



Regular Article

Involvement of K⁺ channels in the augmented nasal venous responsiveness to nitric oxide in rat model of allergic rhinitisHiroyasu Sakai^{*}, Jun Enzaka, Michiko Sakai-Oshita, Futa Uto, Yoshihiko Chiba, Miwa Misawa

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ARTICLE INFO

Article history:

Accepted 15 November 2010

Available online 23 November 2010

Keywords:

Venodilatation

Nasal mucosa

Nitric oxide

Nasal hyperresponsiveness

ABSTRACT

Objective: One of the factors of nasal obstruction observed in allergic rhinitis is thought to be a dilatation of microveins in nasal mucosa, although the exact mechanism(s) is not fully understood. In nasal mucosae of repeatedly antigen challenged rats, NO-induced venodilatation itself is augmented. In the present study, the roles of K⁺ channels in sodium nitroprusside (NO donor; SNP)-induced venodilatation of nasal mucosae in antigen-challenged rats were investigated.

Methods: Actively sensitized rats were repeatedly challenged with aerosolized antigen. Twenty-four hours after the final antigen challenge, nasal septum mucosa was exposed surgically and observed directly *in vivo* under a stereoscopic microscope. The 20 μ l reagents were administered onto the exposed septal mucosal surface, and the venous diameters of nasal mucosa were observed.

Results: The SNP-induced venodilatation of septal mucosa was markedly and significantly increased in the antigen-challenged rats. The SNP-induced venodilatation was significantly inhibited by pretreatment with either tetraethylammonium [TEA; a large-conductance Ca²⁺ activated-K⁺ (K_{Ca}) and voltage dependent K⁺ (K_v) channel inhibitor] or glibenclamide [an ATP sensitive K⁺ (K_{ATP}) channel inhibitor].

Conclusions: These findings suggest that NO-induced venodilatation is augmented in nasal mucosae of challenged rats, and K⁺ channels play an important role in the augmented venous responsiveness to NO in nasal mucosae of repeatedly antigen challenged rats.

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Introduction

Allergic rhinitis is a common inflammatory disease with an increasing worldwide prevalence. Its major nasal symptoms, nasal blockage, sneezing, rhinorrhea and pruritus are thought to be induced by several chemical mediators released from mast cells and other 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 also plays a crucial role in nasal obstruction in allergic rhinitis.

It is now well established that the endothelium plays a critical role in the control of peripheral vascular resistance and blood pressure via the release of NO. 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 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 vascular relaxation (Waldman and Murad, 1987). Electrophysiological and pharmacomechanical studies in a variety of vascular tissues have pointed out to a pivotal role of plasmalemmal K⁺ channels in PKG-induced relaxation. NO also involves hyperpolarization due to the activation of smooth muscle K⁺ channels (Brayden, 2002; Chen and Cheung, 1997; Plane and Garland, 1996; Tare et al., 1990). NO can activate one or more of at least three different types of smooth muscle K⁺ channels, including large conductance Ca²⁺-activated potassium channels (BK_{Ca}), ATP-sensitive potassium channels (K_{ATP}), and voltage-gated potassium (K_v) channels in vessels (Waldron and Cole, 1999).

In the present study, to determine the roles of K⁺ channels in the augmentation of responsiveness to NO observed in nasal mucosae of repeatedly antigen challenged rats, the effects of K⁺ channels inhibitors on the SNP-induced venodilatation in nasal septal mucosa were examined.

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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 at 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 diameter of nasal mucosal veins

Venous diameter of nasal septal mucosae in anesthetized rats was measured by the method of previous paper (Chiba et al., 2006, 2007). 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×. 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 diameter (50–80 μm) and then pre-warmed (37 °C) 20 μl of 10^{−4} M sodium nitroprusside (SNP) was applied to the exposed mucosal surface of nasal septum directly. Vehicle of SNP was used saline. After the application of the agent, the nasal mucosa was photographed continuously at 5-min interval for 60 min to examine the time-course change in the diameter of veins. In some experiments, pre-warmed (37 °C) 20 μl of 10^{−8} M tetraethylammonium (TEA) or 10^{−5} M glibenclamide was pretreated to the exposed mucosal surface 20 min before the application of SNP. The vehicles of TEA and glibenclamide used were saline and dimethyl sulfoxide (DMSO), respectively. The diameter 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 diameter. Three randomly selected veins were analyzed in each animal and the average was determined as $N=1$. In our preliminary study, venous diameter of septal mucosae at basal state was not changed by 20 μl of saline or DMSO for 60 min.

Reverse transcriptase chain reaction (RT-PCR)

The isolated nasal septa tissues were quickly frozen with liquid nitrogen, and these tissues were crushed to pieces by Cryopress™

(CP-100W; Niti-on, Co. Ltd., Japan: 15 s×3). Total RNA was isolated from each frozen tissue powder by using TRI reagent (Sigma-Aldrich) and stored at −85 °C until use. cDNAs were prepared from the total RNA (1 μg) by reverse transcriptase reaction in a total volume of 40 μl reaction buffer containing 50 mM Tris–HCl, pH 8.3, 4 mM MgCl₂, 2 mM dNTP mixture, 1 U/μl RNase inhibitor, 0.01 mg/ml random hexamers, and 5 U/μl avian M-MuLV reverse transcriptase. The reaction mixture was incubated for 5 min at 25 °C, and then for 60 min at 42 °C to initiate the synthesis of the cDNAs. Reverse transcriptase was inactivated at 70 °C for 5 min. Then to the RT reaction mixture (10 μl) was added 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 5 μl of 2× PCR Master Mix (0.05 U/ml Taq DNA polymerase, 4 mM MgCl₂ and 0.4 mM of each dNTP; Fermentas Life Science) in a total volume of 20 μl. RT-generated cDNAs encoding the gene sequences were amplified by PCR using specific primers (Table 1), which were designed from published sequences. The thermal cycle profile used in the present study was 1) denaturing for 3 min at 95 °C, 2) annealing primers for 15 s at 55 °C, and 3) extending the primers for 1 min at 72 °C. The PCR amplifications were performed for 30 or 35 cycles. The PCR mixtures were subjected to electrophoresis on 1% agarose gel with DNA molecular weight standard marker (100 bp DNA ladder, Takara Bio Inc.) and visualized by ethidium bromide staining.

Statistical analyses

Statistical significance of difference was determined by one- or two-way analysis of variance (ANOVA) and 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). One possible mechanism of the nasal obstruction is thought to be a dilatation of veins in nasal mucosa, although the exact mechanism(s) is not fully understood.

We have previously demonstrated that the increase in venous diameters induced by leukotriene D₄ (LTD₄) in antigen-challenged rats was blocked by pretreatment with L-NMMA, an NO synthase inhibitor (Chiba et al., 2007). The results indicate an involvement of NO in the venodilatation induced by LTD₄ in challenged rats. NO is known as a potent vasodilator. In nasal airways, expression of constitutive (cNOS) and inducible NO synthases (iNOS) has 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 in nasal mucosa (Kawamoto et al., 1998, 1999).

The venous constriction induced by administration of vasoconstrictor, phenylephrine (10^{−3} M), was examined. The phenylephrine-induced

Table 1
PCR primers for K⁺ channels and GAPDH.

	Accession number	Primer Deoxyribonucleotide sequences	Product size (base pairs)
Kv2.1	NM_013186	Forward 5'-TTCCTCAGCAATCCAACAAGAGC-3' Reverse 5'-GGGTAGATGTCTCCATAGCCAACAG-3'	323
Kv2.2	NM_054000	Forward 5'-ATGTAAAGAGGGAGGAGGAAAGC-3' Reverse 5'-GGAAGAGAAAGTGTGACCTGGATG-3'	365
Kir6.1	NM_017099	Forward 5'-GAGTGAAGTGTGACACCAAG-3' Reverse 5'-CGATCACCAGAACTCAGCAAC-3'	248
Kir6.2	NM_031358	Forward 5'-TCCAACAGCCGCTCTAC-3' Reverse 5'-GATGGGGACAAAACGCTG-3'	168
Slo	NM_031828	Forward 5'-GGACTTAGGGGATGGTGGTT-3' Reverse 5'-GGGATGGAGTGGACAGAGGA-3'	297
GAPDH	NM_017008	Forward 5'-CCATCACTGCCACTCAGAAGAC-3' Reverse 5'-TACTCCTTGGAGGCCATGTAGG-3'	469

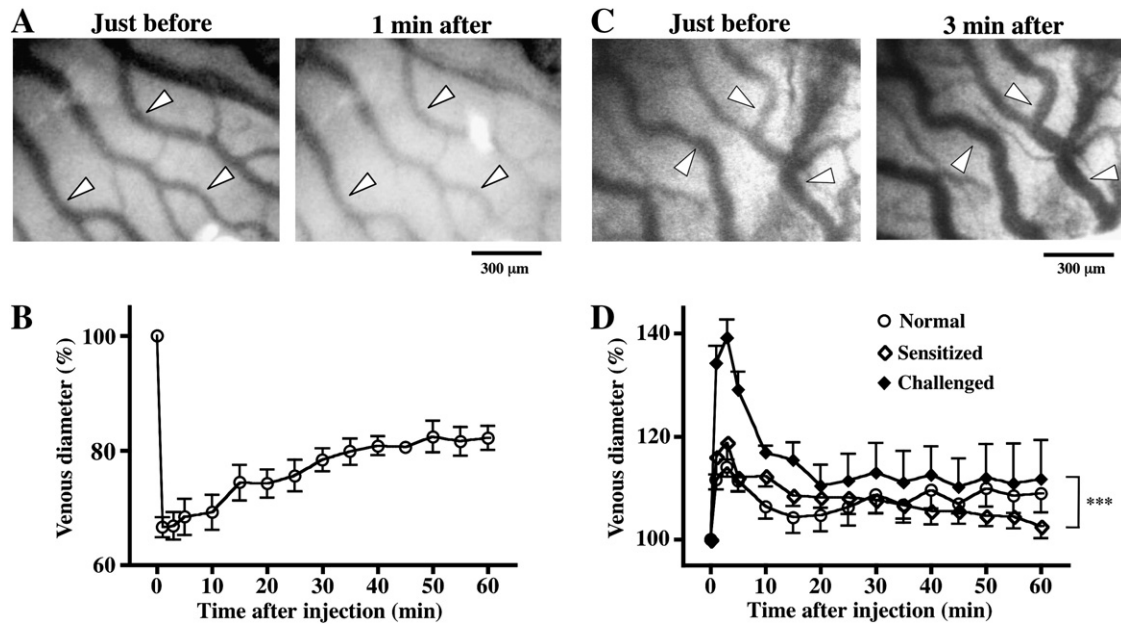


Fig. 1. Time course changes in phenylephrine (10^{-3} M)- or sodium nitroprusside (SNP; 10^{-4} M)-induced venoconstriction or venodilatation of nasal septum in rats. Under anesthesia, the nasal cavity of rat was opened surgically, and then the nasal septal mucosa was observed under a stereoscopic microscope at a magnification of $40\times$ as described in the [Materials and methods](#) section. The photograph was taken for measurement of venous diameter. (A) Typical photographs of veins of nasal septum just before (left) and 1 min (right) after administration of phenylephrine (10^{-3} M) in normal rats. (B) Time course changes in phenylephrine (10^{-3} M)-induced venoconstriction of nasal septum in normal rats. (C) Typical photographs of veins of nasal septum just before (left) and 3 min (right) after administration of SNP (10^{-4} M) in challenged rats. (D) The time-course of SNP-induced venodilatation of nasal septa in nonsensitized normal (Normal), sensitized-control (Sensitized) and repeatedly antigen challenged (Challenged) rats. The 10^{-4} M SNP-induced venodilatation of nasal septa was augmented in challenged animals ($***P<0.001$ vs. Sensitized). Each point represents the mean $\pm$ SEM from 5 different animals. $***P<0.001$ vs. Sensitized.

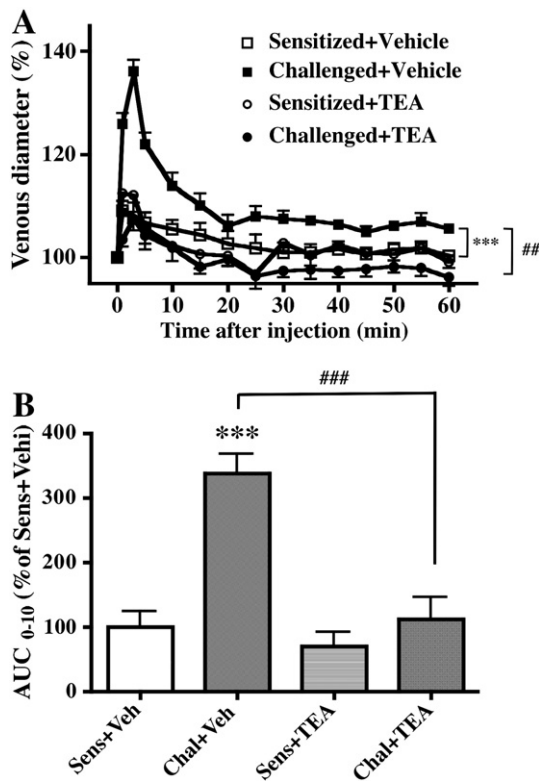


Fig. 2. Effects of tetraethylammonium (TEA; 10^{-5} M) on the SNP (10^{-4} M)-induced venodilatation in nasal septum of the sensitized (Sensitized) and repeatedly antigen challenged (Challenged) rats. (A) Time course of SNP-induced venodilatation of nasal septum. $***P<0.001$ vs. Sensitized + Vehicle, $##P<0.05$ vs. Challenged + Vehicle. (B) Area under the curves (AUC) of the SNP-induced venodilatation between 0 and 10 min. Each point represents the mean with SEM from 5 experiments. $***P<0.001$ vs. Sensitized (Sens) + Vehicle (Veh), $###P<0.05$ vs. Challenged (Chal) + Vehicle.

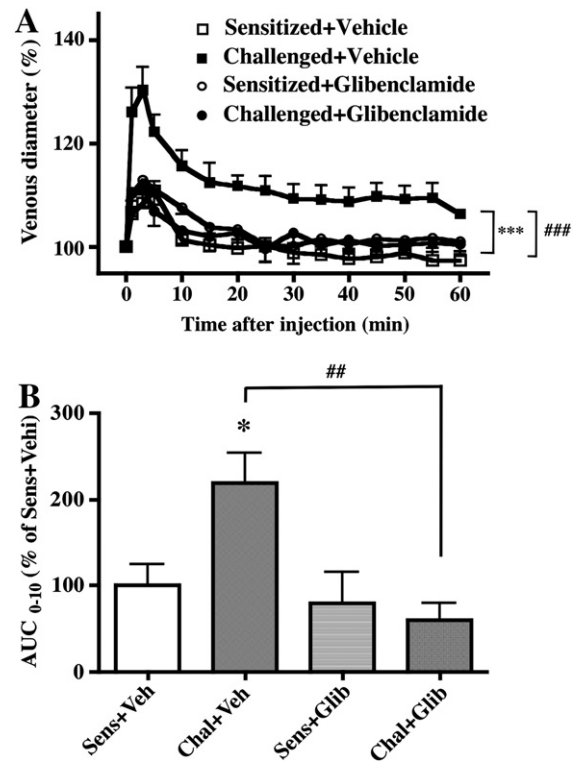


Fig. 3. Effects of glibenclamide (Glib; 10^{-8} M) on the SNP (10^{-4} M)-induced venodilatation in nasal septum of sensitized (Sensitized) and repeatedly antigen challenged (Challenged) rats. (A) Time course of the SNP-induced venodilatation of nasal septum. $***P<0.001$ vs. sensitized + Vehicle, $###P<0.001$ vs. Challenged + Vehicle. (B) Area under the curves (AUC) of the SNP-induced venodilatation between 0 and 10 min. Each point represents the mean with SEM from 5 experiments. $*P<0.05$ vs. sensitized (Sens) + Vehicle (Veh), $##P<0.01$ vs. Challenged (Chal) + Vehicle.

venoconstriction was markedly increased (Figs. 1A and B). Therefore, this method is suitable for observing the reagents-induced venous responses. To examine the role of NO-induced venodilation, the 10^{-4} M SNP (sodium nitroprusside; an NO donor)-induced relaxation of venous vessels in nasal mucosae of antigen-challenged rats was investigated. The SNP-induced venodilation was significantly augmented in the nasal mucosae of antigen-challenged rats as compared with those of normal and sensitized-control animals ($P < 0.001$, Figs. 1C and D). The finding is consistent with our previous report (Sakai et al., 2010).

Broad voltage-dependent K^+ (Kv) channel expression has been detected in vascular smooth muscle cells (Cox and Petrou, 1999). The Kv channels open to allow an efflux of K^+ in response to depolarization of the membrane potential, resulting in repolarization and a return to the resting membrane potential (Ko et al., 2008). The α -subunits of Kv channel structurally have cytoplasmic N- and C-termini and contain poreforming six transmembrane domains with an S4 voltage-sensing transmembrane domain (Korovkina and England,

2002). Each α -subunit is associated with ancillary β -subunits, which influences the characteristics of the channel (Bähring et al., 2001). Although more than 30 genes encoding several subfamilies of Kv α -subunit are currently recognized (Korovkina and England, 2002), the expression of Kv2.1 and Kv2.2 was confirmed in vascular smooth muscle (Tanaka et al., 2006). Kv2.1 also plays an important role in downstream effectors of NO-signaling cascade regulating vascular relaxation in rats (Tanaka et al., 2006).

On the other hand, the large-conductance Ca^{2+} activated K^+ channel (BK_{Ca}) channels, which are activated by changes in the intracellular Ca^{2+} concentration and membrane depolarization, are believed to contribute to the maintenance of the membrane potential in small myogenic vessels (Nelson and Quayle, 1995; Park et al., 2007; Waldron and Cole, 1999). The efflux of K^+ that results from BK_{Ca} channel activation can be used to counteract pressure- or chemical-induced depolarization and vasoconstriction (Brayden and Nelson, 1992; Tanaka et al., 2004). Similar to Kv channels, BK_{Ca} channels are composed of a

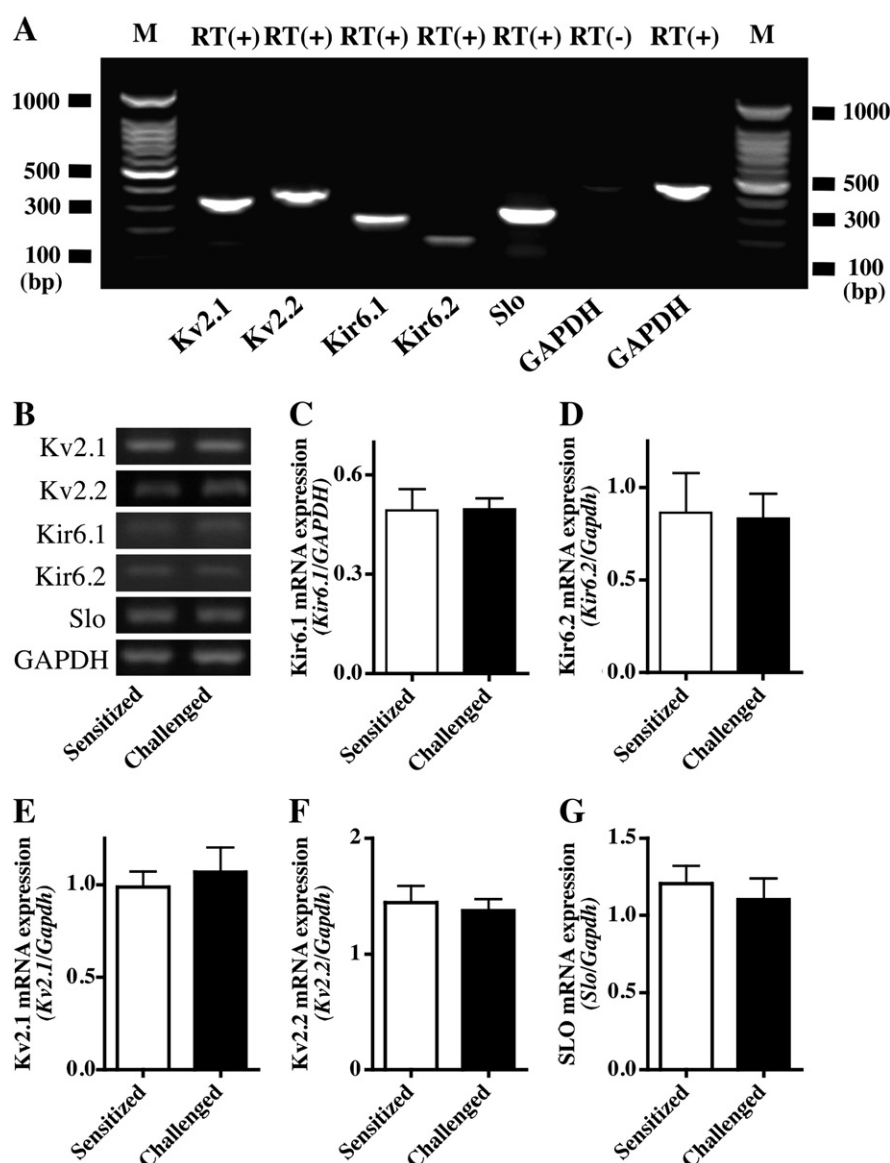


Fig. 4. The mRNA levels of K^+ channels in nasal septa from normal (Normal), sensitized control (Sensitized) and repeatedly antigen-challenged (Challenged) rats. (A) Typical photographs of RT-PCR products for Kv2.1, Kv2.2, Kir6.1, Kir6.2, Slo and GAPDH mRNAs from nasal septa of normal rat. The PCR amplifications for all were 35 cycles. The size of each PCR product is shown in Table 1. (B) Typical photographs of RT-PCR products for each mRNA from nasal septa of sensitized-control (Sensitized) and repeatedly antigen challenged (Challenged) rats. The PCR amplifications were 30 (Kv2.1, Kv2.2, Kir6.1, Kir6.2 and Slo) and 35 (GAPDH) cycles. The bands were scanned, and the levels of K^+ channel mRNA expressions were expressed as density ratios of the K^+ channel to the GAPDH bands. The data are summarized in C–G. The experiments were done by using the total RNA from 5 to 6 different animals. Each column represents the mean $\pm$ SEM from 5 to 6 different animals.

pore-forming α -subunit and a regulatory β -subunit (Knaus et al., 1994; Tanaka et al., 2004; Toro et al., 1998). The α -subunits contain six transmembrane-spanning domains, including a voltage sensor, which form the pore (Nelson and Quayle, 1995). The α -subunit is produced from a single gene (Slo) (McCobb et al., 1995; Standen and Quayle, 1998; Waldron and Cole, 1999). The vasodilator effects of NO are partly mediated by the activation of vascular BK_{Ca} channels (Ghatta et al., 2006). Activation of BK_{Ca} channels by NO is likely to involve phosphorylation of the channels or their regulatory subunits by cGMP-dependent protein kinase G, based on the findings using selective inhibitors of this kinase and/or direct application of biochemically purified kinase to the cytoplasmic face of excised, inside-out membrane patches containing BK_{Ca} (Robertson et al., 1993; Taguchi et al., 1995). NO has also been reported to activate BK_{Ca} channels directly (Bolotina et al., 1994).

To determine the roles of BK_{Ca} and K_v channels in the augmentation of NO-induced nasal mucosal venodilatation in antigen-challenged rats, the effect of tetraethylammonium (TEA) on the SNP-induced relaxation of venous vessels in nasal mucosae was examined. TEA had no effect on the SNP-induced mucosal venodilatation of sensitized-control rats. Interestingly, the augmented SNP-induced venodilatation of septal mucosa was significantly inhibited by presence of TEA (Fig. 2A). Similar results were also observed when the areas are under the curves during 0–10 min (AUC_{0–10}) were analyzed (Fig. 2B).

The K_{ATP} channels are hetero-octameric complexes containing four pore-forming, inwardly rectifying channel subunits (Kir6.1 or Kir6.2), together with four sulfonylurea receptors (SUR), which are ATP-binding cassette family proteins (Babenko et al., 1998; Standen and Quayle, 1998; Teramoto, 2006). The molecular diversity that exists between species and tissues in terms of their K_{ATP} channel is magnified by the presence of multiple isoforms of SUR (SUR1, SUR2A and SUR2B) (Brayden, 2002). The K_{ATP} channels in vascular smooth muscle most likely contain Kir6.X/SUR2B, as co-expression of SUR2B with Kir6.1 or Kir6.2 has been shown to produce channels with the properties of native K_{ATP} channels in smooth muscle (Cui et al., 2002; Koh et al., 1998; Standen and Quayle, 1998; Yamada et al., 1997). Pharmacological evidence of activation of K_{ATP} channels by NO has been obtained using vascular smooth muscle cells (Quayle and Standen, 1994).

To investigate the role of ATP sensitive K⁺ (K_{ATP}) channel in the augmentation of NO-induced nasal mucosal venodilatation in antigen-challenged rats, the effect of glibenclamide on the SNP-induced relaxation of venous vessels in nasal mucosae was examined. As well as the effect of TEA, glibenclamide inhibited the augmentation of SNP-induced venodilatation in septal mucosa of antigen-challenged rats ($P < 0.001$, Fig. 3A). The SNP-induced increase in AUC_{0–10} was also significantly attenuated by glibenclamide in antigen-challenged rats.

The augmentation of SNP-induced nasal mucosal venodilatation in challenged rats was significantly inhibited by pretreatment with TEA or glibenclamide, suggesting that K⁺ channels play an important role in the enhanced NO-induced nasal venodilatation in antigen-challenged rats. The findings remind us of upregulations of these K⁺ channels in septal mucosa of challenged rats. The gene expression of K⁺ channels was therefore examined in septal mucosae of normal rats by RT-PCR. mRNAs of K_v channels (Kv2.1 and Kv2.2), K_{ATP} channels (Kir6.1, Kir6.2) and BK_{Ca} channel (Slo) were expressed in the septal mucosae of normal rats (Fig. 4A). The expression levels of mRNAs for these K⁺ channels were not changed even in antigen-challenged group (Figs. 4B–G). It is thus possible that the NO-mediated activation of these K⁺ channels is augmented in mucosal veins of antigen-challenged rats. The expression level and/or activity of NO pathway, such as sGC and PKG, also might be augmented in nasal mucosae of antigen-challenged rats. Further studies are needed to make clear the mechanism of the induction of nasal hyperresponsiveness (Fig. 5).

In conclusion, the current study clearly showed that K⁺ channel plays a pivotal role in the augmentation of NO-induced venodilatation

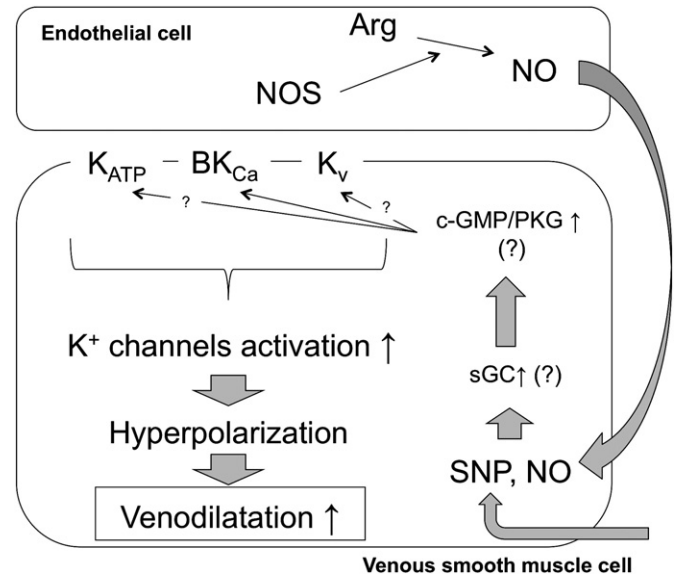


Fig. 5. Schema of the augmented NO-induced venodilatation in nasal mucosa of antigen-challenged rats. Upon the stimulation of NO or SNP (NO donor), c-GMP/PKG-mediated pathway is activated in venous smooth muscle cells. The PKG pathway activates K⁺ channels (K_{ATP}, BK_{Ca}, and K_v channels). Activation of K⁺ channels thus causes the membrane hyperpolarization, resulting in the venodilatation. NO-induced augmentation of venodilatation might involve the activation of K⁺ channels via c-GMP/PKG pathway. NO, nitric oxide; SNP, sodium nitroprusside; NOS, NO synthase; sGC, soluble guanylate cyclase.

of nasal mucosa, which might be involved in the development of nasal obstruction in allergic rhinitis.

Acknowledgments

We thank Ms Mayuko Suzuki, Mr Yuhei Sako and Ms Ari Hayakawa for their help in technical assistance.

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