Regulator of G protein signaling 2 is a key modulator of airway hyperresponsiveness

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Background: Drugs targeting individual G protein–coupled receptors are used as asthma therapies, but this strategy is limited because of G protein–coupled receptor signal redundancy. Regulator of G protein signaling 2 (RGS2), an intracellular selective inhibitor of multiple bronchoconstrictor receptors, may play a central role in the pathophysiology and treatment of asthma.

Objective: We defined functions and mechanisms of RGS2 in regulating airway hyperresponsiveness (AHR), the pathophysiologic hallmark of asthma.

Methods: Real-time PCR and Western blot were used to determine changes in RGS2 expression in ovalbumin-sensitized/-challenged mice. We also used immunohistochemistry and real-time PCR to compare RGS2 expression between human asthmatic and control subjects. The AHR of RGS2 knockout mice was assessed by using invasive tracheostomy and unrestrained plethysmography. Effects of loss of RGS2 on mouse airway smooth muscle (ASM) remodeling, contraction, intracellular Ca^{2+}, and mitogenic signaling were determined *in vivo* and *in vitro*.

Results: RGS2 was highly expressed in human and murine bronchial epithelium and ASM and was markedly downregulated in lungs of ovalbumin-sensitized/-challenged mice. Lung tissues and blood monocytes from asthma patients expressed significantly lower RGS2 protein (lung) and mRNA (monocytes) than from nonasthma subjects. The extent of reduction of RGS2 on human monocytes correlated with increased AHR. RGS2 knockout caused spontaneous AHR in mice. Loss of RGS2 augmented Ca^{2+} mobilization and contraction of ASM cells. Loss of RGS2 also increased ASM mass and stimulated ASM cell growth via extracellular signal-regulated kinase and phosphatidylinositol 3-kinase pathways.

Conclusion: We identified RGS2 as a potent modulator of AHR and a potential novel therapeutic target for asthma. (J Allergy Clin Immunol 2012;130:968-76.)

Key words: *Asthma, airway hyperresponsiveness, airway smooth muscle mass, airway smooth muscle contraction, Ca^{2+} mobilization, extracellular signal-regulated kinase, G protein–coupled receptors, knockout mice, ovalbumin-sensitized/-challenged murine model of asthma, phosphatidylinositol 3-kinase, regulator of G protein signaling 2*

Airway hyperresponsiveness (AHR) is exaggerated constriction of the airways in response to bronchoconstrictor stimuli. It is a key diagnostic criterion of asthma, and improvement in AHR is associated with better asthma control.[1] One major challenge in asthma treatment is the ability to reduce AHR. G protein–coupled receptors (GPCRs) are important regulators of multiple cell types involved in asthma. Numerous agonists for pro-contractile GPCRs such as muscarinic receptors (mAChRs), serotonin, endothelin B, leukotriene, and proton-sensing OGR1 receptors expressed on airway smooth muscle are present or upregulated in the airway during allergic inflammation, and are potential mediators of bronchoconstriction and AHR.[2,3] Drugs targeting specific GPCRs are used as therapies for AHR in asthma,[4,5] yet asthma still causes significant morbidity. The strategy of inhibiting a single GPCR is limited because airway constriction in asthma can be induced by different GPCRs simultaneously, thereby having constrictor signal redundancy. A GPCR signaling unit has 4 components: receptor, G protein (classified into G_s, G_i, G_q, and G_{12}), effector, and regulator of G protein signaling (RGS) protein.[6] Since RGS proteins act just downstream from the points of GPCR signaling convergence by increasing the rate of the signal-terminating GTP hydrolysis of G protein up to 1000-fold, 1 RGS protein can potentially inhibit the responses from multiple types of bronchoconstrictor GPCRs acting through the same signaling cascade. Thus, targeting RGS proteins could be an effective alternative strategy for a broader-based suppression of unwanted bronchoconstrictor GPCR signaling in airways.

About 20 different mammalian RGS proteins that share a conservative RGS domain have been identified.[6] Although several reviews have postulated potential pathophysiological roles of RGS proteins in allergic asthma,[7,8] their regulation of airway smooth muscle (ASM) cell contraction and proliferation in asthma was demonstrated only recently.[9-12] We have been investigating how RGS proteins modulate GPCRs mediating AHR in allergic asthma. During our initial screening, we found that at least 14 RGS gene transcripts were detectable in mouse lung

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Abbreviations used
AHR: Airway hyperresponsiveness
ASM: Airway smooth muscle
ERK: Extracellular signal-regulated kinase
GM: Growth medium
GPCRs: G protein–coupled receptors
mAChR: Muscarinic receptor
PI3K: Phosphatidylinositol 3-kinase
RGS: Regulator of G protein signaling
WT: Wild-type

tissues. Interestingly, RGS2 was the one that was significantly downregulated in lung tissues of an ovalbumin-sensitized/-challenged murine model of asthma.

RGS2 is a member of the RGS R4 subfamily.[6] Compared with other members of this subfamily that regulate both G_i- and G_q-coupled GPCRs, RGS2 is highly selective in the regulation of G_q-coupled receptors including mAChRs and serotonin receptors.[13-15] RGS2 repression has been implicated in many human diseases such as hypertension, schizophrenia, and cancer.[16-18] RGS2 is abundantly expressed in human lungs,[19] and a recent study suggests that β2-adrenergic receptor agonist-induced RGS2 expression in ASM cells is a mechanism of bronchoprotection.[10] The human *RGS2* gene maps to chromosome 1q31, and single-nucleotide polymorphisms at this region were strongly associated with asthma in children.[20,21] However, the expression profile and the pathological importance of RGS2 in asthma are by and large unknown.

In the present study, we found that subjects with asthma have reduced expression of RGS2 that correlates with the severity of AHR. RGS2 knockout mice exhibit spontaneous AHR during methacholine challenge. In addition, the loss of RGS2 increases ASM mass *in vivo* and augments Ca^{2+} oscillations in mouse ASM cells, causing enhanced cell contraction. Our findings suggest dysregulation of GPCRs due to the loss of RGS2 as a unique mechanism underlying AHR in asthma. Further investigation could lead to the development of RGS2 as a biomarker for AHR and asthma and to new therapeutic avenues that target this pathway.

METHODS

Selection of subjects and methacholine airway challenge

The study was approved by the Creighton University institutional review board, and all subjects provided written informed consent prior to any procedures. We enrolled 15 normal nonsmoking subjects as controls—no history of asthma, negative skin prick testing to a battery of common aeroallergens, FEV_1 of more than 80% predicted, and negative methacholine challenge (PD_{20} > 10 mg/mL)—and 15 nonsmoking treatment-naive subjects with mild-to-moderate asthma: FEV_1 of more than 70% predicted, and positive methacholine challenge (PD_{20} < 10 mg/mL). There were no significant differences between the normal and asthmatic subjects regarding age, sex, and baseline FEV_1 though there were more African Americans in the asthmatic group (see Table E1 in this article's Online Repository at www.jacionline.org). Methacholine challenge was performed as described.[22]

Isolation of monocytes and quantitative real-time PCR analysis of RGS2 expression

Untouched monocytes were negatively isolated by using MACS Monocyte Isolation Kit II (Miltenyi Biotec, Cambridge, Mass). Total RNA was extracted from monocytes, and procedures for quantitative real-time PCR of the *RGS2* gene were described previously.[18,23]

Immunohistochemistry analysis of human lung tissues

Archived formalin-fixed, paraffin-embedded lung tissue blocks of subjects with asthma (n = 3) and healthy controls (n = 5) were from Dr Reynold A. Panettieri. Immunohistochemistry was performed by using a polyclonal anti-RGS2 C-terminal peptide antibody as previously described.[18] The negative control used nonimmune rabbit IgG as the primary antibody. The intensity of RGS2 protein staining was determined as an average optical density by Image-Pro Plus in 5 randomly chosen airways for each sample.

Measurement of AHR in mice

Animal studies were approved by the Creighton University IACUC. Dr David Siderovski kindly provided a RGS2 knockout C57BL/6j mouse breeding colony.[24] AHR was determined by the direct measurement of lung resistance and dynamic compliance in response to nebulized methacholine in anesthetized, ventilated mice by using FinePointe apparatus (Buxco, Wilmington, NC).[25] Airway constriction to nebulized methacholine was also measured noninvasively in conscious, unrestrained mice by using whole-body plethysmography (Buxco). Results of plethysmography are a percentage of the respective basal values in response to PBS.

Preparation and contractile response of airways of mouse lung slice to acetylcholine, serotonin, and endothelin-1

Mouse lungs were inflated with agarose, and thin lung slices (150 μm) were cut with a vibratome as described.[26] Changes in the luminal area of the airways in lung slices were measured in response to bronchoconstrictor ligands with a Nikon TE200 inverted microscope (Melville, NY). A decrease in the cross-sectional area was considered to be airway constriction.[26]

Western blot analysis

Protein was extracted from ASM cells and subjected to Western blot analysis by using antibodies for phosphorylated and total extracellular signal-regulated kinase (ERK) 1/2 and Akt (Cell Signaling Technology, Inc, Danvers, Mass) as described.[23]

Histopathologic analysis and α-smooth muscle actin immunostaining of lung sections

Lungs were collected and fixed in formalin (n = 6-8 mice per group). Parasagittal sections of tissue representing central and peripheral airways were embedded in paraffin, cut at 5-μm thickness, and stained with hematoxylin and eosin for the evaluation of inflammatory infiltrate. Lung sections were also processed for α-smooth muscle actin immunostaining as described.[26] The area of peribronchial α-smooth muscle actin staining was outlined and quantified by Image-Pro Plus. Results are expressed as the area of α-smooth muscle actin staining per micrometer length of basement membrane of bronchioles.

Isolation and measurement of intracellular Ca^{2+} and contraction of mouse ASM cells

Mouse tracheal ASM cell isolation, culture and measurement of intracellular Ca^{2+}, and contraction were described previously.[26] Ca^{2+} oscillation frequency was measured as the inverse of the average time between 2 oscillations. Data are averages from at least 25 cells.

Images of ASM cells on coverslips were taken by using a Nikon TE200 microscope equipped with a digital camera before (time 0 minutes) and 5 minutes after acetylcholine (1 μM) stimulation. Surface area of 50 cells was