ONLINE DATA SUPPLEMENT

Sex Hormone-Dependent Regulation of Cilia Beat Frequency in Airway Epithelium

Raksha Jain, Jennifer M. Ray, Jie-hong Pan and Steven L. Brody

Supplemental Materials and Methods

Immunofluorescent staining and microscopy

Human lung samples fixed in 10% buffered formalin and human tracheal epithelial cells (hTEC) on membranes were fixed with 4% paraformaldehyde. Both types of samples were embedded in paraffin and processed for sectioning. Deparaffinized sections were incubated in Antigen Unmasking Solution, pH 6.0 (Vector Laboratories, Burlingame, CA), in an electric pressure cooker (Decloaking Chamber, Biocare Medical, Concord, CA) for 10 minutes then allowed to cool to room temperature. These samples were treated with 5% hydrogen peroxide in phosphate buffered saline (PBS) at room temperature (RT) for 5 min, washed in PBS, then blocked in a solution of 2% fish gelatin (Sigma Aldrich) with 5% donkey serum in PBS for 30 min. Other hTEC preparations were also fixed in 4% paraformaldehyde but processed for immunofluorscent antibody staining without paraffin embedding as previously described (1). These membranes were blocked in 2% bovine serum albumin with 5% donkey serum in PBS for 30 min. Fixed samples from all preparations were then incubated overnight at 4°C in primary antibody (and dilutions), including mouse anti-acetylated α-tubulin (1:10,000, Sigma-Aldrich), rabbit anti-PR (sc-539; 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-Foxj1 (1:1,000) (2). Antibodies were detected by Alexa Fluor labeled secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA). In paraffin embedded tissues, PR was detected by biotinylated secondary antibody (Vector Laboratories), followed by avidin, biotin (Vector Laboratories) and then horseradish peroxidase (HRP)-streptavidin with tyramide-Alexa Fluor 594 (Invitrogen) amplification, following the manufacturer's protocol. DNA was counterstained with 4'6 diamidino-2-phenylindole (DAPI). Immunofluorescent and differential interference (DIC) images were obtained using a DM5000B Leica microscope (Wetzlar, Germany) equipped with a Retiga 2000R camera and QCapture Pro software (both from Q Imaging, Surrey, BC, Canada). Captured images were globally adjusted for contrast and brightness and composed using Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

Protein blot analysis

Cells were lysed using a modified RIPA buffer (1× PBS pH 7.4; 1%; IPEGAL CA630; 0.5% sodium deoxycholate; 0.1% sodium dodecylsulfate) containing proteinase inhibitors (Complete Mini Cocktail, Roche, Mannheim, Germany). Proteins were resolved by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% powdered milk solution in PBS and 0.2% triton-X100. Membranes were blocked for one hour at room temperature, then incubated at 4°C overnight with rabbit anti-PR antibody (dilution 1:200, sc-539, Santa Cruz Biotechnology) or mouse anti-β-actin (1:2000, Sigma). After incubation with an HRP-labeled secondary antibody, enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) was used. Autoradiography was used to identify primary antibody binding (2). Densitometry of scanned immunoblot images was performed using Image J software (3). PR band intensity was calculated as a fraction of the actin band.

Cilia beat frequency assay

Well-differentiated hTEC (ALI culture over 21 days) were imaged on Transwell Clear membranes using an inverted microscope with a 20X phase contrast objective (Eclipse Ti-U, Nikon, Melville NY). The microscope was enclosed within a custom environmental chamber that maintained cells at 37°C (In Vivo Scientific, Saint Louis, MO). Cilia beat imaging was captured using a high-speed video camera (602f, Basler, Ahrensburg, Germany) sampled at 85 frames per second for 3 seconds and analyzed using the Sisson-Ammons Video Analysis system (4). At least 5 fields per cell preparation per hTEC sample were captured for whole field analysis. Baseline readings were acquired from all samples at time 0 (prior to the addition of any vehicle or treatment. The percent of baseline for each sample then used this time zero reading

as a comparison for the treatment or vehicle. The addition of 2% NuSerum to basic medium for the culture of cells did not affect CBF.

Supplement References

(E1) You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am J Physiol Lung Cell Mol Physiol 2002;283:L1315-1321.

(E2) Pan J, You Y, Huang T, Brody SL. RhoA-mediated apical actin enrichment is required for ciliogenesis and promoted by Foxj1. J Cell Sci 2007;120:1868-1876.

(E3) Abramoff MM, PJ; Ram, SJ. Image Processing with ImageJ. Biophotonics International 2004;11:36-42.

(E4) Sisson JH, Stoner JA, Ammons BA, Wyatt TA. All-digital image capture and whole-field analysis of ciliary beat frequency. J Microsc 2003;211:103-111.

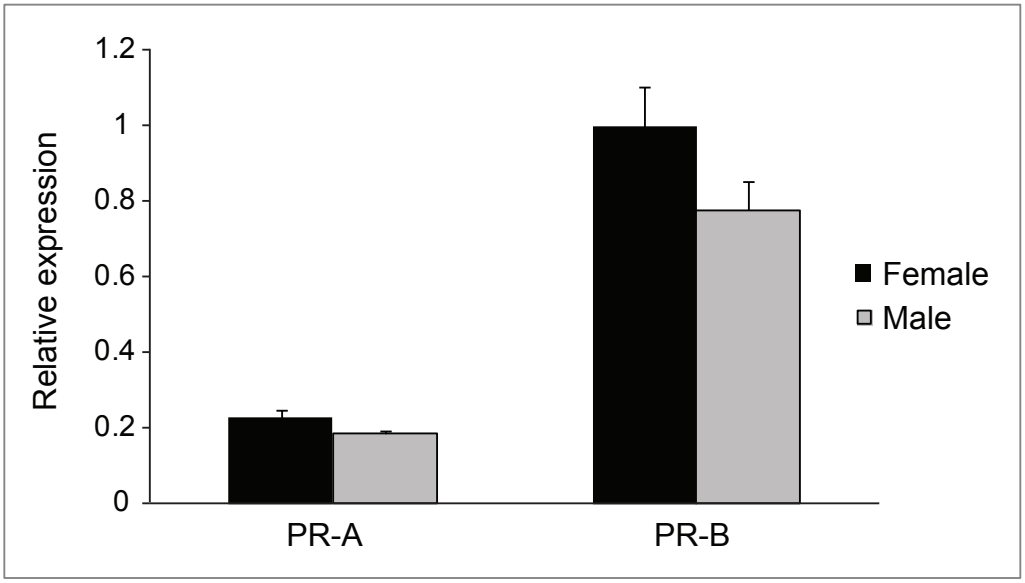
Supplemental Results

Supplemental Figure E1. Relative expression of progesterone receptor isoforms A and B in human tracheal epithelial cell (hTEC) preparations from male compared to female donors. Protein was isolated from at least 3 male and 3 female and subjected to protein blot analysis using a rabbit anti-PR antibody (Santa Cruz Biotechnology). Immunoblots (n= 3) were scanned and the density of specific bands for progesterone receptor isoforms A and B (PR-A, PR-B) analyzed using Image J software. The relative expression of each PR isoform was normalized to the mean of the female PR-B samples. Shown are the mean and SD of image density. There was a trend toward decreased expression of PR-A and PR-B in males, but no significant difference in expression of PR-A ($p = 0.05$) or PR-B ($p = 0.08$) between hTEC preparations from male and female donors.

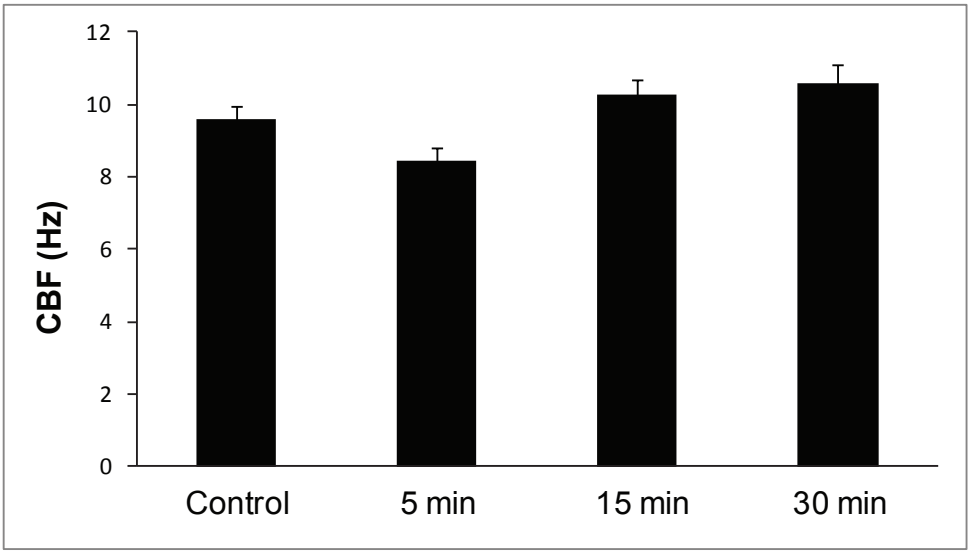
Supplemental Figure E2. Effect of progesterone on cilia beat frequency (CBF) within the first 30 minutes of treatment. hTEC preparations derived from male (n=3) and female (n=2) donors were treated with vehicle or progesterone (P4, 20 μM) then assayed for CBF using whole field analysis (5 independent fields from each Transwell insert, 3 inserts for each preparation at each time point). Shown are the mean and SD of CBF. There was no significant difference in CBF when compared to control with t-test or when analyzed using ANOVA ($p = 0.467$).

Supplemental Figure E3. Densitometry of protein blot analysis of progesterone receptor expression in human tracheal epithelial cell preparations following treatment with Actinomycin D. hTEC preparations derived from at least 3 male and 3 female donors were

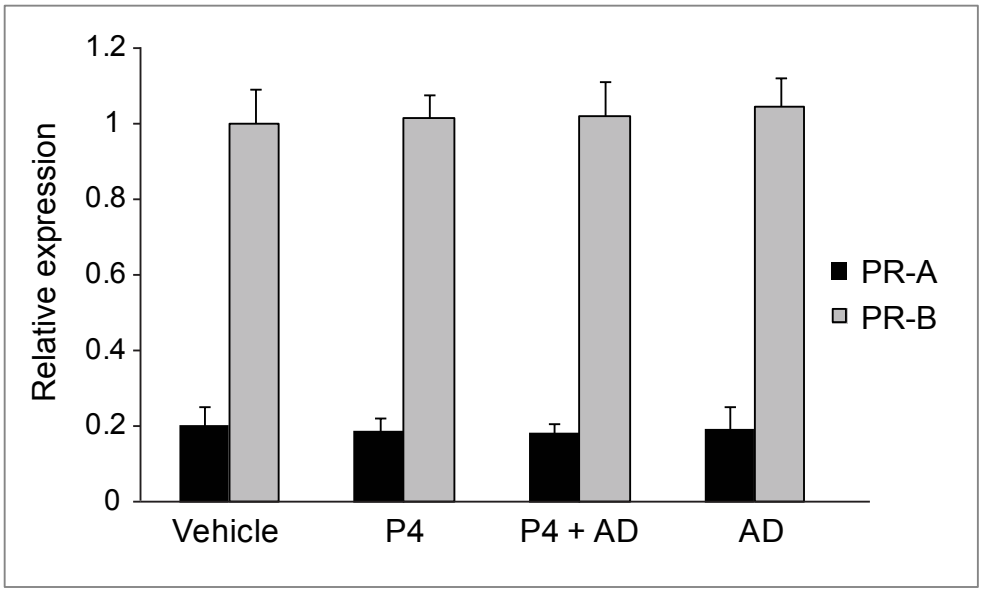
treated with vehicle and progesterone (P4, 20 μM) with and without Actinomycin D (AD) for 24 h. Cells were lysed and subjected to protein blot analysis using a rabbit anti-PR. Immunoblots (n=3) were scanned and the density of specific bands for progesterone receptor isoforms A and B (PR-A, PR-B) analyzed using Image J software. The relative expression of each PR isoform was normalized to the density of the vehicle treated band for PR-B. Shown are the mean and SD of image density. There was no significant difference between the expression of isoforms in the different treatment conditions using ANOVA analysis ($p > 0.05$).



Jain, et al. Supplementary Figure E1



Jain et al., Supplemental Figure E2



Jain et al., Supplemental Figure E3