A_{2B} Adenosine Receptors Regulate the Mucus Clearance Component of the Lung's Innate Defense System

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Online Data Supplement

Materials and Methods

Laser capture microscopy, qPCR and primer sequences

Excess specimens of human normal human bronchi obtained immediately after lung transplant surgery were dissected and snap frozen on dry ice in OCT media (Tissue-Tek). The tissue was sectioned at 8 µm in a cryostat and the frozen sections placed on Leica PEN membrane slides. ~20 ciliated cells were identified by light microscopy and laser captured onto HS LCM caps (Arcturus) and RNA was isolated using PicoPure RNA Isolation Kit (Arcturus). RNA was converted to cDNA and measured by LightCycler as described previously (21).

The gene specific primer sequences used for qPCR were as follows (5' to 3'): A_1 – forward, cat tgggccacagacctact and reverse, cagattgttccagccaaaca; A_{2A}– forward, tcaacagcaacctgc agaac and reverse, agtggttcttgccctccttt; A_{2B} – forward, ctccatcttcagccttctgg and reverse, acaaggcagcagctttcatt; A_3 – forward, gggcatcacaatccactt ct and reverse, agggccagccatattcttct.

In vivo nasal potential differences (PDs).

The study protocol was approved by the University of North Carolina Committee on the Protection of Rights of Human Subjects, and written informed consent was obtained. Mean age of subjects was 27 ± 3 years (n=6). PD was measured between a subcutaneous reference electrode and an exploring electrode placed against the inferior turbinate as previously described (22). In brief, electrodes are connected via calomel half-cells to a high-impedance voltmeter electrically isolated from the subject. The modified

exploring (nasal) catheter was designed with a single lumen catheter (3 cm length of polyethylene 10 tubing threaded over a 30G needle) that acts as a flowing bridge. Four perfusion lines (Polyethylene 50 tubing; Becton Dickinson) were connected to the perfusion catheter (identical polyethylene tubing). Ringer solution was perfused (0.02 ml/min). After maximal stable basal PDs were detected, the perfusing solution was sequentially changed whilst simultaneously recording PD. For each measurement, PD was recorded in each nostril and the mean was taken.

Measurement of In vivo ASL ADO.

Nasal [ADO] was measured in separate nasal cavities during basal conditions in a humidified 37°C room. Samples were collected as follows: lavage of 5 ml 37°C saline was instilled into each nasal cavity over a period of 30 s; the lavage remained in place for 30 s and the lavage was retrieved over 15 s and placed on ice. The ADO concentration was measured by adenosine derivatization followed by HPLC as previously described (23).

Human airway epithelial cultures

Human excess donor lungs and excised recipient lungs were obtained at the time of lung transplantation from portions of main stem or lumbar bronchi under a protocol approved by the UNC medical school institutional review board, and cells were harvested by enzymatic digestion seeded directly as primary cultures on 12 mm Transwell Clear inserts (Costar, USA) in modified BEGM media under air-liquid interface conditions, and

studied when fully differentiated (2-5 weeks) as described previously (24). For Ussing chamber experiments, cultures were seeded on 12 mm Snapwell inserts (Costar, USA), but otherwise cultured under identical conditions to cultures grown on T-clears.

RT-PCR and primer sequences

cDNA was obtained from HBECs as previously described (14). The gene specific primer sequences were as follows (5' to 3'): A_1 – forward, ttgggccacagacctacttc and reverse, cagccaaacataggggtcag; A_{2A}– forward, tcttcagtctcctggccatc and reverse, tccaacctagcatgggagtc; A_{2B} – forward, ctccatcttcagccttctgg and reverse, acaaggcagcagctttca tt; A_3 – forward, gggcatcacaatccacttct and reverse, agggccagccatattcttct.

Ussing Chamber measurements of transepithelial potential difference

Primary bronchial epithelial cultures were mounted in modified Ussing chambers (Physiologic Instruments) as previously described (25). The epithelia were bathed with bilateral KRB warmed at 37 °C circulated by gas lift with 95% O2/5%. Data were acquired and analyzed using Acquire and Analysis (version 1.2) software (Physiological Instruments). The EC_{50} was calculated from apical drug additions ranging from 10^{-8} to 10^{-4} M (half-log increments) and analyzed using nonlinear regression.

Confocal microscopy measurements of ASL height

To label the ASL, 20 µl PBS containing 2 mg/ml Texas Red or FITC conjugated to dextran (10 kDa) was added to the mucosal surface of the bronchial cultures. Prior to secretagogue addition, excess PBS was aspirated with a Pasteur pipette to acutely set ASL height at ~6 µm. For all studies, 100 µl PFC was added mucosally to prevent evaporation of the ASL. Cultures were then placed in a chamber which had a serosal reservoir containing 80 µl of modified TES-buffered Ringer solution and placed on the stage of an inverted confocal microscope (Leica SP5). This amount of PFC was selected since it was sufficient to cover the ASL during recordings yet, evaporated soon after the cultures were returned to the highly-humidified incubator. Cultures were imaged using a 63 x glycerol immersion lens. This approach yields a good working distance (3000 µm) whilst maintaining a sufficiently high numerical aperture (1.2) to obtain high resolution XZ images suitable for resolving <3 µm changes in ASL height. To measure the average height of the ASL, five predetermined points on the culture (one central, 4 circumferential) were XZ-scanned. To increase the contrast between the ASL and the image background, the gain on the confocal microscope photomultiplier tube was adjusted to give an image intensity mid-way on an 8 bit image (i.e. ~128 intensity units). The offset was then reduced up to the point where the background was zero. This approach gave a high contrast image suitable for determination of ASL height. N.B., cultures which spontaneously produced grossly visible mucus "hurricanes" and exhibited rotational mucus transport were excluded from this study to remove the confounding effects of the mucus reservoir effect.

Images were analyzed using ImageJ software (NIH freeware) by placing several regions of interest around the ASL which were then averaged for each image and the mean height calibrated based on a 512^2 pixel image corresponding to 125 μm^2 as previously described (21).

Intracellular Ca^{2+} and cAMP measurements

HBECs were loaded with free Fura-2 by incubation in 2.5 μM Fura-2/AM at 37°C for 45 min. Cells were then washed and the fluorescence intensity ratio (excitation 340/380, emission >450 nm) was collected from a field of 30-40 cells (18).

To measure cAMP, the mucosal surface of airway cultures was washed in PBS containing 200 μM papaverine. The assay was carried out at room temperature for 10 minutes after which the media was aspirated and the assay was stopped by addition of 0.1 N HCl. The cells were allowed to lyse completely then were triturated and transferred to microfuge tubes and centrifuged. The resulting supernatant was assayed for cAMP determined via enzyme immunoassay (Sigma-Aldrich). Cellular cAMP was normalized to protein as determined by the BCA method (Pierce Biotechnology Inc.). The results represent triplicate determinations for each condition as previously described (23).

Solutions and chemicals

For confocal microscopy experiments and measurements of intracellular cAMP and Ca^{2+}, cultures were bathed serosally in a modified Ringer solution (116 mM NaCl, 10 mM $NaHCO_3$, 5.1 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 20 mM TES, 10 mM

glucose, pH 7.4). All nasal PD and Ussing chamber experiments were performed in Krebs-Ringer bicarbonate solution (KRB), pH 7.4, containing: 120 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM $NaHPO_4$, 0.4 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 5 mM glucose. At all other times, cultures were maintained in a modified BEGM growth medium which contained 24 mM $NaHCO_3$ and gassed with 5% CO_2. Phosphate buffered saline (PBS) was used as an apical volume challenge and for washing the apical surface. Fura 2-AM and Texas red-dextran were obtained from Molecular Probes (USA). Adenosine, adenosine deaminase, alloxazine, amiloride, aprotinin, ATP, CGS21680, DIDS, DPCPX, glibenclamide, IB-MECA, MRS1191, NECA, papaverine, R-PIA and all salts and buffers were obtained from Sigma-Aldrich (USA). ZM241385 was purchased from Tocris Bioscience (USA).

Perfluorocarbon (PFC; FC-77) was obtained from 3M Company. Rather than add compounds to the ASL in a liquid vehicle which would disturb ASL volume, ADO, CGS21680, IBMECA, NECA, R-PIA, were added as dry powder suspended in PFC (FC-72) to give a final concentration of ~300 μM as previously described (53). Adenosine deaminase and aprotinin were added at 1-2 units/ml in the initial wash of PBS with the Texas red-dextran. In some cases, ADO, NECA and papaverine were made as a 10^{-1} M stock in H_2O then serially diluted prior to their addition to mucosal surfaces. Alloxazine, amiloride, ATL-801, DPCPX, MRS1191 and ZM241385 were made up as 10^{-2} M solutions in DMSO and diluted in PBS.