Inhibitory Action of OKY-046-HCl, a Specific TXA2 Synthetase Inhibitor, on Platelet Activating Factor (PAF)-Induced Airway Hyperresponsiveness of Guinea Pigs: Role of TXA2 in Development of PAF-Induced Nonspecific Airway Hyperresponsiveness

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Abstract—We studied a role of TXA2 in the development of PAF-induced nonspecific airway hyperresponsiveness in guinea pigs using a TXA2 synthetase inhibitor (OKY-046-HCl) and a stable TXA2 mimetic agent (STA2). Inhalation of PAF (1 μg/ml) and STA2 (1 or 10 ng/ml) increased the airway response to acetylcholine (ACh), histamine, leukotriene D4 and electrical vagal stimulation. Intraduodenal administration (i.d.) of OKY-046-HCl (100 mg/kg) inhibited PAF-induced airway hyperresponsiveness. However, OKY-046-HCl (30 mg/kg, i.v.) did not suppress STA2-induced airway hyperresponsiveness. Neither hexamethonium (1 mg/kg, i.v.) nor hemicholinium-3 (10 mg/kg, i.v.) prevented the increase in the airway response to ACh after inhalation of PAF and STA2. In the presence of atropine (0.5 mg/kg, i.p.), PAF-induced airway hyperresponsiveness to histamine did not change. OKY-046-HCl (100 mg/kg, i.d.) inhibited the increase in ACh (10^{-8} M)-induced 45Ca uptake into the lung tissue from PAF-inhaled guinea pigs. Inhalation of STA2 increased the number (Bmax) of muscarinic and H1-histaminergic receptors in the lung tissue from guinea pigs, but no changes were found on β-adrenoceptors. These results suggest that TXA2 should act on the smooth muscle cells or alter functions of muscarinic and H1-histaminergic receptors, except β-adrenoceptors, and then increase the membrane permeability to extracellular Ca^{2+}. We also assume that OKY-046-HCl can inhibit PAF-induced nonspecific airway hyperresponsiveness by suppressing the generation of TXA2.

Airway hyperresponsiveness is a characteristic and deteriorative feature of bronchial asthma (1, 2). Involvements of the vagal cholinergic nervous systems including muscarinic and irritant receptor, β-adrenoceptor blockade, non-adrenergic inhibitory nerve and non-cholinergic excitatory nerve are considered to be closely associated with the development of airway hyperresponsiveness (3).

PAF, which is released from various cells including platelets, macrophages, mast cells, eosinophils and basophils, caused nonspecific bronchial hyperresponsiveness in both experimental animals (4–9) and humans (10). PAF was observed to elicit a down-regulation of β-adrenoceptors in rat brain (11) and in human lung (12). PAF also released acetylcholine (ACh) from the cholinergic nerve (13) and TXA2 from various tissues or cells (14). So, we paid attention to the involvements of the vagal nervous systems and alterations of the smooth muscle in airway hyperresponsiveness.
On the other hand, recently, TXA₂, generated from arachidonic acid by the cyclooxygenase pathway, was noted to play an important role in airway hyperresponsiveness. Indeed, U-46619, a TXA₂ mimetic drug, increases the airway response to ACh in the isolated bronchial preparations of dogs (15). However, the essential mechanism of TXA₂ in the development of airway hyperresponsiveness remains unknown.

In this study, we investigated the role of TXA₂ in the development of PAF-induced nonspecific airway hyperresponsiveness in guinea pigs using OKY-046•HCl, a specific TXA₂ synthetase inhibitor and STA₂, a stable TXA₂ mimetic agent.

Materials and Methods

1. Animals
Male Hartley guinea pigs were purchased from Nippon SLC Co., Ltd., Hamamatsu, Japan. They (body weight: 350–700 g) were used after breeding under a temperature of 22±2°C and humidity of 55±15%.

2. Drugs
Drugs used were as follows: (E)-3-[p-(1H-imidazol-1-ylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046•HCl, Kissei), platelet activating factor (PAF, Funakoshi, Tokyo), epothiomethano-thromboxane A₂ (a stable TXA₂ mimetic drug=STA₂, Ono, Osaka), acetylcholine chloride (ACh, Daiichi, Tokyo), histamine dihydrochloride (ACh, Daiichi, Tokyo), histamine dihydrochloride (Wako, Osaka), leukotriene D₄ (LTD₄, Funakoshi), hexamethonium bromide (Nacalai Tesque, Kyoto), hemicholinium-3 hydrate (Aldrich, WI, U.S.A.), mepyramine maleate (Pfaltz & Bauer, CT, U.S.A.), propranolol hydrochloride (Sumitomo, Osaka) and atropine sulfate, monohydrate (Wako).

Drugs were suspended in 0.5% carboxymethyl cellulose for intraduodenal administration and were dissolved in 0.9% NaCl solution for intravenous administration.

3. Airway hyperresponsiveness models
After anesthesia with intraperitoneal administration of urethane (500 mg/kg) and α-chloralose (20 mg/kg), an endotracheal cannula was inserted into the animal. The endotracheal cannula was connected to a respirator (SN-480-7, Shinano Seisakujo, Tokyo), and then the animal was ventilated at a tidal volume of 10 ml/kg and at a frequency of 60 breaths/min. A side-hole catheter through the endotracheal cannula was linked with a pressure transducer (P-23ID, Gould, U.S.A.) for the measurement of an endotracheal pressure.

PAF and STA₂ were dissolved in 0.25% bovine serum albumin and in 99.5% ethanol, respectively, and diluted with 0.9% NaCl solution. PAF (1 μg/ml) and STA₂ (1 or 10 ng/ml) were inhaled to guinea pigs using a nebulizer (NE-U1013, Tateishi Electrics, Kyoto) linked to the respirator.

ACh, histamine or LTD₄ aerosol was also generated for 10–15 sec with the nebulizer system described above. Electrical stimulation (60 Hz, 1 msec, for 5 sec) was added to the ipsilateral vagal nerve in uni-vagotomized guinea pigs with an electric stimulator (2907, NEC Sanei, Tokyo). Airway responses were induced by increasing concentrations of aerosol ACh and histamine, by inhalation of LTD₄ at 100 ng/ml and by increasing voltage of electric stimulation.

Airway hyperresponsiveness was determined by PC₁₀ cmH₂O: provocative concentrations of ACh and histamine increasing intratracheal pressure by 10 cmH₂O above the base line, PC₅₀%: a provocative concentration of ACh increasing intratracheal pressure by a half of the maximal response of ACh, and PV₅ cmH₂O: a provocative voltage increasing intratracheal pressure by 5 cmH₂O above the base line. The log PC₁₀ cmH₂O and PC₅₀% showed the logarithms of PC₁₀ cmH₂O and PC₅₀% of ACh before inhalation of PAF and STA₂, PC₁₀ cmH₂O and PC₅₀% of ACh after inhalation of PAF and STA₂. The PV₅ cmH₂O showed PV₅ cmH₂O before PAF inhalation minus PV₅ cmH₂O after PAF inhalation.

4. Receptor binding assay
1) Tissue preparation: The animals were set as described for airway hyperresponsiveness models. The animals were killed by bleeding 30 min after STA₂ (10 ng/ml) inhalation. After adequate intrapulmonary washing with 0.9% NaCl solution, the lung tissues were removed and chopped into small pieces, then homogenized in ice-cold of 0.32 M sucrose solution. The homogenated lung was centrifuged at 500 x g for 10 min at 4°C, and then
the supernatant was centrifuged at 100,000× g for 20 min at 4°C. The resulting pellet was suspended in 50 mM Tris-HCl buffer solution (pH 7.5). The final membrane pellets were resuspended in the buffer solution. Protein concentration in the lung membrane suspension was determined using a Tonein II assay kit (Otsuka Assay, Tokyo).

2) Muscarinic, H₁-histaminergic and β-adrenergic receptor assay: Aliquots of the lung membrane suspensions (containing protein at a concentration of about 1 mg/ml) were incubated in duplicate with ³H-quinuclidinylbenzilate (³H-QNB, specific activity=43.3 Ci/mmol, NEN, England) for 60 min, [pyridinyl-5-³H]pyrilamine (³H-pyrilamine, specific activity=20–30 Ci/mmol, NEN) for 30 min or ³H-dihydroalprenolol (³H-DHA, specific activity=95 Ci/mmol, NEN) for 30 min at 25°C, respectively. Nonspecific binding was determined in the presence of 10⁻⁵ M of atropine, 10⁻⁶ M of mepyramine and 10⁻⁶ M of propranolol. The dissociation constant (Kᵦ) and maximal binding capacity (Bₒ) of muscarinic, H₁-histaminergic and β-adrenergic receptors were determined from the regression lines (correlation coefficient=r>0.69) of Scatchard plots.

5. ⁴⁵Ca uptake into guinea pig lungs

The animals were set as described for airway hyperresponsiveness models and then killed by bleeding 30 min after PAF (1 μg/ml) inhalation. After adequate intrapulmonary washing with a Locke Ringer’s solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 2.1 mM MgCl₂·6H₂O, 2.0 mM glucose and 6.0 mM NaHCO₃), the lung tissues were removed and cut finely with a tissue chopper (Mickle Laboratory Engineering). The suspended lung fragments (100 mg in 1.0 ml of the Locke Ringer’s solution) containing ⁴⁵CaCl₂ (0.1 μCi/ml, specific activity=35.1 mCi/mg, NEN) and ACh (final concentration of 10⁻⁸ M) were incubated for 3 min at 37°C. The ⁴⁵Ca uptake reaction was stopped by chilling in ice. The lung fragments were washed with a buffer (80.8 mM LaCl₃, 11.0 mM glucose and 6.0 mM tris (hydroxy methyl) aminomethane) as described by Karaki and Weiss (16). The radioactivity of ⁴⁵Ca in the lung fragments was measured with a scintillation counter (TriCarb 4640, Packard, Downers Grove, U.S.A.).

6. Drug administration

OKY-046·HCl was administered intraduodenally 30 min before PAF inhalation and intravenously 5 min before STA₂ inhalation. Hexamethonium (1 mg/kg) and hemicholinium-3 (10 mg/kg) were intravenously pretreated 5 and 10 min, respectively, before inhalation of PAF and STA₂. Atropine (0.5 mg/kg) was intraperitoneally pretreated 30 min before PAF inhalation.

7. Statistics

Data were expressed as the mean±S.E. of the mean. Statistical significance was determined by Student’s t-test.

Results

1. Effects of OKY-046·HCl on PAF-induced airway hyperresponsiveness in guinea pigs

Figure 1 shows the airway response to ACh, histamine and electrical vagal stimulation 60 min after PAF inhalation. There were a significant increase in 4 log PC₁₀ cmH₂O of ACh and J PV₅ cmH₂O of voltage and a decrease in PC₁₀ cmH₂O of histamine after PAF inhalation as compared to those of saline (0.9% NaCl solution) inhalation. Intraduodenal administration of OKY-046·HCl (100 mg/kg) suppressed such hyperresponsiveness (Fig. 1). OKY-046·HCl showed no antagonistic actions to ACh, histamine and vagal stimulation.

2. Effects of OKY-046·HCl on STA₂-induced airway hyperresponsiveness in guinea pigs

Inhalation of STA₂ caused a significant increase in 4 log PC₅₀ cmH₂O of ACh, decrease in PC₁₀ cmH₂O of histamine and the potentiation of airway response to LTD₄ (100 ng/ml) compared to those of saline (0.9% NaCl solution) inhalation. Intravenous injection of OKY-046·HCl (30 mg/kg) did not inhibit such hyperresponsiveness (Fig. 2).

3. Effects of autonomic nervous blocking agents on PAF- and STA₂-induced airway hyperresponsiveness in guinea pigs

1) Effect of hexamethonium: The intravenous injection of hexamethonium before inhalation of PAF and STA₂ did not suppress the increase in 4 log PC₁₀ cmH₂O of ACh. Hexamethonium had no antimuscarinic action (Fig. 3).

2) Effect of hemicholinium-3: The intravenous injection of hemicholinium-3, which had
no antimuscarinic action, did not suppress the increase in $\Delta \log PC_{10 \text{cmH}_2\text{O}}$ of ACh induced by PAF and STA$_2$. Hemicholinium-3 conversely potentiated PAF-induced airway hy-

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**Fig. 1.** Effects of OKY-046-HCl on PAF-induced airway hyperresponsiveness (AHR) to acetylcholine aerosol (ACh, A), histamine aerosol (B) and electrical vagal stimulation (C) in anesthetized guinea pigs. OKY-046-HCl was administered intraduodenally (i.d.) 30 min before PAF inhalation. $PC_{10 \text{cmH}_2\text{O}}$ indicates the concentration of ACh that increased the airway pressure by 10 cmH$_2$O above the baseline value. $PV_{5 \text{cmH}_2\text{O}}$ indicates the voltage of electrical vagal stimulation that increased airway pressure by 5 cmH$_2$O above the baseline value. Each column shows the mean±S.E. of 4 to 13 experiments. Each P value represents the statistical significance of the difference from the control. A: AHR to ACh 60 min after PAF inhalation. B: AHR to histamine 60 min after PAF inhalation. C: AHR to electrical vagal stimulation 60 min after PAF inhalation.

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**Fig. 2.** Effects of OKY-046-HCl on STA$_2$-induced airway hyperresponsiveness (AHR) to aerosol of acetylcholine (ACh, A), histamine (B) and leukotriene (LT) $D_4$ (C) in anesthetized guinea pigs. $PC_{50\%}$ shows the concentration of ACh that increased the airway pressure by a half of the maximal response of ACh. $PC_{10 \text{cmH}_2\text{O}}$ indicates the concentration of histamine that increased the airway pressure by 10 cmH$_2$O above the baseline value. LTD$_4$ at 100 ng/ml was inhalated. OKY-046-HCl was administered intravenously 5 min before STA$_2$ inhalation. Each column shows the mean±S.E. of 4 to 5 experiments. Each P value represents the statistical significance of the difference from the control. A: AHR to ACh 30 min after STA$_2$ inhalation. B: AHR to histamine 30 min after STA$_2$ inhalation. C: AHR to LTD$_4$ 30 min after STA$_2$ inhalation.
3) Effect of atropine: The PAF-induced significant decrease in the threshold concen-

Fig. 3. Effects of hexamethonium (C6) on PAF- and STA2-induced airway hyperresponsiveness (AHR) to acetylcholine aerosol (ACh) in anesthetized guinea pigs. C6 (1 mg/kg) was intravenously administered 5 min before inhalation of PAF and STA2. PC10 cmH2O indicates the concentration of ACh that increased airway pressure by 10 cmH2O above the baseline value. Each column shows the mean±S.E. of 3 to 13 experiments. Each P value represents the statistical significance of the difference from the control. A: PAF-inhalated AHR, B: STA2-inhalated AHR.

Fig. 4. Effects of hemicholinium-3 (HC-3) on PAF- and STA2-induced airway hyperresponsiveness (AHR) to acetylcholine aerosol (ACh) in anesthetized guinea pigs. HC-3 (10 mg/kg) were intravenously administered 10 min before inhalation of PAF and STA2. PC10 cmH2O indicates the concentration of ACh that increased airway pressure by 10 cmH2O above the baseline value. Each column shows the mean±S.E. of 4 to 13 experiments. Each P value represents the statistical significance of the difference from the control. A: PAF-inhalated AHR, B: STA2-inhalated AHR.
Fig. 5. Effect of atropine on PAF-induced airway hyperresponsiveness to histamine in anesthetized guinea pigs. Atropine (0.5 mg/kg) was intraperitoneally administered 30 min before PAF inhalation. PC_{10 cmH2O} indicates the concentration of histamine that increased the airway pressure by 10 cmH_2O above the baseline value. Each column shows the mean±S.E. of 3 to 4 experiments. Each P value represents the statistical significance of the difference from the control.

4. Effect of OKY-046-HCI on 45Ca uptake into lung fragments from PAF-inhalated guinea pigs

In PAF-inhalated guinea pigs, 45Ca uptake into lung fragments significantly increased in the presence of ACh (10^{-8} M), which was suppressed by the intraduodenal administration of OKY-046-HCI (100 mg/kg). On the other hand, no significant changes of 45Ca uptake into lung fragments were found in the presence of ACh in saline-inhalated animals (Fig. 6).

5. The binding assay of muscarinic, H_1-histaminergic and β-adrenergic receptors (B_{max} and K_d) of the lung tissue from STA_2-inhaled guinea pigs

Significant increase in the B_{max} of muscarinic and H_1-histaminergic receptors were found in the lung tissues from STA_2-inhaled guinea pigs, but the K_d of these receptors did not change. No significant alterations of β-adrenergic receptor binding sites were seen after STA_2 inhalation (Table 1).

Discussion

Nadel and his colleagues have already demonstrated that intravenous injection of OKY-046, a specific TXA_2 synthetase inhibitor, suppresses airway hyperresponsiveness and TXB_2 generation induced by inhalation of an antigen, ozone, LTB_4 and PAF (4, 17-19). In this study, inhalation of PAF and STA_2 increased the airway response to ACh, histamine, LTD_4 and electrical vagal stimulation in guinea pigs. Intraduodenal administration of OKY-046-HCI suppressed PAF-induced nonspecific airway hyperresponsiveness; however, OKY-046-HCI is not a PAF antagonist (data not shown), and OKY-046-HCI did not inhibit STA_2-induced nonspecific airway hyperresponsiveness. From those facts, we conclude that TXA_2 plays an important role for the development of PAF-induced nonspecific airway hyperresponsiveness.

Recently, it has been reported that U-46619 might cause the release of ACh from the postganglionic vagal efferent nerve in airway hyperresponsiveness (5, 15, 20). Also, it was suggested that TXA_2 and PGF_{2α} induce airway hyperresponsiveness by stimulating sensory nerve endings and by activating the reflex pathway (21). In this study, however, neither PAF- nor STA_2-induced nonspecific airway hyperresponsiveness was prevented by hexamethonium or hemicholinium-3. Furthermore, the increase in the airway response to histamine was not changed in the presence of atropine after PAF inhalation. From these results, it is unlikely that TXA_2 acts on the preganglionic and postganglionic site of the autonomic nervous system in such hyperresponsiveness. Therefore, we think that PAF-induced nonspecific airway hyperresponsiveness should be due to the alteration of postjunctional mechanisms including receptor functions and post-receptor processes of the
Fig. 6. Effect of OKY-046-HCl on $^{45}$Ca uptake into the lung fragments from PAF-inhalated guinea pigs. OKY-046-HCl (100 mg/kg) was intraduodenally administered 30 min before PAF inhalation. The lung fragments (100 mg), $^{45}$CaCl$_2$ (0.1 $\mu$Ci/ml) and acetylcholine (ACh, $10^{-8}$ M) were incubated for 3 min at 37°C. Each column shows the mean±S.E. of 5 experiments. Each P value represents the statistical significance of the difference between in the absence of ACh (-) and in the presence of ACh ($10^{-8}$ M).

Table 1. Binding parameters of muscarinic, H$_1$-histaminergic and $\beta$-adrenergic receptors in the lung parenchyma of guinea pigs after STA$_2$ inhalation

|                          | B$_{\text{max}}$ (fmole/mg protein) | K$_d$ (nM) |
|--------------------------|-------------------------------------|------------|
|                          | Saline        | STA$_2$     | Saline        | STA$_2$     |
| Muscarinic receptor      | 38±13         | 91±17*      | 0.91±0.09     | 1.97±0.48  |
| H$_1$-histaminergic receptor | 1431±168     | 2066±189*   | 0.67±0.13     | 1.29±0.31  |
| $\beta$-Adrenergic receptor | 575±113       | 485±89      | 0.85±0.13     | 0.70±0.14  |

B$_{\text{max}}$ (maximal binding capacity) and K$_d$ (dissociation constant) were determined by the Scatchard plot analysis. Each value shows the mean±S.E. of 4-8 experiments. *: Significant difference from the saline at P<0.05.

It was reported that PAF elicited a down-regulation of $\beta$-adrenoceptors in human lung in vitro and rat brain (11, 12). In guinea pigs, however, the response of airway smooth muscle to isoproterenol and the $\beta$-adrenoceptor function of the trachea and lung did not change after PAF application (7). No detectable down-regulation of $\beta$-adrenoceptors was found in lung tissues from PAF-
inhalated guinea pigs (data not shown). Changes of receptor functions induced by PAF acether were reported to be based on TXA2 release (14); in this study, however, no significant changes in β-adrenoceptor functions were found in lung tissues from STA2-inhaled animals. This demonstrates that PAF-induced nonspecific airway hyperresponsiveness is not related to β-adrenoceptor blockade in airway tissues.

Recently, Robertson et al. (9) have reported that no changes are found in muscarinic and histaminergic receptor functions of the airway smooth muscle in guinea pigs after PAF inhalation. However, PAF acts on a variety of cell types and smooth muscles. Based on this fact, we assume that PAF should have complicated actions on lung tissues containing airway smooth muscle and vascular muscle. We investigated the effect of STA2 on muscarinic and H1-histaminergic receptors. When STA2 was used, increases in the binding capacity of muscarinic and H1-histaminergic receptors were seen. Furthermore, we think that the discrepancy between the results of Robertson et al. (9) and ours can be attributed to a difference in methodology: they used the lung preparation immediately after the end of induction of the airway response to ACh and histamine for their receptor binding assay; our preparation was obtained without exposure to ACh and histamine after STA2 inhalation. It is likely that PAF-induced nonspecific airway hyperresponsiveness is due to the generation of TXA2 which increases the binding capacity of muscarinic and H1-histaminergic receptors.

It is widely accepted that calcium ion (Ca2+) is mobilized for the activation of contractile proteins after drug-receptor interaction. The contraction of airway smooth muscle to ACh is dependent on extracellular Ca2+ (22, 23). If the potentiation of smooth muscle contraction happens nonspecifically and originates from changes of postjunction sites, it has been suggested that the mechanism of hyperresponsiveness should be closely related to the enhancement of mobilization of cytoplasmic Ca2+ which is presumably supplied from intracellular, membrane bound and extracellular sources (24, 25). Additionally, changes in the membrane permeability of smooth muscle should be essential to hyperresponsiveness (26). In this study, inhalation of PAF increased ACh-induced 45Ca uptake into the lung fragments of guinea pigs, which was suppressed by OKY-046-HCl. These results suggest that TXA2 should change the membrane permeability of the airway smooth muscle to extracellular Ca2+. It has been currently reported that TXA2 may act on the smooth muscle like a calcium ionophore (27). However, we assume that the action of TXA2 as a calcium ionophore is not essential to the development of nonspecific airway hyperresponsiveness as described by Fujimura and Matsuda (28), because STA2 modified the number of muscarinic and H1-histaminergic receptors without being accompanied by contraction of airway smooth muscle. Therefore, we assume that the modification of membrane permeability to extracellular Ca2+ should result from the alteration of the number of muscarinic and H1-histaminergic receptors induced by TXA2.

In conclusion, we assume that the action of TXA2 on receptor functions and on Ca uptake in airway smooth muscle should be essential to the development of PAF-induced nonspecific airway hyperresponsiveness: TXA2 should act on airway smooth muscle cell or alter the number of muscarinic, H1-histaminergic and leukotriene receptors that are responsible for the contraction of the smooth muscle. Those actions of TXA2 should increase the membrane permeability to extracellular Ca2+ and finally cause nonspecific airway hyperresponsiveness. We also conclude that OKY-046-HCl can prevent PAF-inhaled nonspecific airway hyperresponsiveness by suppressing the generation of TXA2.

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