



Brief Communications

Elevated guanylate cyclase and cyclic-guanosine monophosphate-dependent protein kinase levels in nasal mucosae of antigen-challenged rats



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ABSTRACT

Objective: In patients with severe allergic rhinitis, the most serious symptom is rhinostenosis, which is considered to be induced by a dilatation of plexus cavernosum. The vascular relaxing responses to chemical mediators are mainly mediated by the production of nitric oxide (NO). However, the exact mechanism(s) in nasal venoresponsiveness of allergic rhinitis is not fully understood. In the present study, we investigated the roles of soluble guanylate cyclase (sGC) and cyclic-guanosine monophosphate (c-GMP)-dependent protein kinase G (PKG) in venodilatation of nasal mucosae of antigen-challenged rats.

Methods: Actively sensitized rats were repeatedly challenged with aerosolized antigen (2,4-dinitrophenylated *Ascaris suum*). Twenty-four hours after the final antigen challenge, nasal septum mucosa was exposed surgically and observed directly in vivo under a stereoscopic microscope. The sodium nitroprusside (SNP) and 8-Br-cGMP (a PKG activator) were administered into arterial injection, and the venous diameters of nasal mucosa were observed.

Results: The intra-arterial injections of SNP and 8-Br-cGMP-induced venodilatation were significantly augmented in the nasal mucosae of repeatedly antigen-challenged rats. Furthermore, protein expressions of sGC and PKG were significantly increased in nasal mucosae of the antigen-challenged rats.

Conclusion: The present findings suggest the idea that the promoted cGMP/PKG pathway may be involved in the enhanced NO-induced venodilatation in nasal mucosae of antigen-challenged rats.

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Introduction

Allergic rhinitis is a common inflammatory disease with an increasing worldwide prevalence. The nasal blockage, sneezing, rhinorrhea and pruritus are thought to be induced by several chemical mediators and inflammatory cytokines released from various inflammatory cells after provocation of relevant allergen (Bousquet et al., 1996; Naclerio and Baroody, 1994; Naclerio et al., 1983). In addition to the specific antigen-induced nasal symptoms, the state of nasal hyperresponsiveness to non-specific stimuli other than the relevant antigen is one of the characteristic features of patients with allergic rhinitis.

Nitric oxide (NO) is released following the antigen-antibody reaction (Miadonna et al., 1999; Shaw et al., 1985), and has an ability to induce nasal obstruction in a guinea pig model of allergic rhinitis (Mizutani et al., 2001). One of the functions of NO is the relaxation of vascular smooth muscle cells, regulating blood flow through vessels (Bredt and Snyder, 1994; Moncada et al., 1991). Thus, NO plays important and crucial roles in nasal obstruction in allergic rhinitis. The NO is thought to be responsible for endothelium-dependent vascular smooth muscle relaxation in many vessels. Nitrovasodilators, including sodium nitroprusside (SNP), release NO and increase cyclic-guanosine monophosphate-dependent protein (cGMP) levels via activation of soluble guanylate cyclase (sGC). As a consequence, cGMP-dependent protein kinase (PKG) activity is enhanced causing modulation (phosphorylation) of various intracellular proteins, and resulting vascular relaxation (Waldman and Murad, 1987). In the present study, to determine the roles of sGC and PKG in the augmentation of responsiveness to NO observed in nasal mucosae of repeatedly antigen challenged rats, we investigated the effects of 8-Br-cGMP on the venoresponsiveness in nasal septal mucosa.

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Materials and methods

Animals and antigen sensitization

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g; Charles River Japan, Inc.) were used. All experiments were approved by the Animal Care Committee of Hoshi University (Tokyo, Japan). Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated *Ascaris suum* antigen (DNP-Asc) by the method described in the previous paper (Chiba et al., 2008; Misawa and Chiba, 1993). In brief, the rats were sensitized with DNP-Asc together with *Bordetella pertussis*, and were boosted 5 days later. Eight days after the first immunization, the rats were challenged by inhaling DNP-Asc for 40 min under conscious state. The animals were subjected to a total of three antigen-challenges, spaced 48-h apart. The sensitized-control animals received the same immunization procedure but inhaled saline aerosol instead of antigen challenge.

Measurement of changes in area of nasal mucosal veins

Venous area of nasal septal mucosae in anesthetized rats was measured by the method of previous paper (Chiba et al., 2006, 2007; Sakai et al., 2010, 2011a). In brief, the rats were anesthetized with urethane (2 g/kg, i.p.) and nasal mucosa was exposed surgically. Then the nasal septal mucosa was observed under a stereoscopic microscope (SZX9; Olympus Optical Co., Ltd., Tokyo, Japan) at a magnification of 40 $\times$. A digital camera (Camedia C-4040 Zoom; Olympus Optical Co., Ltd.,

Tokyo, Japan) was equipped with the microscope to obtain photographs of nasal septum blood vessels. After an equilibration period, a photograph was taken for measurement of baseline venous area and then 30 μ g sodium nitroprusside (SNP)/0.1 mL/animal or 300 μ g 8-Br-cGMP/0.1 mL/animal was administered into the external carotid artery (i.a.). After the application of the agent, the nasal mucosa was photographed continuously at 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 min to examine the time-course changes in the area of veins. In case of dose-response experiment, after the application of the 1–100 μ g SNP/animal or 10–1000 μ g 8-Br-cGMP/animal, the nasal mucosa was photographed continuously at 3 min to examine the dose-response changes in the diameter of veins. The area of veins was measured using Image-Pro Plus (Media Cybernetics, San Diego, CA, USA) application with a computer and expressed as percentage of the baseline venous area. The basal areas of vasculature in nasal mucosae were not statistically different between control and antigen-challenged rats (control: 45,962 $\pm$ 8894 pixels, antigen-challenged: 40,889 $\pm$ 9036 pixels).

Immunoblotting

The isolated nasal septa tissues were quickly frozen with liquid nitrogen, and these tissues were crushed to pieces by CryopressTM (CP-100W; Niti-on, Co. Ltd., Japan: 15 $\times$ 3). The tissue was then homogenized in ice-cold T-PERTM Tissue Protein Extraction Reagent (Pierce). The tissue homogenate was centrifuged (1 000 $\times$ g, 4 $^{\circ}$ C for 1 min), and supernatants were stored at -85° C until use. To

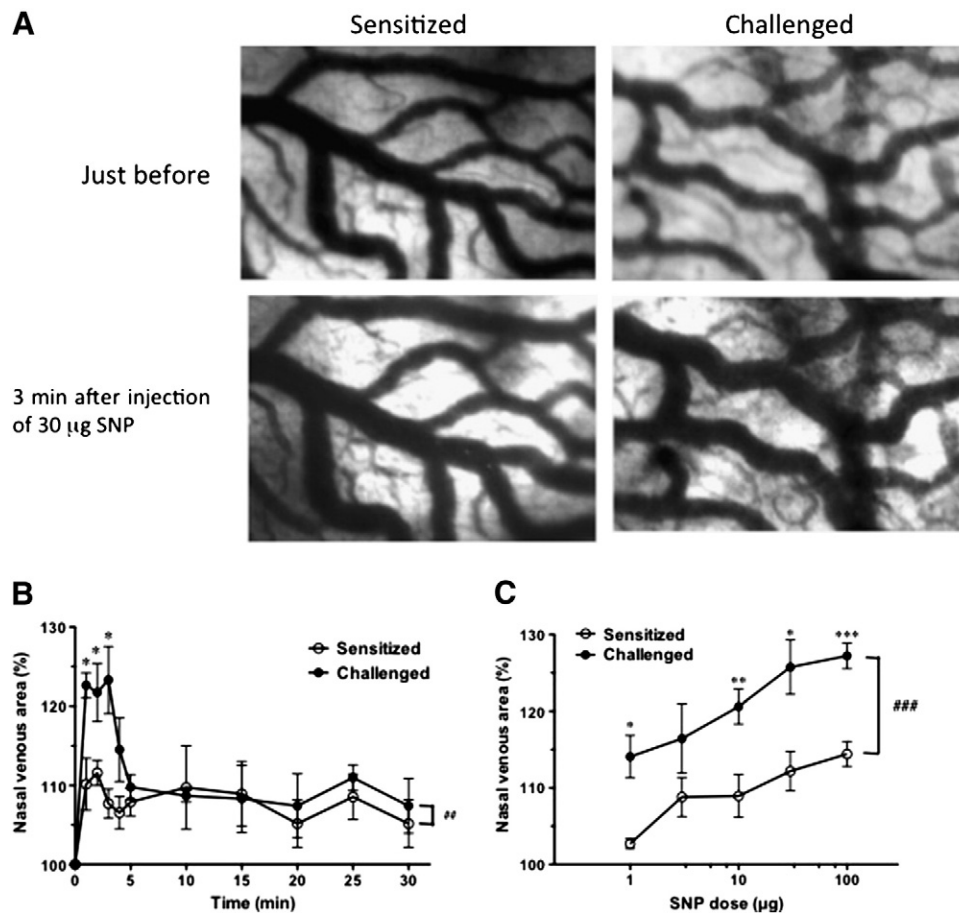


Fig. 1. Sodium nitroprusside (SNP)-induced venodilation in nasal septum of sensitized control and challenged rats. **A.** Typical stereomicroscopic photos of veins of nasal septum just before (upper panels) and 3 min (lower panels) after intra-arterial (i.a.) injection of SNP (30 μ g) into the external carotid artery in sensitized control (Sensitized; left panels) and challenged (Challenged; right panels) rats. **B.** Time course changes in the SNP (30 μ g)-induced venodilation in nasal septum of sensitized control and challenged rats. **C.** Dose-response curves of the SNP-induced venodilation in sensitized control and challenged rats. Each point represents the mean with S.E. from 4 experiments. * p < 0.05, ** p < 0.01 or *** p < 0.001 and ### p < 0.001 (ANOVA) vs. sensitized.

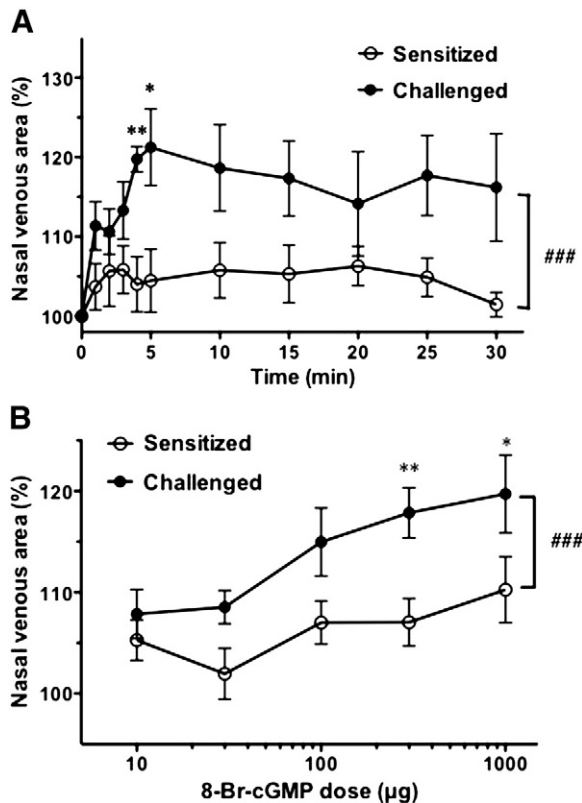


Fig. 2. 8-Br-cGMP-induced venodilation in nasal septum of sensitized control and challenged rats. A. Time course changes in venodilation of nasal septum just before (upper panels) and 3 min (lower panels) after intra-arterial (*i.a.*) injection of 8-Br-cGMP (300 µg) into the external carotid artery in sensitized control (Sensitized) and challenged (Challenged) rats. B. Dose-response curves of the 8-Br-cGMP-induced venodilation in sensitized control and challenged rats. Each point represents the mean with S.E. from 4 experiments. * $p < 0.05$ or ** $p < 0.01$ and ### $p < 0.001$ (ANOVA) vs. sensitized.

determine the level of sGC and PKG protein in nasal mucosa, the samples (10 µg of total protein per lane) were subjected to SDS-PAGE, and the proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% gelatin, the PVDF membrane was incubated with polyclonal rabbit anti-soluble guanylate cyclase $\beta 1$ subunit antibody (1:1000 dilution) (Cayman Chemical, Ann Arbor, MI, USA) or anti-PKG antibody (1:1000 dilution) (Stressgen Biotechnologies Inc., Philadelphia, PA, USA). The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2500 dilution) (GE Healthcare, Buckinghamshire, UK), detected by an enhanced chemiluminescent system (GE Healthcare), and analyzed by a densitometry system. The detection of house-keeping gene was performed on the same membrane by using monoclonal mouse anti GAPDH antibody (1:5000 dilution) (Sigma Aldrich) to confirm the same amount of proteins loaded.

Statistical analyses

Statistical significance of difference was determined by unpaired Student *t*-test and two-way analysis of variance (ANOVA) with Bonferroni/Dunn's *post hoc*-test. A value of $p < 0.05$ was considered significant.

Results and discussion

The nasal obstruction is one of the characteristics of allergic rhinitis. In patients with seasonal allergic rhinitis, nasal obstructive symptom has been induced by nasal allergen challenge (Wang et al., 1997). Although the exact mechanism(s) is not fully understood, one possible mechanism of the nasal obstruction is thought to be a dilatation of veins in nasal mucosa. We have previously demonstrated that the increase in venous diameters induced by leukotriene D_4 (LTD_4), one of the chemical mediators, in antigen-challenged rats is blocked by pretreatment with L-NMMA, an NO synthase inhibitor (Chiba et al., 2007). The results indicate an involvement of NO, a potent vasodilator, in

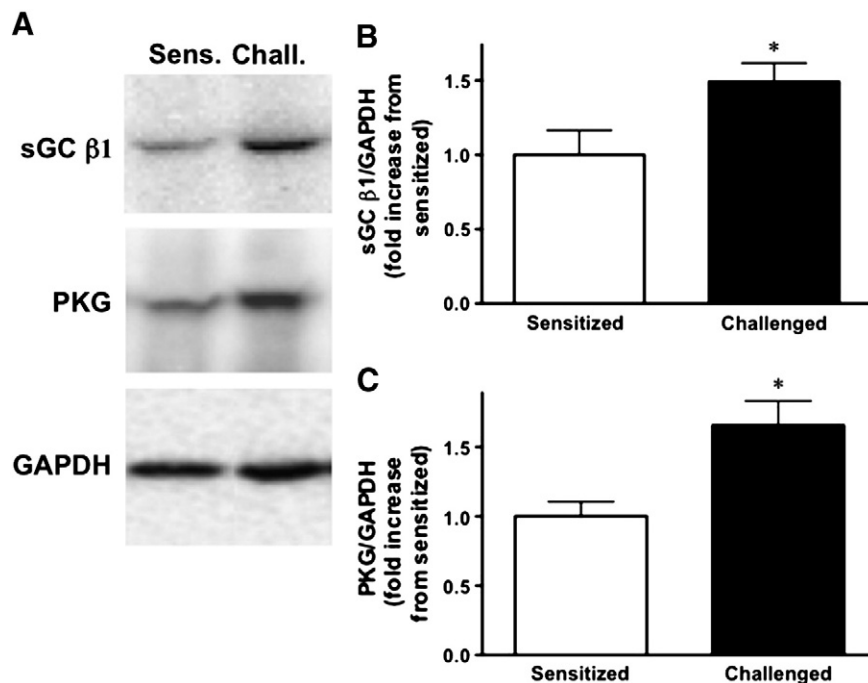


Fig. 3. The expression levels of soluble guanylate cyclase (sGC) and PKG proteins in nasal mucosae of sensitized and challenged rats. A. Typical photos of immunoblotting for sGC $\beta 1$, PKG and GAPDH. B and C. The level of sGC and PKG proteins was expressed as the density ratio of sGC or PKG to GAPDH bands. Each column represents the mean with S.E. from 8 independent animals. * $p < 0.05$ vs. sensitized.

the venodilatation induced by LTD₄ in challenged rats. In nasal airways, the expressions of constitutive (cNOS) and inducible NO synthases (iNOS) have been demonstrated in normal subjects and animals (Chiba et al., 2006; Kawamoto et al., 1998, 1999; Oh et al., 2003). Immunohistochemical studies also demonstrated the localization of constitutive NO synthases in endothelial and epithelial cells of nasal mucosa (Kawamoto et al., 1998, 1999).

In this study, to examine the role of NO-induced venodilatation, the sodium nitroprusside (SNP; an NO donor)-induced relaxation of venous vessels in nasal mucosae of antigen-challenged rats was investigated. The 30 µg SNP-induced venodilatation was significantly augmented in the nasal mucosae of antigen-challenged rats as compared to those of sensitized-control animals ($p < 0.01$, Fig. 1). These findings are consistent with our previous report (Sakai et al., 2010). It is also possible that the expression level or/and activity of NO pathway including sGC and PKG is augmented in nasal mucosae of antigen-challenged rats. Therefore, to examine the roles of sGC, 8-Br-cGMP (a PKG activator)-induced venodilatation was investigated in nasal mucosae of antigen-challenged rats. The 8-Br-cGMP-induced venodilatation also significantly augmented in the nasal mucosae of antigen-challenged rats as compared to those of sensitized-control animals (Fig. 2). Under these conditions, the levels of sGC and PKG proteins were augmented in antigen-challenged rats (Fig. 3). Secrest et al. (Secrest et al., 1988) showed that LTD₄ increased cyclic GMP (cGMP) accumulation in canine vessels. Taken together, these observations suggest that the relaxations may be via the liberation of NO. sGC was reported to catalyze the conversion of GTP to the second messenger molecule cGMP. Consequently, PKG activity is enhanced causing modulation of various intracellular proteins, and eliciting vasorelaxation (Waldman and Murad, 1987; Warner et al., 1994). We previously demonstrated that leukotriene D₄ (LTD₄)-induced generation of NO in nasal cavity lavage fluid was significantly augmented in antigen-challenged rats. However, basal NO level was not different between control and antigen-challenged rats. Furthermore, the level of iNOS mRNA was augmented in antigen-challenged rats, although the levels of eNOS and nNOS mRNAs in nasal mucosae were not different between control and antigen-challenged rats (Sakai et al., 2010). Interestingly, SNP (an NO donor)-induced venodilatation itself was also significantly augmented in nasal mucosae of repeatedly antigen challenge rats in this study. Taken together, not only increased NO production but also enhanced NO responsiveness might be involved in the development of nasal hyperresponsiveness in allergic rhinitis.

We previously examined the alteration of profile pattern of the transcription factors after the antigen challenge in bronchi with the use of protein/DNA arrays. Many transcription factors (upstream transcription factor-1; USF-1, Heat shock transcription factor; HSE, Signal transducer and activator of transcription factor 4 and 6; STAT4 and 6, Nuclear factor-kappa B; NF-kB) exhibited activation after the last antigen challenge in rat bronchial tissue (Sakai et al., 2011b). Although these transcriptional factors may be involved in the mechanism of sGC and PKG upregulation induced by antigen challenges in the mucosa of nasal, it is still unclear in this study. Therefore, further studies are needed for its mechanism.

In conclusion, the present study clearly provides evidence that sGC and PKG upregulated by antigen challenge play a pivotal role in the augmentation of NO-induced venodilatation of nasal mucosa, which might be involved in the development of nasal obstruction in allergic rhinitis.

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References

- Bousquet, J., et al., 1996. Pathophysiology of allergic rhinitis. *Int. Arch. Allergy Immunol.* 110, 207–218.
- Bredt, D.S., Snyder, S.H., 1994. Nitric oxide: a physiologic messenger molecule. *Annu. Rev. Biochem.* 63, 175–195.
- Chiba, Y., et al., 2006. Comparison of norepinephrine responsiveness of mucosal veins in vivo with that of isolated mucosal tissue in vitro in guinea pig nasal mucosa. *Am. J. Rhinol.* 20, 349–352.
- Chiba, Y., et al., 2007. Involvements of cysteinyl leukotrienes and nitric oxide in antigen-induced venodilatation of nasal mucosa in sensitized rats in vivo. *J. Smooth Muscle Res.* 43, 139–144.
- Chiba, Y., et al., 2008. Glucocorticoids ameliorate antigen-induced bronchial smooth muscle hyperresponsiveness by inhibiting upregulation of RhoA in rats. *J. Pharmacol. Sci.* 106, 615–625.
- Kawamoto, H., et al., 1998. Localization of nitric oxide synthase in human nasal mucosa with nasal allergy. *Acta Otolaryngol. Suppl.* 539, 65–70.
- Kawamoto, H., et al., 1999. Increased expression of inducible nitric oxide synthase in nasal epithelial cells in patients with allergic rhinitis. *Laryngoscope* 109, 2015–2020.
- Miadonna, A., et al., 1999. Nasal response to a single antigen challenge in patients with allergic rhinitis – inflammatory cell recruitment persists up to 48 hours. *Clin. Exp. Allergy* 29, 941–949.
- Misawa, M., Chiba, Y., 1993. Repeated antigenic challenge-induced airway hyperresponsiveness and airway inflammation in actively sensitized rats. *Jpn. J. Pharmacol.* 61, 41–50.
- Mizutani, N., et al., 2001. Markedly increased nasal blockage by intranasal leukotriene D₄ in an experimental allergic rhinitis model: contribution of dilated mucosal blood vessels. *Jpn. J. Pharmacol.* 86, 170–182.
- Moncada, S., et al., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43, 109–142.
- Naclerio, R.M., Baroody, F.M., 1994. Observations on the response of the nasal mucosa to allergens. *Otolaryngol. Head Neck Surg.* 111, 355–363.
- Naclerio, R.M., et al., 1983. Mediator release after nasal airway challenge with allergen. *Am. Rev. Respir. Dis.* 128, 597–602.
- Oh, S.J., et al., 2003. Expression of nitric oxide synthases in nasal mucosa from a mouse model of allergic rhinitis. *Ann. Otol. Rhinol. Laryngol.* 112, 899–903.
- Sakai, H., et al., 2010. Augmented venous responsiveness to leukotriene D₄ in nasal septal mucosae of repeatedly antigen-challenged rats. *Eur. J. Pharmacol.* 644, 215–219.
- Sakai, H., et al., 2011a. Involvement of K(+) channels in the augmented nasal venous responsiveness to nitric oxide in rat model of allergic rhinitis. *Microvasc. Res.* 81, 129–134.
- Sakai, H., et al., 2011b. Antigen challenge influences various transcription factors of rat bronchus: protein/DNA array study. *Int. Immunopharmacol.* 11, 1133–1136.
- Secrest, R.J., et al., 1988. Relationship between LTD₄-induced endothelium-dependent vasomotor relaxation and cGMP. *J. Pharmacol. Exp. Ther.* 245, 47–52.
- Shaw, R.J., et al., 1985. Allergen-induced release of sulphidopeptide leukotrienes (SRS-A) and LTB₄ in allergic rhinitis. *Allergy* 40, 1–6.
- Waldman, S.A., Murad, F., 1987. Cyclic GMP synthesis and function. *Pharmacol. Rev.* 39, 163–196.
- Wang, D., et al., 1997. An approach to the understanding of the nasal early-phase reaction induced by nasal allergen challenge. *Allergy* 52, 162–167.
- Warner, T.D., et al., 1994. Effects of cyclic GMP on smooth muscle relaxation. *Adv. Pharmacol.* 26, 171–194.