Adenosine Deaminase 1 and Concentrative Nucleoside Transporters 2 and 3 Regulate Adenosine on the Apical Surface of Human Airway Epithelia: Implications for Inflammatory Lung Diseases†

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ABSTRACT: Adenosine is a multifaceted signaling molecule mediating key aspects of innate and immune lung defenses. However, abnormally high airway adenosine levels exacerbate inflammatory lung diseases. This study identifies the mechanisms regulating adenosine elimination from the apical surface of human airway epithelia. Experiments conducted on polarized primary cultures of nasal and bronchial epithelial cells showed that extracellular adenosine is eliminated by surface metabolism and cellular uptake. The conversion of adenosine to inosine was completely inhibited by the adenosine deaminase 1 (ADA1) inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA). The reaction exhibited K_m and V_{max} values of 24 μM and 0.14 nmol·min^{-1}·cm^{-2}. ADA1 (not ADA2) mRNA was detected in human airway epithelia. The adenosine/mannitol permeability coefficient ratio (18/1) indicated a minor contribution of paracellular absorption. Adenosine uptake was Na^+-dependent and was inhibited by the concentrative nucleoside transporter (CNT) blocker phloridzin but not by the equilibrative nucleoside transporter (ENT) blocker dipyridamole. Apparent K_m and V_{max} values were 17 μM and 7.2 nmol·min^{-1}·cm^{-2}, and transport selectivity was adenosine = inosine = uridine > guanosine = cytidine > thymidine. CNT3 mRNA was detected throughout the airways, while CNT2 was restricted to nasal epithelia. Inhibition of adenosine elimination by EHNA or phloridzin raised apical adenosine levels by >3-fold and stimulated IL-13 and MCP-1 secretion by 6-fold. These responses were reproduced by the adenosine receptor agonist 5′-(*N*-ethylcarboxamido)-adenosine (NECA) and blocked by the adenosine receptor antagonist, 8-(p-sulfophenyl) theophylline (8-SPT). This study shows that adenosine elimination on human airway epithelia is mediated by ADA1, CNT2, and CNT3, which constitute important regulators of adenosine-mediated inflammation.

In human airways, extracellular adenosine is normally considered beneficial as it regulates mucociliary clearance, the primary defense mechanism against infection (*1*−*3*). Activation of A_{2B} receptors stimulates cilia beating activity (*4*) and maintains the airway surface liquid (ASL)[1] layer through the ion transport activity of the cystic fibrosis transmembrane regulator (CFTR) (*5*−*8*). However, high adenosine levels in bronchoalveolar fluid of asthmatic patients (*9*) reproduce several features of this disease in animal models, including airway inflammation and remodeling (*10*). Excess adenosine exacerbates lung inflammation through surface receptors expressed on immune (*11*−*15*), epithelial (*16*), and smooth muscle (*17*) cells. These studies suggest that abnormalities in airway adenosine regulation contribute to inflammatory lung diseases.

Adenosine triphosphate constitutes the main source of airway adenosine (*18*). Continuously released by the epithelium under basal conditions and in response to mechanical or hypotonic stress (*19*), ATP is rapidly dephosphorylated into adenosine by cell surface enzymes named ectonucleotidases (*18*, *20*). The enzymes responsible for the surface conversion of AMP to adenosine were identified as ecto-5′-nucleotidase (ecto-5′-NT; CD73) and nonspecific alkaline phosphatase (NS AP) (*18*). The fact that extracellular adenosine levels are maintained constant despite the continuous release and surface metabolism of ATP suggested that airway epithelia both generate and eliminate the nucleoside. In human tissues, extracellular adenosine is converted to inosine by two ADA isoforms: ubiquitous ADA1 and ADA2 secreted by monocytes and macrophages (*21*). These two cytosolic enzymes are also functionally distinguished by the specific ADA1 inhibitor EHNA (*21*). Whereas both isoforms are released in body fluids, only ADA1 binds to cell surface

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[1] Abbreviations: ADA, adenosine deaminase; ASL, airway surface liquid; CNT, concentrative nucleoside transporter; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; ecto-5′-NT, ecto-5′-nucleotidase; ENT, equilibrative nucleoside transporter; HPLC, high-pressure liquid chromatography; IL-13, interleukin 13; K_m, Michaelis constant; KRB, Krebs−Ringers bicarbonate buffer; MCP-1, monocyte chemoattractant protein 1; NECA, 5′-(*N*-ethylcarboxamido)adenosine; NS AP, nonspecific alkaline phosphatase; PCR, polymerase chain reaction; P_{coeff}, permeability coefficient; P_{ADO}, permeability coefficient of adenosine; P_{MAN}, permeability coefficient of mannitol; RT-PCR, reverse transcriptase−polymerase chain reaction; 8-SPT, 8-(sulfophenyl)theophylline; V_{max}, maximal velocity.

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proteins, namely, dipeptidyl peptidase IV (CD26) (*22*) and adenosine receptors (*23*−*25*). Extracellular adenosine is also eliminated by cellular uptake through nucleoside/nucleobase transporters (*26*). Widely distributed in human tissues, they provide nucleoside salvage for cells lacking de novo ATP synthesis, such as airway epithelia (*18*). Two transporter families have been identified: high-affinity Na^+-dependent CNTs facilitating uptake against a concentration gradient and ENTs acting as low-affinity facilitated carrier proteins (*26*). While ENTs are ubiquitous, CNTs are generally restricted to the apical surface of polarized epithelia (*27*).

This study identifies and characterizes the mechanisms responsible for the elimination of airway adenosine and establishes their contribution to adenosine-mediated inflammatory responses. Functional and expression assays were conducted on polarized primary cultures of human nasal and bronchial epithelial cells maintained in air−liquid interface conditions. Adenosine elimination was measured by liquid scintillation counting and high-pressure liquid chromatography (HPLC). Airway ADA isoforms were identified on the basis of pharmacological and kinetic properties, as well as mRNA expression. The contribution of paracellular diffusion was tested in Ussing chambers by comparing the permeability coefficients of adenosine and mannitol. The functional expression of CNTs and ENTs was investigated by use of the broad-range inhibitors phloridzin (*28*−*30*) and dipyridamole (*31*, *32*), respectively. The CNT isoforms were identified by competition assays, kinetic properties, and mRNA expression. Finally, we tested the impact of each component on the regulation of ASL adenosine levels and adenosine-mediated cytokine release from human airway epithelia. This study provides evidence for the critical role of epithelial ADA1 and CNTs in the regulation of adenosine-mediated airway inflammation.

MATERIALS AND METHODS

Cell Culture. Human nasal and bronchial epithelial cells were provided by the Tissue Culture Core of the Cystic Fibrosis Center (UNC-CH) under the auspices of protocols approved by the Institutional Committee on the Protection of the Rights of Human Subjects. Polarized primary cultures were prepared as previously described (*33*). In brief, nasal turbinate and main bronchus sections were harvested from donors and as excess tissue during lung transplant, respectively. They were incubated (4 °C; 24 h) in MEM medium containing 0.1% protease (Sigma type XIV) and 1 μg/mL DNase. The epithelial cells were sedimented (5 min; 500*g*) and seeded (0.25×10^6/cm^2) on collagen-coated (human placenta type VI) porous Transwell or Snapwell membranes (12 mm; 0.4 μm pore size) for Ussing chamber experiments. The cultures were kept at air−liquid interface in serum-free medium (50:50 mixture of LHC basal and DMEM-H) containing 50 nM retinoic acid, 0.8% bovine pituitary extract, 10 μg/mL insulin, 1 μM hydrocortisone, 30 nM triiodothyronine, 5 μg/mL transferrin, 3.8 μg/mL endothelial cell growth factor, 25 ng/mL epidermal growth factor, 50 units/mL penicillin, and 50 μg/μL streptomycin (*33*). After 4 weeks of confluence, the polarized cultures exhibited transepithelial resistance >200 $\Omega \cdot$cm^2.

Incubation Conditions. All functional assays were conducted on nasal or bronchial cultures washed (3×) with phosphate-buffered saline and incubated in bilateral Krebs−Ringers bicarbonate buffer (KRB; pH 7.4) containing (millimolar) 140 Na^+, 120 Cl^-, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 2.4 HPO_4^{2+}, 0.4 $H_2PO_4^-$, 25 HCO_3^- and 5 glucose, unless specified otherwise. Low-Na^+ KRB (pH 7.4) was prepared by replacing NaCl and $NaHCO_3$ with *N*-methyl-D-glucamine, titrated with HCl and choline bicarbonate, respectively. The Transwell cultures were kept in an incubator (37 °C; 5% CO_2−95% O_2) during the experiment and retrieved for sample collection.

Characterization of Adenosine Elimination. Nasal cultures were incubated in KRB (0.04 mL/0.5 mL apical/basolateral) and apical reactions were initiated with 1 μM [^{3}H]adenosine (1 μCi). Buffer samples (5 μL) collected over 60 min were transferred to 150 μL of ice-cold 5 mM EDTA, boiled 3 min, filtered, and analyzed by HPLC, as previously described (*20*).

Kinetic Properties of ADA1. The reactions were initiated in KRB (0.35 mL/0.5 mL apical/basolateral) by the addition of [^{3}H]adenosine (0.003−1 mM; 1 μCi) to the apical surface. Buffer samples (10 μL), collected over incubation periods limiting substrate deamination to ≤10%, were processed for HPLC analysis as indicated above. The assays were repeated on the same cultures after a 30 min preincubation with apical 10 μM EHNA to inhibit ADA1 (*21*). ADA1 activity was calculated from the difference between adenosine elimination rates measured in the absence and presence of EHNA. Michaelis constants (K_m) and maximal velocities (V_{max}) were calculated from the slope and ordinate of a Woolf−Augustinson Hoftsee transformation. Catalytic efficiency was calculated from the velocity (V_0) at K_m divided by K_m (*34*).

Unidirectional Permeability Coefficients. Nasal cultures seeded on Snapwells were mounted in Ussing chambers (Physiologic Instruments Inc., San Diego, CA) and voltage-clamped. The epithelium was bathed on both sides with 5 mL of KRB (37 °C; pH 7.4) circulated by gas lift (95% O_2−5% CO_2). Measurements of apical-to-basolateral permeability began 45 min after the addition of [^{3}H]adenosine (1 μM; 1 μCi) and [^{14}C]mannitol (0.2 μCi) to the apical bath. Buffer samples were collected from the apical bath every 60 min (50 μL; source) and from the basolateral bath every 30 min (500 μL, sink, replaced by 500 μL of KRB) for scintillation counting (Beckman LS 6500 counter). The permeability coefficients of adenosine (P_{ADO}) and mannitol (P_{MAN}) were calculated from the equation $P_{coeff} = (\Delta Q/\Delta t/SA)$ where $\Delta Q/\Delta t$ is the steady-state rate of appearance of radiotracer in the sink (counts per minute per second), S is the source of radioactivity (counts per minute per cubic centimeter), and A is the surface area (square centimeters) (*35*). P_{MAN} constitutes a measure of paracellular permeability.

Kinetic Properties of Apical Adenosine Uptake. Nasal cultures were preincubated 30 min in KRB (0.04 mL/0.5 mL apical/basolateral) containing 10 μM EHNA. The reactions were initiated by the addition of [^{3}H]adenosine (0.1−100 mM; 1 μCi) to the apical surface. Buffer samples (1 μL) were transferred to ice-cold 5 mM EDTA containing 10 μM EHNA, and [^{3}H]adenosine concentration was measured by liquid scintillation counting. The passive diffusion component of adenosine uptake was measured in low-Na^+ KRB−EHNA and 2 mM phloridzin. The kinetic parameters, corrected for passive diffusion, were calculated as for ADA_1.

Table 1: Specific Primers for ADA and CNT Isoforms, IL-13, and MCP-1

strand[a]	sequence	position	fragment size (bp)
	ADA1, Accession No. NM_000022		
S	CCTACCAGGAGGCTGTGAAG	727	181
AS1	TCGAAGTGCATGTTTTCCTG	907	
AS2	GTGGCATCCCATAGGCTTTA	1191	
	ADA2, Accession No. NM_017424		
S	CTG GAT GCT CTG ATG CTG aac	468	167
AS1	GCT ACA GGG TGG TTC CTC aag	634	
AS2	CCT CAT AGA AAT CAT AGG aca	726	
	CNT1, Accession No. NM_004213		
S	CCT CGA GAC GAA GAG AGT cca	216	115
AS1	CTC AAG TCA CTC CTA GGG agc	330	
AS2	CTC GCT CCA GCT GCT CCT	361	
	CNT2, Accession No. NM_004212		
S	CCA CTT ACC AGA GGA GGA gtc	217	138
AS1	AGA TGC AAG CTG CCA GGA	354	
AS2	TCA CCA AGC AGG TGA TGA caa	402	
	CNT3, Accession No. NM_022127		
S	TTG ATG AGA TGC TGT CTC ctg	556	118
AS1	GCA GTG TCA AAG GCC AAC	673	
AS2	TAC ATT ATG AGC CCA CCG aag	721	
	IL-13, Accession No. NM_002188		
S	TGA CCA CGG TCA TTG CTC	70	216
AS1	TGC AGC CTG ACA CGT TGA	285	
AS2	CTC AGC ATC CTC TGG GTC ttc	316	
	MCP-1, Accession No. NM_002982		
S	GCT CAT AGC agc cac ctt cat	93	201
AS1	TCT CCT TGG CCA CAA TGG	293	
AS2	TCC TGA ACC CAC TTC TGC	327	

[a] S, sense strand; AS1, inner antisense strand; AS2, outer antisense strand.

Specificity of Nucleoside Transporters. All reactions were initiated in KRB (0.04 mL/0.5 mL apical/basolateral) containing 10 μM EHNA by the apical addition of 1 μM [^{3}H]-adenosine (1 μCi) and excess (100 μM) competitor (uridine, cytidine, guanosine, inosine, thymidine, or hypoxanthine) or 2 mM phloridzin. Apical samples (5 μL) were collected over 60 min and processed for HPLC analysis, as previously described (*20*).

Adenosine and ATP Concentrations. The impact of 10 μM EHNA and/or 2 mM phloridzin on ASL adenosine and ATP levels was determined on primary cultures of nasal epithelial cells. After an incubation of 1 or 24 h [0.3 mL of apical Hanks' basal salt solution (HBSS) containing the inhibitor-(s)/0.3 mL of basolateral ALI culture medium], 0.2 mL of apical HBSS was collected to be processed by ethenyl derivatization and HPLC analysis, as previously described (*5*).

Reverse Transcriptase−Polymerase Chain Reaction. Total RNA from primary cultures of human nasal and bronchial epithelial cells was extracted with an RNeasy RNA extraction kit (Qiagen, Valencia, CA). The RT-PCR reactions (melting points 58−61 °C) used specific primer sets for ADA1, ADA2, CNT1, CNT2, CNT3, IL-13, and MCP-1 spanning exon−intron boundaries (Table 1), as previously described (*18*). The PCR products, visualized on a 2% agarose gel, were extracted and the identity was verified by sequence analysis. Positive RT-PCR controls were commercially available for total RNA from kidney, colon, and lung parenchyma (Clontech, Palo Alto, CA). Negative PCR control reactions were performed without cDNA.

Measurements of IL-13 and MCP-1. Epithelial cultures were incubated for 1 or 24 h with 10 μM EHNA, 2 mM phloridzin, 0.01−0.1 μM NECA, and/or 0.1 mM 8-SPT added to the apical surface in a final volume maintaining air−liquid culture conditions. The ASL was collected by a 0.1 mL KRB wash and filtered to eliminate mucin, and then IL-13 and MCP-1 concentrations were determined by cytokine BioPlex assays (Biorad Laboratories, CA) according to the manufacturer's recommendations. The assays were conducted in 96-well plates and cytokine concentrations were quantified against standard curves by use of a Luminex-100 (xMAP Technology).

Materials. [2-^{3}H]Adenosine (1.0 mCi/mL) was from Amersham Biosciences (Piscataway, NJ), and [1-^{14}C]mannitol (0.1 mCi/mL) was from Perkin-Elmer Life Sciences (Wellesley, MA). Culture media, bovine serum albumin, fetal bovine serum, bovine pituitary extract, epidermal growth factor, penicillin, retinoic acid, DNase, human placenta collagen VI, NECA, streptomycin, EHNA, phloridzin, adenosine, uridine, inosine, guanosine, thymine, hypoxanthine, and cytidine were from Sigma Chemical Co. (St. Louis, MO). Salts and solvents were of analytical grade.

Data Analysis. All experiments were performed on nasal or bronchial cultures from ≥3 donors. HPLC chromatograms were analyzed with the software Flo-One (Perkin-Elmer, Boston, MA). All values were expressed as mean ± SEM. Statistical significance was tested by unpaired *t* tests for two data sets from different cultures. Multiple sets were compared by one-way analysis of variance followed by a post-hoc Newman Keuls test. Differences were significant at $p < 0.05$.

RESULTS

Airway Epithelia Eliminate Extracellular Adenosine. The regulation of airway adenosine was investigated in well-differentiated polarized primary cultures of human nasal epithelial cells. While we previously showed that ASL adenosine is produced by dephosphorylation of released ATP (*18*), this work addresses mechanisms regulating adenosine elimination. Nasal cultures, incubated in bilateral KRB buffer, were tested via a physiological [^{3}H]adenosine concentration (1 μM) added to the apical surface. HPLC analysis of apical buffer samples indicated that adenosine concentrations decrease over time, as inosine and hypoxanthine accumulate (Figure 1A). The presence of inosine supported the involvement of a cell surface ADA activity. On the other hand, the rate of adenosine decay was found to be significantly higher than the rate of inosine + hypoxanthine accumulation (Figure 1B), suggesting that airway epithelia are permeable to nucleosides and/or nucleobases. Altogether, these results suggested that adenosine elimination is regulated by metabolic and transport activities on the apical surface of human airway epithelia.

Identification of the Airway Adenosine Deaminase(s). Two ADA isoforms have been identified in human body fluids and tissues: ADA1 and ADA2 (*21*). While specific ADA2 inhibitors remain unavailable, the contribution of ADA1 to total ADA activity is classically measured by use of EHNA (*21*). Dose−response curves constructed on the apical surface of nasal cultures with 100 μM adenosine showed that 10 μM EHNA completely inhibits the cell surface conversion of adenosine to inosine (Figure 2A). The ADA isoform

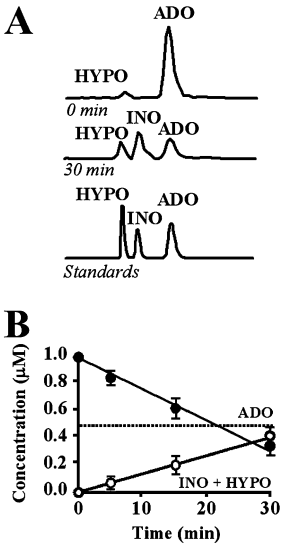

FIGURE 1: Adenosine elimination on the apical surface of human nasal epithelia. (A) Representative HPLC traces for buffer (KRB, pH 7.4) samples collected 0 or 30 min after addition of 1 μM [^{3}H]-adenosine to the apical surface. (B) Linear regression comparing the rates of adenosine elimination (●) and inosine + hypoxanthine accumulation (○) over 30 min ($R^2 = 0.99$). ADO, adenosine; INO, inosine; HYPO, hypoxanthine ($N = 3-5$).

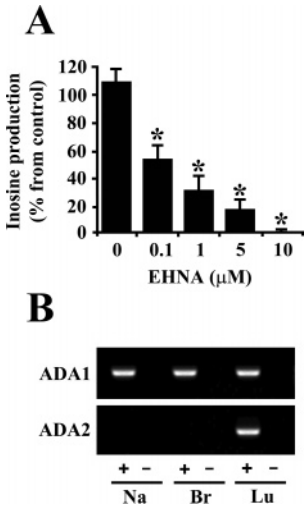

FIGURE 2: Identification of the airway adenosine deaminase (ADA) isoforms. (A) Dose-dependent inhibition by the ADA1 inhibitor EHNA. Nasal epithelial cultures were preincubated 30 min in bilateral KRB (pH 7.4) with 0−10 μM EHNA and then assayed on the apical surface with 0.1 mM adenosine. Total ADA activity was measured by the rate of inosine production. (B) Messenger RNA distribution of the ADA isoforms. Representative agarose gels show RT-PCR products generated with primers specific to ADA1 or ADA2 and total RNA from nasal and bronchial epithelial cultures. Tissue control: human lung parenchyma. Negative controls (−): PCRs in the absence of cDNA ($N = 4$; *, $p < 0.05$).

expressed by human airway epithelia was confirmed at the mRNA level by RT-PCR. Figure 2B shows that ADA1 (not ADA2) is expressed by nasal and bronchial epithelia. The detection of both ADA isoforms in lung parenchyma is consistent with their reported coexpression in monocytes and macrophages (*21*, *36*, *37*). These functional and expression data demonstrate that ADA1 is responsible for the metabolic elimination of ASL adenosine.

Airway Epithelia Are Permeable to Adenosine. The permeability of the epithelial barrier to ASL adenosine was investigated to determine the relative contribution of transporters and passive diffusion. The permeability coefficients of [^{3}H] adenosine and [^{14}C]mannitol were measured by Ussing chamber experiments conducted in the presence of 10 μM EHNA to inhibit ADA_1 activity (Figure 2A). Figure 3A shows that P_{ADO} was 18 times higher than P_{MAN}, suggesting a minimal (<5%) involvement of passive diffusion. On the other hand, two families of nucleoside/nucleobase transporters have been identified in human tissues: Na^+-independent ENTs and Na^+-dependent CNTs (*26*). The broad-range ENT inhibitor dipyridamole had no significant effect on adenosine uptake at concentrations used in other tissues (1−100 μM) (*31*, *32*). In contrast, the CNT inhibitor, phloridzin, induced a dose-dependent decrease in adenosine uptake (Figure 3B). Optimal phloridzin concentration (2 mM) reduced adenosine uptake by 80%. The importance of CNTs was further demonstrated in Ussing chambers by measuring the apical-to-basolateral permeability of [^{3}H]adenosine. Since nucleosides and nucleobases leave passively airway epithelia on the basolateral side through ENTs (*38*), we further addressed the identity of apical CNTs by measuring the accumulation of radiolabeled compounds in the basolateral bath following the addition of apical [^{3}H]-adenosine. Basolateral radioactivity was reduced by 85% in

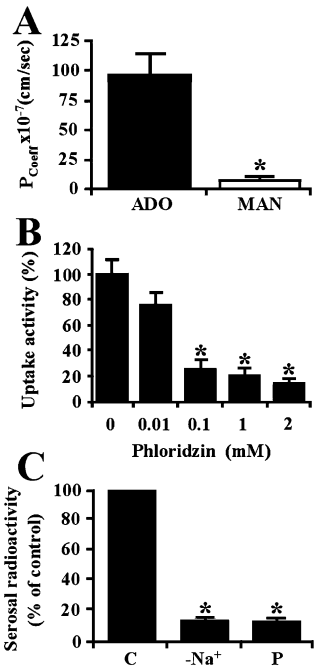

FIGURE 3: Adenosine uptake on the apical surface of human airway epithelia. (A) Ussing chamber measurements of paracellular diffusion across cultured nasal epithelia. Reactions were initiated in bilateral KRB-EHNA (pH 7.4) with apical [^{3}H]adenosine (0.01 μCi/μL; 10 μM) and [^{14}C]mannitol (0.2 μCi/μL). Basolateral buffer samples were collected over 120 min to calculate the permeability coefficients (P_{coeff}) of [^{3}H]adenosine (ADO) and [^{14}C]mannitol (MAN). (B) Dose-dependent inhibition by the CNT inhibitor phloridzin. Nasal cultures were preincubated for 30 min in bilateral KRB (pH 7.4) with 0−2 mM phloridzin and then assayed on the apical surface with [^{3}H]adenosine (0.01 μCi/μL; 10 μM). Adenosine uptake was measured by the rate of apical radioactivity decay. (C) Contribution of CNTs to apical adenosine uptake. Nasal cultures in Ussing chambers were preincubated in KRB-EHNA (C), KRB-EHNA-low Na^+ ($-Na^+$), or KRB-EHNA + 2 mM phloridzin (P) and then incubated with apical [^{3}H]adenosine (0.01 μCi/μL; 10 μM). Basolateral radioactivity was measured after 120 min ($N = 3-5$; *, $p < 0.01$).

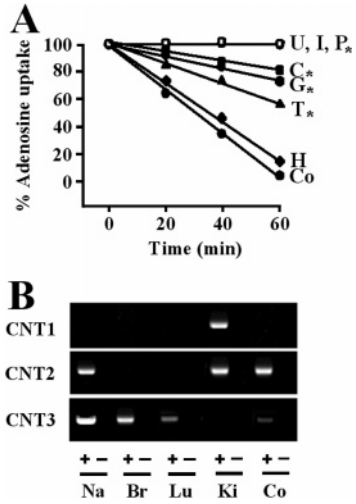

FIGURE 4: Identification of the CNTs expressed by human airway epithelia. (A) Competitive inhibition of adenosine uptake. Primary nasal cultures were preincubated in bilateral KRB-EHNA and then assayed with [^{3}H]adenosine (0.01 μCi/μL; 1 μM) and 100 μM competitor (cytidine, C; thymidine, T; inosine, I; hypoxanthine, H; guanosine, G; uridine, U) or 2 mM phloridzin (P). Adenosine uptake was quantified by HPLC from apical buffer samples collected over 60 min. (B) Representative agarose gels for RT-PCR products generated with primers specific for CNT1, CNT2, and CNT3 and total RNA from primary cultures of human nasal (Na) or bronchial (Br) epithelial cells. Tissue controls: human lung parenchyma (Lu), kidney (Ki), and colon (Co). Negative controls (−): PCRs in the absence of cDNA ($N = 3$; *, $p < 0.05$).

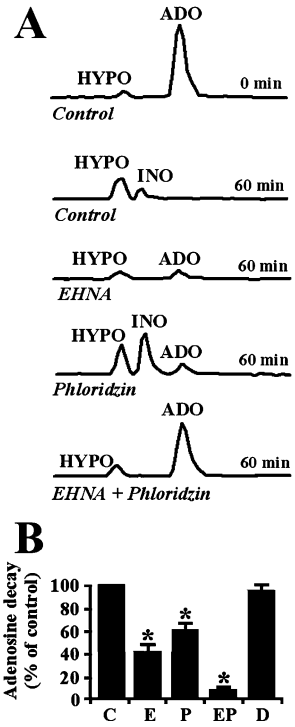

FIGURE 5: Relative contribution of ADA1 and CNTs to the elimination of adenosine on nasal epithelia. (A) Representative HPLC traces for apical buffer samples collected from epithelial cultures preincubated for 30 min without (C; control) or with 10 μM EHNA (E; ADA1 inhibitor), 2 mM phloridzin (P; CNTs inhibitor) or 10 μM dipyridamole (D; ENT inhibitor) and then for 60 min with apical [^{3}H]adenosine (0.01 μCi/μL; 1 μM). ADO, adenosine; INO, inosine; HYPO, hypoxanthine. (B) Quantification of adenosine decay, based on the HPLC traces, and expressed as percent adenosine elimination rate from control (C) conditions ($N = 4$; *, $p < 0.05$).

low Na^+ buffer or by 2 mM phloridzin (Figure 3C). Collectively, these functional assays suggest that adenosine uptake occurs primarily through Na^+-dependent CNTs on the apical surface of human nasal epithelia.

The nucleoside transporter activities were also characterized by competition assays on nasal epithelia. Adenosine uptake was monitored on EHNA-pretreated cultures by HPLC analysis of apical buffer samples collected over 60 min after simultaneous addition of 1 μM [^{3}H]adenosine and excess (100 μM) competitor. Whereas adenosine uptake was not affected by hypoxanthine, all other competitors significantly reduced adenosine uptake in the following order: inosine = uridine > cytidine = guanosine > thymidine (Figure 4A), consistent with CNT2 and/or CNT3 activities (*39*−*44*). The identity of the CNT isoforms was confirmed by RT-PCR using total RNA from nasal and bronchial epithelial cultures. The kidney isoform, CNT1, was not detected in nasal or bronchial epithelia (Figure 4B). In contrast, CNT2 was highly expressed in nasal epithelia but not in bronchial epithelia or lung parenchyma. Strong signals were obtained for CNT3 in nasal and bronchial epithelia and weaker bands in lung parenchyma. These data suggest that CNT2 and CNT3 mediate apical adenosine uptake on airway epithelia.

Relative Contributions of ADA and CNTs to ASL Adenosine Regulation. The impact of cell surface metabolism and cellular uptake on ASL adenosine elimination was investigated on nasal epithelial cultures by HPLC analysis with a physiological adenosine concentration (1 μM [^{3}H]adenosine), and optimum concentrations of EHNA (10 μM; Figure 2A) and phloridzin (2 mM; Figure 3B). Analysis of apical buffer samples, collected 60 min after the addition of 1 μM [^{3}H]-adenosine, showed that the nucleoside is replaced by [^{3}H]-inosine and [^{3}H]hypoxanthine (Figure 5A). The ADA1 inhibitor, EHNA, completely inhibited inosine formation without preventing the loss of total HPLC counts. In contrast, the CNT inhibitor maintained total HPLC counts without affecting ADA1 activity. Simultaneous exposures to EHNA and phloridzin generated HPLC traces similar to those at the onset of experiment. Quantitative analysis of the HPLC traces showed that ADA1 and CNTs account for 40% (EHNA-sensitive) and 60% (phloridzin-sensitive) of the decay rate of 1 μM adenosine on nasal epithelia (Figure 5B). Simultaneous exposure to both inhibitors reduced adenosine elimination by >90%. In contrast, the ENT inhibitor, 100 μM dipyridamole (*31*, *32*), had no effect on adenosine decay. These experiments demonstrate that two mechanisms contribute significantly to the elimination of ASL adenosine on nasal epithelia: surface metabolism by ADA1 and cellular uptake by CNTs.

The relative efficiency of CNTs and ADA1 in regulating physiological or pathological adenosine concentrations depends on their kinetic properties, we determined on nasal epithelia. The EHNA-sensitive rate of adenosine decay, measured over a wide range of substrate concentrations, generated a saturable Michaelis−Menten relationship for ADA1 activity (Figure 6A). The K_m and V_{max} values calculated from a Woolf−Augustinson Hoftsee transformation were 24.1 ± 3.5 μM and 0.14 ± 0.04 nmol·min^{-1}·cm^{-2}. On the other hand, uptake measurements for 1−100 μM adenosine, in EHNA-pretreated nasal cultures, generated a

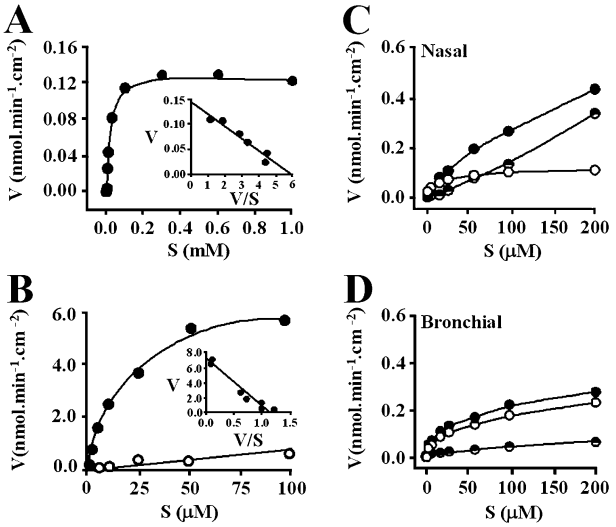

FIGURE 6: Kinetic properties of ASL adenosine metabolism and uptake. (A) Michaelis−Menten saturation curve for ADA1. Nasal epithelial cultures were assayed with 0.001−1.0 mM [^{3}H]adenosine and then washed and re-assayed following a 30 min preincubation with 10 μM EHNA (ADA1 inhibitor). ADA1 activity was calculated at each adenosine concentration from the difference between these two assays. (Inset) K_m (24 μM) and V_{max} (0.14 nmol·min^{-1}·cm^{-2}) calculated from the slope and ordinate of a Woolf−Augustinson Hoftsee transformation. (B) Michaelis−Menten saturation curve for apical adenosine uptake. All assays were initiated with [^{3}H]adenosine in KRB-EHNA (●) or in low-Na^+ KRB containing 2 mM phloridzin to calculate passive diffusion (○). (Inset) Woolf−Augustinson Hoftsee transformation providing K_m and V_{max} values of 17 μM and 7.2 nmol·min^{-1}·cm^{-2}, corrected for passive diffusion. (C, −D) Dose−response curves of ADA1 and CNT activities on nasal and bronchial epithelia. (N = 3−5; error bars < symbols).

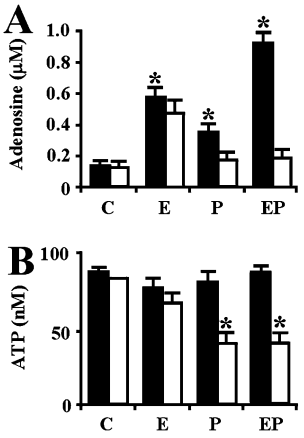

FIGURE 7: Physiological role of ADA1 and CNTs in the regulation of airway adenosine. Measurements of endogenous (A) adenosine and (B) ATP concentrations on the apical surface of nasal epithelial cultures after 1 h (solid bars) or 24 h (open bars) pretreatments without (C; control) or with 10 μM EHNA (E; ADA1 inhibitor), 2 mM phloridzin (P; CNTs inhibitor), or both are shown. Apical adenosine and ATP concentrations were determined by ethenyl derivatization and HPLC analysis (N = 4; *, p < 0.05 for differences between control and treatments).

typical saturation carrier-mediated transport kinetic (Figure 6B). Passive diffusion, assessed in low-Na^+ KRB containing 10 μM EHNA and 2 mM phloridzin, was linear over the concentration range. The Woolf−Augustinson Hoftsee transformation of the data, corrected for passive movement, generated K_m and V_{max} values of 17.3 ± 5.3 μM and 7.2 ± 0.6 nmol·min^{-1}·cm^{-2}. These experiments revealed a 7-fold higher catalytic efficiency for CNTs over ADA1 on nasal epithelial surfaces, with values of 0.023 min^{-1} and 0.003 min^{-1}, respectively. These data predict that both mechanisms contribute significantly to adenosine elimination at physiological levels (0.1−1 μM) on nasal epithelia, as shown in Figure 1B, owing to their similar substrate affinities (K_m). On the other hand, CNT uptake activities may dominate at higher adenosine levels encountered under pathological conditions. This hypothesis was verified by comparing the activities of ADA1 and CNTs at adenosine concentrations encountered under basal conditions (*5*) and in airway diseases (>100 μM) (*5*, *9*). Figure 6C shows that ADA1 activity was higher than CNT activities for adenosine concentrations <50 μM, while higher adenosine levels were eliminated mainly by cellular uptake. Bronchial epithelia regulated ASL adenosine primarily through ADA1 activity, as CNT activities accounted for <20% of total adenosine decay over the entire concentration range (Figure 6D). The activity of ADA1 toward 200 μM adenosine was 2-fold higher on bronchial than on nasal epithelia, whereas CNT activity was reduced by >10-fold, in part due to the loss of one isoform (Figure 4B). These data suggest that CNTs are most effective in nasal epithelia, whereas ADA1 activity plays a key role in regulating ASL adenosine in the lower airways.

Impact of ADA and CNTs on Adenosine-Mediated Inflammation. The physiological importance of ADA1 and CNTs was demonstrated by testing the impact of their specific inhibitors on ASL adenosine levels. Nasal epithelial cultures were incubated for 1 or 24 h on the apical surface with 10 μM EHNA (Figure 2A), 2 mM phloridzin (Figure 3B), or both, and then apical endogenous adenosine concentrations were measured by ethenyl derivatization and HPLC. Figure 7A shows that ADA1 inhibition raises ASL adenosine levels by 3-fold. Similar findings were obtained with 1 and 24 h treatments. On the other hand, the CNT blocker phloridzin induced a 2-fold increase in surface adenosine level after 1 h but not after 24 h. Simultaneous inhibition of ADA1 and CNTs generated additive effects on adenosine after 1 h but not 24 h. The transient response to phloridzin reiterates the importance of nucleotide salvage pathways for the maintenance of ASL adenosine levels on airway epithelia (*18*). Surface adenosine and inosine are continuously returned to the cytosol to regenerate ATP, the later being released to maintain basal ASL adenosine levels through dephosphorylation by ectonucleotidases (*20*, *45*). This hypothesis was further tested by concomitant measurements of endogenous ATP levels on the apical surface of the same cultures. Figure 7B shows that short-term (1 h) treatments with EHNA or phloridzin did not significantly affect ATP. However, prolonged inhibition of adenosine/inosine uptake (not ADA1 activity) resulted in a 2-fold decrease in surface ATP concentration (Figure 7B) without inhibiting cell surface ATPase activity (data not shown). Altogether, these results illustrate the role of ADA1 and CNTs in the regulation of ASL adenosine, as well as ATP, through the nucleotide salvage pathway. Similar manipulations of surface adenosine were also reported to affect intracellular ATP concentrations on other cell types depending on the nucleotide salvage pathway (*46*, *47*).

The contribution of chronically elevated ASL adenosine to airway inflammation was demonstrated by testing the

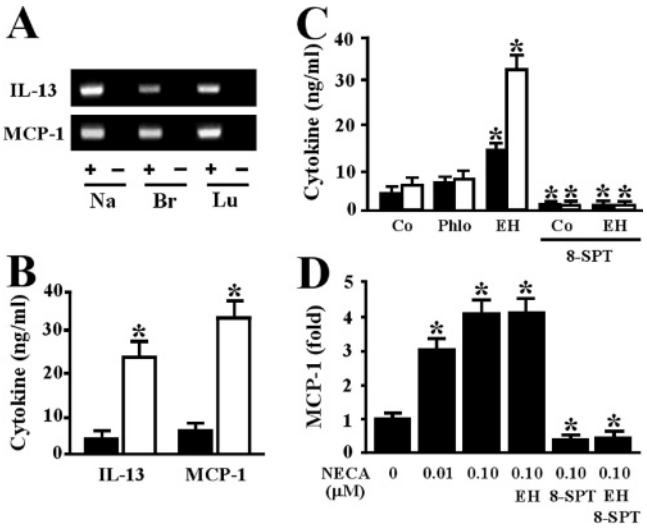

FIGURE 8: Chronically elevated airway adenosine stimulates apical cytokine secretion through adenosine receptors. (A) Representative agarose gels for RT-PCR products generated with primers specific for IL-13 and MCP-1 and RNA from nasal (Na) or bronchial (Br) epithelial cultures. Tissue control: human lung parenchyma (Lu). Negative controls (−): PCRs in the absence of cDNA. (B) Bronchial epithelial cultures incubated 24 h without (solid bars) or with (open bars) 2 mM phloridzin and 0.01 mM EHNA. Simultaneous inhibition of adenosine elimination by EHNA and phloridzin raised ASL IL-13 and MCP-1 levels by >8-fold. (C) Bronchial cultures incubated 24 h without (Co; control) or with 2 mM phloridzin (Phlo), 0.01 mM EHNA (EH), and/or a broad-range adenosine receptor antagonist (0.1 mM 8-SPT) and then assayed for apical IL-13 (solid bars) or MCP-1 (open bars). (D) Dose−response of bronchial cultures to the stable adenosine receptor agonist NECA (24 h; 0−0.1 μM), 0.1 μM NECA and/or 8-SPT, or 0.01 mM EHNA (EH). ADA1 inhibition increases apical cytokine release through adenosine receptor activation ($N = 5$; *, $p < 0.01$).

impact of ADA1 or CNTs inhibition on the secretion of two markers of eosinophilic airway inflammation: IL-13 and MCP-1 (*48*). The presence of these cytokines in airway epithelia was first verified by RT-PCR (Figure 8A). We then demonstrated, for the first time, that human bronchial epithelia secrete IL-13 and MCP-1 on the apical surface under basal conditions (Figure 8B). Furthermore, combined 24 h treatments with apical 0.01 mM EHNA and 2 mM phloridzin raised IL-13 and MCP-1 concentrations by ≥8-fold. Figure 8C shows that individual 24 h exposures to EHNA, but not phloridzin, also induced significant responses for both cytokines. The lack of effect of phloridzin is in agreement with the small contribution of CNTs on bronchial epithelia, compared to ADA1 (Figure 6D) (*18*). Interestingly, the adenosine receptor antagonist 8-SPT (*49*) significantly reduced both basal and EHNA-induced IL-13 and MCP-1 secretion. The involvement of adenosine receptors was further tested by use of the stable adenosine receptor agonist NECA (*50*). Figure 8D shows that NECA induced a dose-dependent increase in MCP-1 secretion over a concentration range known to stimulate cilia beating activity on these cells (*4*). In addition, this response to NECA was completely abrogated by 8-SPT. Altogether, these data suggest that EHNA stimulates cytokine secretion through increased availability of apical adenosine for receptor activation. The specificity of EHNA-mediated responses for adenosine-dependent mechanisms was demonstrated by the lack of additivity of optimal NECA and EHNA concentrations on MCP-1 secretion. These results demonstrate the pivotal role of ADA1 and CNTs in the regulation of ASL adenosine, as well as adenosine-mediated inflammatory responses, on the apical surface of human airway epithelia.

DISCUSSION

Adenosine, a biologically active purine nucleoside detected in the ASL layer of human airway epithelia (*5*), plays an important role in the maintenance of epithelial functions essential for bacterial clearance (*4*−*8*). Chronically elevated adenosine, however, is implicated in airway inflammation (*9*, *11*−*15*, *17*, *51*−*55*). An imbalance in the rates of production/elimination of ASL adenosine may contribute to the severity of chronic lung diseases, such as asthma (*11*, *55*). Our present knowledge of the mechanisms regulating ASL adenosine was incomplete. The main source of adenosine on apical epithelial surfaces was clearly identified as the dephosphorylation (*18*, *20*) of released ATP (*19*), and the ectonucleotidases directly responsible for adenosine production were identified as ecto-5′-NT and NS AP (*18*). However, the mechanisms regulating the elimination of airway adenosine remained unknown.

This study demonstrates that ASL adenosine is eliminated by a combination of surface metabolism and cellular uptake. We show that adenosine, added to the apical surface of nasal epithelia, is gradually replaced by inosine and hypoxanthine. It is widely accepted that extracellular adenosine is eliminated through deamination by ADA activities in human tissues (*21*). The subsequent conversion of inosine to hypoxanthine is generally mediated by purine nucleoside phosphorylase, a cytosolic enzyme of the nucleotide salvage pathways (*56*) detected in various body fluids (*57*, *58*). Incidentally, cultured A549 alveolar epithelial cells incubated with inosine were shown to generate extracellular hypoxanthine (*59*). These findings support the linear conversion of ASL adenosine to inosine and inosine to hypoxanthine.

The identity of the ADA isoform(s) responsible for the metabolic elimination of airway adenosine was determined by expression and functional assays. First, RT-PCR experiments demonstrated that nasal and bronchial epithelia express ADA1 but not ADA2. These data are consistent with the wide distribution of ADA1 (*21*) and the localization of ADA2 limited to monocytes/macrophages (*21*, *36*, *37*). Second, the conversion of adenosine to inosine was completely inhibited by the ADA1 inhibitor EHNA (*21*). Finally, the K_m of airway ADA (24 μM) is in the range reported for cell surface ADA1 activities (20−50 μM) (*21*). In contrast, the K_m of ADA2 (2 mM) (*60*) is several orders of magnitude higher than the K_m of ADA1 and physiological adenosine concentrations (*21*). The gene encoding ADA2 was recently identified as the cat eye syndrome critical region candidate 1, member of a novel family of growth factors (*60*). Altogether, these results demonstrate that ASL adenosine is metabolically eliminated by ADA1 on human airway epithelia.

Comparative analysis of adenosine decay and inosine production revealed that cell surface metabolism does not account for the total rate of adenosine elimination from apical epithelial surfaces. Complete inhibition of adenosine deamination by EHNA (*21*) was unable to prevent the disappearance of exogenous adenosine. These data suggested that airway epithelia are permeable to adenosine. The contribution

of paracellular passive diffusion across the epithelial barrier was found to be negligible on the basis of the relatively low permeability of mannitol compared to adenosine (P_{ADO}/P_{MAN} = 18:1). On the other hand, we provide evidence that nucleoside/nucleobase transporters are responsible for the permeability of airway epithelia to ASL adenosine. Numerous reviews have described the properties and distribution of two transporter families: Na^+-independent ENTs and Na^+-dependent CNTs (*26*). The contribution of apical ENTs to ASL adenosine elimination was previously ruled out because airway epithelia are unable to transport basolateral and cytosolic adenosine to the apical surface (*18*). In this work, this finding was confirmed by the inability of the ENT inhibitor dipyridamole to reduce apical adenosine uptake (*31*, *32*). In contrast, inhibition of apical CNT activities by low-Na^+ buffer or phloridzin reduced adenosine uptake by 85%. Collectively, these data demonstrate that Na^+-dependent CNTs are responsible for the permeability of airway epithelia to ASL adenosine.

The CNT isoforms expressed on the apical surface of human airway epithelia were identified on the basis of kinetic properties, substrate specificity, and mRNA expression. All functional assays were conducted in the presence of EHNA to prevent adenosine decay by surface metabolism. Adenosine uptake, corrected for passive diffusion, exhibited a K_m (17 μM) in the range reported for CNT2 (8−23 μM) (*61*, *62*) and CNT3 (5−15 μM) (*39*, *40*) but significantly lower than those of ENTs (40−1900 μM) (*26*). Although originally viewed as a substrate for CNT1, adenosine was recently identified as a blocker of the transporter (*63*).

The substrate specificity of the Na^+-dependent adenosine transporters supported the coexpression of CNT2 and CNT3 on the apical surface of nasal epithelia. Adenosine uptake was not affected by hypoxanthine, as reported for CNT2 and CNT3 (*41*, *42*). In contrast, all other competitors inhibited adenosine transport [% inhibition: inosine (100) = uridine (100) > cytidine (89), guanosine (81) > thymidine (60)]. The broad substrate specificity of nasal CNTs for purines and pyrimidines supported the expression of CNT3 (*40*). In addition, thymidine and cytidine are substrates of CNT3 but not CNT2 (*40*). On the other hand, the potency order reflected more closely the specificity of CNT2 (adenosine = guanosine > uridine) (*43*, *44*) than CNT3 (uridine > adenosine = guanosine) (*39*). Incidentally, two high-affinity ($K_m \sim 10$ μM) CNTs have been colocalized on renal brush-border membranes, displaying broad substrate specificity toward purines and pyrimidines and selectivity for purines, respectively (*64*). These data suggest that CNT2 and CNT3 both contribute to adenosine uptake on the apical surface of human nasal epithelia. These findings were corroborated at the mRNA level by RT-PCR. The expression of CNT2 was restricted to nasal passages, whereas CNT3 mRNA was detected in nasal and bronchial epithelial cells as well as lung parenchyma. These data are in agreement with the previous report of CNT3 in human trachea by Northern blot (*40*). Taken together, functional and expression data support a major role for CNT2 and CNT3 in apical adenosine uptake by human airway epithelia.

Earlier studies using cell lines suggest that ENTs regulate apical adenosine uptake on human airway epithelia. For instance, alveolar A549 cells were shown to express ENT1 and ENT2 (*65*, *66*). The Calu-3 airway submucosal gland cell line exhibited mRNA for ENT2 and CNT3, although adenosine uptake was supported mainly by ENT2 on both surfaces (*67*). Similar discrepancies have been reported for intestinal epithelia between studies that used fresh preparations and cell lines. Intestinal CNT-mediated nucleoside transport activity was detected in enterocytes (*68*) and brush-border vesicles (*69*) but not in the IEC-6 cell line (*70*). It is important to mention that this cell line fails to differentiate in conventional media (*71*). The addition of hormones and growth factors to the medium allowed IEC-6 cells to differentiate, and CNTs became the predominant adenosine transporters on the apical surface (*70*). In this study, nasal and bronchial cultures were maintained in medium containing hormones and growth factors promoting differentiation and the establishment of a polarized epithelium (*18*). These studies, therefore, emphasize the need for a well-differentiated polarized epithelial barrier for the apical expression of CNTs.

The relative importance of ADA1 and CNTs on nasal and bronchial epithelia was determined by comparing the effects of optimal concentrations of EHNA (*21*) (10 μM; Figure 2B) and phloridzin (*28*−*30*) (2 mM; Figure 3B) on the elimination of ASL adenosine over a concentration range including basal (*5*, *72*, *73*) and pathological (>100 μM) (*5*, *9*) conditions. We showed that ADA1 and CNT activities account for 40% and 60% of the elimination of a physiological (1 μM) adenosine concentration on nasal epithelia. On the other hand, the 7-fold higher catalytic efficiency of CNTs over ADA1 suggested that CNTs may dominate at high adenosine levels encountered under pathological conditions. Incidentally, we demonstrated that ASL adenosine concentrations <50 μM are regulated more efficiently by ADA1, whereas higher adenosine levels are eliminated mainly by cellular uptake. Given the importance of lower airways for chronic lung diseases, we also examined ASL adenosine regulation on bronchial epithelia. While both mechanisms were functionally detected, adenosine was primarily (>80%) regulated by ADA1 over a wide concentration range. These findings are in agreement with the presence of only one CNT isoform in bronchial epithelia and higher ADA activity levels compared to nasal epithelia. These data suggest that CNTs are most effective in nasal epithelia, whereas ADA1 activity plays a key role in regulating ASL adenosine in the lower airways.

This study completes the regulatory model of ASL adenosine in human airways. The epithelium releases ATP (*5*), which is dephosphorylated into AMP by ectonucleotidases (*20*), and then AMP is converted to adenosine by ecto-5′-NT and NS AP (*18*). In this work, we show that ASL adenosine is eliminated by a combination of surface metabolism and cellular uptake. ADA1 converts adenosine into inosine, which are both transported to the cytosol through CNT2 and CNT3. The fact that airway ecto-5′-NT (K_m = 15 μM) (*18*), CNTs (K_m = 17 μM), and ADA1 (K_m = 24 μM) all exhibit similar high substrate affinities supports the presence of a highly integrated system for the regulation of ASL adenosine. On the other hand, the higher catalytic efficiency of ecto-5′-NT (0.041 min^{-1}) (*18*) compared to CNTs (0.023 min^{-1}) and ADA1 (0.003 min^{-1}) would maintain the basal adenosine level found to be essential for the regulation of ASL height through A_{2B} receptor-mediated CFTR activity (*7*). In contrast, chronically elevated airway

adenosine has been reported in asthmatic patients (*9*) and causes airway inflammation in animal models (*10*). However, the contribution of airway epithelia in adenosine-mediated inflammation had not been addressed. In this study, we demonstrate that a reduction in ASL adenosine elimination dramatically enhances the apical secretion of IL-13 and MCP-1, two markers of eosinophilic lung diseases (*48*). The involvement of adenosine receptor activation was demonstrated by the stimulating effects of NECA (*50*) on cytokine release, which was completely inhibited by the broad-range adenosine receptor antagonist 8-SPT (*49*). The lack of additivity of optimum NECA and EHNA concentrations and the lack of response of the epithelia to EHNA in the presence of 8-SPT both support the specificity of the ADA1 inhibitor for adenosine-dependent mechanisms. The parallel increase in apical IL-13 and MCP-1 secretion is consistent with the reported upregulation of MCP-1 secretion induced by IL-13 in primary cultures of human bronchial epithelial cells (*74*). Interestingly, IL-13 expression is strongly induced by adenosine in ADA-null mice (*75*). Hence, the existence of adenosine-mediated IL-13 secretion on apical surfaces suggests that the epithelium contributes to the inflammatory phenotype of asthmatic patients. On the other hand, ADA1 and CNT activities in healthy airways may suppress airway inflammation while maintaining adenosine-mediated bacterial clearance (*1*−*8*). The identification of the proteins regulating ASL adenosine and the pharmacological tools regulating their activities open a new field of research on the role of airway adenosine in chronic inflammatory lung diseases.

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REFERENCES

1. Wanner, A., Salathe, M., and O'Riordan, T. G. (1996) Mucociliary clearance in the airways, *Am. J. Respir. Crit. Care Med. 154*, 1868−1902.
2. Adler, K. B., Wooten, O., and Dulfano, M. J. (1973) Mammalian mucociliary clearance, *Arch. Environ. Health 27*, 364−369.
3. Boucher, R. C. (1999) Molecular insights into the physiology of the 'thin film' of airway surface liquid, *J. Physiol. 516*, 631−638.
4. Morse, D. M., Smullen, J. L., and Davis, C. W. (2001) Differential effects of UTP, ATP, and adenosine on ciliary activity of human nasal epithelial cells, *Am. J. Physiol. 280*, C1485−C1497.
5. Lazarowski, E. R., Tarran, R., Grubb, B. R., van Heusden, C. A., Okada, S., and Boucher, R. C. (2004) Nucleotide release provides a mechanism for airway surface liquid homeostasis, *J. Biol. Chem. 279*, 36855−36864.
6. Clancy, J. P., Ruiz, F. E., and Sorscher, E. J. (1999) Adenosine and its nucleotides activate wild-type and R117H CFTR through an A_{2B} receptor-coupled pathway, *Am. J. Physiol. 276*, C361−C369.
7. Huang, P., Lazarowski, E. R., Tarran, R., Milgram, S. L., Boucher, R. C., and Stutts, M. J. (2001) Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells, *Proc. Natl. Acad. Sci. U.S.A. 98*, 14120−14125.
8. Cobb, B. R., Ruiz, F., King, C. M., Fortenberry, J., Greer, H., Kovacs, T., Sorscher, E. J., and Clancy, J. P. (2002) A_2 adenosine receptors regulate CFTR through PKA and PLA2, *Am. J. Physiol. 282*, L12−L25.
9. Driver, A. G., Kukoly, C. A., Ali, S., and Mustafa, S. J. (1993) Adenosine in the bronchoalveolar lavage fluid in asthma, *Am. Rev. Respir. Dis. 148*, 91−97.
10. Blackburn, M. R., Kellems, R. E., and Frederick, W. A. (2005) Adenosine deaminase deficiency: metabolic basis of immune deficiency and pulmonary inflammation, *Adv. Immunol. 86*, 1−41.
11. Wasserman, S. I. (1987) The regulation of inflammatory mediator production by mast cell products, *Am. Rev. Respir. Dis. 135*, S46−S48.
12. Holgate, S. T., Church, M. K., and Polosa, R. (1991) Adenosine: a positive modulator of airway inflammation is asthma, *Ann. N.Y. Acad. Sci. 629*, 227−236.
13. Walker, B. A., Jacobson, M. A., Knight, D. A., Salvatore, C. A., Weir, T., Zhou, D., and Bai, T. R. (1997) Adenosine A_3 receptor expression and function in eosinophils, *Am. J. Respir. Cell Mol. Biol. 16*, 531−537.
14. Nagano, Y., Tamaoki, J., Nishimura, K., Yamawaki, I., and Nagai, A. (2000) Adenosine A_3 receptor-mediated airway microvascular leakage: role of mast cells and tachykinins, *Res. Commun. Mol. Pathol. Pharmacol. 108*, 96−107.
15. Chunn, J. L., Young, H. W. J., Banerjee, S. K., Colasurdo, G. N., and Blackburn, M. R. (2001) Adenosine-dependent airway inflammation and hyperresponsiveness in partially adenosine deaminase-deficient mice. *J. Immunol. 167*, 4676−4685.
16. Zhong, H., Wu, Y., Belardinelli, L., and Zeng, D. (2006) A_{2B} adenosine receptors induce IL-19 from bronchial epithelial cells and results in TNFα increase, *Am. J. Respir. Cell Mol. Biol. 35*, 587−592.
17. Zhong, H., Belardinelli, L., Maa, T., Feoktistov, I., Biaggioni, I., and Zeng, D. (2004) A_{2B} adenosine receptors increase cytokine release by bronchial smooth muscle cells, *Am. J. Respir. Cell Mol. Biol. 30*, 118−125.
18. Picher, M., Burch, L. H., Hirsh, A. J., Spychala, J., and Boucher, R. C. (2003) Ecto 5′-nucleotidase and nonspecific alkaline phosphatase. Two AMP-hydrolyzing ectoenzymes with distinct roles in human airways, *J. Biol. Chem. 278*, 13468−13479.
19. Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules, *Mol. Pharmacol. 64*, 785−795.
20. Picher, M., Burch, L. H., and Boucher, R. C. (2004) Metabolism of P2 receptor agonists in human airways: implications for mucociliary clearance and cystic fibrosis, *J. Biol. Chem. 279*, 20234−20241.
21. Franco, R., Casado, V., Ciruela, F., Saura, C., Mallol, J., Canela, E. I., and Lluis, C. (1997) Cell surface adenosine deaminase: much more than an ectoenzyme, *Prog. Neurobiol. 52*, 283−294.
22. Martin, M., Huguet, J., Centelles, J. J., and Franco, R. (1995) Expression of ecto-adenosine deaminase and CD26 in human T cells triggered by the TCR-CD3 complex. Possible role of adenosine deaminase as co-stimulatory molecule, *J. Immunol. 155*, 4630−4643.
23. Herrera, C., Casado, V., Ciruela, F., Schofield, P., Mallol, J., Lluis, C., and Franco, R. (2001) Adenosine A_{2B} receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells, *Mol. Pharmacol. 59*, 127−134.
24. Saura, C., Ciruela, F., Casado, V., Canela, E. I., Mallol, J., Lluis, C., and Franco, R. (1996) Adenosine deaminase interacts with A_1 adenosine receptors in pig brain cortical membranes, *J. Neurochem. 66*, 1675−1682.
25. Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluis, C., and Franco, R. (1996) Adenosine deaminase affects ligand-induced signaling by interacting with cell surface adenosine receptors, *FEBS Lett. 380*, 219−223.
26. King, A. E., Ackley, M. A., Cass, C. E., Young, J. D., and Baldwin, S. A. (2006) Nucleoside transporters: from scavengers to novel therapeutic targets, *Trends Pharmacol. Sci. 27*, 416−425.
27. Griffith, D. A., and Jarvis, S. M. (1996) Nucleoside and nucleobase transport systems of mammalian cells, *Biochim. Biophys. Acta 1286*, 153−181.
28. Huang, Q. Q., Harvey, C. M., Paterson, A. R., Cass, C. E., and Young, J. D. (1993) Functional expression of Na^+-dependent nucleoside transport systems of rat intestine in isolated oocytes of *Xenopus laevis*, *J. Biol. Chem. 268*, 20613−20619.
29. Hirsh, A. J., Yao, S. Y., Young, J. D., and Cheeseman, C. I. (1997) Inhibition of glucose absorption in the rat jejunum: a novel action of alpha-D-glucosidase inhibitors, *Gastroenterology 113*, 205−211.

30. Leung, G. P. H., Cheung, K. H., Tse, C. M., and Wong, P. Y. D. (2001) Na^+ reabsorption in cultured rat epididymal epithelium via the Na^+/nucleoside cotransporter, *Biol. Reprod. 64*, 764−769.
31. Cass, C. E., Young, J. D., Baldwin, S. A., Cabrita, M. A., Graham, K. A., Griffiths, M., Jennings, L. L., Mackey, J. R., Ng, A. M., Ritzel, M. W., Vickers, M. F., and Yao, S. Y. (1999) Nucleoside transporters of mammalian cells, *Pharm. Biotechnol. 12*, 313−352.
32. Pastor-Anglada, M., Cano-Soldado, P., Molina-Arcas, M., Lostao, M. P., Larrayoz, I., Martinez-Picado, J., and Casado, F. J. (2005) Cell entry and export of nucleoside analogues, *Virus Res. 107*, 151−164.
33. Gray, T. E., Guzman, K., Davis, C. W., Abdallah, L. H., and Nettesheim, P. (1996) Mucociliary clearance of serially passaged normal human tracheobronchial epithelial cells, *Am. J. Respir. Cell Mol. Biol. 14*, 104−112.
34. Fedde, K. N., Lane, C. C., and Whyte, M. P. (1988) Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells, *Arch. Biochem. Biophys. 264*, 400−409.
35. Gatzy, J. T. (1975) Ion transport across the excised bullfrog lung, *Am. J. Physiol. 228*, 1162−1171.
36. Perez-Rodriguez, E., and Jimenez Castro, D. (2000) The use of adenosine deaminase and adenosine deaminase isoenzymes in the diagnosis of tuberculous pleuritis, *Curr. Opin. Pulm. Med. 6*, 259−266.
37. Conlon, B. A., and Law, W. R. (2004) Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses, *Clin. Exp. Immunol. 138*, 14−20.
38. Hirsh, A. J., Picher, M., and Boucher, R. C. (2001) Adenosine metabolism and transport by human airway epithelium, *Ped. Pulmonol. 22*, 241.
39. Toan, S. V., To, K. W., Leung, G. H., Souza, M., Ward, J., and Tse, C. M. (2003) Genomic organization and functional characterization of the human concentrative nucleoside transporter-3 isoform (hCNT3) expressed in mammalian cells, *Pfleugers Arch. Eur. J. Physiol. 447*, 195−204.
40. Ritzel, M. W. L., Ng, A. M. L., Yao, S. Y. M., Graham, K., Loewen, S. K., Smith, K. M., Ritzel, R. G., Mowles, D. A., Carpenter, P., Chen, X.-Z., Karpinski, E., Hyde, R. J., Baldwin, S. A., Cass, C. E., and Young, J. D. (2001) Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside transporter proteins (hCNT3 and mCNT3) selective for purine and pyrimidine nucleosides, *J. Biol. Chem. 276*, 2914−2927.
41. Chang, C., Swaan, P. W., Ngo, L. Y., Lum, P. Y., Patil, S. D., and Unadkat, J. D. (2004) Molecular requirements of the human nucleoside transporters hCNT1, hCNT2, and hENT1, *Mol. Pharmacol. 65*, 558−570.
42. Mangravite, L. M., Badagnani, I., and Giacomini, K. M. (2003) Nucleoside transporters in the disposition and targeting of nucleoside analogs in the kidney, *Eur. J. Pharmacol. 479*, 269−281.
43. Lang, T. T., Selner, M., Young, J. D., and Cass, C. E. (2001) Acquisition of human concentrative nucleoside transporter 2 (hCNT2) activity by gene transfer confers sensitivity to fluoropyrimidine nucleosides in drug-resistant leukemia cells, *Mol. Pharmacol. 60*, 1143−1152.
44. Lang, T. T., Young, J. D., and Cass, C. E. (2004) Interactions of nucleoside analogs, caffeine and nicotine with human concentrative nucleoside transporters 1 and 2 stably produced in a transport-defective human cell line, *Mol. Pharmacol. 65*, 925−933.
45. Picher, M., and Burch, L. (2006) E-NTPDases in human airways: regulation and relevance for chronic lung diseases, *Purin. Signal. 2*, 399−408.
46. Fujimori, H., Yasuda, M., and Pan-Hou, H. (2002) Enhancement of cellular adenosine triphosphate levels in PC12 cells by extracellular adenosine, *Biol. Pharm. Bull. 25*, 307−311.
47. Schulze, K., Duschek, C., Lasley, R. D., and Bünger, R. (2007) Adenosine enhances cytosolic phosphorylation potential and ventricular contractility in stunned guinea pig heart: receptor-mediated and metabolic protection, *J. Appl. Physiol. 102*, 1202−1213.
48. Romagnani, S. (2002) Cytokines and chemoattractants in allergic inflammation, *Mol. Immunol. 38*, 881−885.
49. Poulsen, S. A., and Quinn, R. J. (1998) Adenosine receptors: new opportunities for future drugs, *Bioorg. Med. Chem. 6*, 619−641.
50. Cristalli, G., Lambertucci, C., Taffi, S., Vittori, S., and Volpini, R. (2003) Medicinal chemistry of adenosine A_{2A} receptor agonists, *Curr. Top. Med. Chem. 3*, 387−401.
51. Polosa, R., Rorke, S., and Holgate, S. T. (2002) Evolving concepts on the value of adenosine hyperresponsiveness in asthma and COPD, *Thorax 57*, 649−654.
52. Rorke, S., and Holgate, S. T. (2002) Targeting adenosine receptors: novel therapeutic targets in asthma and chronic obstructive pulmonary disease, *Am. J. Respir Med. 1*, 99−105.
53. Tilley, S. L., and Boucher, R. C. (2005) A_1 antagonism in asthma: better than coffee? *J. Clin. Invest. 115*, 13−16.
54. Zhong, H., Belardinelli, L., Maa, T., and Zeng, D. (2005) Synergy between A_{2B} adenosine receptors and hypoxia in activating human lung fibroblasts, *Am. J. Respir. Cell Mol. Biol. 32*, 2−8.
55. Blackburn, M. R. (2003) Too much of a good thing: adenosine overload in adenosine-deaminase-deficient mice, *Trends Pharmacol. Sci. 24*, 66−70.
56. Bzowska, A., Kulikowska, E., and Shugar, D. (2000) Purine nucleoside phosphorylases: properties, functions and clinical aspects, *Pharmacol. Ther. 88*, 349−425.
57. Silva, G. R., Santos, D. S., Basso, L. A., Oses, J. P., Wofchuk, S., Portela, L. V., and Souza, D. O. (2004) Purine nucleoside phosphorylase activity in rat cerebrospinal fluid, *Neurochem. Res. 29*, 1831−1835.
58. Roberts, E. L., Newton, R. P., and Axford, A. T. (2004) Plasma purine nucleoside phosphorylase in cancer patients, *Clin. Chim. Acta 344*, 109−114.
59. Yamamoto, T., Moriwaki, Y., Fujimura, Y., Takahashi, S., Tsutsumi, Z., Tsuitsui, T., Higashino, K., and Hada, T. (2000) Effect of TEI-6720, a xanthine oxidase inhibitor, on the nucleoside transport in the lung cancer cell line A549, *Pharmacology 60*, 34−40.
60. Zavialov, A. V., and Engstrom, A. (2005) Human ADA2 belongs to a new family of growth factors with adenosine deaminase activity, *Biochem. J. 391*, 51−57.
61. Li, J. Y., Boado, R. J., and Pardridge, W. M. (2001) Differential kinetics of transport of 2′,3′-dideoxyinosine and adenosine via concentrative Na^+ nucleoside transporter CNT2 cloned from rat blood-brain barrier, *J. Pharmacol. Exp. Ther. 299*, 735−740.
62. Ritzel, M. W., Yao, S. Y., Ng, A. M., Mackey, J. R., Cass, C. E., and Young, J. D. (1998) Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na^+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine, *Mol. Membr. Biol. 15*, 203−211.
63. Larrayoz, I. M., Casado, F. J., Pastor-Anglada, M., and Lostao, M. P. (2004) Electrophysiological characterization of the human Na^+/nucleoside cotransporter 1 (hCNT1) and role of adenosine on hCNT1 function, *J. Biol. Chem. 279*, 8999−9007.
64. Williams, T. C., and Jarvis, S. M. (1991) Multiple sodium-dependent nucleoside transport systems in bovine renal brush-border membrane vesicles, *Biochem. J. 274*, 27−33.
65. Lu, X., Gong, S., Monks, A., Zaharevitz, D., and Moscow, J. A. (2002) Correlation of nucleoside and nucleobase transporter gene expression with antimetabolite drug cytotoxicity, *J. Exp. Ther. Oncol. 2*, 200−212.
66. Szkotak, A. J., Ng, A. M. L., Sawicka, J., Baldwin, S. A., Man, S. F. P., Cass, C. E., Young, J. D., and Duszyk, M. (2001) Regulation of K^+ current in human airway epithelial cells by exogenous and autocrine adenosine, *Am. J. Physiol. 281*, C1991−2002.
67. Szkotak, A. J., Ng, A. M., Man, S. F., Baldwin, S. A., Cass, C. E., Young, J. D., and Duszyk, M. (2003) Coupling of CFTR-mediated anion secretion to nucleoside transporters and adenosine homeostasis in Calu-3 cells, *J. Membr. Biol. 192*, 169−179.
68. Vijayalakshmi, D., and Belt, J. A. (1988) Sodium-dependent nucleoside transport in mouse intestinal epithelial cells, *J. Biol. Chem. 263*, 19419−19423.
69. Jarvis, S. M. (1989) Characterization of sodium-dependent nucleoside transport in rabbit intestinal brush-border membrane vesicles, *Biochim. Biophys. Acta 979*, 132−138.
70. Jakobs, E. S., Van Os-Corby, D. J., and Paterson, A. R. (1990) Expression of sodium-linked nucleoside transport activity in monolayer cultures of IEC-6 intestinal epithelial cells, *J. Biol. Chem. 265*, 22210−22216.
71. Quaroni, A. (1985) Expression of YBB 3/10 antigen in human tumor colon cell lines and its induction by *N,N*-dimethylformamide, *J. Natl Cancer Inst. 74*, 591−602.
72. Melani, A., De Micheli, E., Pinna, G., Alfieri, A., Corte, L. D., and Pedata, F. (2003) Adenosine extracellular levels in human brain gliomas: an intraoperative microdialysis study, *Neurosci. Lett. 346*, 93−96.

73. Robertson, C. L., Bell, M. J., Kochanek, P. M., Adelson, P. D., Ruppel, R. A., Carcillo, J. A., Wisniewski, S. R., Mi, Z., Janeski, K. L., Clark, R. S., Marion, D. W., Graham, S. H., and Jackson, E. K. (2001) Increased adenosine in cerebrospinal fluid after severe traumatic brain injury in infants and children: association with severity of injury and excitotoxicity, *Crit. Care Med. 29*, 2390−2391.
74. Ip, W. K., Wong, C. K., and Lam, C. W. K. (2006) Interleukin (IL)-4 and IL-13 up-regulate monocyte chemoattractant protein-1 expression in human bronchial epithelial cells: involvement of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 and Janus kinase-2 but not c-Jun NH2-terminal kinase 1/2 signaling pathways, *Clin. Exp. Immunol. 145*, 162−172.
75. Blackburn, K., Lee, C. G., Young, H. W. J., Zhu, Z., Chunn, J. L., Kang, M. J., Banerjee, S. K., and Elias, J. A. (2003) Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway, *J. Clin. Invest. 112*, 332−344.

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