Effect of thromboxane A\(_2\) inhibitors on allergic pulmonary inflammation in mice

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ABSTRACT: Thromboxane (Tx)A\(_2\) synthase inhibitors and thromboxane prostanoid (TP) receptor antagonists have been developed as anti-asthma drugs. TxA\(_2\) may contribute to airflow limitation and bronchial hyperresponsiveness, and these compounds have been demonstrated to improve them. However, it is not known whether TxA\(_2\) is involved in bronchial inflammation.

To address this question, we explored the influences of OKY-046 (a TxA\(_2\) synthase inhibitor) and S-1452 (a TP receptor antagonist) on eosinophilic inflammation of the airways using a murine model. BALB/c mice sensitized with ovalbumin and challenged by repeated exposure to ovalbumin yielded marked eosinophilia in bronchoalveolar lavage fluid (BALF).

Treatment with either compound significantly reduced the number of total cells and eosinophils in BALF in a dose-dependent manner. The production of interleukin (IL)-5, IL-2 and interferon (IFN)γ was increased in untreated mice. Furthermore, both compounds inhibited proliferation and cytokine production of SMNC in vitro.

These results suggest that both OKY-046 and S-1452 are capable of inhibiting production of cytokines, which in turn inhibits eosinophil infiltration into the murine airway. Thus, both thromboxane A\(_2\) synthesis inhibitors and thromboxane prostanoid antagonists may be effective as anti-inflammatory drugs in the treatment of asthma.


Thromboxane (Tx)A\(_2\), an unstable cyclo-oxygenase product of arachidonic acid metabolism, has been shown to be an important mediator of thrombosis, vasospasm and bronchoconstriction [1]. As a potent bronchoconstrictor, it is involved in acute bronchoconstriction after antigen inhalation in asthmatic patients, as demonstrated by a markedly elevated level of thromboxane (Tx)B\(_2\), a stable metabolite of TxA\(_2\) [2–4]. TxA\(_2\) has an important role in bronchial hyperreactivity, a key feature of bronchial asthma [5]. Moreover, there is accumulating evidence indicating that TxA\(_2\) could be an immunomodulator.

The immunomodulatory role of TxA\(_2\) has been considered by the tissue distributions of the TxA\(_2\) synthase and thromboxane prostanoid (TP) receptor. TxA\(_2\) synthase in humans has been shown to be expressed abundantly in blood monocytes, lung (especially alveolar macrophages) and liver (especially Kupffer cells) [6]. Antigen-presenting cells such as macrophages and dendritic cells, but not lymphocytes, in the spleen and thymus also express this enzyme [7]. The content of TxA\(_2\) synthase was dramatically increased in inflammatory tissues by up to 16 fold [7]. On the other hand, TP receptor messenger ribonucleic acid (mRNA) is expressed abundantly in thymus, spleen and lung [8], and thymic and splenic T-cells, but not B-cells, in mice [9].

Therefore, antigen-presenting cells express TxA\(_2\) synthase, whereas T-cells express TP receptor, suggesting that TP receptor is involved in, and modulates, T-cell responses.

T-cells have a pivotal role in the pathogenesis of bronchial asthma [10]. Although the above evidence suggests a connection between TxA\(_2\) and inflammation, there is no evidence showing that TxA\(_2\) may also be involved in underlying airway inflammation.

To investigate this point, the effects of two TxA\(_2\) inhibitors were studied using a murine model of allergic pulmonary inflammation. The two inhibitors used were: OKY-046, a TxA\(_2\) synthase inhibitor ((E)-3-(4-(1-imidazolylmethyl)-phenyl)-2-propenoic acid hydrochloride monohydrate), and S-1452, a TP receptor antagonist (calcium (1 R, 2S, 3S, 4S)-(5Z)-7(((phenylsulphonyl) amino)-bicyclo-(2.2.1)-hept-2-yl) hept-5-heptenoate dihydrate). The former has been found to suppress bronchoconstriction, at least in part, caused by antigen and prostaglandin (PG)D\(_2\) [2, 11]. It also suppresses bronchial hyperresponsiveness caused by allergen both in dogs and guinea pigs, and by ozone, platelet-activating factor, and leukotriene (LT)B\(_4\) in dogs [12–16]. OKY-046 can reduce bronchial microvascular leakage and cough response to capsaicin [17, 18]. The latter has recently been reported to exert an inhibitory effect on antigen-induced bronchoconstriction and bronchial
hypersensitiveness induced by antigen or lipopolysaccharide (LPS) exposure in guinea-pigs [19–21].

Methods

Mouse sensitization and challenge

Six week old BALB/c mice (Charles River Laboratories, Shizuoka, Japan) were immunized intraperitoneally three times with 10 μg ovalbumin (Grade V; Sigma Chemical Co., St. Louis, MO, USA) plus 20 mg aluminium hydroxide (Al(OH)₃) in 0.2 mL saline at 2 week intervals. Immediately after the third immunization, the sensitized mice were individually placed in 50 mL plastic tubes and exposed to aerosolized ovalbumin (50 mg·mL⁻¹) dissolved in saline, delivered by a DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, PA, USA) driven by compressed air at 5 L·min⁻¹ for 20 min, once a day for six consecutive days. For negative controls, saline alone was used for exposure instead of ovalbumin.

Administration of OKY-046 and S-1452

OKY-046 (Ono Pharmaceutical Co., Osaka, Japan) and S-1452 (Shionogi Pharmaceutical Co., Osaka, Japan) were kindly provided by each company. Both compounds were dissolved in sterile saline and the highest dose to be administered to the mice was determined by the maximum solubility of each compound (100 mg·kg⁻¹ for OKY-046 and 20 mg·kg⁻¹ for S-1452). In the OKY-046- and S-1452-treated groups, the sensitized mice were given an intraperitoneal injection of the indicated dose (1, 10 or 100 mg·kg⁻¹ for OKY-046; 0.2, 2 or 20 mg·kg⁻¹ for S-1452) of compound in a volume of 300 μL at 5 min before each challenge. Additionally, some mice were treated with both OKY-046 and S-1452 at the same time. In control groups, saline alone was injected intraperitoneally instead of the compounds.

Eosinophil count in bronchoalveolar lavage fluid

Twenty four hours after the final challenge, mice were sacrificed by cervical dislocation. The spleens of treated mice were removed aseptically and used for culture (see below). Bronchoalveolar lavage fluid (BALF) was obtained by slow injection of phosphate-buffered saline (PBS) (0.8 mL) into the trachea followed by recovery using a cannula six times (total 4.8 mL). This procedure always yielded >85% recovery of the infused fluid. The total number of cells in BALF was counted by a haemocytometer. A differential count was made from cytosin preparations (150×, 2 min, at room temperature). The cells were fixed and stained with May-Giemsa. Differential counts of at least 300 cells were performed using standard morphological criteria to identify eosinophils. The absolute number of eosinophils in BALF was then calculated.

Cytokine production and proliferative response of splenic mononuclear cells

The spleens of treated mice were removed aseptically and pressed through a wire mesh screen to separate cells. The splenic mononuclear cells (SMNCs) were purified by a density-gradient method (using Lympholyte-M; Cedarlane Lab, Ontario, Canada), and suspended in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Gibco, Grand Island, NY, USA), 100 U·mL⁻¹ penicillin G, 100 mg·mL⁻¹ streptomycin, 50 μg·L⁻¹ 2-mercaptoethanol (Sigma) and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Sigma). They were seeded at 2×10⁵ cells·mL⁻¹ on 24-well culture dishes. For the stimulation of splenic cells, 200 μg·mL⁻¹ ovalbumin was added to the culture wells. SMNCs were cultured at 37°C in a humidified incubator in a 5% CO₂ atmosphere for 24–48 h. The supernatant was then collected and stored at -80°C until use. Interleukin (IL)-5, IL-2 and interferon (IFN)-γ enzyme-linked immunosorbent assay (ELISA) kits were purchased from Endogen (Boston, MA, USA). The concentrations of these cytokines in the supernatant were assayed according to the procedures recommended by the manufacturer.

For proliferation assay, SMNCs (2×10⁵ cells-well⁻¹) from positive control mice were seeded in triplicate in flat-bottomed 96-well microplates in the presence or absence of OKY-046 (10⁻³–10⁻⁵ M) or S-1452 (10⁻⁴–10⁻⁶ M) [22]. At these concentrations, neither compound was toxic to SMNC, as determined by trypan blue dye exclusion test. Cells were cultured for 3 days under the above conditions. 3H-thymidine was added to each well 14 h prior to harvest for counting using a scintillation counter. At the same time, parallel wells were run and the supernatant was collected after 24 h of culture for IL-5 assay.

Flow cytometric analysis

A single cell suspension (1×10⁶) in 100 μL PBS with 5% normal mouse serum was incubated with 1 μg fluorescein isothionate (FITC)-labelled monoclonal antibodies (moAbs) (Cedarlane, Ontario, Canada) as follows for 30 min at 4°C: anti-L3/T4 (CD4; YTS 191.1.2); Ly-2 (CD8; YTS 169.4); CD3ε (145-2C11); and rat immunoglobulin (Ig)G2a control moAb. Then these cells were washed three times with 100 μL PBS with 0.5% bovine serum albumin. Stained cells were analysed using a fluorescence activated cell sorter (FACScan; Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data are expressed as means±SD, or median (range). The significance of differences between data from each group was determined using Student's t-test following one-way analysis of variance (ANOVA) when applicable. Mann-Whitney U-test was applied for the comparison of cytokine concentrations. A p-value of less than 0.05 was considered statistically significant.

Results

As shown in table 1, the total cell number in BALF from the positive control was markedly higher than that in the negative control group (p<0.001). Since 77% of BALF cells were eosinophils, the absolute number of eosinophils...
was markedly increased. Treatment with OKY-046 (100 mg·kg\(^{-1}\)) or S-1452 (20 mg·kg\(^{-1}\)) resulted in significant decreases in total cell number and eosinophil number in BALF. The reductions of eosinophils induced by both compound were dose-dependent (fig. 1). The absolute number of eosinophils was significantly decreased even with a low dose of either compound (1 mg·kg\(^{-1}\) for OKY-046, 0.2 mg·kg\(^{-1}\) for S-1452), although there was no significant reduction of the percentage of eosinophils in BALF. Only the highest dose of each compound induced a small, but significant, reduction of the eosinophils. The reduction of the absolute number of eosinophils in BALF was significantly greater in mice treated with S-1452 (20 mg·kg\(^{-1}\)) than in mice treated with OKY-046 (100 mg·kg\(^{-1}\)). Under these conditions, an additional treatment with the other compound induced a further significant reduction of eosinophils in the mice treated with OKY-046 (additional treatment with S-1452), but not in the mice treated with S-1452 (additional treatment with OKY-046) (fig. 2).

We could not detect IL-5, IL-2 or IFN-\(\gamma\) either in BALF or the sera, from any group of mice in our experimental system. The level of IL-5 in the supernatant of ovalbumin-stimulated SMNC was significantly decreased in mice treated with either compound compared with that in positive control mice (OKY-046, \(p<0.05\); S-1452, \(p<0.01\)). IL-5 is the most important cytokine in eosinophilic inflammation [23–25]. However, this effect was not restricted to IL-5. Release of both IL-2 and IFN-\(\gamma\) was also significantly inhibited (table 2). Essentially the same result was observed in the supernatant obtained at 48 h (data not shown). The total number of SMNCs tended to be decreased, but not significantly, by treatment with these compounds. There were no significant changes in the percentage of CD3-, CD4- and CD8-positive T-cells in SMNCs by treatment (table 2). Essentially the same result was observed in the supernatant obtained at 48 h (data not shown). The total number of SMNCs tended to be decreased, but not significantly, by treatment with these compounds. There were no significant changes in the percentage of CD3-, CD4- and CD8-positive T-cells in SMNCs by treatment (table 2). These observations (production of three cytokines, and surface expression of these markers) were confirmed in two other separate sets of experiments.

SMNCs from positive control mice could proliferate and produce IL-5 in response to ovalbumin \textit{in vitro}. Addition of either compound to the culture resulted in significant inhibitory effects on both proliferation and IL-5 production \textit{in vitro}. The representative results are shown in figure 3.

### Discussion

Our results clearly demonstrated that BALF eosinophilia, after chronic ovalbumin-challenge in sensitized mice, was significantly inhibited by either a TxA\(_2\) synthase inhibitor or its receptor antagonist in a dose-dependent manner. The precise mechanisms by which these drugs inhibit eosinophil recruitment are currently uncertain. However, our results suggest that the suppression of eosinophils
cytokine production is involved. As a consequence of decreased cytokine production, cellular infiltration in the lung would be inhibited by treatment with TxA2 inhibitors. Since the production of cytokines by SMNCs was inhibited without alteration in the populations of T-cells, these drugs directly affect SMNCs in vivo. The observation that TxA2 inhibitors inhibit both proliferation and IL-5 production of splenic T-cells in vitro supports this notion.

Cummings et al. [26] reported that TxA2 does not appear to directly regulate the proliferation of peripheral blood mononuclear cells stimulated with mitogens. However, they mentioned that dazoxiben (a TxA2 synthase inhibitor)
inhibits immunoglobulin production by peripheral blood mononuclear cells, which is highly dependent on cytokine production. The effect of inhibition of TxA2 on cytokine production by human alveolar macrophages [27] and monocytes [28] has been reported. TxA2 itself stimulates the release of tumour necrosis factor (TNF-α) and/or IL-1 in a dose-dependent manner, without an in-creased production of PGE2.

OKY-046 (100 µg·kg⁻¹·min⁻¹, i.v.) inhibits bronchial hyperreactivity induced by ozone and platelet-activating factor in dogs, but does not alter the increase in cells in BALF [12, 13, 15]. AERMURA et al. [19] reported that S-1452 at 10 mg·kg⁻¹ can completely suppress bronchial hyper-reactivity induced by LPS in guinea-pigs, but cannot reduce the concentration of cytokines (TNF-α and IL-6) in BALF, nor inflammatory cell infiltration into the lung. These studies cannot simply be compared with our study, because the inflammatory responses in their model were much more severe, and the predominant cells infiltrating the lungs were neutrophils. On the other hand, NAGAI and co-workers [16, 20, 21] described the efficacy of OKY-046 (25 and 50 mg·kg⁻¹, i.p.) [16] and S-1452 [20, 21] against bronchial hyperresponsiveness induced by ovalbumin in guinea-pigs, but they did not significantly affect the number of inflammatory cells in BALF. The reason for the discrepancy between their results and those of the present study is not clear, but the protocol (more eosinophils appeared in BALF in our system) and species (i.e. guinea-pig versus mouse) used for the model may account for these different results. It should be noted, however, that oral administration of S-1452 at 10 mg·kg⁻¹ tended to decrease the eosinophils in BALF (by about 20%) [20].

Our results demonstrated that S-1452 (TP receptor antagonist) was more potent than OKY-046 (TxA2 synthase inhibitor). The effect of S-1452 (molecular weight (MW) 829) at 0.2 mg·kg⁻¹ was comparable to that of OKY-046 (MW 282) at 10 mg·kg⁻¹ in terms of the resultant reduction of eosinophils in BALF. Concerning the effect on bronchoconstriction, TP receptor antagonists are more potent than TxA2 synthase inhibitors, because the TP receptor mediates the constrictive response not only to TxA2, but also to other prostanoids such as PGD2 and prostaglandin F2α (PGF2α). In fact, S-1452 inhibited bronchial constriction induced not only by TxA2, but also by PGD2 and PGF2α, whereas OKY-046 had no effect on the action of any of these agents [29]. Therefore, the potential of TxA2 synthase inhibitors may be limited because synthesis of endoperoxidase still occurs, with the consequent transformation to these prostanoids, which activate TP receptor just as TxA2 does [30]. Such a mechanism may explain the difference in the inhibitory effects on cytokine production between synthase inhibitor and receptor antagonist, if the TP receptor is involved in the cytokine production.

Although TxA2 inhibitors are effective in experimental animals as described above, these compounds are much less effective in human asthma [31]. However, in our experience, some patients, who we could not identify prior to treatment, were improved by them. The same mechanism demonstrated in the present study may possibly take place in such patients, although it has not been proved.

In conclusion, our results disclosed that these compounds, which attenuate thromboxane A2, may have a role as anti-inflammatory drugs in the treatment of asthma.

References