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Lipoxin A_4 inhibits cholinergic neurotransmission through nitric oxide generation in the rabbit trachea

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Abstract

The effect of lipoxin A_4 and lipoxin B_4 on cholinergic neurotransmission in rabbit tracheal segments was studied under isometric conditions in vitro. Lipoxin A_4 attenuated the contractile responses to electrical field stimulation and caused a rightward shift of the frequency-response curves, so that the stimulus frequency required to produce a half-maximal effect (ES_{50}) increased from 8.1 ± 0.8 to 25.7 ± 1.9 Hz ($P < 0.001$), whereas lipoxin B_4 had no effect. In contrast, lipoxin A_4 did not alter the contractile responses to acetylcholine. Pretreatment of tissues with N^G-nitro-L-arginine methylester inhibited the effect of lipoxin A_4 on electrical field stimulation, but N^G-nitro-D-arginine methylester did not. This inhibition by N^G-nitro-L-arginine methylester was reversed by L-arginine but not by D-arginine. These results suggest that lipoxin A_4 prejunctionally reduces the vagal nerve-mediated contraction of airway smooth muscle, probably by inhibiting the release of acetylcholine, and that this effect may be exerted through stimulation of nitric oxide generation.

Keywords: Lipoxin A_4; Cholinergic neurotransmission; Nitric oxide (NO); Smooth muscle, airway

1. Introduction

Lipoxins are a class of biologically active trihydroxy lipids with a conjugated tetraene and are formed by interactions between the 5- and 15-lipoxygenases within the arachidonic acid cascade (Serhan et al., 1984). The two major compounds, lipoxin A_4 and lipoxin B_4, is 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid and 5*S*,14*R*,15*S*-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid, respectively (Serhan et al., 1986a,b). Lipoxin A_4 is generated by neutrophils, alveolar macrophages, eosinophils and, possibly, epithelial cells (Sigal and Nadel, 1988), and significant amounts of lipoxin A_4 can be detected in bronchoalveolar lavage fluid obtained from patients with inflammatory pulmonary diseases (Lee et al., 1990). In addition, lipoxins display biological actions in the airways. For example, lipoxin A_4 contracts lung parenchymal strips but not tracheal smooth muscle preparations in guinea pigs (Dahlén et al., 1987; Jacques et al., 1988), stimulates airway sensory C-fibers (Manzini and Meini, 1991), and enhances or inhibits neutrophil migration (Palmblad et al., 1987; Lee et al., 1989), whereas lipoxin B_4 does not produce such effects. However, the effect of lipoxins on airway cholinergic neurotransmission is unknown.

There is increasing evidence that nitric oxide (NO) may regulate airway smooth muscle and vascular smooth muscle tone, pulmonary neurotransmission and host defence (Gaston et al., 1994). Although an interaction between lipoxin A_4 and the NO-generating system remains uncertain, Dahlén et al. (1987) showed that lipoxin A_4 induced arteriolar dilatation in the hamster cheek pouch by stimulating the release of endothelium-derived relaxing factor, which was consequently identified as NO. Therefore, in the present study, to determine whether lipoxins alter cholinergic neurotransmission in the airways and, if so, to assess a possible contribution of NO generation, we studied rabbit tracheal ring segments under isometric conditions in vitro.

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2. Materials and methods

2.1. Preparation of tracheal segments

This study was approved by the ethical committee of animal research in Tokyo Women's Medical College. Japanese white rabbits weighing 2.3–2.6 kg were anesthetized with intravenous pentobarbital sodium (35 mg/kg). The trachea was removed and immersed in oxygenated Krebs-Henseleit (KH) solution of the following composition (in mM): NaCl, 118; KCl, 5.9; $CaCl_2$, 2.5; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25.5; and glucose, 5.6. Transverse ring segments of the trachea, 7 mm in length, were dissected free from underlying connective tissues and mounted in organ chambers filled with 14 ml KH solution maintained at 37°C and continuously aerated with 95% O_2-5% CO_2 to obtain a pH of 7.4, a PCO_2 of 38 mmHg and a PO_2 of > 500 mmHg (Tamaoki et al., 1987). The lower end of the preparation was attached to a force-displacement transducer (model TB-652T, Nihon Kohden, Tokyo, Japan) for continuous recording of isometric tension by a pen recorder (model WT-685G, Nihon Kohden). Each organ chamber was fitted with two rectangular platinum electrodes (6 × 25 mm) placed alongside the tissue for transmural electrical field stimulation, using biphasic pulses (pulse duration 0.5 ms, supramaximal voltage of 20 mV for 20 s). A contractile response was measured as the difference between peak tension developed and resting tension. For all experiments, to avoid a possible contribution of a β-adrenergic effect, propranolol (10^{-6} M) was added to the chamber 10 min before the starting point. Under this experimental condition, contraction of rabbit tracheal segment induced by electrical field stimulation was abolished by either tetrodotoxin (10^{-6} M) or atropine (10^{-6} M), indicating that the response was entirely dependent on the release of acetylcholine from the cholinergic nerve endings.

2.2. Effects of lipoxins on contractile responses

The tissues were allowed to equilibrate for 60 min, while they were washed with fresh KH solution every 15 min and the resting tension was adjusted to 2 g (Yamawaki et al., 1992). To test the effects of lipoxins on cholinergic neurotransmission, contractile responses to electrical field stimulation were assessed before and after the addition of either lipoxin A_4 or lipoxin B_4 at 10^{-7} M (Wako Pure Chemical Co., Tokyo, Japan). We first obtained the baseline responses to electrical field stimulation at increasing stimulus frequencies (1–50 Hz); we then added each lipoxin to the chamber and, 5 min later, we made the second frequency-response curves. To analyze the frequency-response curves, the stimulus frequency that produced a half-maximal baseline response (ES_{50}) was determined by linear regression analysis. In evaluating the concentration-response relationship, we first obtained the baseline response to electrical field stimulation at 10 Hz; we then cumulatively added lipoxin A_4 or lipoxin B_4 to the chambers in half-molar increments (10^{-9} to 10^{-6} M). The responses to electrical field stimulation were measured 5 min after each addition.

To determine whether the site of action of lipoxins is pre- or postjunctional in the vagal motor pathway, we assessed the contractile responses to exogenous acetylcholine. We first obtained concentration-response curves for acetylcholine (10^{-8} to 10^{-3} M, Nacalai Tesque, Kyoto, Japan); we then washed tissues with KH solution, added lipoxin A_4 or lipoxin B_4 at 10^{-7} M, and determined the second concentration-response curves 5 min later.

2.3. Effects of pharmacologic blocking agents

Because endogenous prostaglandin E_2 plays a protective role against bronchoconstrictor responses by inhibiting the release of acetylcholine (Walters et al.,

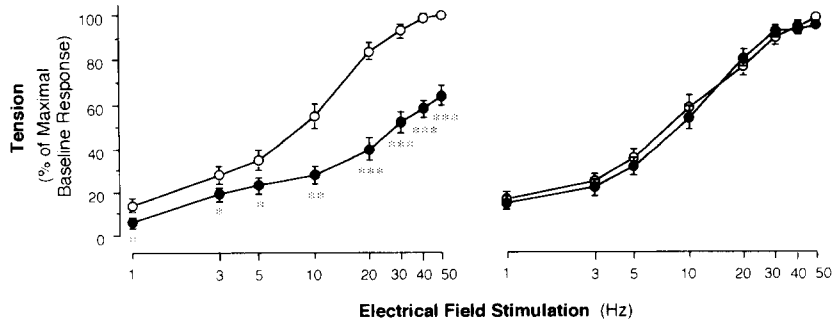

Fig. 1. Effects of lipoxin A_4 (left panel) and lipoxin B_4 (right panel) on contractile responses of rabbit tracheal segments to electrical field stimulation at increasing impulse frequencies. After obtaining baseline responses (open circles), each lipoxin was added at 10^{-7} M and, 5 min later, the measurements were repeated (closed circles). Values are expressed as percentages of the maximal baseline responses obtained before administration of lipoxins. Each point represents mean ± S.E.; $n = 12$ for lipoxin A_4 and $n = 10$ for lipoxin B_4. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from corresponding baseline response.

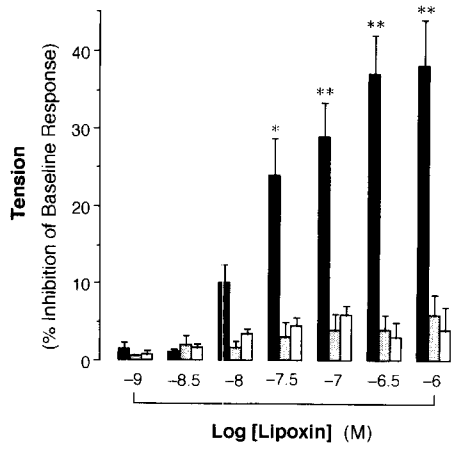

Fig. 2. Concentration-dependent effect of lipoxin A_4 (solid columns), lipoxin B_4 (stippled columns) and their vehicle alone (open columns) on contractile responses to electrical field stimulation at 10 Hz. Values are expressed as percent inhibition of the baseline response obtained before administration of lipoxins. Data are means ± S.E.; $n = 9$ for lipoxin A_4 and $n = 8$ for lipoxin B_4. * $P < 0.05$, ** $P < 0.01$, significantly different from baseline response.

1984), we assessed the possible involvement of prostaglandin E_2 synthesis in the lipoxin A_4 action. To do so, contractile responses to electrical field stimulation at 10 Hz were determined before and 5 min after the addition of lipoxin A_4 (10^{-7} M) in the absence and presence of indomethacin (3×10^{-6} M, Sigma Chemical Co., St. Louis, MO). Moreover, to assess whether NO generation was involved, tissues were pretreated for 15 min with N^G-nitro-L-arginine methylester (L-NAME, 10^{-3} M, Sigma), an inhibitor of NO synthase (Rees et al., 1990), or its inactive enantiomer N^G-nitro-L-arginine methylester (D-NAME, 10^{-3} M, Sigma), and the effect of lipoxin A_4 (10^{-7} M) on the electrical field stimulation (10 Hz)-induced contraction was determined. Additionally, after the response to lipoxin A_4 in the presence of L-NAME was determined, D-arginine or L-arginine at 10^{-2} M (Sigma) was added and, 5 min later, the measurements of the contractile response to electrical field stimulation were repeated. In this series of experiments, indomethacin and L-NAME per se increased the electrical field stimulation-induced contraction by 8.6 ± 2.0% ($P < 0.05$, $n = 12$) and 12.3 ± 2.3% ($P < 0.01$, $n = 12$), respectively, whereas D-NAME, D-arginine and L-arginine were without effect by themselves, and the contractile responses to electrical field stimulation in the presence of a pharmacologic blocking agent obtained before addition of lipoxin A_4 served as the baseline responses. Further, to test whether airway epithelial cells were involved in the release of NO, the effect of lipoxin A_4 (10^{-7} M) on the contractile responses to electrical field stimulation (10 Hz) and its modulation by arginine analogues were likewise determined in tissues in which the epithelial cells had been mechanically removed by rubbing with a cotton swab. At the end of this experiment, successful removal of the epithelium was histologically confirmed.

2.4. Statistics

All values were expressed as means ± S.E. Statistical analysis was performed by analysis of variance or Newman-Keuls multiple comparison test, and $P < 0.05$ was considered statistically significant.

3. Results

Addition of lipoxin A_4 or lipoxin B_4 at 10^{-7} M did not alter the resting tension of rabbit tracheal segments. As demonstrated in Fig. 1, lipoxin A_4 attenuated the contractile responses to electrical field stimulation at all stimulus frequencies (the mean decrease for all frequencies was 24.6 ± 3.8% below baseline responses; $P < 0.001$, $n = 12$), so that the ES_{50} value increased from 8.1 ± 0.8 to 25.7 ± 1.9 Hz ($P < 0.001$, $n = 12$), whereas lipoxin B_4 had no effect. Cumulative addition of lipoxin A_4 but not lipoxin B_4 reduced the

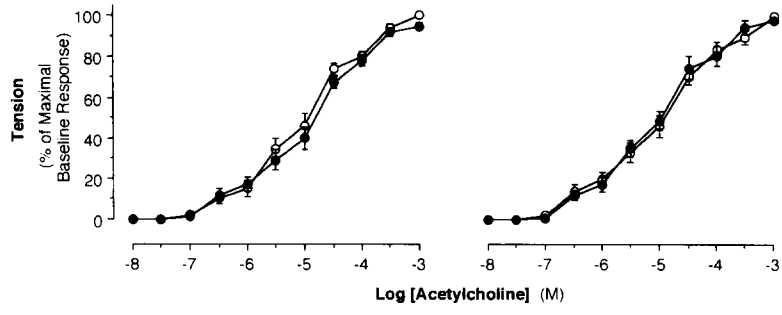

Fig. 3. Effects of lipoxin A_4 (left panel) and lipoxin B_4 (right panel) on contractile responses to acetylcholine. After obtaining baseline responses (open circles), tissues were washed, each lipoxin was added at 10^{-7} M and, 5 min later, the measurements were repeated (closed circles). Values are expressed as percentages of the maximal baseline responses obtained before administration of lipoxins. Each point represents mean ± S.E.; $n = 11$ for lipoxin A_4 and $n = 9$ for lipoxin B_4.

contractile responses to electrical field stimulation at 10 Hz in a concentration-dependent manner: the threshold concentration was 3×10^{-8} M, and the maximal inhibition of the baseline response was $38.2 \pm 5.9\%$ ($P < 0.01$, $n = 9$) observed at 10^{-6} M (Fig. 2).

In contrast to the effect on the electrical field stimulation-induced contraction, neither lipoxin A_4 nor lipoxin B_4 at 10^{-7} M altered the contractile responses to acetylcholine at concentrations ranging from 10^{-9} to 10^{-3} M (Fig. 3).

Pretreatment of tissues with indomethacin (3×10^{-6} M) did not affect the inhibitory effect of lipoxin A_4 (10^{-7} M) on the contractile responses to electrical field stimulation at 10 Hz. Addition of L-NAME (10^{-3} M) inhibited the effect of lipoxin A_4 (percent inhibition of the baseline response was $33.5 \pm 4.7\%$ for lipoxin A_4 alone vs. $9.8 \pm 2.2\%$ for lipoxin A_4 plus L-NAME; $P < 0.01$, $n = 8$), but D-NAME (10^{-3} M) did not. This inhibition by L-NAME was completely reversed by L-arginine but not by D-arginine at 10^{-2} M (Fig. 4). In tissues in which the epithelial cells had been removed, lipoxin A_4 likewise decreased the contractile responses to electrical field stimulation. Pretreatment of tissues with L-NAME inhibited the lipoxin A_4 action, an effect that was reversed by L-arginine.

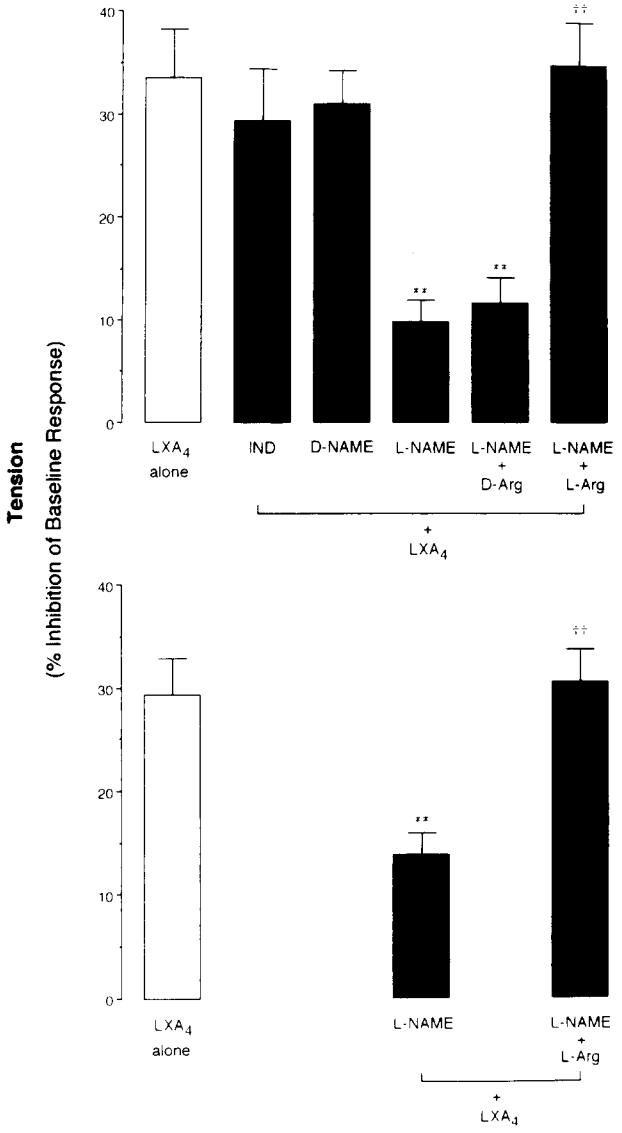

Fig. 4. Effects of pharmacologic blocking agents on the lipoxin A_4(LXA_4)-induced inhibition of the contractile responses to electrical field stimulation at 10 Hz in epithelium-intact (upper panel) and epithelium-denuded tissues (lower panel). The responses were determined in the absence and presence of the following drugs: IND, indomethacin (3×10^{-6} M); D-NAME, N^G-nitro-D-arginine methylester (10^{-3} M); L-NAME, N^G-nitro-L-arginine methylester (10^{-3} M); D-Arg, D-arginine (10^{-2} M); L-Arg, L-arginine (10^{-2} M). Values are expressed as percent inhibition of the baseline response obtained before administration of LXA_4. Data are means ± S.E.; $n = 8$ for each column. $^{**}P < 0.01$, significantly different from the response to LXA_4 alone. $^{\dagger\dagger}P < 0.01$, significantly different from the response to LXA_4 in the presence of L-NAME.

4. Discussion

Our in vitro studies demonstrate that lipoxin A_4 does not change the resting tone of rabbit tracheal smooth muscle but reduces the neurally mediated contraction, probably by inhibiting cholinergic neurotransmission, and that lipoxin B_4 is without such an effect. This result suggests that the presence of the hydroxyl groups at the C-5 and C-6 positions of lipoxin A_4 may be essential for the expression of the neuromodulatory activity.

It has been recognized that transmural electrical field stimulation of rabbit isolated tracheal smooth muscle stimulates postganglionic nerve fibers and produces excitatory responses as a result of activation of vagal motor nerves and inhibitory responses as a result of activation of β-adrenergic mechanisms (Tanaka and Grunstein, 1986). Because the β-adreneroceptor antagonist propranolol was present throughout the present experiments, the inhibition of the contractile responses to electrical field stimulation by lipoxin A_4 is dependent on cholinergic mechanisms. Then, to determine whether the site of lipoxin A_4 action is pre- or postjunctional in the parasympathetic motor pathway, we compared the effects of lipoxin A_4 on the airway contraction induced by electrical field stimulation with that induced by exogenously administered acetylcholine, and found that, in contrast to the effect on the response to electrical field stimulation, the contractile response to acetylcholine was not changed by lipoxin A_4. This implies that lipoxin A_4-induced inhibition may not be related to alterations in smooth muscle function, such as decreased sensitivity to acetylcholine or increased degradation of acetylcholine. Therefore,

attenuation of the contractile response to electrical field stimulation by lipoxin A_4 may be attributable to a prejunctional mechanism, probably involving the inhibition of the exocytotic release of acetylcholine from cholinergic nerve terminals.

Isolated airway smooth muscle preparations synthesize and release prostaglandin E_2 in response to electrical field stimulation, which in turn inhibits acetylcholine release (Walters et al., 1984). It is thus possible that the lipoxin A_4 action was mediated by stimulation of prostaglandin E_2 synthesis, but this possibility seems unlikely because pretreatment of tissues with indomethacin did not alter the inhibitory effect of lipoxin A_4 on the contractile responses to electrical field stimulation. The lack of effect of indomethacin on lipoxin A_4 action is in accordance with previous result obtained with guinea pig lung strips (Dahlén et al., 1987). It has been shown that substance P increases acetylcholine release from airway cholinergic nerve terminals (Tanaka and Grunstein, 1986) and that lipoxin A_4 may stimulate capsaicin-sensitive sensory fibers in the airway (Manzini and Meini, 1991). However, our observation that lipoxin A_4 did not augment but rather decreased the electrical field stimulation-induced contraction excludes a possible contribution of tachykinins. Recent evidence suggests that NO is generated in the airway by various cell types, such as vascular endothelial cells, airway epithelial cells, alveolar macrophages, and neuronal cells (Kobzik et al., 1993), and may play a protective role against bronchoconstrictor responses (Gaston et al., 1994). In the present study, pretreatment of tissues with the NO synthase inhibitor L-NAME (Rees et al., 1990) inhibited the effect of lipoxin A_4, whereas its inactive enantiomer D-NAME had no effect. Furthermore, this inhibitory effect of L-NAME was reversed by L-arginine but not by D-arginine. In the epithelium-denuded tissues, the inhibitory effect of lipoxin A_4 on the contractile responses to electrical field stimulation and its modulation by arginine analogues were likewise observed. These results indicate that lipoxin A_4 may have reduced the electrical field stimulation-induced contraction through generation of NO and that airway epithelial cells may not be involved in the NO generation. However, the inhibition by L-NAME was only partial, and the mechanism of the remaining response is unknown. One possibility would be that the effect of lipoxin A_4 might be mediated by suppression of neuronal calcium mobilization, as has been shown in neutrophils (Lee et al., 1989).

In conclusion, the present studies indicate that lipoxin A_4 attenuates the neurally mediated contraction of airway smooth muscle, probably by inhibiting the release of acetylcholine from the postganglionic vagal nerve fibers, and that this effect may be exerted at least in part by NO generation.

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