BOMBESIN INHIBITS ALVEOLARIZATION AND PROMOTES PULMONARY FIBROSIS IN NEWBORN MICE: A ROLE FOR GRP RECEPTORS

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MATERIALS AND METHODS

Animals:

The NIH Guide for the Care and Use of Laboratory Animals was strictly adhered to throughout this study.

Timed-pregnant outbred Swiss Webster mice were received from Charles River Laboratories (Wilmington, MA). GRPR-, NMBR-, and BRS3-null mice (knockouts, KOs) were inbred against a C57BL/6 background for at least 10 generations. Wild-type ("normal") littermates (WT) were used as controls for the KOs for all experiments. Mice were sacrificed on P14 using CO_2, lungs were inflated in situ and fixed 18 hrs in 4% paraformaldehyde, then processed into paraffin. At 2 weeks of age, the lungs are very small (less than ~1 gm total) and the most reliable method for fully inflating the lungs with fixative without over-inflation is to inject fixative transtracheally into the lungs in situ without opening the thoracic cavity. The trachea is then tied off in order to retain the fixative at the complete in situ filling pressure. The lungs are visualized as fully inflated by viewing them through the diaphragm, which is a thin membranous structure at this age. Lung weights and volumes were measured using standard methods (E1).

For experiments, mice were divided into 4 groups. Groups (1) and (2) are referred to as "prenatal", and Groups (3) and (4) are referred to as "postnatal":

(1) Bombesin was given at a dose of 200 µg/kg (20 µg/ml in phosphate-buffered saline [PBS]) administered intraperitoneally (i.p.) twice a day to pregnant mice at gestational ages E14-E16 (E2). [PBS = **154** mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4]. Fresh vials of dessicated peptide were used to prepare stock aqueous solutions (1 mg/ml) for each 3-day series of injections, with 6 aliquots of the stock aqueous solution stored at –80^{o}C until use.

(2) An equivalent volume (~0.4ml) of PBS was given i.p. following the same treatment schedule at E14-E16.

(3) Similarly, bombesin in PBS was given i.p. twice a day to pups beginning at 24 hours after birth = 1st through 3rd postnatal days (P1-P3). In selected experiments, GRP and neuromedin B were tested in a similar fashion, but using a dose of equivalent molarity as 200 µg/kg bombesin: (GRP @346 µg/kg [2805 g/mole] ; NMB @140 µg/kg [1132 g/mole]; BRS3 ligand [DTyr^{6},(R-Apa^{11}, Phe^{13}, Nle^{14}]Bombesin-(6-14) from David Coy (peptide #14 in Table I) (30) @150 µg/kg [1210 g/mole]. Bombesin, GRP, and NMB were purchased from Peninsula Laboratories. The synthetic BRS3 ligand was provided by David Coy at Tulane University Coy (30).

(4) An equal volume (~50 µl) of PBS was administered I.P. twice a day from P1-P3. The mice were weighed daily to determine the proper dose of BLP to be administered (200 µg/kg/injection). This dose was previously determined to elicit significant responses in fetal mouse lung (E2).

Immunostaining:

The procedure was a variation of the avidin-biotin complex (ABC) technique (E3). Four µm sections were deparaffinized and rehydrated. Slides used for alpha-smooth muscle actin (SMA) immunostaining were pre-treated with 0.3% Triton X-100 for 5 minutes (min). Prior to immunostaining for proliferating cell nuclear antigen (PCNA), slides were incubated in methanol at –20^{o}C for 30 min. All slides were then blocked for 60 min in 10% normal horse serum in 2% bovine serum albumin (BSA) in PBS (PBS/BSA). The primary antibodies were mouse monoclonal antibodies: anti-SMA: clone 1A4, mouse IgG2a isotype (Sigma, St. Louis, MO) used at 1:250 dilution in PBS/BSA; and anti-PCNA: clone PC10 (Dako, Carpinteria, CA) used at 1:30 dilution in PBS/BSA. Slides were incubated with primary antibodies overnight at 4^{o}C in a humid

chamber then washed with PBS. The biotinylated secondary antibody, horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) was used at a 1:200 dilution and incubated for 2 hrs at 4^{o}C. Following 15 min of blocking in 30% H_2O_2 in methanol, the ABC reagent (Vector Laboratories) was applied for 1 hr at room temperature. After washing, slides were developed using the chromogen diaminobenzedine (DAB, 0.03% solution) (Sigma) for 8 min. Slides were counterstained with 2% methyl green prior to coverslipping with Permount.

For immunofluorescence, the antibodies were primary murine monoclonal anti-SMA (@1/20) and rabbit anti-surfactant protein C (SPC, donated by Jeff Whitsett {Vorbroker, 1995 #6591}) (@1/100). We used methodologies as previously described (E6), summarized as follows: both primary antibodies were incubated for 1 hour at 20^{o}C on the sections together. This was followed by co-incubation of secondary antibodies for 1 hour at 20^{o}C including Alex Fluor 568 goat anti-rabbit IgG (Cat. #A11011, Molecular Probes, Eugene, Oregon, USA) and fluorescein-labeled anti-mouse IgG (Alexa Fluor 488 goat anti-mouse IgG, Cat. #A11001 from Invitrogen, Carlsbad, CA). Secondary antibodies were both used at 1:200 dilution in a solution containing DAPI 300 nM (Molecular Probes, Eugene, Oregon, USA) to label nuclei. The wavelengths for fluorescence microscopy are as follows:

1) Alexa Fluor 568 (PE-red): excitation @579 nm and emission @604 nm;
2) Alexa Fluor 488 (FITC-green): excitation @495 nm and emission @519 nm;
3) DAPI (blue): excitation @358nm and emission @461 nm.

Brightfield slides were visualized using a Nikon Optiphot microscope and images captured using the greatest digitizer interfaced with a Dell computer using ACT-I software (Nikon). Images were saved as TIFF files. Contrast was optimized automatically by Adobe Photoshop 6.0.

Morphometric analyses

a) General: Digitized images were captured at 20x magnification using a Nikon Labphot camera connected to a light microscope. TIFF images were transferred to the computer (MAC) using Scion image 1.62 software program. Eight to twelve 20x images were shot randomly from each lung lobe per mouse, using non-overlapping fields at 3, 6, 9, and 12 o'clock.

b) PCNA: Cells with dark brown nuclei are defined as PCNA-positive. The % of nuclei with PCNA staining was calculated for lung parenchymal cells as follows: (#PCNA-positive cells)/(total # nucleated cells in a given compartment, both positive & negative) x 100%.

c) SMA: A grid with 365 points (crosshairs) was superimposed on each captured field. The number of crosshairs overlying SMA-positive cells in the parenchyma was quantified, excluding vascular and airway smooth muscle. The percent volume of lung tissue positive for SMA was obtained using the formula: **% volume of SMA positive cells =** [Total number of SMA-positive points]/[365 – (positive points over airspaces)] x 100%.

d) Alveolar Wall Thickness: Using the same images captured above, ~60-80 lines per field were drawn across the narrowest segment of primary and secondary alveolar septa at 90^{o} using 4-5 randomly picked fields per mouse. The mean length of the lines crossing the septa was determined in μm (E4).

e) Mean Linear Intercept: Four to eight non-overlapping 20x images were captured from each of the lobes as described above; but under-inflated lobes were not included in this analysis. Mean linear intercept (MLI) was determined by superimposing a predetermined grid on the image, with set randomly placed lines totaling 1 mm in actual length at 20x. A pathology technologist trained in this method (A.S.) but with no knowledge of the experimental groups counted the number of times the lines cross an air-tissue interface. The actual MLI was calculated as the inverse of the number of air-tissue interfaces per mm x 1000, yielding the average distance from one air-tissue interface to the next in units of microns.

Genotyping

Genomic tail DNA was prepared as described (5, 6). Samples were stored at 4°C until used for PCR. Mice were genotyped using PCR for GRPR, NMBR, and BRS3 as described previously (E5, E6).

Statistical Analysis

All data are reported as mean ± Standard Error of the Mean (SEM). Data analysis was performed using Statistical Analysis System software. Group means were compared using the unpaired Students' t-test. Significant differences were defined as those with $P < 0.05$ between the experimental groups.

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