Thromboxane causes airway hyperresponsiveness after cigarette smoke-induced neurogenic inflammation

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Matsumoto, Koichiro, Hisamichi Aizawa, Hiromasa Inoue, Mutsumi Shigyo, Shohei Takata, and Nobuyuki Hara. Thromboxane causes airway hyperresponsiveness after cigarette smoke-induced neurogenic inflammation. J. Appl. Physiol. 81(6): 2358–2364, 1996.—We investigated the role of neurogenic inflammation and the subsequent mechanisms in cigarette smoke-induced airway hyperresponsiveness in guinea pigs. Exposure to cigarette smoke was carried out at tidal volume for 3 min. Airway responsiveness to histamine was determined before and after smoke exposure followed by bronchoalveolar lavage (BAL). Plasma extravasation was evaluated by measuring the extravasation of Evans blue dye in the airway. Cigarette smoke produced significant airway hyperresponsiveness and plasma extravasation, with an influx of neutrophils in BAL fluid. FK-224 (10 mg/kg iv), a tachykinin antagonist at NK1 and NK2 receptors, significantly inhibited these changes. The thromboxane (Tx) B2 concentration was increased in BAL fluid after smoke exposure and was significantly inhibited by FK-224. OKY-046 (10 mg/kg iv), a Tx synthase inhibitor, significantly inhibited airway hyperresponsiveness but had no effect on neutrophil influx or plasma extravasation. The results suggest that neurogenic inflammation and the subsequent generation of Tx in the airway are important in the development of the airway hyperresponsiveness induced by cigarette smoke.

guinea pigs; plasma extravasation; neutrophils

CIGARETTE SMOKE is a major irritant present in the environment and a known risk factor for airway diseases, including asthma (5). Clinical studies suggest that cigarette smoke contributes to airway hyperresponsiveness, a characteristic feature of asthma (10, 28, 32). It is thus important to clarify the mechanism of the airway hyperresponsiveness induced by exposure to cigarette smoke.

A previous study revealed that cigarette smoke-induced airway hyperresponsiveness in guinea pigs was reduced by pretreatment with capsaicin (7). Capsaicin pretreatment reportedly inhibits a neurogenic extravasation of plasma in the rodent airway exposed to cigarette smoke through depleting tachykinins (25). Airway hyperresponsiveness induced by cigarette smoke may therefore be linked to the neurogenic inflammation mediated by tachykinins. However, capsaicin treatment may have effects other than the depletion of tachykinin. For example, it may deplete the afferent nerves of other mediators (24). Confirmation of the role of tachykinins by other methods would therefore seem desirable. Furthermore, little is known about the mechanism by which neurogenic inflammation leads to airway hyperresponsiveness.

To clarify the role of tachykinins in the airway hyperresponsiveness and inflammation induced by exposure to cigarette smoke in guinea pigs, we investigated the effect of FK-224 (14, 27), a tachykinin antagonist at NK1 and NK2 receptors. During this study, we found that cigarette smoke increases the concentration of thromboxane (Tx) B2 in bronchoalveolar lavage (BAL) fluid (BALF) and that FK-224 prevents this increase in Tx B2. Because Tx appeared to be involved in the development of airway hyperresponsiveness, we evaluated the effects of a specific Tx synthase inhibitor, OKY-046 (16), in this condition.

METHODS

Study protocol. Fifty Hartley strain male guinea pigs weighing 450–550 g (Kyudo, Kumamoto, Japan) were used. Thirty animals were used in the determination of airway hyperresponsiveness and BAL and were randomly divided into six groups as follows: 1) sham exposure with vehicle treatment (n = 5; control group); 2) sham exposure with FK-224 treatment (n = 5); 3) sham exposure with OKY-046 treatment (n = 5); 4) cigarette smoke exposure with vehicle treatment (n = 5); 5) cigarette smoke exposure with vehicle treatment (n = 5); and 6) cigarette smoke exposure with OKY-046 treatment (n = 5). The study protocol is shown in Fig. 1A. After determination of the preexposure concentration of histamine required to produce a 200% increase in pulmonary resistance (RL) (PC200; see below), FK-224 (10 mg/kg), OKY-046 (10 mg/kg), or vehicle (saline) was administered intravenously. Ten minutes later, the animals were exposed to cigarette smoke or to room air for 3 min. Postexposure PC200 was measured after 30 min. BAL was performed after total R L returned to its baseline value. The dose of FK-224 was based on a previous study that sought to abolish the effects of exogenous tachykinins on the cardiopulmonary functions of guinea pigs (14). The dose of OKY-046 was based on our preliminary study and on previous reports (1, 16) conducted to inhibit the generation of Tx induced by various stimuli, including cigarette smoke, in vivo.

Twenty animals were used in the measurement of plasma extravasation (Fig. 1B). They were randomly divided into four groups as follows: 1) sham exposure with vehicle treatment (n = 5; control group), 2) cigarette smoke exposure with vehicle treatment (n = 5), 3) cigarette smoke exposure with FK-224 treatment (n = 5), and 4) cigarette smoke exposure with OKY-046 treatment (n = 5). FK-224, OKY-046, or vehicle was intravenously administered 10 min before the cigarette smoke or sham exposure. Evans blue dye (20 mg/kg) was intravenously administered 2 min before each exposure. The measurement of extravasated Evans blue dye was performed as described below.

Measurement of total RL. Guinea pigs were anesthetized with 50 mg/kg of pentobarbital sodium administered intraperitoneally. Animals were intubated via tracheostomy and mechanically ventilated with a respirator (model 680, Har-
Airway responsiveness and bronchoalveolar lavage (BAL) study. After measurement of preexposure concentration of histamine required to produce a 200% increase in pulmonary resistance (RL) (prePC200), FK-224 (10 mg/kg), OKY-046 (10 mg/kg), or vehicle (saline) was administered intravenously. Ten minutes later, animals were exposed to cigarette smoke or room air for 3 min. Postexposure PC200 (postPC200) was measured at 30 min after exposure. BAL was performed after RL returned to baseline.

Exposure to cigarette smoke. Cigarette smoke was supplied by a smoke generator that consisted of a respirator (model 681, Harvard Apparatus) and a 1.26-liter volume chamber for dilution with fresh air. The chamber consisted of two holes of 3.5 ml and a rate of 60 breaths/min. On completion, the cigarette butt was detached, and the smoke was delivered to the animal by using the respirator for 3 min at a constant tidal volume flow rate of 10.2 ± 0.3 ml/min and a rate of 60 breaths/min. Cigarettes were purchased from Japan Tobacco (Tokyo, Japan). According to the manufacturer’s specifications, each cigarette contained 2.7 mg of nicotine and 26 mg of tar.

Bal. Animals were killed by exanguination. The lung was gently lavaged three times with normal saline via the tracheal cannula at a pressure of 25 cm H2O. Total cell counts were determined under light microscopy by using a standard hemocytometer. The lavage fluid was centrifuged at 200 g for 10 min at 4°C. The cell pellet was resuspended in normal saline to obtain a suspension of 10^6 cells/ml. Cytosin preparations (Cytosin 3, Shandon, Pittsburgh, PA) were made, and the cells were visualized with a modified Wright-Giemsa stain (Diff-Quick, Baxter, McGaw Park, IL). Differential counts on 200 cells were performed under light microscopy by using a single-blind method. The remaining supernatant from the BALF was mixed with 5 mM indomethacin and stored frozen at −80°C for the measurements of TxB2, a stable metabolite of TxA2.

Table 1. Baseline RL, baseline RL ratio, and prePC200 values

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Cigarette Smoke</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>FK-224</td>
</tr>
<tr>
<td>Baseline RL, cmH2O·ml⁻¹·s⁻¹</td>
<td>0.102 ± 0.007</td>
<td>0.108 ± 0.003</td>
</tr>
<tr>
<td>Baseline RL ratio</td>
<td>1.162 ± 0.065</td>
<td>1.030 ± 0.047</td>
</tr>
<tr>
<td>log (prePC200 &gt; 100)</td>
<td>1.106 ± 0.129</td>
<td>1.242 ± 0.142</td>
</tr>
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</table>

Values are means ± SE. RL, pulmonary resistance; prePC200, preexposure concentration of histamine required to produce 200% increase in RL.
dissolved in benzene-ethylacetate (60:40). The solution was evaporated by stirring for 30 min, and its supernatant was processed for assay by using the radioimmunoassay kits (Daiichi Kagaku, Tokyo, Japan). Samples were briefly incubated with $^{125}$I-labeled TxB$_2$ and were incubated with an antiserum for 16 h at 4°C. After this incubation, the antibody-bound fraction was separated with centrifugation. The radioactivity of the antibody-bound fraction was determined by using a gamma scintillation counter (model ARC-950, Aokha, Tokyo, Japan). The sensitivity of this assay is 3.0 pg/ml, and the reproducibility, expressed as coefficient variance, is 8.19–9.66%.

Measurement of plasma extravasation. As shown in Fig. 1B, 10 min after initiation of smoke exposure 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 in 1 ml of formamide at 37°C for 18 h to extract the extravasated Evans blue dye. The extravasation was quantified by measuring the optical density of the formamide extracts with a spectrophotometer at a wavelength of 620 nm (model UV-2200A, Shimadzu Scientific Instruments, Tokyo, Japan). The amount of dye extravasated in the tissues was interpolated from a standard curve and was expressed in nanograms per milligram of wet weight of the tissues.

Drugs. Histamine diphosphate and formamide were obtained from Sigma Chemical (St. Louis, MO), and pentobarbital sodium was obtained from Abbott (North Chicago, IL). FK-224 was provided by Fujisawa Pharmaceutical (Osaka, Japan) and was suspended in 0.9% saline at a concentration of 20 mg/ml. OKY-046 was provided by Ono Pharmaceutical (Osaka, Japan) and was dissolved in 0.9% saline at a concentration of 20 mg/ml.

Data analysis. PC$_{200}$ values were expressed as the geometric mean and SE. Other values are expressed as the arithmetic mean and SE. Values of baseline RL and PC$_{200}$ before exposure were compared among all groups by using two-way analysis of variance followed by Scheffé's F-test, if overall significance was found by analysis of variance. To evaluate baseline RL changes after cigarette smoke, the postexposure RL was divided by the preexposure RL (baseline RL ratio). To evaluate the changes in airway responsiveness after exposure, the postexposure PC$_{200}$ was divided by the preexposure

![Fig. 2. Dose-response curves to histamine aerosols for 20 animals. O, Dose-response curves obtained before exposure; ●, dose-response curves obtained after exposure. Animals 1–5 were exposed to sham procedure and treated with vehicle; animals 6–10 were exposed to cigarette smoke (cig) and treated with vehicle, and animals 11–15 were exposed to cigarette smoke and treated with FK-224. Animals 16–20 were exposed to cigarette smoke and treated with OKY-046. There seemed to be no consistent change in dose-response curve before vs. after sham exposure. In contrast, dose-response curve was markedly shifted to left in all animals after exposure to cigarette smoke. Pretreatment with FK-224 or OKY-046 seemed to suppress this leftward shift of dose-response curve induced by exposure to cigarette smoke. S, saline.](http://jap.physiology.org/doi/10.1038/jn.2007.331)
Thus a decrease in this value indicated an increase in airway responsiveness. The changes in airway responsiveness, baseline RL ratio, BAL cell counts, concentrations of TxB₂, and tissue content of Evans blue dye were compared among all groups by the Kruskall-Wallis H-test followed by the Mann-Whitney U-test. A level of \( P, 0.05 \) was accepted as statistically significant.

### RESULTS

Baseline RL and preexposure PC₂₀₀ among groups. There were no significant differences in baseline RL, baseline RL ratio, and preexposure PC₂₀₀ values between groups (Table 1).

Effects of FK-224 and OKY-046 on cigarette smoke-induced airway hyperresponsiveness. Figure 2 illustrates the dose-response curves to inhaled histamine aerosols in each animal. Vehicle-treated animals showed no consistent change in dose-response curves after sham exposure. The dose-response curve was markedly shifted to the left in all animals after exposure to cigarette smoke. Pretreatment with FK-224 or OKY-046 suppressed the leftward shift of the dose-response curve induced by cigarette smoke. These results are summarized in Fig. 3. Vehicle-treated cigarette smoke-exposed animals exhibited a significantly lower PC₂₀₀ ratio than the sham-exposed group (\( P < 0.05 \)). Treatment with FK-224 or OKY-046 significantly inhibited the cigarette smoke-induced airway hyperresponsiveness (\( P < 0.01 \) and \( P < 0.05 \), respectively). In the three groups of sham-exposed animals, airway responsiveness was not altered by treatment with FK-224 or OKY-046.

Effects of FK-224 and OKY-046 on cell counts in BALF. The recovery rate of BALF did not differ significantly between groups, with a range of 88–92%. The cell counts shown in Fig. 4 illustrate a significant increase in neutrophils after the exposure to cigarette smoke in the vehicle-treated animals vs. the control group (\( P < 0.05 \)). Treatment with FK-224 significantly inhibited the neutrophilia (\( P < 0.05 \)). Treatment with OKY-046 had no effect on the change in cell counts induced by the exposure to cigarette smoke. In the three groups of sham-exposed animals, the cell counts in BALF were not altered by treatment with FK-224 or OKY-046 (Table 2).

Effects of FK-224 and OKY-046 on TxB₂ concentration in BALF. Figure 5 illustrates the effect of cigarette smoke on TxB₂ concentration in BALF. The concentration of TxB₂ was significantly higher in the cigarette smoke-exposed vehicle-treated group vs. the sham-exposed vehicle-treated group (\( P < 0.05 \)). The concentration of TxB₂ was significantly lower in the cigarette smoke-exposed animals treated with FK-224 or OKY-046 compared with the vehicle-treated group (\( P < 0.05 \) and \( P < 0.05 \), respectively).
Effects of FK-224 and OKY-046 on the extravasation of Evans blue dye induced by exposure to cigarette smoke. As shown in Fig. 6, a significant increase in the amount of Evans blue dye was noted in both the trachea and the main bronchi of the cigarette smoke-exposed vehicle-treated animals compared with the control group (P < 0.01 in trachea, P < 0.05 in main bronchi). Treatment with FK-224 significantly inhibited the extravasation (P < 0.05 in trachea, P < 0.05 in main bronchi). In contrast, treatment with OKY-046 had no effect on the extravasation of dye induced by cigarette smoke. It has been reported that cigarette smoke-induced extravasation of plasma reaches a plateau within 15 min after the exposure to smoke in guinea pigs (22). To confirm this, we examined the effect of OKY-046 on the plasma extravasation 30 min after exposure to cigarette smoke in a preliminary study. Treatment with OKY-046 also had no effect on the extravasation of dye (data not shown).

**DISCUSSION**

The present study demonstrated that acute exposure to cigarette smoke caused airway hyperresponsiveness, neutrophilia, and plasma extravasation in the airway. These changes were significantly inhibited by FK-224, suggesting an essential role of the tachykinins in the development of airway inflammation and hyperresponsiveness. In addition, the exposure to cigarette smoke increased the concentration of TxB2 in BALF and FK-224 inhibited the increase in TxB2. OKY-046 inhibited the airway hyperresponsiveness without any effect on the neutrophilia in BALF or on plasma extravasation. These results indicate that TxA2 is important in the development of airway hyperresponsiveness after the neurogenic inflammation caused by exposure to cigarette smoke.

**Table 2. Cell counts in BALF among sham groups**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.70 ± 0.41</td>
<td>2.29 ± 0.35</td>
<td>0.19 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>FK-224</td>
<td>2.30 ± 0.21</td>
<td>1.93 ± 0.21</td>
<td>0.16 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>OKY-046</td>
<td>2.64 ± 0.52</td>
<td>2.12 ± 0.49</td>
<td>0.21 ± 0.05</td>
<td>0.14 ± 0.01</td>
<td>0.18 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE in cell counts × 10⁵/ml of recovered bronchoalveolar lavage fluid (BALF).
The exposure to cigarette smoke has been shown to cause airway hyperresponsiveness in guinea pigs (7, 8, 12, 15, 18, 29). Daffonchio et al. (7) reported that such airway hyperresponsiveness was inhibited by capsaicin pretreatment. This suggests a possible role of tachykinins in cigarette smoke-induced airway hyperresponsiveness. However, capsaicin may cause the depletion of other neuropeptides stored in afferent nerves (24) and probably causes damage to some nerve fibers. Therefore, confirmation of the role of tachykinins would be of interest. In the present study, a tachykinin antagonist, FK-224, significantly inhibited airway hyperresponsiveness. This finding confirms the important role of tachykinins in the airway hyperresponsiveness induced by cigarette smoke.

The precise mechanism of the development of airway hyperresponsiveness with neurogenic inflammation has not been elucidated. Narrowing of the airways caused by mucosal edema may be responsible (19, 20). However, in the present study, the airway hyperresponsiveness was inhibited without an inhibition of plasma extravasation by OKY-046. This finding indicates that plasma extravasation was of lesser importance in causing the airway hyperresponsiveness in this model. Another possible mechanism for the airway hyperresponsiveness is the release of mediators into the airway after neurogenic inflammation. TxA2 is a plausible candidate in this regard, since the concentration of TxB2 in BALF was increased after exposure to cigarette smoke and this increase was significantly inhibited by FK-224. The prevention of airway hyperresponsiveness by administration of a Tx synthase inhibitor strongly suggested a key role of TxA2.

TxA2 is reported to facilitate the cholinergic contraction of airway smooth muscle in vitro (6). Because the bronchoconstriction induced by histamine is mediated in part by a cholinergic reflex in vivo (17), the TxA2-mediated airway hyperresponsiveness to histamine may involve this mechanism.

The source of TxA2 was not confirmed in the present study. A previous report showed that TxB2 is increased in BALF immediately after the acute exposure to acrolein, a component of cigarette smoke (23). Thus TxA2 may be released from the resident cells in the airway immediately after the exposure. Alternatively, TxA2 may arise from recruited inflammatory cells, since previous studies have reported the neutrophil to be a potential source of TxA2 (1, 13, 31). In this study, significant neutrophilia was observed in BALF within 2 h after exposure to smoke. Although it generally takes more than a few hours to recruit inflammatory cells into the airway, our previous study showed that the number of neutrophils in BALF increased immediately after the exposure to ozone in dogs (3 parts/million for 30 min) (1) and in guinea pigs (3 parts/million for 2 h) (21). Therefore, neutrophils may be rapidly recruited into the airway lumen in this condition.

It is not known how tachykinins cause an influx of neutrophils into the airway. Tachykinins reportedly possess chemotactic activity on neutrophils and eosinophils in vitro (11, 30). However, concentrations of tachykinins >1 µM are required for such chemotaxis. It seems unlikely that such high levels of endogenous tachykinins would be released into the airway mucosa in vivo. Alternatively, tachykinins may stimulate the epithelium, the endothelium, the T-lymphocytes, or the mast cells to release mediators that are responsible for chemotaxis and the transmigration of inflammatory cells from the vessels into the mucosa (4, 9, 33, 35). Previous studies demonstrated that substance P induces rapid expression of endothelial cell adhesion molecules and elicits granulocytic inflammation in human skin (33) and that NK1 receptors mediate leukocyte adhesion in neurogenic inflammation in the rat trachea (4). Thus the binding of tachykinins to endothelium is likely to be the first step of tachykinin-mediated granulocyte migration into the airway. The next steps may be mediated by other factors such as leukotriene B4, since epithelium reportedly releases granulocyte chemotactic factor, including leukotriene B4, in response to tachykinins (35).

There have been several reports suggesting possible interaction between tachykinins and various eicosanoids, cysteiny1-leukotrienes in particular. Prior study has demonstrated that superfusion of isolated guinea pig lungs with leukotriene D4 resulted in elevated tachykinin concentrations in the perfusate (24). Another study has shown that tachykinins can liberate leukotrienes in isolated guinea pig tracheal preparations (34). Furthermore, cysteinyl-leukotrienes are known to elicit Tx generation in the airways (3). Thus multiple mechanisms may be involved in the development of airway hyperresponsiveness induced by tachykinins.

In summary, the exposure of guinea pigs to cigarette smoke increased the activity of the tachykinins that produced neurogenic inflammation in the airway. The latter condition was characterized by the extravasation of plasma and the influx of neutrophils. The release of TxA2 after the neurogenic inflammation contributed to the development of the airway hyperresponsiveness produced by exposure to cigarette smoke.

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Cigarette smoke and thromboxane


