Soluble guanylyl cyclase expression is reduced in allergic asthma

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Asthma is a chronic inflammatory lung disease characterized by airway hyperactivity and mucus hypersecretion that results in intermittent airway obstruction (3). Asthmatic patients use β2-adrenergic receptor agonists to alleviate bronchoconstriction (2). The occupancy of β2-adrenergic receptors by an agonist leads to activation of adenyl cyclase, which in turn catalyzes the conversion of ATP to cAMP, producing bronchial muscle relaxation.

Although present in smaller concentrations than cAMP, cGMP is an important regulator of smooth muscle tone (11, 18). cGMP induces smooth muscle relaxation by lowering intracellular Ca2+ concentration. The cGMP-induced smooth muscle relaxation is mediated by several mechanisms: 1) inhibition of myosin-light chain kinase activation, 2) phosphorylation and opening of Ca2+-activated maxi-K+ channels, and 3) inhibition of inositol 1,4,5-trisphosphate-stimulated Ca2+ release from the endoplasmic reticulum (17). cGMP is generated through the action of guanylyl cyclases (GCs), of which two isoforms exist (18). One is mostly soluble (sGC) and is maximally activated following the binding of nitric oxide (NO) to its heme prosthetic group (16). The other GC isoform is particulate and serves as a receptor for natriuretic peptides (21).

sGC is a heterodimeric enzyme composed of a large (α) and small (β) subunit (22). Two isoforms for each subunit are known to exist, termed α1, α2, β1, and β2 (8, 16). The most abundant form of sGC is the ubiquitously distributed α1/β1 (4, 19); a second form of sGC, α2/β1, has been shown to occur naturally in the placenta and brain, the latter being the tissue with highest α2 expression (25, 26). The α2/β1 sGC exhibits indistinguishable biochemical and pharmacological properties with α1/β1 but differs in its ability to associate with plasma membrane; in neuronal tissue α2/β1 localizes to the synapse through an interaction with the postsynaptic density 95 (26).

Lungs of asthmatic patients and animals in which an asthma-like response has been triggered express high levels of inducible NO synthase (iNOS) (24). However, despite the presence of ample amounts of NO that could activate sGC in the smooth muscle and cause relaxation, airway tone is significantly elevated in asthma. This observation would be consistent with reduced expression and/or responsiveness of signaling molecules downstream of NO synthase. Thus the aim of this study was to determine the expression of the “NO receptor” sGC in a murine model of allergic airway disease. To this end, mice were sensitized and challenged with ovalbumin (OVA), and sGC subunit levels were determined. We have shown that mice exhibiting many of the characteristics of asthma also displayed reduced mRNA and protein levels of the sGC subunits. Moreover, treatment of naïve mice with a selective inhibitor of sGC was accompanied by the development of airflow hyperreactivity (AHR) to methacholine, suggesting that sGC inhibition could contribute to the AHR seen in asthma.

MATERIALS AND METHODS

Reagents. Reagents for SDS-polyacrylamide gel electrophoresis and Western blotting were obtained from Bio-Rad (Hercules, CA). The Supersignal Chemiluminescent Substrate was obtained from Eastman Kodak (Rochester, NY). The IL-13 ELISA kit was obtained from R&D Systems (Minneapolis, MN), and the total IgE ELISA kit from BD Biosciences (San Jose, CA). TRIZol, SuperScript First-Strand Synthesis System for RT-PCR, DNase I, dNTPs, and platinum Taq poly-

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merase were purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal antibodies for α1 and β-actin were purchased from Sigma-Aldrich (St. Louis, MO), the β1 antibody was obtained from Cayman Chemicals (Ann Arbor, MI), and the α2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-rabbit horseradish peroxidase-labeled secondary antibody for Western blotting was purchased from NEN Life Science Products (Boston, MA). The secondary biotinylated anti-rabbit secondary antibody for immunohistochemistry (IHC) as well as the 3,3'-diaminobenzidine (DAB) substrate kit were obtained from Vector Laboratories (Burlingame, CA); Perlmunt was purchased from Fisher (New York, NY). Alum (Aguell-S) was obtained from Boehringer-Ingelheim (Ridgefield, CT). All other reagents, including OVA, methacholine, 1H-1,2,4 oxadizolo[4,3-e]quinolxalin-1-one (ODQ), and SYBR Green I, were obtained from Sigma-Aldrich.

Sensitization and challenge protocol. Animal protocols were approved by the local committee for animal care and use. Eight- to ten-wk-old (25–30 g) BALB/c (Pasteur Hellenic Institute, Athens, Greece) were transferred to our animal facility and housed for at least 1 wk before use. Mice were sensitized with OVA at a concentration of 0.01 mg/mouse in 0.2 ml of alum, intraperitoneally on 1 wk before use. Mice were sensitized with OVA, at concentration of ten-wk-old (25–30 g) BALB/c (Pasteur Hellenic Institute, Athens, proved by the local committee for animal care and use. Eight- to

Noninvasive assessment of airway reactivity. The degree of airway responsiveness to cholinergic stimulation was measured in unrestrained conscious mice 24 h after the final OVA challenge (day 24) or 16 h after ODQ treatment by barometric plethysmography (Buxco Technologies, Sharon, CT) (14). To inhibit sGC, naïve BALB/c mice were injected intraperitoneally with ODQ (10 mg/kg) dissolved in dimethyl sulfoxide (DMSO); animals receiving an equivalent volume of DMSO served as controls. The ODQ dose was chosen based on data available in the literature (1) and data obtained during our prior studies. After the treatments, mice were placed in whole body plethysmography chambers and exposed for 1 min to aerosolized methacholine at a concentration of 3–100 mg/ml for 1 min. Recordings were obtained for 5 min. AHR was expressed as enhanced passive (Penh), a calculated value that correlates with measurements of airway resistance, impendence, and intrapleural pressure (10).

Bronchoalveolar lavage, IL-13, and IgE measurements. On day 26, the airways of the mice were lavaged via a tracheal cannula with 1 ml of PBS. The resulting bronchoalveolar lavage (BAL) fluid was immediately centrifuged (700 g, 5 min at 4°C), supernatant was collected, and cells were resuspended in 1 ml of PBS. Total BAL cell counts were performed, and aliquots (5 x 10^5 cells/slide) were pelleted on glass slides by cytocentrifugation. Differential counts were performed on Giemsa-stained cytospins, and percentages of eosinophils lymphocytes, neutrophils, and macrophages were determined by counting their number in 400 cells. To obtain the absolute number of each leukocyte subtype in BAL, these percentages were multiplied by the total number of cells recovered from BAL fluid. To determine IL-13, lung tissue (100 mg) was homogenized in 2 ml of Hank's balanced salt solution containing 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM phenylmethylsulfonyl fluoride; it was then centrifuged (1,900 rpm for 10 min), and the supernatant was collected. IL-13 levels were determined in lung homogenates by ELISA according to the manufacturer’s instructions. Serum levels of total IgE were measured by ELISA using paired antibodies according to the manufacturer’s instructions.

Lung histology. Lung lobes were fixed in 10% formalin, dehydrated, and mounted in paraffin, and sections (4 μm) were stained with hematoxylin-eosin according to standard protocols. A semiquantitative scoring system was used to grade the size of lung infiltrates, where +5 signifies a large (>3 cells deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci. Goblet cells were counted on periodic acid-Schiff (PAS)-stained lung sections with an arbitrary scoring system. PAS-stained goblet cells in the airway epithelium were measured with a numerical scoring system (0 = <5% goblet cells; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = >75%). The sum of PAS airway scores from each lung was divided by the number of airways examined (20–30 per mouse) and expressed as mucus score in arbitrary units.

Quantitative real-time PCR. Lungs were homogenized in TRIzol. Subsequently, total RNA was extracted, photometrically quantified (260 nm), and adjusted to a concentration of 1 μg/μl. We determined RNA quality by running the samples on agarose gels and by determining their optical density ratios at 260 and 280 nm; only RNA samples with a ratio of 1.85 or higher were used for further analysis. To eliminate residual genomic DNA, the RNA samples were treated with DNase I. The cDNA was synthesized from 1 μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR according to the manufacturer’s instructions and diluted 1:5 with water. Two microtiter of each cDNA sample were used as template for the amplification reaction. Each PCR reaction included 5 μl of 1× PCR buffer with 2.5 mM MgCl2, 80 nM dNTPs, 0.3 μM of each primer, 2.5 units of platinum Taq polymerase, and SYBR Green I at final concentration of 0.1×. Primers and PCR cycling conditions were as described by Mergia et al. (19). PCR amplifications were performed in triplicates in a LightCycler System (Bio-Rad) and analyzed with LightCycler IQ software 3.0. The threshold cycle (Ct value) was chosen as the first amplification cycle giving a signal above background. To calculate the relative quantity of the respective subunit, we used the ΔΔCt method; 18S rRNA was used for normalization.

Western blot and immunohistochemical detection of GC subunits. After killing the animals, we froze lung tissue in liquid nitrogen and stored it at −80°C until use. One lobe was homogenized in 10 volumes (wt/vol) of a lysis buffer containing 1% Triton-X, 1% SDS, 150 mM NaCl 50 mM NaF, 1 mM Na3VO4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA, and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM PMSF). Samples were subjected to SDS-PAGE followed by blotting with antibodies raised against the α1 (1:5,000), α2 (1:1,000), or β1 (1:2,000) and visualized using a chemiluminescent substrate. For IHC detection of the sGC subunits, lung sections (4 μm) were deparaffinized, rehydrated, and fixed in 2% paraformaldehyde; they were then treated with avidin-biotin complex, blocked with 10% donkey-horse serum, and incubated overnight at 4°C with rabbit polyclonal antibodies for sGC α1 (1:500) or β1 (1:200). Next day, sections were incubated with secondary biotinylated anti-rabbit antibody. Subunits were visualized with the DAB substrate kit, which produces a dark brown color. Sections were counterstained briefly with hematoxylin before mounting.

Statistical analysis. Results are presented as means ± SE of the number of observations. Statistical comparisons between groups were made by two-tailed unpaired t-test, Mann-Whitney sign test, or analysis of variance followed by an appropriate post hoc test using SPSS software. Differences were considered significant when P < 0.05.

RESULTS

Experimental asthma. Mice sensitized and challenged with OVA displayed many of the characteristics of asthma. BAL fluid obtained from OVA mice contained increased numbers of eosinophils, lymphocytes, and macrophages. Histological scoring of sections obtained from these animals confirmed the presence of large numbers of inflammatory cells in the lung (Fig. 1, A and B). Moreover, IL-13 levels were found to be increased in lung homogenates of mice challenged with OVA (Fig. 1C). In addition to the inflammatory response, mice
developed increased airway reactivity to methacholine throughout the concentration range used (Fig. 1D).

**Steady-state mRNA and protein levels for the sGC subunits are decreased in mice with allergic asthma.** To determine whether sGC subunit expression is altered in mice sensitized and challenged with OVA, total RNA was isolated from the lung, and sGC subunit levels were determined by real-time PCR (Fig. 2). Steady-state levels for α1, α2, and β1 were reduced by 84, 63, and 79%, respectively. It should be mentioned that we could not detect the β2 subunit in the lung under the conditions used.

To test whether the changes observed at the mRNA also extended to the protein level, lung homogenates were analyzed by Western blotting. Representative Western blots are shown in Fig. 3A. Based on densitometric analysis, we determined that expression of α1, α2, and β1 was significantly reduced for all sGC subunits (20.1 ± 6.3, 48.9 ± 18.2, and 36.5 ± 15.5% respectively).

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**Fig. 1.** Induction of allergic airway disease in mice. Mice were sensitized and challenged with ovalbumin (OVA) as described in MATERIALS AND METHODS. The presence of inflammation was determined 24 h after the final OVA challenge by differential cell counts in bronchoalveolar lavage (BAL, A) and also by histological analysis of infiltrates in lung sections stained with hematoxylin and eosin (HE, left y-axis) and periodic acid–Schiff (PAS) (right y-axis, B). IL-13 (left y-axis) in lung homogenates and serum IgE (right y-axis, C) were measured by ELISA. Airway hyperreactivity (AHR) in response to increasing concentrations of methacholine was measured after the final allergen challenge on day 24 (D). Values are expressed as means ± SE; n = 20 (2 separate experiments); *P < 0.05 from control mice. Macs, macrophages; Eos, eosinophils; Linfs, lymphocytes; Neuts, neutrophils.

**Fig. 2.** Steady-state mRNA levels of soluble guanylyl cyclase (sGC) subunits are decreased in mice with allergic asthma. Lung homogenates from control and mice sensitized and challenged with OVA were analyzed by real-time PCR. Results are presented as means ± SE of the relative expression of the α1, α2, and β1 subunits normalized for endogenous 18S rRNA. Expression for each subunit was set at 100% for control mice; n = 5–10; **P < 0.01 and *P < 0.05.

**Fig. 3.** Protein levels for soluble guanylyl cyclase (sGC) subunits are decreased in OVA mice with allergic asthma. Representative Western blots for the α1, α2, and β1 sGC subunit and β-actin in lung homogenates of control (lanes 1–4) and mice sensitized and challenged with OVA (lanes 5–8) mice (A). Data quantified by densitometric analysis (B). Expression for each subunit set at 100% for control mice. Data are expressed as means ± SE; α1, n = 11–14; α2, n = 4; β1, n = 11–14. **P < 0.01 and *P < 0.05.
of control, respectively; Fig. 3B). To evaluate the cellular distribution of α1 and β1 sGC subunits and to evaluate the cell types in which sGC was downregulated, we performed IHC staining of lung sections. In control lung, α1 and β1 were ubiquitously present at high levels (Fig. 4). In agreement to our results from Western blotting experiments, expression of both subunits was reduced in mice with allergic airway disease with lower sGC levels being detected in most cell types of the lung, including bronchial smooth muscle cells and epithelial cells.

**Inhibition of sGC and AHR.** To investigate whether inhibition of sGC per se could increase airway reactivity to cholinergic stimulation, mice were treated with the sGC inhibitor ODQ, and Penh was measured 16 h later. As shown in Fig. 5, airway reactivity to methacholine was increased in mice treated with ODQ compared with vehicle-treated animals, suggesting that sGC inhibition results in AHR.

**DISCUSSION**

Asthma is a chronic disease characterized by airway inflammation and hyperreactivity that cause reversible episodes of airway obstruction (3). During the course of the disease, structural changes also occur in the airways that are known as remodeling; these include subepithelial fibrosis, increase in airway smooth muscle mass, and excessive mucus secretion from hyperplastic goblet cells (6). Data from animal models have implicated Th2 cytokines, like IL-4, IL-5, and IL-13, in the pathogenesis of asthma and allergic airway disease (3, 6).

In the presence of such a proinflammatory environment, most of the cells in the lung parenchyma cells are primed to synthesize larger than normal amounts of NO through transcriptional activation of iNOS (24). NO is a bronchodilator and upregulation of its production in the absence of other inflammatory stimuli decreases airway resistance and responsiveness (15). However, the role of NO in asthma is elusive, as it remains unclear whether the excessive NO production associated with this disease is protective or destructive for lung tissue. Apart from increased degradation of NO due to inactivation by superoxide anions in asthmatic subjects, one hypothesis that could reconcile the increased tone and responsiveness of the airways with the large amounts of NO observed in asthma is that NO is a better bronchodilator when present as S-nitrosothiol (S-NO). Some of the evidence in line with this hypothesis is that S-NOs are reduced in asthmatic airways (5, 9) and that genetic ablation of a dehydrogenase involved in S-NO metabolism in mice protects against the development of airway hyperactivity (23). Alternatively, aberrant sGC expression could underlie the inability of NO to act as an effective bronchodilator in asthmatic airways. To determine whether downregulation of NO-sensitive guanylyl cyclase (sGC) occurs in asthma, we investigated the expression of steady-state mRNA levels of sGC subunits in mice sensitized and challenged with OVA. From the known sGC subunits, α1, α2, and β1 were found to be present in the lung, confirming previous observations (4, 19). Interestingly, experimental asthma resulted in a substantial decrease in the steady-state levels of sGC subunit mRNA. We and others have reported that treatment of cultured cells with inflammatory stimuli including lipopolysaccharide, IL-1β, or cytokine mixtures leads to reduction in sGC mRNA levels (20, 28). The above-mentioned cytokines have been shown to be increased in asthma (3) and might contribute to the reduction in sGC levels. An additional pathway contributing to sGC downregulation in asthmatic airways could be iNOS-derived NO: uncontrolled

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Fig. 4. Immunohistochemical staining of lung tissue for α1 and β1 sGC subunits. Photomicrographs shown are representative of experiments performed at least twice. Both sGC α1 and β1 were visualized with 3,3′-diaminobenzidine, which produces a dark brown color. Notice the stronger staining in control mice, as well as the ubiquitous distribution of the enzyme. Arrows point to smooth muscle cells. Hematoxylin was used as a counterstain (light purple). ×40 Magnification.

Fig. 5. sGC inhibition causes AHR. Naïve mice were treated with either 10 mg/kg ip 1H-[1,2,4] oxadiazolo[4,3-ea]quinazolin-1-one (ODQ) or with DMSO as vehicle. Airway reactivity to aerosolized methacholine was estimated by measuring enhanced pause (Penh) 16 h after drug injection. Values are expressed as means ± SE; n = 7; *P < 0.05 from DMSO.
production of NO has been shown to decrease the mRNA stability of both the α1 and the β1 subunits (7).

Any reduction in mRNA levels, if it were to have an impact on function, would have to extend to the protein level. To determine sGC subunit levels in our animal model, we analyzed lung homogenates and sections by Western blotting and IHC, respectively. Densitometric analysis revealed that all three sGC subunits measured were significantly reduced. Especially for the α2 subunit, this is the first time that its presence at the protein level has been shown in the lung; in addition, this is the first time to our knowledge that regulation of α2 expression at the mRNA or protein level has been shown to occur in relation to a disease process. sGC activity is expected to be greatly reduced in allergic asthma, not only because of the low levels of sGC subunit expression, but also due to desensitization that occurs upon exposure of the enzyme to excessive amounts of NO produced from iNOS (8).

As sGC is ubiquitously expressed, determination of protein levels in lung homogenates does not reveal the cell types in which sGC is decreased. To test whether reduced sGC protein levels are present in the cell type relevant to AHR (i.e., smooth muscle), we stained lung sections with α1 and β1 antibodies. These experiments demonstrated a marked reduction in both subunits of the most common isoform of sGC in bronchial smooth muscle cells of mice with allergic airway disease. The expression of α2 was not evaluated by IHC, as sGC is an obligate heterodimer and decreased β1 levels would suffice to cause a reduction in sGC activity, irrespectively of any changes in the α subunits.

To study whether reduced sGC activity mimics the asthmatic phenotype with respect to AHR, mice were treated with the sGC inhibitor ODQ, and airway reactivity to methacholine stimulation was determined. Although baseline values were not significantly different between ODQ- and DMSO-treated animals, cholinergic stimulation of airway smooth muscle elicited greater responses in animals in which sGC was pharmacologically inhibited. Increased airway reactivity to methacholine after sGC inhibition has been previously reported to occur but was marginal and was only observed at the highest concentration of methacholine used (50 mg/ml) (1); this is likely due to the fact that Penh in these experiments was determined 48 h after ODQ administration. Our findings on the role of sGC in AHR are in line with those of Sadeghi-Hashjin et al. (27), who showed that treatment of guinea pig trachea in vitro with methylene blue (an agent that prevents activation of sGC) caused an eightfold increase in the sensitivity to histamine contractile responses. Our data on the development of AHR in naïve mice receiving the sGC inhibitor, taken together with the observation that allergic airway disease is characterized by reduced expression of α1, α2, and β1, suggest that AHR in asthma might result, at least in part, from the downregulation of sGC in bronchial smooth muscle.

In conclusion, we have shown that sGC expression is reduced in allergic asthma both at the mRNA and protein level. We have also shown that sGC inhibition results in AHR. Evidence from the literature also implicates the NO/sGC/cGMP pathway in the regulation of proliferation in airway smooth muscle. Hamad et al. (12) have shown that the NO donor S-nitroso-N-acetylpenicillamine reduces proliferation of airway smooth muscle cells in response to serum and thrombin.

The antiproliferative effect of NO could be enhanced by phosphodiesterase-5 inhibition and mimicked by a cell-permeable cGMP analog. A newer study has demonstrated the NO-mediated arrest at the G1 phase to be cGMP dependent (13). Thus long-term downregulation of sGC in asthma not only has the potential to contribute to the increased airway reactivity to constrictors but might also be implicated in the hyperplastic smooth muscle response and remodeling that occurs with the disease.

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REFERENCES


