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The effect of phosphoramidon and epithelium removal on toluene diisocyanate-induced contractions in guinea-pig bronchi

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ABSTRACT: To evaluate the role of airway neutral endopeptidase 24.11 (NEP) and epithelium removal in the contraction of airway smooth muscle in response to toluene diisocyanate (TDI), we studied the effects of the NEP inhibitor, phosphoramidon, on TDI-induced contractions of guinea-pig bronchial rings with intact epithelium and without epithelium.

In preparations with intact epithelium, phosphoramidon (10 μM) potentiated the contractile response to TDI (0.3 mM) (mean±SEM, 23.7±2.5% *versus* 67.9±10.3%, p<0.01). Phosphoramidon also increased TDI-induced contractions in tissues without epithelium (36.9±4.9% *versus* 52.5±7.1%, p<0.05). Removal of the epithelium increased the contractile response to TDI (23.7±2.5% *versus* 36.9±4.9%, p<0.05).

These results demonstrate the response to TDI is increased in epithelium-free compared to intact bronchi and that NEP 24.11 modulates the effects of endogenously released tachykinins by TDI at all of the sites where NEP is found in the airways.

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Toluene diisocyanate (TDI) is a chemical widely used in industries, known as a potent sensitizer, able to induce occupational asthma in exposed subjects [1]. Recently, we have shown that toluene diisocyanate activates the "efferent" function of capsaicin-sensitive sensory nerves in guinea-pig airways [2]. Thus, toluene diisocyanate causes contraction of airway smooth muscle in guinea-pigs. This effect is diminished or abolished by a high dose of capsaicin and by a tachykinin antagonist.

Several enzymes degrade tachykinins [3]. One of these, neutral endopeptidase 24.11 (NEP), (also known as encephalinase or EC 3.4.24.11), a membrane-bound enzyme, is located on the surfaces of multiple cells, including smooth muscle, epithelium, nerves and glands [4, 5]. It has been shown that a NEP-like enzyme is present on guinea-pig airway smooth muscle and that it modulates the effects of endogenously released tachykinins [6].

In the present study, we investigated the effect of the NEP inhibitor phosphoramidon on smooth muscle contraction produced by toluene diisocyanate in tissues with and without epithelium. The results indicate that a NEP-like enzyme is present on guinea-pig airway smooth muscle and that it modulates the effects of tachykinins released by toluene diisocyanate.

Methods

Male Hartley-outbred guinea-pigs (Rodentia Laboratoires, Torre Pallavicina, Bergamo, Italy), weighing 300–400 g, were anaesthetized with pentobarbital sodium (50 $mg \cdot kg^{-1}$ *i.p.*). The lungs were rapidly removed and immersed in oxygenated Krebs-Henseleit solution containing the following (in mM): 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, and 11.1 D(+)-glucose. The main bronchi were dissected free of loose connective tissue and were prepared in two rings. The rings were mounted in a double jacketed organ bath filled with 15 ml of Krebs-Henseleit solution that was maintained at 37°C and aerated continuously by bubbling it with a mixture 95% O_2 and 5% CO_2, which produced a pH of 7.4. We measured isometric tension by connecting the tissue to a force-displacement transducer (Grass FTO3), and we recorded the responses on a Battaglia Rangoni model KV380 polygraph recorder. The rings were allowed to equilibrate for 90 min while resting tension was adjusted to 5 mN. During equilibration the medium was changed every 20 min. Contractions were normalized as a percentage of the active tension obtained in response to acetylcholine (ACh) 1 mM.

To study the effect of the NEP inhibitor phosphoramidon on TDI-induced contractions, parallel studies were performed on paired bronchial rings from the same animal, both in tissues with and without epithelium. The responses induced by 1 mM acetylcholine were measured. The rings were washed until the tension returned to resting values. Phosphoramidon (10 μM, contact time 15 min) was added to one of the two bronchial rings, whereas nothing was added to the second ring. Then the responses induced by 0.3 mM TDI in both rings were monitored. The concentration of TDI was chosen on the basis of the results obtained in previous studies [2].

Removal of the epithelium was confirmed histologically: the epithelium was removed by rubbing the luminal surface gently with a gauze. The gauze was cut into strips, and one side was tied to the suture. The suture was used as a guide to rub the gauze inside the bronchial ring. At the end of the experiment, control rings and rings without epithelium were fixed in 4% formaldehyde in 0.1 M phosphate buffer at pH 7.2. After fixation, samples were dehydrated through ethanol, passed through xylene and embedded in paraffin; 6 μm thick sections were cut perpendicularly to the minor internal diameter of the bronchi and tissue blocks were oriented for light microscopy analysis. Four sections at a regular interval of 100 μm were stained with haematoxylin-eosin; the length of the intact epithelium and of disepithelized mucosa was measured on each slide. The percentage of the basement membrane covered with epithelium was assessed by an observer who was unaware of the previous treatment of the preparations. Light microscopy measurements were performed with a Jenamed 30G0040 microscope by using an eyepiece graticule at a magnification of ×160. The final results were expressed as percentage of length of disepithelized mucosa for the total mucosal length and they represented the average of all the measurements performed for each specimen.

Materials and reagents

Phosphoramidon was obtained from Peninsula Labs (Belmont, CA, USA). Acetylcholine was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Toluene diisocyanate consisted of an 80:20 mixture of the 2,4 and 2,6 isomers (obtained from Montedison, Porto Marghera, Venezia, Italy), and dissolved in dimethyl sulphoxide. The maximal final concentration of dimethyl sulphoxide in the organ bath was 0.3%.

Statistical analysis

Each value is mean±SEM. The effect of phosphoramidon on contraction induced by TDI was compared by two-tail Student's test for paired data. The effect of the removal of epithelium on contraction induced by TDI was compared by two-tail Student's test for unpaired data. $p<0.05$ was considered significant.

Results

In tissues with epithelium, incubation with phosphoramidon (10 μM, 15 min), significantly potentiated the contraction to 0.3 mM TDI (23.7±2.5% *versus* 67.9±10.3%, n=6, $p<0.01$) (fig. 1). In tissues without epithelium, incubation with phosphoramidon (10 μM, 15 min) significantly potentiated the response to 0.3 mM TDI (36.9±4.9%*versus* 52.5±7.1%, n=6, $p<0.05$). The removal of the epithelium significantly potentiated the response to 0.3 mM TDI (23.7±2.5% *versus* 36.9±4.9%, n=6, $p<0.05$). Histological analysis confirmed that 97.3±1.7% of the epithelium had been removed.

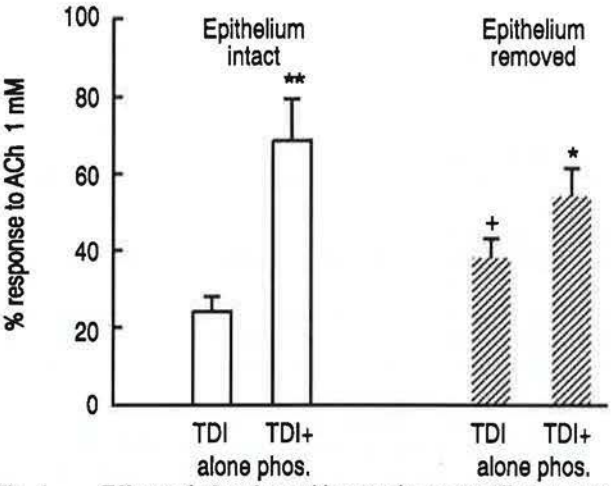

Fig. 1. – Effects of phosphoramidon on the contractile response to 0.3 mM TDI in guinea-pig bronchi with and without epithelium. Data are expressed as percentage of muscle contraction (mean±SEM) induced by 1 mM acetylcholine. Phosphoramidon (10 μM, preincubation 15 min) increased the response to TDI in tissues with and without epithelium (Student's paired t-test, **: $p<0.01$; *: $p<0.05$; n=6 each). Removing the epithelium significantly increased the TDI-induced contraction (unpaired Student's test, +: $p<0.05$). TDI: toluene diisocyanate; ACh: acetylcholine; phos.: phosphoramidon.

Discussion

Our results suggest that a NEP-like enzyme on the airway smooth muscle of guinea-pigs has a role in modulating the contractile effects of neuropeptides released by toluene diisocyanate. Thus, we showed that contraction induced by TDI was potentiated by a NEP inhibitor, an effect that was also observed after removing the airway epithelium. Since pretreatment with capsaicin and with a tachykinin receptor antagonist inhibits responses to TDI [2], it is possible that TDI acts on the airways *via* the release of substance P or other tachykinins or other peptides from capsaicin-sensitive primary afferents. NEP 24.11 is localized in airways at different sites including smooth muscle, nerves, epithelium and glands [7]. Biochemical studies have shown that NEP 24.11 inactivates tachykinins such as substance P and neurokinin A [8]. NEP 24.11 also cleaves and modulates effects of mediators such as kinins, neurotensin, encephalins, endothelin and the synthetic chemotactic peptide N-formyl--methionyl--leucyl--phenylalanine (fMLP) [7].

Recently, it has been shown that thiorphan, a NEP inhibitor, increased not only the contractile response to exogenously administered tachykinins but also the outflow of both substance P-like immunoreactivity (LI) and tachykinin-like immunoreactivity (LI) released by capsaicin, providing biochemical evidence for inactivation of these peptides by NEP in guinea-pig bronchi [9]. This degradation may occur at sites of release of neuropeptides and at sites of action on target cells.

The present findings, in conjunction with those of DJOKIC *et al.* [6], and of MAGGI *et al.* [9], provide evidence for a significant role of NEP 24.11 in modulating the effects of endogenously released tachykinins mediated by capsaicin, electrical field stimulation, and toluene diisocyanate. These studies have also shown that the effect of NEP inhibitors such as phosphoramidon and thiorphan is still present after removing the bronchial epithelium, providing further evidence for the localization of NEP 24.11 outside of epithelium. Removing airway epithelium, decreases NEP 24.11 and thereby modulates the motor response of smooth muscle to tachykinins [10–12]. Since calcitonin gene-related peptide (CGRP) is also present in sensory nerves, CGRP could be a favourable substrate for NEP 24.11, but recent work suggests that the effects of neuropeptides such as substance P and neurokinin A are more likely to be influenced by NEP 24.11, whereas the effects of CGRP may be less affected [13].

In the absence of phosphoramidon, the response to toluene diisocyanate was higher in epithelium-free than in intact bronchi. The hypothesis that an epithelium-derived airway relaxing factor could explain the different motor responses to a variety of agents in tissues with and without epithelium has been debated [14]. Recent work suggests that prostanoids such as prostaglandin E_2 (PGE_2) may be involved [15]. However, since a classic inhibitor of cyclooxygenase, such as indomethacin, inhibits TDI-induced contractions [16], it is unlikely that dilator prostaglandins are involved. It is possible that removing the epithelium has effects which are more complex and not completely understood, and perhaps the action of an epithelium-derived relaxing factor is on other structures, such as sensory nerves and blood vessels, which in turn act on airway muscle. Recently, it has been reported that the removal or damage of epithelium releases mast cell components, which may affect the function of various cells in the airway [17].

Previous studies showed that after removal of the airway epithelium, NEP 24.11 is an important modulator of responses to exogenously or endogeneously produced tachykinins [6, 9]. The present findings, in conjunction with those of DJOKIC *et al.* [6], suggest that about one third, to one fourth of NEP activity is localized to the epithelium.

In conclusion, we have demonstrated that the response to toluene diisocyanate is increased in epithelium-free bronchi compared to intact bronchi and that it is possible that NEP modulates the effects of tachykinins released by TDI at all sites where NEP is found in airways.

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