Opsin 3-$G_{\alpha s}$ Promotes Airway Smooth Muscle Relaxation Modulated by G Protein Receptor Kinase 2

Amy D. Wu, BS; William Dan, BS; Yi Zhang, MD, PhD; Shruti Vemaraju, PhD; Brian A. Upton, BS, Richard A. Lang; Ethan D. Buhr; Dan E. Berkowitz; George Gallos; Charles W. Emala; and Peter D. Yim

ONLINE DATA SUPPLEMENT

SUPPLEMENTAL METHODS

Reagents and Chemicals: G-protein receptor kinase 2 inhibitor (GRK2i) (Methyl-5-[(E)-2-(5-nitrofuran-2-yl)ethenyl]furan-2-carboxylate) was purchased from Santa Cruz (Dallas, TX, USA). 9-*cis* retinal was purchased from Sigma Aldrich (St. Louis, MO, USA). GRK2i and 9-*cis*-retinal was used to minimize receptor desensitization and chromophore bleaching, respectively, to environmental light. 9-*cis* retinal is commonly used instead of 11-*cis* retinal, the endogenous chromophore in the retina, due to increased chromophore stability of 9-*cis* retinal in presence of ambient light (18, 19). We have previously demonstrated significantly enhanced photorelaxation upon inclusion of GRK2i and 9-*cis*-retinal in our previous study (3).

Cell Culture: Primary ASM cells were kindly gifted from Dr. Reynold A. Panettieri and were previously characterized(20). Cells were cultured in 5% CO_2 at 37 °C with Ham's F12 basal media with 10% FBS and antibiotics (10 units/ml penicillin, 10 µg/ml streptomycin, 25 pg/ml fungizone, 1 ng/ml human fibroblast growth factor, 0.25 ng/ml human epidermal growth factor, 1 µg/ml insulin, 0.55 µg/ml transferrin, and 0.67 ng/ml selenium; Gibco, Waltham, MA, USA). ASM cells stably transfected with human telomere reverse transcriptase were kindly gifted from Dr. William Gerthoffer and have been previously characterized (21). These cells were also maintained in 5% CO_2 at 37°C, with M199 basal media supplemented with 10% FBS and antibiotics. Immortalized cells were used for transfection (see below).

OPN3 Null Mouse Model: Opn3 null mice ($Opn^{lacZ/lacZ}$) were established and characterized by Dr. Richard Lang's group (22). Excised tracheas were shipped overnight in media along with trachea from control wild type mice for wire myography and western blotting.

Tracheal Ring Preparation and Wire Myography: C57BL/6 male and female mice were sacrificed with 50 mg/kg pentobarbital delivered through an i.p. injection. Animal tissue procedures were approved by Columbia University Institutional Animal Care and Use Committee. Tracheas were excised and dissected in ice cold Krebs Henseleit (KH) buffer (115 mM NaCl, 2.5 mM KCl, 1.91 $CaCl_2$, 2.46 mM $MgSO_4$, 1.38 mM NaH_2PO_4, 25 mM $NaHCO_3$, 5.56 mM D-glucose, pH 7.4). OPN3 null mouse tracheas were shipped overnight from the University of Washington. Tracheal rings were mounted in a 620M Myograph (DMT, Ann Arbor, MI, USA) in KH buffer oxygenated with 95% O_2/5% CO_2, at 37 ^oC. Rings were set at a resting tension of 0.5 g and kept in the dark. Buffer was exchanged every 15 minutes for 1 hour to equilibrate the tissue. Tracheal rings were contracted with 3 dose-response cycles of increasing log_{10} concentrations acetylcholine from 100 nM to 1 mM to determine an EC_{50} concentration of each ring. Rings were then contracted with EC_{50} acetylcholine.

OPN3 Null Mice Experiment. Rings were pretreated with 28 μM 9-*cis* retinal to minimize chromophore bleaching for 1 hour. Afterwards, rings were exposed to 405 nm blue light from a light emitting diode.

cAMP Experiment. Rings were pretreated with both 95 μM GRK2i and 28 μM 9-*cis* retinal for 30 minutes to minimize bleaching and desensitization. Afterwards, rings were exposed to 435 nm blue light (Thorlabs, Newton, NJ, USA) or kept dark. Then, the tracheal rings were flash frozen in liquid nitrogen and ground with a tissue pulverizer chilled in liquid nitrogen. 0.1 M HCl was added to solubilize the tissue (1 mL per 0.1 g) which was then centrifuged at 500 Xg for 10 minutes at 4 °C to pellet insolubilized components. Then, the supernatant (100 μL per assay well) was assayed according to followed according to the manufacturer.

Beta Agonist Potentiation Experiment. Rings were pretreated with 28 μM 9-*cis* retinal and 20 μM GRK2i for 30 minutes. Afterwards, rings were exposed to 450 nm blue light from a light emitting diode (Thorlabs, Newton, NJ, USA) concurrent an isoproterenol dose curve ($10^{-10.0}$ to $10^{-4.0}$ M, in increments of $10^{-0.5}$ M).

PKA Inhibitor Experiment. Tracheal rings were pretreated with 500 nM GRK2i and 28 μM 9-*cis* retinal with or without 100 μM Rp-cAMPS (Tocris, Bristol, UK), an enantiomer and competitive inhibitor of cAMP for 30 minutes. Tracheas were then exposed to a 435 nm blue light intensity curve (1-5%, 20%, 27%) (Thorlabs, Newton, NJ, USA). As positive control, separately prepared tracheas were treated with 1 μM forskolin (Tocris, Bristol, UK), an adenylyl cyclase activator, instead of light with and without Rp-cAMPS in the presence of 500 nM GRK2i and 28 μM 9-*cis* retinal.

Isoproterenol ("Hetero") Desensitization Experiment. Tracheal rings were incubated with or without 1 μM isoproterenol (Sigma Aldrich, St. Louis, MO, USA) overnight at 4 °C oxygenated with 95% O_2/5% CO_2 prior to wire myography the next day. On the day of the experiment, tracheal rings were prepared as before. Rings were exposed to an isoproterenol dose curve ($10^{-10.0}$ to $10^{-5.0}$ M, in increments of $10^{-0.5}$ M) to confirm desensitization. Then, rings were re-contracted with EC_{50} acetylcholine and exposed to 405 nm blue light from a light-emitting diode.

Mouse Trachea ASM Lysate Preparation: C57BL/6 wildtype or OPN3 null mice were sacrificed and tracheas were isolated (9 each). Under a microscope, airway smooth muscle from the tracheal posterior wall was carefully dissected out and placed in a minimal volume of a pre-chilled lysis buffer (150mM NaCl, 1% NP-40, 50mM Tris-HCl, protease inhibitor (1 cOmplete tablet per 10mL (Roche, Basel, Switzerland), pH 8). On ice, the sample was blended with the

Ultra-Turrax T24 basic homogenizer (speed 6, approximately 5x 10 second pulses; IKA-WERKE, Staufen, Germany). The lysate was centrifuged (300 Xg, 20 minutes, 4°C) and denatured with Laemelli's buffer (final concentration: 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 0.0625 M Tris-HCl, pH 6.8) at 100°C for 10 minutes.

Western Blot: Maximal and equivalent (when applicable) amounts of denatured protein was loaded and electrophoresed through a 4-15% acrylamide gradient gel (Mini Protean TGX, BioRad; Basel, Switzerland) in running buffer (25 mM Tris, 192 mM glycine, and 0.2% (w/v) SDS, pH 8.3). Gels were run at 100 V for 1-2 hours. Proteins were transferred to a methanol-activated PVDF membrane in 4 °C overnight at 5V in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at pH 8.3. The resulting PVDF membranes were blocked with 5% milk protein in Tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) for 1 hour at room temperature and incubated overnight in 4°C with primary antibodies diluted in 1% milk in TBS. The antibody against OPN3 (LS-C120583; LS Biosciences, Seattle, WA, USA) was diluted 1:300. The antibody against β-actin (PA1-16889; ThermoFisher, Waltham, MA, USA) was diluted 1:5000. Membranes were washed four times for 7 minutes each in TBS with Tween-20 (0.1% (v/v) Tween-20) with rocking, and incubated with 1:3000 goat anti-rabbit secondary antibody diluted in 1% milk in TBS for 1 hour at room temperature, and washed again four times for 7 minutes each in TBST with rocking. Images were developed using Supersignal West Femto reagents (ThermoFisher, Waltham, MA, USA) and visualized through chemiluminescence (Biospectra UVP, Cambridge, UK). Protein loading control was performed on a parallel membrane.

Proximity Ligation Assay: Primary human ASM cells were grown on slides to sub-confluency. Cells were washed with PBS and were fixed with 4% paraformaldehyde for 10 minutes and washed again. Cells were permeabilized with 0.1% Triton-X in PBS for 10 minutes and washed

three times with ice cold PBS. The Duolink PLA kit in red with rabbit/mouse secondary antibodies (Sigma Aldrich, St. Louis, MO, USA) were used. The included blocking solution was applied for 1 hour. All incubations were performed in a humidity chamber at 37°C. Then, primary antibody binding pairs (e.g. OPN3 and $G_{\alpha s}$) were diluted using the kit's antibody diluent. Antibody dilutions are as follows: OPN3 1:100 (rabbit anti-human; LS-C120583; LS Biosciences, Seattle, WA, USA), $G_{\alpha s}$ 1:50 (mouse anti-human; sc-55545; Santa Cruz, Dallas, TX, USA), $G_{\alpha s}$ 1:100 (rabbit anti-human; ab83735; Abcam, Cambridge, UK), GRK2 1:250 (mouse anti-human; G7670; Sigma Aldrich, St. Louis, MO, USA), β2AR 1:100 (mouse anti-human; sc-81577; Santa Cruz, Dallas, TX, USA), MLCK 1:50 (mouse anti-human; SAB4200808, Sigma Aldrich, St. Louis, MO, USA). Of note, the GRK2 antibody has been demonstrated to have minimal cross-reactivity with GRK3 (34). The β2AR-$G_{\alpha s}$ (rabbit) antibody combination was used as a positive control due to the well-established co-localization of these proteins. OPN3-MLCK was used as a negative control due to the expectation that membrane associated OPN3 and myosin light chain kinase would not co-localize. Additionally, for every binding pair, each primary antibody was individually omitted to demonstrate the lack of cross reactivity between the secondary antibodies in the presence of only a single primary antibody. For example, to control for the OPN3-$G_{\alpha s}$ experimental group, one well was incubated with only the OPN3 antibody and another was incubated with only the $G_{\alpha s}$ antibody. All primary incubations were 2 hours. Afterwards, the kit's oligomer-conjugated secondary antibodies were incubated, ligated, and amplified according to manufacturer instructions. Amplified product generates a fluorescent signal (Texas Red) if antigens are within 40 nm proximity. Slides were mounted with a DAPI-containing mounting media. Images were visualized on a Nikon A1

confocal microscope at 20x magnification (DAPI: λ_{ex} 405 / λ_{em} 488; Texas Red [visualized using mCherry filter]: λ_{ex} 561 / λ_{em} 640).

OPN3-GRK2 proximity permutations were also pretreated with 10 μM isoproterenol (Tocris, Bristol, UK) overnight for desensitization. All PLA permutations were assessed in the absence of light.

Co-Immunoprecipitation: Primary human ASM cells were grown to 80-95% confluence in 150 mm dishes. The cells were first incubated for 30 minutes in the dark (5% CO_2, 37 °C). Afterwards, cells were treated with GRK2i at 95 μM and 9-*cis* retinal at 28 μM for 15 minutes. Cells were treated with 435 nm light (Thorlabs, Newton, NJ, USA) and lysed with chilled 1.5 mL lysis buffer (final concentration: 40 mM Tris-HCl, 500 mM NaCl, 1% Nonidet P-40, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, protease inhibitor (1 cOmplete tablet per 10mL (Roche, Basel, Switzerland)) and phosphatase inhibitor (Phosphatase Inhibitor 3, Sigma Aldrich, St. Louis, MO, USA), pH 8) per 150 mm dish, scraped, and combined. Cell lysates were centrifuged for 5 minutes at 300 Xg at 4 °C to remove cell debris. The supernatant was pre-cleared of non-specific protein binding by a 10-minute incubation of the supernatant with non-linked, protein A-based Dynabeads at a 1:10 (v/v) (ThermoFisher, Waltham, MA, USA). Beads were magnetically removed.

New beads were linked to primary antibodies directed against the protein of interest through a co-incubation in the kit's "binding and washing buffer" with rotation for 10 minutes. Mouse anti-human $G_{\alpha s}$ targeted antibody was used at 1:30 (v/v) (sc-55545, Santa Cruz, Dallas, TX, USA) and mouse anti-human GRK2 targeted antibody was used at 1:600 (v/v) (G7670, Abcam, Cambridge, UK). Bead washing and bead-lysate incubation was performed according to

manufacturer's instruction. The bead-antibody-antigen complex was then eluted with 0.1 M citrate and denatured at 70 °C with Laemmli's buffer for 10 minutes and samples were analyzed by western blot in the absence or presence of a primary antibody against OPN3 as described above.

Cell Transfection: OPN1LW was transfected into immortalized human ASM cells using lentiviruses encoding OPN1LW, GFP and hygromycin resistance (Origene, Rockville, MD, USA). To transfect immortalized human ASM cells, the cells were seeded at 50% confluence in a 6 well plate. The day afterwards, cells were transduced with a multiplicity of infection of 50 alongside 8 μg/mL polybrene for a total volume of 500 μL (control cells were treated with polybrene alone). The 6 well plates were then centrifuged at 800 Xg for 1 hour to enhance transfection. After 24 hours, the media was changed. Transduced cells and control cells were treated with hygromycin (2 μg/ml) and cultured for 10 days. Successful cell transfection was confirmed by green fluorescence protein expression under UV microscopy. Cells not transfected (negative control) were removed by hygromycin selection and lacked a green fluorescence signal. The optimal hygromycin concentration was determined by preliminary kill curve.

OPN1LW transfected immortalized human ASM cells were grown in 6-well plates to 80-90% confluence. Cells were incubated in the dark for 30 minutes and then pretreated with 15 μM GRK2i and 28 μM 9-*cis* retinal in the dark. Afterwards, cells were treated with 660 nm red light (Thorlabs, Newton, NJ, USA) or 1 μM forskolin. Cells were then lysed with 200 μL of 0.1 M HCl and physical scraping. Protein concentration was determined through bicinchoninic acid assay (BCA, ThermoFisher, Waltham, MA, USA). 100 μL of the sample was used to for each well in the cAMP assay, which was carried out according to the manufacturer's directions.

Statistical Analysis: Student's two-tailed T-test, one-tailed ANOVA with Bonferroni post-hoc, and nonlinear curve fitting was performed using Prism 4 (GraphPad, San Diego, CA, USA). Data are presented as mean ± SEM with $\alpha=0.05$.