RGS5 inhibits bronchial smooth muscle contraction in severe asthma

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Online Data Supplement

Supplemental Methods

Mouse allergen challenge

6 12-week-old female BALB/c mice (Jackson Laboratories) were injected intraperitoneally with 20 μg *Aspergillus fumigatus* ("*Af*", Bayer Pharmaceuticals) together with 20 mg alum (Imject Alum, Pierce) in PBS (100 μl) on Days 0 and 14, followed by intranasal challenge with either PBS + 21% glycerol ("naïve") or 25 μl *Af* extract in PBS ("Ag-challenged) (3 mice in each group) on Days 25, 26, and 27 as previously described (22). All mice were sacrificed on day 28, 24 h after the last intranasal treatment. All mice used in this study were housed under pathogen-free conditions. The protocol (803093) was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Immunohistochemistry and imaging

For immunohistochemical staining of lung, sequential 2 μm sections were cut from glycomethacrylate-embedded bronchial biopsies and stained using monoclonal antibodies against α-smooth muscle actin (Dako, Ely, UK) and RGS5 (ProSci, Inc.) or appropriate isotype controls (Chicken IgY, Dako). $RGS5^+$ cells were enumerated/mm^2 ASM in the ASM bundle. A minimum area of 0.1 mm^2 was considered assessable as described previously (20). For staining of mouse lung, naïve and *Af*-challenged mice were anesthetized by ketamine and xylazine and euthanized by gentle cervical dislocation. Mice were perfused with PBS by intracardiac puncture followed by perfusion with 10% neutral buffered formalin for fixation and embedded in paraffin. 5 μm sections were post-fixed in cold acetone and incubated with chicken anti-RGS5 antibody overnight at room temperature. Sections were then incubated with biotinylated anti-chicken

antibody (Dako) (1:500 for 30 min) followed by HRP-conjugated streptavidin (1:400; Dako) for 30 min. Slides were developed with DAB and counterstained with Hematoxylin. Images were obtained using a Leica DMI400 light microscope equipped with Retiga2000R camera (QImaging, Canada) and analyzed by QCapture™ software.

RNA isolation and quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) per the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using SuperScript III First Strand Synthesis Kit (Invitrogen). Quantitative real-time PCR (qPCR) was performed with TaqMan gene expression assay probes. The 20 μl reaction contained 1× TaqMan Universal Master Mix, 1 μl specific gene or GAPDH gene expression assay probe (final primer concentration 900 nM), and 1 μ1 cDNA. The reactions were run for 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The average cycle threshold (C_t) value from each RGS probe was applied to calculate specific gene expression and normalized by the human GAPDH C_t value. Results represent values from 7-11 individual donors assayed in duplicate.

Gene expression qPCR array

Total RNA (1 μg) was reverse transcribed into cDNA using RT^2 First Strand Kit (SABiosciences). qPCR reaction cocktail consisted of RT^2 SYBR Green/ROX PCR Master Mix (SABiosciences) and pooled cDNAs derived from ASM of 7 healthy donors and 7 subjects with fatal asthma. Samples were analyzed with the Human GPCR Signaling Pathway Finder Array (SA Biosciences catalogue no. PAHS-071) using an ABI StepOnePlus thermal cycler (Applied

Biosystems) as follows: 1 cycle on 95°C, 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Measurement of intracellular Ca^{2+} mobilization

ASM cells (1 x 10^4 cells/well) were seeded in black clear bottom 96 well plates (Corning), allowed to attach overnight and serum-starved in Ham's F-12 medium for 24 h prior to measurement of intracellular Ca^{2+} concentration. Ca^{2+} indicator dye [FLIPR Calcium 3 Assay (Molecular Devices)] was added to each well followed by incubation for 1 h at 37˚C with 5% CO_2. Serial dilutions of agonists [bradykinin, histamine, or thrombin (Sigma-Aldrich)] were prepared in a separate plate at 5x concentrations for robotic addition to cells by the FLEXStation II fluorimeter (Molecular Devices). Relative Fluorescence Units (RFUs) were measured every 1.5 sec for a period of 160 s after agonist addition. Results were plotted as the maximum–minimum signal for each agonist concentration over this time period, with the minimum signal defined as the stable baseline value prior to agonist addition. Graphs represent results (mean ± S.E.M.) of 5 or more independent experiments using cells derived from separate individual donors.

Lentiviral amplification and transduction

The human RGS5 coding sequence was amplified by PCR using pEGFP-RGS5 plasmid as a template and subcloned into pLenti7.3/V5 TOPO (Invitrogen). This construct or pLenti7.3/V5 TOPO-*LacZ* as a negative control was transfected into HEK293T cells together with a lentiviral packaging mix (Sigma) using Lipofectamine 2000. After 72 hr virus was harvested in the supernatant and concentrated 10-20-fold by centrifugation. Concentrated virus was added directly to human lung slices and gene expression and function were assayed 48 h after

transduction. Normal human ASM cells were incubated with lentivirus for 1 h at room temperature and then seeded in Ca^{2+} assay plates. Functional assays were performed 48 h post-transduction after overnight serum starvation.

Immunoblotting

We prepared cell lysates in radioimmunoprecipitation (RIPA) lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Roche). Antibodies were purchased from the following sources: Histamine H1 receptor, Adenosine A2a receptor, Gαq, bradykinin B2 receptor (Santa Cruz); PLCβ1 (BD Biosciences); β-arrestin 1/2, Akt1 (Cell Signaling Technology); PAR1 (R & D Systems); V5 (Invitrogen); β-actin (Sigma). For enhanced detection of RGS5, cell lysates were incubated with recombinant His_6-Gαil (23) (750 ng) coupled to Ni/NTA agarose (Qiagen) essentially as described (24). After 2 washes with RIPA buffer, bead-associated proteins were resuspended in SDS sample buffer and analyzed by immunoblotting. We were unable to detect residual RGS5 in supernatants following G protein pulldown by immunoblotting, indicating that Gαil was present in excess relative to RGS5 (data not shown). Primary antibody staining was detected using near-infrared conjugated secondary antibodies and quantified with the LiCor Odyssey Imaging System (LiCor Biosciences).

PCLS contraction

Lung slice contraction studies were performed on tissue from healthy, non-asthmatic donors as described previously (25).

Supplemental Figure Legends

Fig. E1 RGS5 antibody staining of mouse lung epithelium. Immunohistochemical staining of lung sections from naïve mice was performed as in Fig. 7 using the indicated antibodies.

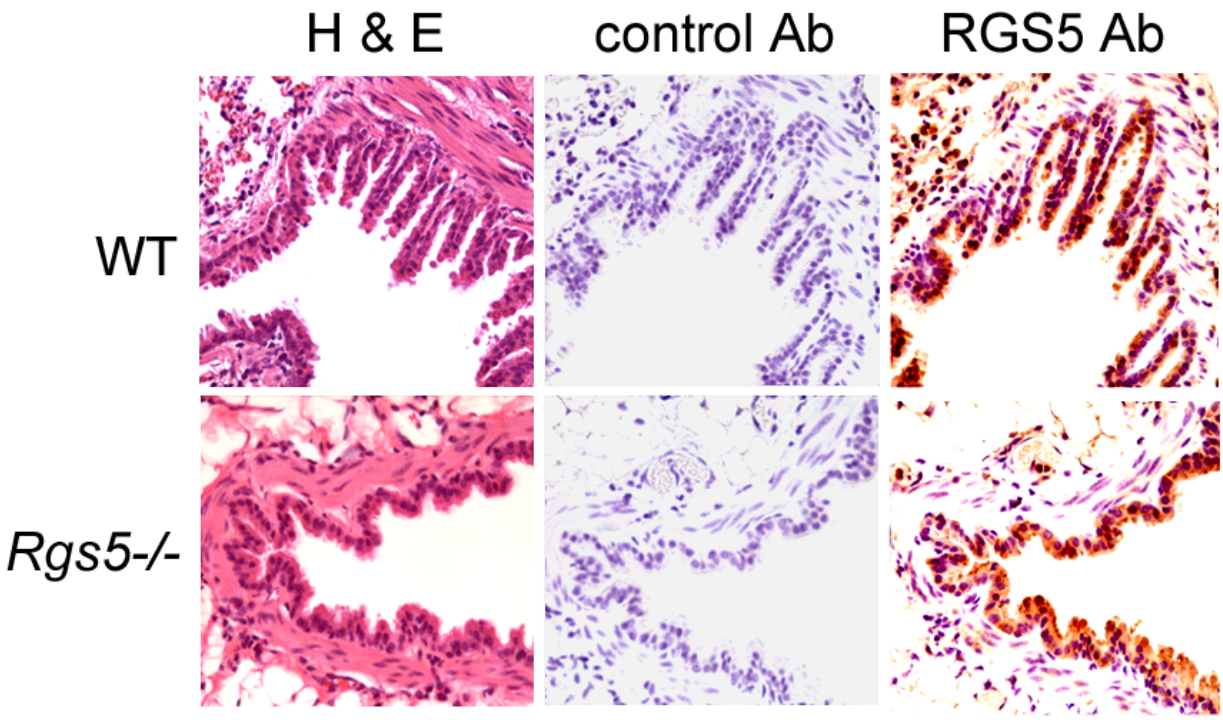

Fig. E1

Supplemental Table

Gene name	Fold change asthmatic v. control
Symbol	1.506243421
ADCY5	24.92200422
ADORA2A	-1.349630588
ADRB1	2.212241559
ADRB2	4.505917189
AGT	7.022803962
AGTR2	12.0870597
AGTRAP	13.2572301
AKT1	15.08571057
ARRB1	14.60358839
ARRB2	8.550017645
BAI1	8.517378354
BCL2	9.796769558
CALCR	16.02694183
CALCRL	6.357359293
CASR	9.958172001
CCL2	33.39987037
CCL4	10.30203779
CCND1	14.20792465
CCNE1	10.46322603
CCNE2	11.08719485
CDKN1A	13.8845765
CDKN1B	6.825967029
CFLAR	11.29582718
COL1A1	6.18343947
CRHR1	4.655647733
CRHR2	10.26953725
CTGF	33.6564319
CYP19A1	10.78379423
DRD1	24.21077328
DRD2	9.261400911
DUSP14	16.24983688
S1PR1	18.12582448
LPAR1	20.32854849
S1PR3	8.261419774
LPAR2	9.292221885
S1PR2	11.71817139
EDN1	1.183455089

EGR1	14.64513816
ELK1	11.81832075
ELK4	16.62044353
FGF2	-5.041155234
FOS	12.33370049
GALR2	24.69575719
GCGR	8.930849577
GNAQ	16.91221136
GRM1	-1.00919395
GRM2	6.329951347
GRM4	7.199114615
GRM5	32.0457482
GRM7	6.240781289
ICAM1	13.56625538
IL1B	16.42965295
IL1R1	17.63855468
IL1R2	12.56414426
IL2	6.544766005
JUN	2.534174201
JUNB	2.678639376
LHCGR	11.65112741
MAX	61.1472901
MMP9	3.939605977
MYC	1.630848573
NOS2	10.61688151
OPRD1	-1.020266977
OPRK1	12.1918607
PIK3CG	11.43829157
PRKCA	14.74784387
PTGDR	20.24700992
PTGS2	18.54286877
PTH1R	4.869412878
RGS2	9.571622324
RHO	6.616436203
SCTR	9.569344805
SERPINE1	8.55726642
SOCS1	5.860162508
TNF	27.48411882
TSHR	21.618746
UCP1	5.807292889
VCAM1	15.64959073

VEGFA	10.23898683
YWHAZ	2.008904316
B2M	1.19296685
HPRT1	1.111876981
RPL13A	1.073176158
GAPDH	1.381787463