,6] However, TK signaling cascades play a critical role in the pathogenesis of allergic airway inflammation. Receptor TKs such as EGFR are important for the pathogenesis of airway remodeling.[7] It has been demonstrated that EGFR expression is increased in asthmatic human airway.[8] In human airway smooth muscle cells, both epidermal and platelet-derived growth factors have been revealed to promote EGFR and platelet-derived growth factor receptor tyrosine autophosphorylation, leading to transcription factor activation and proliferation.[9] However, effects of EGFR signaling on allergic responses induced by erlotinib remain unclear. Using trachea isolated from rats we have developed a simple *in vitro* model to study agents that affect tracheal smooth muscle.[10] This system can provide more evidence of the cholinergic effects in response of the trachea to drugs *in vivo*. To clarify this issue, we used tracheal smooth muscle *in vitro*, which

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has an important role in the response to asthma attacks, to examine the cholinergic effects of erlotinib.

2. Methods

2.1. Reagent sources

This study was approved by the Animal Research Committee of Taipei Medical University (LAC-99-0299; Taipei, Taiwan). Pure erlotinib was obtained from the Roche Company (Taipei, Taiwan). All other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Tissue sampling and preparation

We obtained 18 8-week-old Sprague—Dawley rats from the National Laboratory Animal Breeding and Research Centre (Taipei, Taiwan). Rats were anesthetized via intraperitoneal pentobarbital injections (45 mg/kg), and two 5-mm long pieces of trachea were obtained from each rat; this procedure had been described in detail in previous studies.[11,12] The upper side of the tracheal sample was attached to a Grass FT-03 force displacement transducer (AstroMed, West Warwick, RI, USA) using a steel plate and a 3-0 silk ligature, whereas the other side was fixed to a steel plate attached to a container with 30 mL of Kerb's solution (NaCl, 118 mmol/L; KCl, 4.7 mmol/L; $CaCl_2$, 2.5 mmol/L; $MgSO_4 \cdot 7H_2O$, 1.2 mmol/L; KH_2PO_4, 1.2 mmol/L; $NaHCO_3$, 25.0 mmol/L; and glucose, 10.0 mmol/L) at 37°C. A passive tension of 0.3 g was applied to the strips, and subsequent changes in tension were recorded continuously using Chart V4.2 software (PowerLad; ADI Instruments, Colorado Springs, CO, USA).

2.3. Methacholine challenge

Methacholine (1×10^{-6}M) was used as a tracheal contractor in this study. A preliminary experiment was performed using a tracheal strip to determine the contraction when basal tension was applied. Isolated tracheas were equilibrated in the solution for 30 minutes and aerated with a mixture of 95% O_2 and 5% CO_2. Stepwise increases in erlotinib concentrations (from 1×10^{-8}M to 1×10^{-4}M; dissolved in d-H_2O) were used to investigate the contraction or relaxation responses of the tracheal strips. All treatments were administrated by adding a defined volume of stock solution to the solution. After the experiment, 1×10^{-4}M lidocaine was used to reduce the tension caused by methacholine and/or erlotinib.

2.4. Electrical field stimulation challenge

Electrical field stimulation (EFS; 5 Hz, 5-millsecond pulse duration, voltage of 50 V, stimulation trains of 5-second duration) was applied to the tracheal strip with two wire electrodes placed parallel to the strip and connected to a direct-current stimulator (Grass S44, Grass Instruments, Quincy, MA, USA). A 2-minute interval was imposed between each stimulation period to allow recovery from the response. The stimulation experiment was performed at 37°C.

2.5. Measurements

The effects of erlotinib on tracheal smooth muscle resting tension and 1×10^{-6}M methacholine and erlotinib on electrically induced tracheal smooth muscle contractions were determined. In each experiment, one untreated tracheal strip served as a control. Concentrations of erlotinib were expressed as the concentrations present in the 30-mL solution.

2.6. Statistical analysis

Data for the basal tension and methacholine experiments were presented as the mean tension induced by two different concentrations of the agent. EFS data were presented as the mean EFS peak induced by two different concentrations of the agent. Student *t* test was used to evaluate the differences. All statistical analyses in this study were performed with SPSS 15.0 software (SPSS Inc., Chicago, Illinois, USA). The level of significance for all statistical analyses was $p < 0.05$. Data were presented as the mean ± standard deviation.

3. Results

Tracheal responses to the treatments were determined from the tension applied to the transducer. Control experiments were initially performed to measure the tracheal contraction induced by 1×10^{-6}M methacholine (Figure 1). We then treated the tissue with increasing concentrations (from 1×10^{-8}M to 1×10^{-4}M) of erlotinib and determined the alterations in contraction/relaxation after treatment (Figure 1). A slight decrease was observed in the contractile responses to erlotinib exposure, albeit without significance. The percentages of contraction in the tracheal tissues were 99.0 ± 1.1 (at 1×10^{-8}M erlotinib), 98.4 ± 1.4 (at 1×10^{-7}M erlotinib), 97.8 ± 1.5 (at 1×10^{-6}M erlotinib), 97.3 ± 2.0 (at 1×10^{-5}M erlotinib), and 97.0 ± 1.8 (at 1×10^{-4}M erlotinib), as shown in Figure 2. A relaxant drug (1×10^{-4}M lidocaine) was applied to the tissue to examine the reaction of the tracheal strips.

The effects of erlotinib on electrically induced tracheal smooth muscle contraction were examined. The results revealed that no significant EFS response was induced by the addition of different concentrations of erlotinib (Figure 3). Percentage changes of peak tension in the tissues were reduced slightly from 100 (control) to 98.4 ± 1.9, 98.1 ± 1.6, 97.0 ± 2.0, and 97.1 ± 1.7 at 1×10^{-7}M,

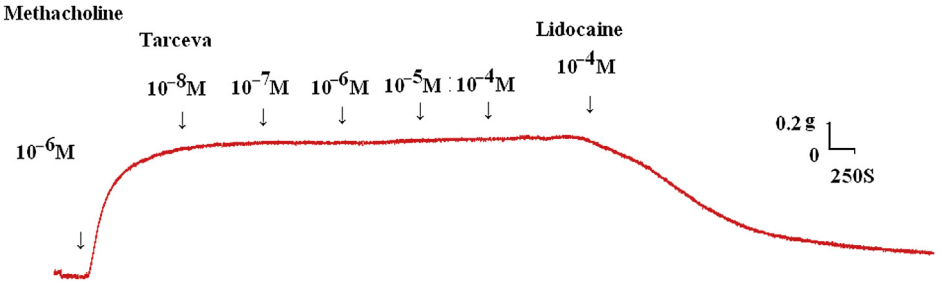

Figure 1 Original recording of the effects of erlotinib on 10^{-6}M methacholine-induced tracheal smooth muscle contractions. Tension changes in tracheal smooth muscle strip were demonstrated after treatment with 10^{-8}M and 10^{-4}M erlotinib. No significant effects were caused by erlotinib. Methacholine was initially used to induce tracheal contraction, whereas lidocaine was used to relax the tracheal muscle after the test.

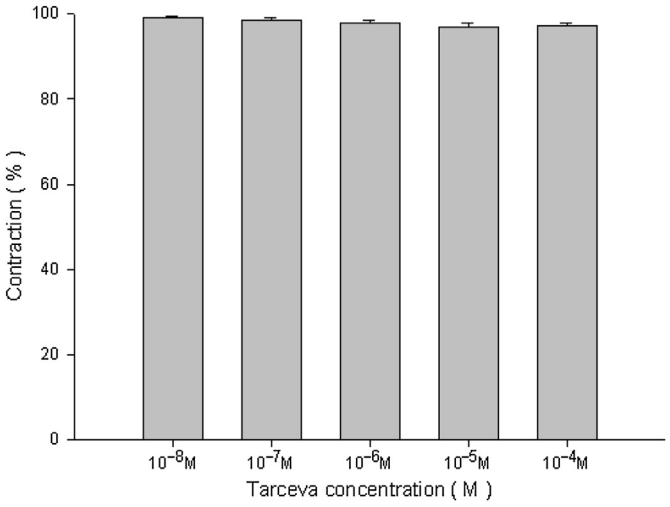

Figure 2 Effects of erlotinib on 10^{-6}M methacholine-induced tracheal contraction (concentration area was calculated at 100% in the absence of erlotinib). The difference in tension induced by 10^{-8}M and 10^{-4}M erlotinib was not statistically significant. Results are presented as mean ± SD ($n = 6$). SD = standard deviation.

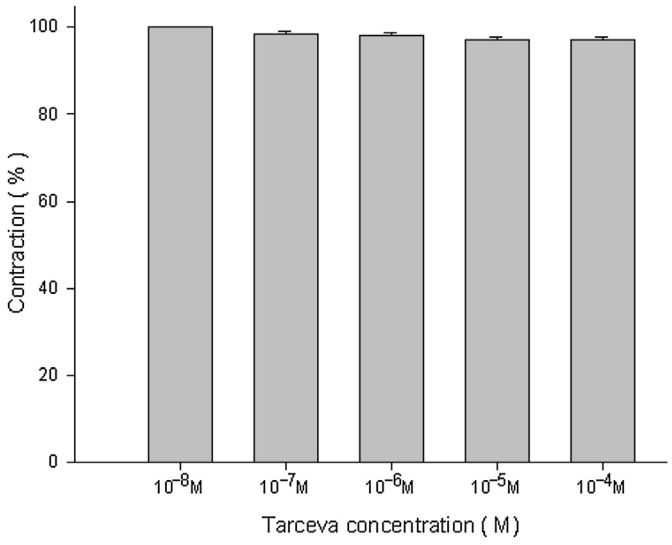

Figure 4 Effects of erlotinib on electrically induced tracheal smooth muscle contractions (concentration area was calculated at 100% in the absence of erlotinib). The difference in tension induced by 1×10^{-8}M and 1×10^{-4}M erlotinib was not statistically significant. Results are presented as mean ± SD ($n = 6$). SD = standard deviation.

1×10^{-6}M, 1×10^{-5}M, and 1×10^{-4}M erlotinib concentrations, respectively (Figure 4).

4. Discussion

Erlotinib is an EGFR-TKI that is mainly used in the treatment of patients with advanced or metastatic NSCLC. However, many adverse effects of erlotinib have been reported previously.[13,14] Epithelial EGFR expression and receptor signaling have been associated with airway remodeling and dysfunction in chronic asthma[8,15]; this finding suggests that there could be an allergic effect in asthma patients who used erlotinib for cancer therapy. However, no significant effects of erlotinib on total immunoglobulin (Ig)G1 and total IgE levels and cytological responses were observed in dust mite-sensitized mice.[16] A better understanding of the cholinergic effects of erlotinib on tracheal smooth muscle is critical for the physiological and toxicological evaluation of erlotinib use in asthma patients. A previous study showed that plasma concentrations of erlotinib in an elderly NSCLC patient ranged from 1.2×10^{-7}M to 9.2×10^{-7}M.[17] To understand the cholinergic effects caused by erlotinib, tracheal smooth muscles were exposed to various concentrations of erlotinib (from 1×10^{-8}M to 1×10^{-4}M). Experimental findings of the present study indicated that no significant cholinergic effects were induced by erlotinib. The main finding supporting the conclusion is the insignificant modification in tension changes and electrical stimulation in the rat trachea after the application of various concentrations of erlotinib.

Investigation of tracheal responses to drugs, in which a relatively long tracheal mucosa strip (8 mm × 20 mm) is attached to an isometric transducer and suspended in a tissue medium (Kerb's solution), has been reported previously.[18,19] To reduce the use of tracheal samples and simplify the experiment, we developed a new tracheal model for drug testing.[11,12] In our modified method, relatively less tracheal, which are excised as an intact ring, are required. Furthermore, an intact tracheal ring is more representative of the physiological setting than smooth muscle strips. Although the mechanisms of tracheal responses to drugs are difficult to determine, we were still able to provide some information based on the nature of specific tissues and their responses to drugs. For example, tracheal smooth muscle is the main tissue component regulating cross-contraction.[12] Furthermore, the isolated tracheal method used in this study did not cause any damage to the endothelium and smooth muscle, allowing us to obtain results that were more physiologically representative of an asthma attack. We used methacholine, a cholinergic contraction-inducing agent, as a control in this study; this agent induced a good contractile response in the tracheal tissue. No significant contractile effects were observed in the tissue after applying different concentrations of erlotinib, suggesting that noncholinergic effects were induced by erlotinib. EFS was used in the presence of various agonists and antagonists to observe drug innervations in the studied muscles.[20,21] In the present study, the results obtained from EFS are useful for understanding the neural control of airway tone in response to different drug-induced cholinergic effects. The EFS findings were in agreement with the results from methacholine challenge, revealing no cholinergic responses to erlotinib.

It is important to evaluate the cholinergic effects induced by erlotinib because it may contain inactive ingredients that can cause

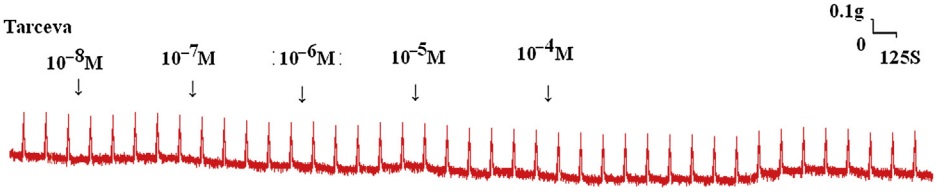

Figure 3 Original recording of the effects of erlotinib on electrically induced tracheal smooth muscle contractions. No significant effects were observed in the spike contraction induced by EFS after treatment.

allergic reactions or other problems. Erlotinib potently inhibits EGFR, which has been associated with airway remodeling and dysfunction in chronic asthma. Erlotinib is an inhibitor of EGFR that reversibly and competitively inhibits the receptor's intracellular TK, causing G1 cell cycle arrest and resulting in reduced proliferation and increased apoptosis in preclinical studies.[22] Although erlotinib inhibits the TK signaling cascade associated with asthmatic responses, our results indicated that its application to tracheal smooth muscle did not result in significant cholinergic effects. Notably, our results demonstrated physiological reactions to erlotinib treatment, the underlying mechanisms of which require further investigation. EGFR overexpression or overactivity has been associated with the development of various type of cancers such as lung cancer. Patients with EGFR mutation-positive NSCLC exhibit marked responses to erlotinib. The present study revealed that noncholinergic effects were induced in tracheal smooth muscle by erlotinib. However, further investigation is required to evaluate the allergic side effects caused by erlotinib in patients. Given this uncertainty, consultation with doctors or pharmacists prior to using this medication is important. The safety of erlotinib therapy in patients should be further examined.

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