Role of thromboxane-A2 and cholinergic mechanisms in bronchoconstriction induced by cigarette smoke in guinea-pigs

K. Matsumoto, H. Aizawa, H. Inoue, S. Takata, M. Shigyo, N. Hara


ABSTRACT: Acute exposure to cigarette smoke elicits bronchoconstriction and increases the concentration of thromboxane (Tx) A2 in bronchoalveolar lavage (BAL) fluid. The purpose of this study was to investigate the role of TxA2 and cholinergic mechanisms in the airway response induced by exposure to cigarette smoke in guinea-pigs.

Anaesthetized animals were exposed to 200 puffs of smoke for 10 min. The amount of Evans blue dye extravasated into the bronchial tissue was then measured. BAL was performed to determine cell counts and the concentration of TxB2, a stable metabolite of TxA2. The effects of pretreatment with a Tx synthase inhibitor, OKY-046 (10 mg·kg⁻¹), and/or atropine (1 mg·kg⁻¹) were evaluated.

Exposure to cigarette smoke caused significant bronchoconstriction (284±33% of baseline pulmonary resistance (RL)), and plasma extravasation (30.0±3.8 vs 16.8±2.6 ng·mg⁻¹ of sham control; main bronchi). OKY-046 or atropine significantly inhibited the bronchoconstriction to a similar extent, without affecting the plasma extravasation. Combined use of these compounds had no additive effect. The cigarette smoke caused significant increase in TxB2 (48±10 vs 14±1 pg·mL⁻¹ of sham control) in BAL fluid, which was abolished by OKY-046 but not by atropine. The cellularity in BAL fluid was not different among groups.

These results suggest that the bronchoconstriction induced by cigarette smoke is partially mediated by thromboxane A2, which is dependent on a cholinergic pathway.


Methods

Study protocol

A total of 53 Hartley-strain male guinea pigs, weighing 450–550 g (Kyudo, Kumamoto, Japan), were anaesthetized with 50 mg·kg⁻¹ of pentobarbital sodium administered intraperitoneally (i.p.). The animals were then intubated through a tracheostomy and mechanically-ventilated with a respirator (model 680; Harvard Apparatus, South Natick, MA, USA), at a tidal volume of 7 mL·kg⁻¹ and a rate of 60 breaths·min⁻¹. A catheter was introduced into the jugular vein to administer drugs. To avoid effects on the adrenergic nervous system, propranolol (1 mg·kg⁻¹) was administered intravenously (i.v.) at the beginning of artificial ventilation.

Bronchoconstriction and plasma extravasation were studied in 28 of the animals. They were randomly divided into four groups (seven per group): 1) vehicle (0.9% saline, 1 mg·kg⁻¹), the control group; 2) OKY-046 (10 mg·kg⁻¹); 3) atropine (1 mg·kg⁻¹); and 4) OKY-046 (10 mg·kg⁻¹) and atropine (1 mg·kg⁻¹). The study protocol is shown in figure 1a. After determining the baseline value of total pulmonary resistance (RL), vehicle, OKY-046, atropine, or a combination of both drugs was...
in the presence of intravenously (i.v.) applied propranolol (1 mg·kg⁻¹).
After determining the baseline value of total pulmonary resistance (R<L), vehicle (1 mL·kg⁻¹), OKY-046 (10 mg·kg⁻¹), atropine (1 mg·kg⁻¹), or a combination of both drugs was administered intravenously. Eight minutes after drug administration, Evans blue dye (20 mg·kg⁻¹) was administered intravenously. Two minutes later, the animals were exposed to cigarette smoke intermittently for 10 min. Immediately after the exposure to smoke, the thorax was opened and a cannula was inserted into the ascending aorta through the left ventricle. The circulatory system was perfused with 500 mL of 0.9% saline at a pressure of 120 mmHg. The lower portion of the trachea and the main bronchi were dissected and incubated with 1 mL of formamide at 37°C for 18 h to extract Evans blue dye. Extravasation was quantified by measuring the optical density of the formamide extracts at a wavelength of 620 nm using a spectrophotometer (model UV-2200A; Shimadzu Scientific Instruments, Tokyo, Japan). The amount of dye that had extravasated into the tissues was interpolated from a standard curve, and expressed as nanograms per milligram of wet weight of tissue. Use of wet weight as the denominator for studies of extravasation might be inappropriate, as wet weight could increase substantially with capillary leakage. This would decrease the signal but might also decrease the ability to see changes with drug intervention. To avoid this, all the specimens were processed for the measurement as strictly in time as possible.

Measurement of plasma extravasation
Immediately after the exposure to smoke, the thorax was opened and a cannula was inserted into the ascending aorta through the left ventricle. The circulatory system was perfused with 500 mL of 0.9% saline at a pressure of 120 mmHg. The lower portion of the trachea and the main bronchi were dissected and incubated with 1 mL of formamide at 37°C for 18 h to extract Evans blue dye. Extravasation was quantified by measuring the optical density of the formamide extracts at a wavelength of 620 nm using a spectrophotometer (model UV-2200A; Shimadzu Scientific Instruments, Tokyo, Japan). The amount of dye that had extravasated into the tissues was interpolated from a standard curve, and expressed as nanograms per milligram of wet weight of tissue. Use of wet weight as the denominator for studies of extravasation might be inappropriate, as wet weight could increase substantially with capillary leakage. This would decrease the signal but might also decrease the ability to see changes with drug intervention. To avoid this, all the specimens were processed for the measurement as strictly in time as possible.

Bronchoalveolar Lavage
Before the lavage, the lung was hyperinflated (three-folds tidal volume) by manual occlusion of the expiratory outlet of the respirator, to decrease the potential occlusion of the airways and atelectasis of the lungs. The lung was gently lavaged three times via the tracheal cannula at a pressure of 2.45 kPa, with 0.9% saline with 5 mM indomethacin added to prevent further production of eicosanoids. The fluid was collected by gentle suctioning by a 20 mL syringe. Total cell counts

Measurement of total pulmonary resistance
A fluid-filled catheter was introduced into the oesophageal cannula at a pressure of 2.45 kPa, with 0.9% saline with 5 mM indomethacin added to prevent further production of eicosanoids. The fluid was collected by gentle suctioning by a 20 mL syringe. Total cell counts
were determined under light microscopy using a standard haemocytometer. The lavage fluid was centrifuged at 200 \( \times \) g for 10 min at 4°C. The cell pellet was resuspended in normal saline to obtain a suspension of 10^5 cells·mL^{-1}. Cytospin preparations were made (Cytospin 3; Shandon, Pittsburgh, PA, USA) and the cells were visualized with a modified Wright-Giemsa stain (Diff-Quick; Baxter, McGaw Park, IL, USA). Differential counts per 500 cells were determined under light microscopy using a single-blind method.

Measurement of TxB_2

The remaining supernatant from the BALF was stored frozen at -80°C for the measurements of TxB_2 (stable metabolite of TxA_2). A 1 mL sample was extracted twice with a double volume of ethylacetate after acidification with 1 N HCl, and was evaporated to dryness under a stream of nitrogen. The residue was dissolved in benzene/ethylacetate (60:40). The solution was evaporated and processed for assay with radioimmunoassay kits (Daiichi Kagaku, Tokyo, Japan). Briefly, samples were incubated with ^125^I-labelled TxB_2 and its antisem for 16 h at 4°C. The antibody-bound fraction was then separated by centrifugation. The radioactivity of the antibody-bound fraction was determined with a gamma scintillation counter.

Materials

The drugs used in the present study were atropine sulphate (Tanabe Pharmaceutical, Osaka, Japan), propranolol hydrochloride (Zeneca Pharmaceutical, Osaka, Japan), indomethacin, formamide (Sigma Chemical, St. Louis, MO, USA), and pentobarbital sodium (Abbott Laboratories, North Chicago, IL, USA). In addition, OKY-046 was provided by Ono Pharmaceutical Co. (Osaka, Japan) and dissolved in 0.9% sterile saline at a concentration of 10 mg·mL^{-1}.

Statistical analysis

Data are expressed as mean±SEM. Baseline RL was compared among all groups by one-way analysis of variance (ANOVA). Values for RL are expressed as the percentage of the baseline RL. Time course curves for RL during the exposure to smoke between each drug-treated group and the control group were assessed by two-way ANOVA, followed by Dunnett’s tests. The amount of Evans blue dye extravasated into the tissues, cell counts, and the concentration of TxB_2 in BALF were compared among all groups by the Kruskal-Wallis H-test, followed by the Mann-Whitney U-test. A p-value of less than 0.05 was accepted as statistically significant.

Results

Baseline pulmonary resistance

There were no significant differences in baseline RL values among the various groups. The values were as follows: 10.75±0.74, 11.72±1.05, 11.18±0.96, 10.03±1.00 (Pa·mL^{-1}·s^{-1}), for vehicle-treated, OKY-046-treated, atropine-treated, OKY-046 and atropine-treated, respectively.

Effects of OKY-046 and atropine on bronchoconstriction induced by cigarette smoke

Effects of the drugs on changes in RL in response to cigarette smoke are shown in figure 2a–c. In vehicle-treated animals, exposure to cigarette smoke caused a time-dependent bronchoconstriction. Treatment with OKY-046 significantly inhibited the bronchoconstriction induced by cigarette smoke (fig. 2a) (p<0.05). Atropine also significantly inhibited the bronchoconstriction (p<0.05) (fig. 2b). However, treatment with OKY-046 combined with atropine did not have any additive effect.
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on the bronchoconstriction (fig. 2c). In our preliminary study, 1 mg·kg⁻¹ of atropine completely prevented the bronchoconstriction induced by acetylcholine (60 µg·kg⁻¹ i.v.), which increased RL by 49±56% (n=5), a greater response than that seen in the present study (the maximum RL change in the present study being 405%).

Effects of OKY-046 and atropine on the extravasation of Evans blue dye

As shown in figure 3, a significant increase in the amount of extravasated Evans blue dye was noted both in: a) the trachea; and b) main bronchi of the cigarette smoke-exposed, vehicle-treated animals (versus the sham-exposed animals). Treatment with OKY-046, atropine, or combined use of both drugs prior to cigarette exposure had no effect on the extravasation.

Effects of OKY-046 and atropine on cell counts in BALF

The recovery rate of BALF did not differ significantly among the study groups, with the range being 94–97%. The cell counts also showed no significant differences among groups (table 1).

Effects of OKY-046 and atropine on concentration of TxB₂ in BALF

The concentration of TxB₂ in BALF was significantly higher in the smoke-exposed, vehicle-treated group compared with the sham-exposed, vehicle-treated group (p<0.01). The concentration of TxB₂ was significantly lower in the animals treated with OKY-046, as compared with the animals treated with vehicle and then exposed to cigarettes (p<0.05). Treatment with atropine had no significant effect on the concentration of TxB₂, compared with treatment with vehicle in smoke-exposed animals (fig. 4).

Table 1. – Bronchoalveolar lavage profile

<table>
<thead>
<tr>
<th>Recovery Rate</th>
<th>BALF cell counts ×10⁵·mL⁻¹</th>
</tr>
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<tbody>
<tr>
<td>%</td>
<td>AM</td>
</tr>
<tr>
<td>Sham</td>
<td>97±1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>95±1</td>
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<tr>
<td>OKY-046</td>
<td>94±2</td>
</tr>
<tr>
<td>Atropine</td>
<td>94±1</td>
</tr>
</tbody>
</table>

Values are presented as mean±SEM. AM: alveolar macrophages; Lymph: lymphocytes; Neu: neutrophils; Eos: eosinophils; BALF: bronchoalveolar lavage fluid.

Fig. 3. – Effects of OKY-046 and/or atropine on the extravasation of Evans blue dye induced by cigarette smoke. A significant increase in the amount of extravasated Evans blue dye was noted both in: a) the trachea; and b) main bronchi of the cigarette smoke-exposed, vehicle-treated animals (versus the sham-exposed animals). Treatment with OKY-046, atropine, or combined use of both drugs prior to cigarette exposure had no effect on the extravasation.

Fig. 4. – Effect of OKY-046 or atropine on concentration of thromboxane B₂ (TxB₂) in bronchoalveolar lavage fluid (BALF). The concentration of TxB₂ was significantly higher in the cigarette smoke-exposed, vehicle-treated group versus the sham-exposed, vehicle-treated group. The concentration of TxB₂ was significantly lower in the cigarette smoke-exposed animals treated with OKY-046 as compared with the vehicle-treated group. Treatment with atropine had no significant effect on the concentration of TxB₂. *: p<0.05; **: p<0.01, significance of comparison between treatment groups.
Discussion

In this study, the bronchoconstriction induced by cigarette smoke was partially inhibited by pretreatment with OKY-046 or with atropine, to a similar extent. This finding clearly indicates that the bronchoconstriction induced by cigarette smoke is partially caused by endogenous TxA2 and acetylcholine. The combined use of OKY-046 and atropine showed no additive effect, which suggests that the effects of both compounds are mediated by the same pathway.

The novel finding of this study is that the inhibition of TxA2 generation by OKY-046 also inhibited the bronchoconstriction induced by cigarette smoke, suggesting a TxA2-mediated component in this type of bronchoconstriction. It is known that TxA2 can elicit bronchoconstriction by at least two mechanisms: 1) by potentiating the cholinergic neurotransmitter, acetylcholine [11–14]; and 2) by direct stimulation of the TP prostanoid receptor on the airway smooth muscle, a cholinergic-independent mechanism [8–10]. The lack of additive effect of OKY-046 and atropine strongly suggests the contribution of a cholinergic-dependent mechanism in the TxA2 action in the present model. Therefore, we consider that TxA2 may cause bronchoconstriction via a cholinergic pathway, presumably by enhancing the release of acetylcholine from the vagus nerve terminals. SAROEA et al. [20] reported that the bronchoconstrictor effect of the inhaled Tx mimetic, U46619, is markedly reduced by a cholinergic antagonist in asthmatic airways. Their finding further suggests that the cholinergic pathway is an important component in TxA2-mediated bronchoconstriction in humans.

The contribution of a cholinergic component to the bronchoconstriction induced by cigarette smoke has been confirmed in numerous studies [5, 6, 21, 22]. We recently reported that the bronchoconstriction induced by cigarette smoke was completely abolished by pretreatment with FK224, a tachykinin antagonist, at the dual neurokinin 1 and neurokinin 2 (NK1 and NK2) receptors [6]. Exposure to cigarette smoke increases the concentration of TxB2, a stable metabolite of TxA2, in BALF, but pretreatment with FK224 prevents this increase [7]. Thus, it is likely that the TxA2-mediated bronchoconstriction induced by exposure to cigarette smoke may depend on a tachykinin-mediated cholinergic pathway.

Unlike bronchoconstriction, the extravasation of plasma into the bronchial tissue was not affected by OKY-046 or atropine. The lack of effect of OKY-046 and atropine on the extravasation was also noted in the present study. Intravenous administration of U46619 has been shown to cause marked bronchoconstriction and plasma extravasation in the airways of guinea-pigs [15]. Another study demonstrated that the plasma extravasation induced by intratracheally instilled leukotriene D4 (LTD4) was partially mediated by Tx generation in guinea-pigs [16]. Our findings on extravasation are inconsistent with those of the previous investigators. Several studies have reported that thromboxane did not affect vascular permeability in the lung [23, 24]. The effect of thromboxane on vascular permeability is still controversial. The discrepancy may be, in part, explained by a dose-response relationship of thromboxane on vascular permeability. Indeed, LOTVALL et al. [15] reported that 2 nmol·kg\(^{-1}\) (i.v.) of U-46619 caused 3.5 fold increase in RL without an effect on the extravasation of Evans blue dye, whereas 20 or 200 nmol·kg\(^{-1}\) of U-46619 elicited marked increases both in RL and extravasation [15]. It seems likely that the amount of endogenous TxA2 may have been insufficient to elicit an extravasation of plasma.

We previously showed that TxB2 was increased in BALF collected 90–120 min after exposure to 3 min of cigarette smoke, and that the number of neutrophils in BALF was also increased [7]. In the present study, we found that TxB2 increased immediately after exposure to cigarette smoke, but cell counts in BALF did not change. A similar report demonstrated that TxB2 is increased in BALF immediately after acute exposure to acrolein, a component of cigarette smoke [25]. Although neutrophils are known to be a potent source of TxA2 [26], the TxA2 in the current study may have been generated from the resident cells other than inflammatory cells in the airway. Whilst we could not confirm the source of TxA2 in the present study, it has been reported that TxA2 can be generated from platelets, airway epithelium, endothelium, alveolar macrophages and fibroblasts, in response to various stimuli [27–29].

In summary, we conclude that the bronchoconstriction induced by acute exposure to cigarette smoke was partially mediated by endogenous thromboxane A2, which was dependent on a cholinergic pathway.

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References


