J Pharmacol Sci **98**, 99 – 102 (2005)

Journal of Pharmacological Sciences

Short Communication

Abrogation of Bronchial Eosinophilic Inflammation and Attenuated Eotaxin Content in Protease-Activated Receptor 2-Deficient Mice

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Received January 21, 2005; Accepted March 19, 2005

Abstract. Protease-activated receptor 2 (PAR2) belongs to the PAR family (PAR1 to PAR4), which is activated by serine proteases (trypsin, tryptase, etc.). In this study, we evaluated the role of PAR2 in allergic inflammation of airways using PAR2-deficient ($PAR2^{-/-}$) mice. In wild- type mice, infiltration of eosinophils and high eotaxin content were found in bronchoalveolar lavage fluid (BALF) after ovalbumin (OA) sensitization and following challenge. In contrast, both OA-induced infiltration of eosinophils and increase of eotaxin content were abrogated in BALF from $PAR2^{-/-}$ mice. The activation of PAR2 might be essential in the production of eotaxin and consequential allergic inflammation in airways.

Keywords: protease-activated receptor 2, eosinophil, eotaxin

Protease-activated receptors (PARs) are a family of G protein-coupled seven transmembrane receptors, currently consisting of four members, PAR1 to PAR4. The activation mechanism of PARs is very unique (1). A neo-terminus tethered ligand produced by proteolytic cleavage of the extracellular N-terminus or a short synthetic peptide based on the tethered ligand sequence, with the exception of PAR3, activates the corresponding receptor. Among these, PAR2 was identified in 1994 (2) and reported to be activated by the tissue factor/factor VIIa, Xa, the sperm protease, acrosin, and a trypsin-like serine protease isolated from rat brain as well as trypsin, tryptase, and synthetic peptides with its tethered ligand sequence (1).

Physiological and pathological roles of PAR2 have been investigated with in vivo animal models with different approaches such as immunohistochemistry, pharmacology with synthetic PAR2-activating peptide, and PAR2-deficient ($PAR2^{-/-}$) mice (3, 4). PAR2 participates in leukocyte rolling, adhesion, and infiltration of neutrophils (3) and induces nuclear factor kappa B-DNA binding (5). In airways, although Cicala et al. showed protective roles of PAR2 (6), there are some reports demonstrating pro-inflammatory roles of PAR2. For example, Vliagoftis et al. suggested proinflammatory roles of PAR2 agonists during airway inflammation by up-regulating the release of matrix metalloproteinase-9 (MMP-9) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes eosinophil survival (7). Furthermore, using $PAR2^{-/-}$ mice, Schmidlin et al. showed a decreased infiltration of airways by eosinophils (8).

Eotaxin is a potent eosinophil attractant. The increase in eotaxin content correlated with infiltration of eosinophils in airways and with severity of asthma (9). On the basis of these reasons, we investigated involvement of eotaxin in the suppression of eosinophil-infiltration by PAR2 deficiency.

Generation of PAR2 homozygous null mice has been described in detail elsewhere (4, 10). Wild-type and homozygous null mice used in the experiments were 6 weeks of age and had a mixed genetic background of C57BL/6 and 129/Ola. All animals were housed under specific pathogen-free conditions and had free access to a standard laboratory diet and water in an air-conditioned room at 23 ± 3°C and a relative humidity of 55 ± 15%. The present experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kowa Co., Ltd. The following reagents were obtained from commercial sources: oval-

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bumin (OA, Grade V; Sigma, St. Louis, MO, USA), bovine serum albumin (BSA, Sigma), Dullbecco's phosphate buffered saline (PBS, Sigma), Diff Quick Solution® (International Reagents, Kobe), a Mouse Eotaxin ELISA kit (R&D Systems, Minneapolis, MN, USA), pentobarbital sodium (Abbott, Abbott Park, IL, USA). Alum (aluminum hydroxide gel suspension) was prepared according to the method described by Nagai (11).

Mice were actively sensitized by intraperitoneal injection of 10 μg OA with 4 mg alum on day 0. The immunized mice were challenged with an aerosol of saline or 1.5% OA solution for 10 min on day 14 as described by Chin et al. (12). To administer an aerosol form, an ultrasonic nebuliser (Model NE-U12; Omron, Kyoto) was employed.

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in bronchoalveolar lavage fluid (BALF) on day 17. Animals were sacrificed with an intraperitoneal injection of pentobarbital (80 mg/kg). The trachea was cannulated and the air lumen was washed 5 times with PBS containing 0.1% BSA. BALF from each animal was centrifuged (5,000 rpm, 30 s) at 4°C. Cell pellets were resuspended in the same buffer (0.5 mL). BALF was stained with Turk solution and the number of nucleated cells was counted in a Burker-Turk chamber. A differential count of 300 cells was made on a smear prepared with a centrifuge (DC8; NeuroProbe, Gaithersburg, MD, USA) and stained with Diff-Quick solution (based on standard morphologic criteria, magnification × 400). The supernatant of BALF was stored at −30°C for determination of eotaxin. The concentration of eotaxin in the supernatant of BALF was measured using the mouse eotaxin ELISA kit, according to the manufacturer's instructions. Results were expressed as the mean ± S.E.M. Data were evaluated by Student's *t*-test, and $P<0.05$ was considered to be significant.

For histopathological observation, wild-type and PAR2$^{-/-}$ mice were sensitized and challenged with saline or OA. On day 17, lung lobes of the mice were dissected and were fixed in 10% neutral buffered formalin. Sections, 3-μm-thick, were stained with hematoxylin-eosin and were observed microscopically (×100 or ×400).

To clarify the role of PAR2 in antigen-induced airway inflammation, we examined accumulation of inflammatory cells in BALF from wild-type mice and PAR2$^{-/-}$ mice 3 days after saline- or OA-challenge. As shown in Table 1, the number of macrophages, eosinophils, neutrophils, and lymphocytes were not different between saline-challenged wild-type and PAR2$^{-/-}$ mice. After OA-challenge, the number of eosinophils was highly increased from 0.2×10^3 cells (saline-challenged) to 275×10^3 cells in wild-type mice. In contrast, in PAR2$^{-/-}$ mice, it was just 21.3×10^3 cells, indicating that PAR2 deficiency suppressed OA-induced eosinophilia by 92%. The number of lymphocytes was also increased 16.9 times by OA-challenge as compared with that in the saline-challenged group in wild type mice, but the increase was suppressed by 90% in PAR2$^{-/-}$ mice. The number of neutrophils was 7 times higher in the OA-challenged group than in the saline-challenged group, but there was no difference between saline- and OA-challenged groups in PAR2$^{-/-}$ mice. A similar tendency was observed in the change in the number of macrophages.

The effect of PAR2 deficiency on eotaxin content in BALF was investigated. Eotaxin content in BALF from saline-challenged wild-type mice was 0.3 ± 0.3 pg/mL. In BALF from OA-challenged wild-type mice, the content increased up to 8.9 ± 3.2 pg/mL (Fig. 1). In contrast, the increase of eotaxin content was abolished in BALF from allergen-challenged PAR2$^{-/-}$ mice.

To clarify the importance of eotaxin content in the mice used in this experiment, we confirmed the correlation between the number of eosinophils and the level of eotaxin in BALF from wild-type mice. There is a positive correlation between these factors with a high correlative index ($r = 0.8289$, $P<0.05$).

Histopathological observation revealed that challenge with OA to sensitized wild-type mice induced hyperplasia of airway epithelium and infiltration of bronchial parenchyma by eosinophils (Fig. 2B). No obvious

Table 1. Effects of PAR2 deficiency on cellular distributions in BALF

Group	Genotype	Challenge	Cell population (×10^3 cells/BALF)			
			Macrophages	Eosinophils	Neutrophils	Lymphocytes
A	+/+	Saline	78.0 ± 10.0	0.2 ± 0.1	0.2 ± 0.1	0.7 ± 0.1
B	+/+	OA	96.6 ± 7.9	275.3 ± 79.1$^{\#\#}$	1.4 ± 0.5$^{\#}$	11.8 ± 4.5$^{\#\#}$
C	−/−	Saline	80.6 ± 9.7	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
D	−/−	OA	80.5 ± 15.9	21.3 ± 6.7$^{\#\#}$.**	0.5 ± 0.2	1.2 ± 0.2$^{\#\#}$.*

Each value represents the mean ± S.E.M. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* group A or C. *$P<0.05$, **$P<0.01$ *vs* group B. N = 13.

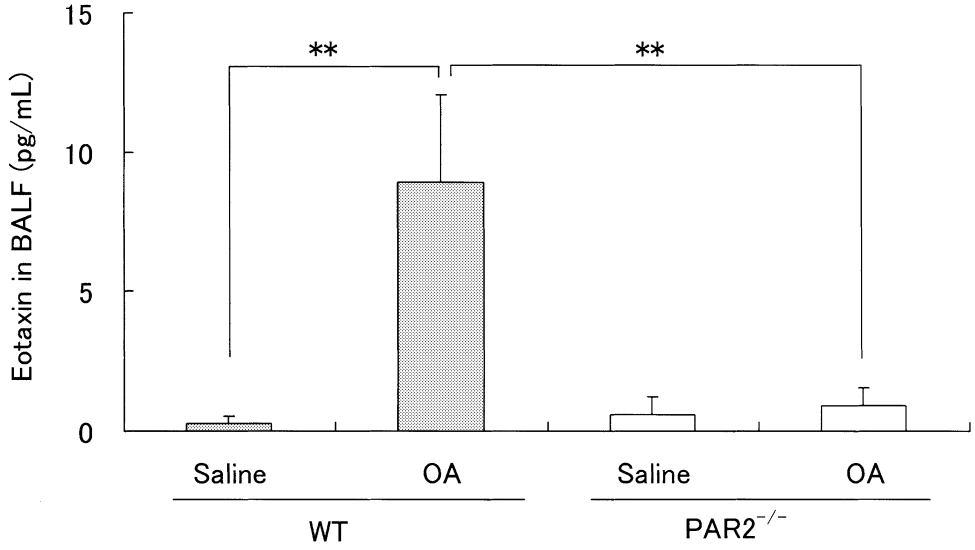

Fig. 1. Effect of PAR2 deficiency on eotaxin level in BALF. BALF was obtained from wild-type (WT) and PAR2$^{-/-}$ mice after treatment with OA or saline. The columns and the bars represent the mean ± S.E.M. of the concentration of eotaxin. ***P*<0.01, N = 13.

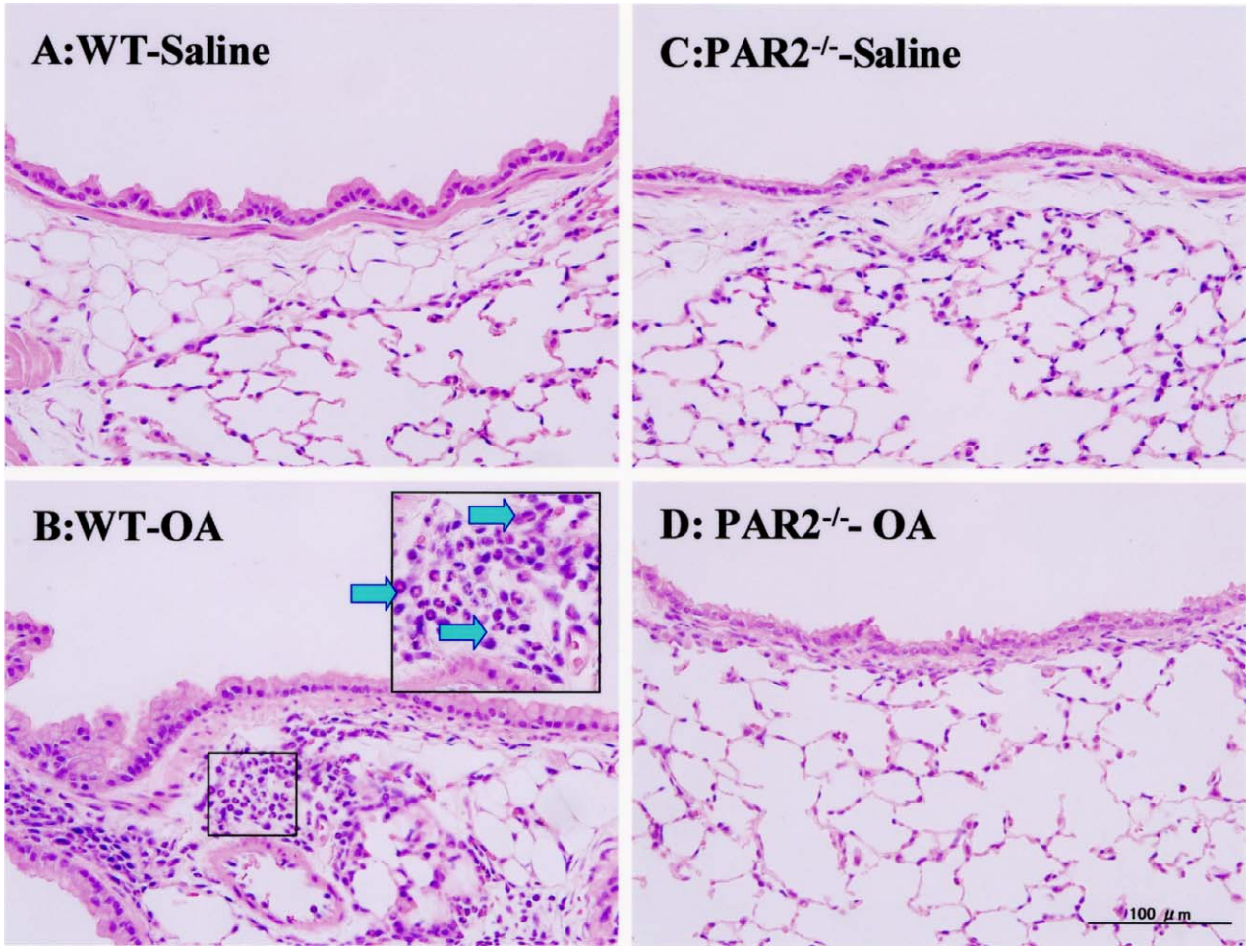

Fig. 2. Hematoxylin and eosin staining of lung sections from wild-type (WT) and PAR2$^{-/-}$ mice. The animals were sensitized and challenged with OA. Three days after the challenge, lung parenchymas were dissected and stained with hematoxylin and eosin. Arrows indicate eosinophils. Bar = 100 μm.

change was observed in saline-challenged mice (Fig. 2: A and C). In contrast, few eosinophils were observed in OA-challenged PAR2$^{-/-}$ mice, although slight impairment of epithelium was observed (Fig. 2D).

In this study, we investigated the role of PAR2 in allergic inflammation of the airways using PAR2$^{-/-}$ mice. At first, we showed that PAR2 deficiency decreased about 90% of the OA-induced infiltration of eosinophils and lymphocytes in BALF. Moreover, histopatholosical observation supported the decrease of the infiltration by eosinophils. This phenomenon was supported by Schumidlin et al., who reported that the deletion of PAR2 diminished inflammatory cell infiltration into airways remarkably (8). They also showed that PAR2 mRNA was readily detected in airway epithelial cells of wild-type mice. Therefore, it seems that PAR2 expressed on epithelial cells participates in the infiltration of inflammatory cells.

In the next step, we examined the mechanism of how PAR2 deficiency reduces OA-induced infiltration of eosinophils and lymphocytes. We focused on eotaxin, because eotaxin is known as the most potent chemokine to eosinophils (13). Eotaxin also acts as a chemoattractant to lymphocytes of the Th2 subset (14). Vliagoftis et al. reported that airway epithelial cells produced eotaxin when stimulated by PAR2 activating peptide (7). As shown in Fig. 1, although the eotaxin content in BALF was increased by OA-challenge in wild-type mice, PAR2 deficiency decreased the increase in eotaxin content by 90%. Furthermore, we found a positive correlation between the number of eosinophils and the eotaxin content. These results strongly suggest that the abrogation of bronchial eosinophilic inflammation in PAR2$^{-/-}$ mice may be caused by the insufficiency of eotaxin production in airway epithelial cells.

Type-2 helper T cell (Th2) cytokines, such as IL-4 and IL-5, and GM-CSF, promote bronchial allergic inflammation (15). Then we evaluated the level of IL-4, IL-5, and GM-CSF. None of these cytokines could be detected in BALF from OA-challenged wild-type and PAR2$^{-/-}$ mice (data not shown). Thus, eotaxin would have more important roles than IL-4, IL-5, and GM-CSF in our experimental asthmatic model. The assumed mechanism of abrogation of eosinophilic inflammation in PAR2$^{-/-}$ mice was as follows: in wild-type mice, antigen binds to IgE on mast cells to evoke the degranulation of mast cells. Tryptase secreted from mast cells activates PAR2 on epithelial cells. Epithelial cells secrete eotaxin that induces chemotaxis of eosinophils and lymphocytes. In PAR2$^{-/-}$ mice, lack of PAR2 would result in low secretion of eotaxin and suppression of the chemotaxis of eosinophils and lymphocytes.

In conclusion, it was suggested that PAR2 played an important role in the production of eotaxin and consequential infiltration of eosinophils and lymphocytes in asthmatic airways.

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