Pre- and postjunctional inhibitory effects of fenspiride on guinea-pig bronchi

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ABSTRACT: Fenspiride is a drug with potential benefits in the treatment of obstructive airways disease. It has antibronchoconstriction and anti-inflammatory properties. The aim of this study was to investigate the effect of this drug on the contractions induced in the guinea-pig isolated main bronchus and perfused lung by electrical field stimulation (EFS) or exogenously added agents.

Bronchi were stimulated transmurally in the presence of indomethacin 10^{-6} M and propranolol 10^{-6} M, and isometric tension was measured. In the perfused lung model, calcitonin gene-related peptide (CGRP) release was determined in the perfusate fractions as a measure of neuropeptide production.

Two successive contractile responses were observed: a rapid cholinergic contraction, followed by a long-lasting contraction due to local release of neuropeptides from C-fibre endings. Fenspiride (10^{-6} to 10^{-4} M) inhibited the nonadrenergic, noncholinergic (NANC) component of the contraction of the guinea-pig isolated main bronchus induced by EFS. Fenspiride significantly affected contractions induced by exogenously added substance P or [Nle¹⁰]-NKA(4-10) only at concentrations higher than 10^{-3} M. In the guinea-pig perfused lung, fenspiride inhibited low pH- but not capsaicin-evoked release of CGRP. At higher concentrations (10^{-4} M to 3×10^{-4} M) fenspiride exhibited a significant inhibitory effect both on the cholinergic component of contractile response induced by EFS in the guinea-pig isolated main bronchus and on exogenously added acetylcholine.

In conclusion, the result of this study suggests that fenspiride, in moderate concentrations, reduces the release of neuropeptides, including tachykinins, from sensory nerve endings at a prejunctional level. At higher concentrations, postjunctional actions on bronchial smooth muscle are also present.

Eur Respir J 1997; 10: 1015–1020.

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Keywords: Fenspiride
guinea-pig
tachykinins

Received: January 11 1996
Accepted after revision January 28 1997

Several experimental studies have demonstrated that fenspiride is a drug that exerts antibronchoconstriction and anti-inflammatory properties. The anti-inflammatory properties have been demonstrated during experimentally-induced inflammation in animals [1]. This effect has been related to an anti-free radical effect and an inhibition of cyclo-oxygenase activity [1, 2]. Antibronchoconstrictor activity has been demonstrated *in vitro* in isolated guinea-pig trachea, with reversal of constriction induced by a variety of factors, and *in vivo* by reversing the bronchoconstriction induced by several agents, including capsaicin and citric acid [1, 3, 4].

In the airways, bronchial C-fibre stimulation causes the release of tachykinins, including substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP) [5]. On the basis of studies of endogenously released or exogenously administered neuropeptides, it has been established that these peptides induce bronchoconstriction, hypersecretion of mucus, vasodilatation, microvascular leakage, release of inflammatory mediators, and inflammatory cell chemotaxis. These proinflammatory

effects may play a role in the pathogenesis of asthma, thereby suggesting that control of the local release of neuropeptides might be effective in the management of this disease [6–9].

It has been demonstrated that isolated guinea-pig main bronchi respond to electrical field stimulation (EFS) *in vitro* with a fast, cholinergic nerve-mediated contraction, followed by a long-lasting contraction that is not inhibited by atropine. This bronchoconstrictor response has been termed the excitatory nonadrenergic noncholinergic (e-NANC) response and is evoked by stimulation of capsaicin-sensitive sensory nerves [10–13], resulting in subsequent neuropeptide release [14]. The aim of the present study was to investigate the effects of fenspiride on the action and the release of sensory neuropeptides induced by EFS in the isolated guinea-pig main bronchus. To determine whether fenspiride acts at a pre- or a postjunctional level, we have also studied the effects of this drug on release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) in the perfused guinea-pig lung, and on the contracting effects

of substance P, [Nle¹⁰]-NKA(4-10), a specific agonist of tachykinin neurokinin-2 (NK₂) receptors, and acetylcholine added exogenously to the organ bath. Finally, we have investigated whether fenspiride inhibited the effects of capsaicin, a drug known to induce the release of neuropeptides from sensory nerves.

Methods

Preparation of bronchial smooth muscle

Guinea-pig main bronchial segments were obtained from tricoloured guinea-pigs of either sex (250–350 g) anaesthetized with urethane (1.25 g·kg⁻¹, *i.p.*), and were suspended under an initial force of 2.0 g in Krebs's solution at 37°C aerated with 95% O₂/5% CO₂. After 1 h of equilibration, resting force was 1.5–2.0 g. Under these conditions, responses to agonists were reproducible over several hours. Tension was measured isometrically with UF-1 Strain Gauges (Pioden, Canterbury, UK) and amplifiers (EMKA, France). The composition of Krebs solution was (mM): NaCl 118.0, KCl 5.4, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, and glucose 11.7.

In all experiments, after 1 h of equilibration, guinea-pig segments were contracted to maximal tension with acetylcholine 1 mM and maximally relaxed with theophylline 3 mM. They were then allowed to equilibrate for 60 min, while they were washed with Krebs solution every 15 min.

Electrical field stimulation

Each organ bath was fitted with two platinum plate electrodes (1 cm²) placed alongside the tissue (10 mm apart) for transmural EFS (biphasic pulse duration 1 ms, constant current of 320 mA for 10 s).

In all experiments, propranolol 10⁻⁶ M and indomethacin 10⁻⁶ M were added to the buffer solution to avoid the influence of adrenergic nerve stimulation and the indirect effects of prostaglandins on neuronal responses, respectively.

After tension had returned to the baseline tone, the preparation was stimulated at 0.5, 1, 4, 8, 16, and 32 Hz for 1 ms, and 320 mA current for 10 s using a stimulator (EMKA, France), where the voltage output was adjusted to give a constant current of 320 mA and biphasic rectangular pulse of alternating polarity. This procedure was repeated at 60 min intervals. Control experiments showed that responses to EFS during the experimental period were reproducible (see Results). These stimulus parameters caused an optimal reproducible biphasic contraction, followed by a sustained contractile response, both abolished by tetrodotoxin. The first, fast component is inhibited by atropine and results from stimulation of cholinergic nerves. The late and prolonged second phase is nonadrenergic noncholinergic (NANC) in nature, and is abolished or strongly reduced by antagonists of tachykinin NK₂-receptors, such as SR 48968 and MEN 10,207, and partially reduced by CP-96,345, an antagonist of neurokinin-1 (NK₁)-receptors [12, 14, 15]. We have previously observed that atropine 10⁻⁶ M did not modify the NANC response and, similarly, SR 48968 (10⁻¹⁰ to 10⁻⁷ M) did not modify

the cholinergic response [15], suggesting that in these experimental conditions no interaction between cholinergic and NANC responses was involved. In each bronchial ring, following a first series (control) of transmural EFS of 1–32 Hz, a second series was performed 60 min later with or without (vehicle) fenspiride (10⁻⁶ to 10⁻⁴ M) added to the bath 15 min before transmural stimulation. The results are expressed as percentages of contraction induced by acetylcholine (1 mM). Results of the second series were compared to the first series.

Cumulative concentration-response curves to acetylcholine, substance P, [Nle¹⁰] NKA(4-10) and capsaicin

The inhibitory effects of fenspiride 10⁻⁴ to 10⁻² M used as preventive treatment were also studied. After 15 min pretreatment with fenspiride, in separate tissues cumulative concentration-response curves to acetylcholine (ACh) (10⁻⁸ to 10⁻³ M), SP (10⁻⁸ to 3×10⁻⁶ M), capsaicin (10⁻⁸ to 10⁻⁵ M) or [Nle¹⁰]-NKA(4-10) (10⁻⁹ to 10⁻⁶ M) were obtained by addition of the compounds every 5–10 min until a plateau was reached.

Contractions were expressed as a percentage of the contraction induced by the ACh (10⁻³ M) initially added to the bath. The experiments with [Nle¹⁰]-NKA(4-10) and SP were performed in the presence of phosphoramidon (10⁻⁵ M) to inhibit their metabolism.

Release of CGRP-LI from perfused guinea-pig lung

The guinea-pigs were anaesthetized with sodium pentobarbital (Pentothal®, 40 mg·kg⁻¹ *i.p.*). After tracheotomy, a tracheal tube connected to a rodent-respirator was inserted into the trachea, and the lung was ventilated with ambient air at a rate of 70 breaths·min⁻¹, using a tidal volume of about 3 mL adjusted according to the size of the animal. Insufflation pressure was recorded as an indicator of bronchoconstriction. The right and left ventricles of the heart were cut open and a tube was introduced into the pulmonary artery and the left ventricle for infusion and collection of perfused solution, respectively. The lung was perfused at a flow rate of 8 mL·min⁻¹, with Krebs-Ringer solution at 37°C and with the following composition (mM): NaCl 118, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 24.9, KH₂PO₄ 1.2, KCl 4.7, glucose 5.6, and hydroxyethylpiperazine ethanesulphonic acid (HEPES) 12.6, aerated with 93.5% O₂ and 6.5% CO₂ to give a pH of 7.4. The β₂-adrenoceptor agonist terbutaline (10⁻⁷ M) was added to all solutions in order to obtain a stable baseline and graded functional response. Preliminary experiments have shown that terbutaline (10⁻⁷ M) did not affect CGRP release. Drugs were added to the perfusion medium. After 15 min of equilibration; when stable contractile conditions had been obtained, the lung was exposed to low pH solution or capsaicin with or without drugs (fenspiride 10⁻⁷ to 10⁻⁵ M, UK14304 10⁻⁶ M or tetrodotoxin 3×10⁻⁷ M, for 15 min). The low pH buffer solution had the following composition (mM): NaCl 140, KCl 0.7, CaCl₂ 2.5, MgSO₄ 1.5, Na₂HPO₄ 0.8, KH₂PO₄ 5.9 and glucose 11, and the pH was adjusted to 5.8 with HCl.

Perfusate fractions (3 min) were collected in a beaker placed on ice, desalted using SEP-PAK C₁₈ cartridges, freeze-dried in a vacuum-centrifuge, and redissolved in appropriate buffer for determination of CGRP-LI by radioimmunoassay (RIA) [14, 16]. Release of CGRP-LI

was monitored, since it is co-released with tachykinins, but it is metabolically more stable.

Drugs

The substances used were: acetylcholine HCl (PCH, Paris); indomethacin (Merck); [Nle¹⁰]-NKA(4-10), phosphoramidon, propranolol HCl, substance P, yohimbine HCl, capsaicin, naloxone HCl, tetrodotoxin (Sigma, St Louis, USA); theophylline sodium anisate (Bruneau, Paris, France); fenspiride (Institut de Recherches Servier, Suresnes, France); terbutaline (Bricanyl®, Astra Draco, Sweden); UK14304 (Pfizer, UK). All drugs were dissolved in distilled water and then diluted in Krebs solution. Theophylline was used as manufactured solution.

Statistical analysis of results

Mean and SEM values were calculated for each experimental series. Statistical differences between mean values were determined using analysis of variance or Student's t-test. A p-value less than 0.05 was considered statistically significant.

Results

Influence of fenspiride on the contractions of the guinea-pig isolated main bronchus induced by EFS

Figures 1 and 2 show the effects of fenspiride 10^{-6} to 10^{-4} M on the two components of the contraction induced in the guinea-pig isolated main bronchus by EFS. Figure 3 shows an original tracing of the effects of fenspiride 10^{-4} M on the two components of the contraction induced by EFS.

Fig. 1. — Histograms illustrating the effects of fenspiride 10^{-6} to 10^{-4} M on the cholinergic response (first contraction) to electrical field stimulation (EFS) (1–32 Hz for 1 ms; constant current of 320 mA for 10 s) on the isolated guinea-pig main bronchus. Values are presented as mean $\pm$ SEM for 6–7 animals. Columns represent contractions expressed as percentage of maximal contraction induced by acetylcholine 10^{-3} M. Experiments were performed in the presence of propranolol (10^{-6} M) and indomethacin (10^{-6} M). □ : control (first series); ▨ : control (second series); ▤ : fenspiride 10^{-6} M; ▧ : fenspiride 10^{-5} M; ▩ : fenspiride 10^{-4} M; *: $p < 0.05$; **: $p < 0.01$, significant difference from control.

Fenspiride inhibited the first cholinergic component only at 10^{-4} M (figs. 1 and 3). Conversely, this drug significantly inhibited the NANC component, even at 10^{-6} M. This inhibitory effect increased from 10^{-6} to 10^{-4} M (figs. 2 and 3).

Influence of fenspiride on the concentration-response curves of ACh, SP, [Nle¹⁰]-NKA(4-10) and capsaicin

Figure 4 shows that fenspiride, at concentrations of 10^{-4} to 10^{-2} M, induced a shift to the right of the concentration response curves of ACh. Fenspiride (10^{-3} M) did not modify the effects of SP or [Nle¹⁰]-NKA(4-10). The effects of these contracting agents were significantly inhibited at higher fenspiride concentrations (10^{-2} and 3×10^{-3} M, respectively); however, fenspiride 10^{-4} M significantly inhibited the contraction caused by lower concentrations of capsaicin (10^{-8} and 10^{-7} M) but not by 10^{-6} M capsaicin. For higher concentrations of fenspiride, the effect of capsaicin was more strongly inhibited (with fenspiride 10^{-3} and 3×10^{-3} M) or completely abolished (fenspiride 10^{-2} M). This greater inhibitory effect of fenspiride (3×10^{-3} and 10^{-2} M) occurs at concentrations that also inhibit contractions evoked by SP and [Nle¹⁰]-NKA(4-10).

Influence of fenspiride on CGRP release

A concentration of fenspiride (10^{-5} M), which had selective action on the NANC contraction, was found to markedly inhibit the CGRP release evoked by low pH (pH 5.8) in the perfused guinea-pig lung (fig. 5a). Furthermore,

Fig. 2. — Histograms illustrating the effects of fenspiride 10^{-6} to 10^{-4} M on the nonadrenergic noncholinergic (NANC) response (second contraction) to electrical field stimulation (EFS) (1–32 Hz for 1 ms; constant current of 320 mA for 10 s) on the isolated guinea-pig main bronchus. Values are expressed as mean $\pm$ SEM for 6–7 animals. Columns represent contractions expressed as percentage of maximal contraction induced by acetylcholine 10^{-3} M. Experiments were performed in the presence of propranolol (10^{-6} M) and indomethacin (10^{-6} M). $\square$: control (first series); ▨ : control (second series); ⋯ : fenspiride 10^{-6} M; ▤ : fenspiride 10^{-5} M; ⊞ : fenspiride 10^{-4} M; *: $p < 0.05$; **: $p < 0.01$, significant difference from control.

Fig. 3. — Representative traces of isometric tension showing the effects of fenspiride 10^{-4} M on the cholinergic and nonadrenergic noncholinergic (NANC) responses to electrical field stimulation (EFS) (1–32 Hz for 1 ms; constant current of 320 mA for 10 s) on the isolated guinea-pig main bronchus.

the $29 \pm 9\%$ increase in insufflation pressure evoked by pH 5.8 was significantly attenuated by fenspiride to $9 \pm 4\%$ ($p < 0.05$). The effects of fenspiride were mimicked by the α_2 -adrenoceptor agonist, UK14304 (10^{-6} M) and tetrodotoxin (3×10^{-7} M) (fig. 5a). In contrast, the CGRP release (fig. 5b) and bronchoconstriction induced by capsaicin (10^{-6} M) were not influenced by fenspiride. Control CGRP-Li release evoked by capsaicin (1 μ M) or low pH (pH 5.8) were 0.9 ± 0.1 pM ($n=4$) and 0.4 ± 0.07 pM ($n=4$).

Fig. 4. — Influence of fenspiride (10^{-4} to 10^{-2} M) on the cumulative concentration-response curves of acetylcholine (ACh), substance P (SP), [Nle¹⁰]-NKA(4-10), and capsaicin on the isolated main bronchus of the guinea-pig. Values are presented as mean $\pm$ SEM for 5–7 animals. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, significant difference from control. $\bullet$ —: control; ⋯ : fenspiride 10^{-4} M; — : fenspiride 10^{-3} M; — : fenspiride 10^{-2} M.

Fig. 5. — a) Influence of fenspiride (10⁻⁷ to 10⁻⁵ M), UK14304 (10⁻⁶ M) and tetrodotoxin (3×10⁻⁷ M) on the CGRP release evoked by low pH (5.8) medium in perfused guinea-pig lung. b) Influence of fenspiride (10⁻⁷ to 10⁻⁵ M) on CGRP release upon capsaicin (1 μM) infusion into perfused guinea-pig lung. Values are presented as mean ± SEM, n=4 each. *: p < 0.05, significant difference from control. CGRP: calcitonin gene-related peptide.

Discussion

Neuronal control of airways is complex. In addition to classic efferent cholinergic and adrenergic nerves, there are NANC sensory mechanisms, which are mediated by the release of neuropeptides. Neuronal responses may be measured *in vitro* using EFS with parameters that selectively activate nerves and not smooth muscle. EFS produced both a rapid cholinergic and a long-lasting noncholinergic contraction of bronchial smooth muscle of the guinea-pig due to release of sensory neuropeptides from C-fibre endings [10–13].

Our results demonstrate that fenspiride inhibits the two components of neurally-mediated biphasic response observed following EFS of the guinea-pig isolated main bronchus. There is, however, a difference between the inhibitory effects of fenspiride on these two components. The inhibition of the cholinergic response appears significant only for concentrations of 10⁻⁴ M and higher, whereas the inhibition of the NANC component is significant even at 10⁻⁶ M for the stimulation at 4 and 8 Hz.

Furthermore, the mechanisms of the effects of fenspiride on these two components seem to be different. The results of the present study are consistent with a postjunctional inhibitory action of fenspiride on the cholinergic response, since we observed an inhibitory effect of this compound on cumulative concentration-response curves to ACh at 10⁻⁴ M (fig. 3). In contrast, fenspiride appears to produce an inhibition of the NANC response predominantly at a prejunctional level, since fenspiride did not modify concentration-response curves to [Nle¹⁰]-NKA(4-10), a specific agonist of tachykinin-NK₂ receptors, or to SP in concentrations lower than 3×10⁻³ M. However, a postjunctional effect occurred at higher concentrations. Furthermore, the low pH-evoked CGRP release from the perfused lung [17, 18] was inhibited by fenspiride 10⁻⁵ M. Since the low pH response was inhibited by tetrodotoxin, it resembles the effects of EFS. The inhibition by fenspiride (10⁻⁴ M) of the effects

of a low but not a high concentration of capsaicin, which induces a release of tachykinins from sensory nerve endings by different mechanisms, is in agreement with this hypothesis [16]. Thus, the failure of fenspiride to inhibit the contraction and CGRP release by a high concentration of capsaicin (10⁻⁶ M) is in agreement with the hypothesis that a high concentration of capsaicin causes peptide release *via* Ca²⁺ entry through receptor-gated channels, which may be less sensitive to prejunctional regulation [16].

Interestingly, in the present study, the effects of fenspiride on EFS are observed at concentrations lower than those reported for the antibronchoconstriction effect in the *in vitro* studies in guinea-pig trachea and human bronchus (-log concentration required to effect half the maximum response (-logEC₅₀) varying from 3.5 to 4 for various contractile agents) [1, 3], or for the anti-inflammatory effect, such as the anti-free radical effect in guinea-pig alveolar macrophage (doses of the order of mmol·L⁻¹) [2]. However, the clinical relevance of this effect remains difficult to specify, since the plasma levels of fenspiride observed during repeated administrations are close to 3×10⁻⁶ M (maximal concentration (C_{max}): 800 ng·mL⁻¹) (unpublished data).

It has been shown by binding studies that this product has an affinity for the adrenergic (α₁) and histaminergic (H₁) receptors [1]. The binding to this type of receptor does not appear to be implicated in this inhibitory effect [19]. Studies are being carried out to determine another mechanism, notably an inhibitory effect of phosphodiesterase.

In conclusion, the present results show that fenspiride inhibits the cholinergic bronchoconstrictor response at high concentrations with a postjunctional mechanism, whereas fenspiride produces an inhibition of the non-adrenergic noncholinergic response at lower concentrations predominantly at a prejunctional level. Inhibition of the nonadrenergic noncholinergic system might be involved in the mechanism of action of fenspiride, but the clinical interest of this effect remains to be determined.

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