Adenosine A$_{2A}$ receptors promote adenosine-stimulated wound healing in bronchial epithelial cells

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Adenosine A$_{2A}$ receptors promote adenosine-stimulated wound healing in bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 290: L849–L855, 2006. First published December 16, 2005; doi:10.1152/ajplung.00373.2005.—Adenosine produces a wide variety of physiological effects through the activation of specific adenosine receptors (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$). Adenosine, acting particularly at the A$_{2A}$ adenosine receptor (A$_{2A}$AR), is a potent endogenous anti-inflammatory agent and sensor of inflammatory tissue damage. The complete healing of wounds is the final step in a highly regulated process. Recent studies on epidermal wounds have identified the A$_{2A}$AR as the main adenosine receptor responsible for altering the kinetics of wound closure. We hypothesized that A$_{2A}$AR promotes wound healing in bronchial epithelial cells (BECs). To test this hypothesis, the human BEC line BEAS-2B and bovine BECs (BBECs) were used. Real-time RT-PCR of RNA from unstimulated BEAS-2B cells revealed transcriptional expression of A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$ receptors. Western blot analysis of lysates from BEAS-2B cells and BBECs detected a single band at 44.7 kDa in both the BECs, indicating the presence of A$_{2A}$AR. In a wound healing model, we found that adenosine-stimulated wound repair in cultured BBECs in a concentration-dependent manner, with an optimal closure rate observed between 4 and 6 h. Similarly, the A$_{2A}$AR agonist 5'-N-cyclopropyl)carboxamidoadenosine (CPA) augmented wound closure, with a maximal closure rate occurring between 4 and 6 h. Inhibition of A$_{2A}$AR with ZM-241385, a known A$_{2A}$AR antagonist, impeded wound healing. In addition, ZM-241385 also attenuated adenosine-mediated wound repair. Kinase studies revealed that adenosine-stimulated airway repair activates PKA by ligation A$_{2A}$AR. Collectively, the data suggest that the A$_{2A}$AR is involved in BEC adenosine-stimulated wound healing and may prove useful in understanding purinergic-mediated actions on airway epithelial repair.

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MATERIALS AND METHODS

Reagents and materials. Laboratory of Human Carcinogenesis (LHC) basal medium and medium 199 were purchased from Biofluids (Rockville, MD). RPMI 1640 was purchased from GIBCO (Chagrin Falls, OH). Streptomycin, penicillin, protease (type IV), fetal calf serum, and fungizone were purchased from Life Technologies (Grand Island, NY). The type I collagen gel matrix Vitrogen 100 was purchased from Cohesion (Palo Alto, CA). Phosphocellulose P-81 paper was purchased from Whatman (Clifton, NJ). Heptapeptide substrates for PKA (LRRASLG) and PKC were purchased from Peninsula Laboratories (San Carlos, CA). 2-Chloro-N$^\circ$-cyclopentyladenosine (CPA; A$_1$ receptor agonist), ZM-241385 (A$_{2A}$ receptor antagonist), 1-[2-chloro-6-[[3-isodophenyl]methyl]amino]-9H-purin-

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9-yl)-1-deoxy-N-methyl-[β-d-ribofururannuronamide (2CI-IB-MECA; A3a receptor agonist) were purchased from Tocris (Ellisville, MO). Adenosine, 5′-(N-cyclopropyl)carboxamidoadenosine (CPA; A3a receptor agonist), 5′-(N-ethylcarboxamido)adenosine (NECA; nonselective adenosine receptor agonist), BSA, and all other reagents not listed were purchased from Sigma (St. Louis, MO).

**Cell preparation.** The transformed human BEAS-2B bronchial epithelial cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured on type I collagen (Vitrogen 100)-coated dishes in serum-free medium (LHC-9-RPMI; Ref. 18). Primary cultured bovine bronchial epithelial cells (BECs) were obtained from bovine lungs by a modification (1, 33) of a method described by Wu and Smith (36). BECs were prepared from bovine lung obtained fresh from a local abattoir. Cells were maintained in culture at 37°C in humidified 95% air-5% CO2 for 48–72 h before the experiment. This technique typically produces a high-viability cell preparation of >95% epithelial cells (28).

**In vitro wound closure (migration) assay.** BECs were grown to confluence in 96-well flat-bottomed or 60-mm tissue culture dishes. Cell monolayers were “woundened” with a small, sterile scriber to remove a circular area of cells, ~1,000 μm². To account for the difference in the rate of wound closure between 96-well and 60-mm dishes, we adjusted the volume as a ratio of volume to surface area as described in Table 1. Adjusting the volume established an equipotential rate in wound closure between different tissue culture vessels. The progress of migration was monitored with a phase-contrast microscope outfitted with a video camera. The camera output was captured with image analysis software (NIH ImageJ version 1.30) on a Macintosh G-3 computer. Each wound was photographed with the video camera and image analysis software at specified times, and the area of the wound was measured. The dishes were returned to the incubator between measurements. This assay is based on migration and not proliferation; therefore, as cells migrate into the wound the open area is reduced (11, 17, 41). The 96-well dish setup allows simultaneous assay of many different treatment conditions in triplicate. For signaling experiments, BECs were grown to confluence on 60-mm tissue culture dishes. Cell monolayers were wounded with a sterile “cell rake,” removing cells in a gridlike pattern (29). The linear wounds averaged 325 μm in width and ~2 mm apart. This process removes ~14.1% of total cells.

**Preparation of membranes.** Cell membranes from BEAS-2B cells or BECs were prepared with a modified method as described previously (19). Cell lysates were sonicated and particulates were removed by centrifugation. Protein concentrations were determined by the Bradford method (4) with Bio-Rad protein reagent. Proteins were separated by SDS-PAGE under reducing conditions on a 10% polyacrylamide gel. The resolved proteins were electroblotted to Immob-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked with buffer containing 20 mM Tris, 150 mM NaCl, 5% nonfat milk, and 0.2% Tween (Tris-buffered saline-Tween-Blotto; pH 7.4). Trans- ferred proteins were probed with rabbit anti-canine adenosine A3a receptor antisem antibody (A3aA; Alpha Diagnostic Intl., San Antonio, TX) overnight at 4°C. Membranes were washed several times and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 90 min at room temperature (Rockland, Gilbertsville, PA). An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) was used to visualize the blotted proteins on X-ray film (Kodak, Rochester, NY).

**Determination of PKA and PKC.** PKA activity was determined in crude whole cell fractions of bronchial epithelial cells. The assay used is a modification of procedures previously described (13), with 130 μM PKA substrate heptapeptide (LRRASLG), 10 μM cAMP, 0.2 mM IBMX, 20 mM Mg-acetate, and 0.2 mM [γ-32P]ATP in a 40 mM Tris-HCl buffer (pH 7.5). PKC activity was also conducted as described above with a reaction mixture of 900 μM PKC substrate peptide. Samples (20 μl) were added to 50 μl of the above reaction mixture and incubated for 15 min at 30°C. Reactions were initiated by the addition of 10 μl of cell fraction. Incubations were halted by spotting 50 μl of each sample onto P-81 phosphocellulose papers. Papers were then washed five times for 5 min each in phosphoric acid (75 mM), washed once in ethanol, dried, and counted in nonaqueous scintillant as previously described (26). Negative control groups consisted of similar assay conditions with or without the appropriate substrate peptide or cyclic nucleotide. Kinase activity is expressed in relationship to total cellular protein assayed and was calculated in picomoles of phosphate incorporated per minute per milligram of total protein. All the samples were assayed in triplicate, and no fewer than three separate experiments (n = 9) were performed per unique parameter.

**RT-PCR of adenosine receptor mRNA in cultured cells.** RT-PCR was conducted on unstimulated BEAS-2B cells, utilizing total cellular RNA. The amplification primers and probes for the adenosine receptor messages were synthesized with Primer Express Software (PE Applied Biosystems, Foster City, CA). We followed a method described previously (14) in which 5 μg of cellular RNA is denatured at 95°C for 5 min and incubated at 42°C for 60 min in 20 μl of mixture consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 1 U/μl RNasin, 100 μM random hexamers, dATP, dGTP, and dTTP at 1 mM, and 200 U of Superscript reverse transcriptase. For each reaction mixture, 2 μl of the reverse transcription product was added to PCR buffer, each primer at 1 mM, dATP, dCTP, dGTP, and dTTP at 1 mM, and 2.5 U of AmpliTaq polymerase. Amplification products were separated electrophoretically on a 1.5% agarose gel and visualized with ethidium bromide staining. We used quantitative PCR (PCR Taqman construction kit; PE Applied Biosystems) utilizing nonhomologous internal standards designed for each primer of interest. RT-PCR was also performed for a control gene (Taqman Ribosomal RNA Control) for each condition to allow a more confident comparison of signals.

**Lactate dehydrogenase assays.** Cell viability and cytotoxicity from concentrations of adenosine receptor agonists and/or antagonists used in all experiments were determined by cell media assay of lactate dehydrogenase (LDH) release with a commercially available kit (Sigma).

**Statistical analysis.** The wound closure assays (96-well format) were performed with triplicate wounds (in separate wells) and repeated in three separate experiments with similar results (n = 3). The data represent means ± SE for these triplicates. Other assays were also performed in triplicate and repeated in three separate experiments.

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**Table 1. Standardization of volume/surface area for wound closure assays**

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Surface Area per Well, cm²</th>
<th>Relative Surface Area (vs. 24-well vessel)</th>
<th>Volume of Plating Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well</td>
<td>0.3</td>
<td>0.2</td>
<td>100 μl</td>
</tr>
<tr>
<td>24 well</td>
<td>2</td>
<td>1</td>
<td>500 μl</td>
</tr>
<tr>
<td>12 well</td>
<td>4</td>
<td>2</td>
<td>1 ml</td>
</tr>
<tr>
<td>6 well (35 mm)</td>
<td>10</td>
<td>5</td>
<td>2 ml</td>
</tr>
<tr>
<td>60 mm</td>
<td>20</td>
<td>10</td>
<td>5 ml</td>
</tr>
<tr>
<td>100 mm</td>
<td>60</td>
<td>30</td>
<td>15 ml</td>
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with similar results (n = 3). To pool data from multiple independent experiments, the effect was tested with paired t-tests to account for varying control results. For the kinase assay, all samples were assayed in triplicate and no fewer than three separate experiments were performed per unique parameter (n = 9). Data were analyzed for significance with one-way ANOVA followed by Tukey multiple-comparison test. Significance was assigned at P ≤ 0.05.

RESULTS

Identification of adenosine receptor(s) present on normal airway epithelium. We first determined whether adenosine receptors were present in airway epithelium to further understand the mechanism(s) by which adenosine could contribute to airway repair. Adenosine receptor profiles were generated by examining adenosine receptor expression as quantified by real-time RT-PCR using the human bronchial epithelial cell line BEAS-2B. Our data revealed that unstimulated cells expressed all four adenosine receptors and that by 30 min levels of all four adenosine receptors were markedly elevated (Fig. 1A). Western blot analysis revealed a 44.7-kDa band representative of A2A adenosine receptor in lysates from both BEAS-2B cells and primary cultured BBECs (Fig. 1B). These findings indicate that adenosine receptors exist on normal airway epithelial cells.

Adenosine stimulates wound repair in BBECs. To determine adenosine involvement in airway wound repair we utilized an in vitro wounding model developed in our laboratory (17, 29, 41). We found that adenosine stimulated wound repair in cultured BBECs with an optimal concentration at 10 μM and an optimal closure rate observed between 4 and 6 h (Fig. 2). In addition, our data demonstrated that adenosine accelerated closure rapidly (within 2 h) and that this closure rate was sustained, resulting in rapid wound closure over the first 24 h (data not shown). To delineate which adenosine receptor(s) is activated, cells were wounded and then stimulated with either serum-free medium (control medium) or specific adenosine receptor analogs in serum-free medium until wounds approached closure as determined by wound digital imaging. Cells stimulated with CCPA (10 μM), a potent, selective A1 receptor agonist, delayed wound closure compared with control cells (Fig. 3A). However, activation of A2A receptor with CPA (10 μM), a selective A2A receptor agonist, revealed an accelerated increase in the rate of wound closure within the first 2 h, and this was maintained up to 6 h (Fig. 3B). Cells stimulated with NECA (10 μM), a potent adenosine receptor agonist that has an EC50 of 3.1 μM for A2A receptor, showed no effect on wound closure (data not shown). In addition, delayed wound closure was detected when cells were stimulated with 2CI-IB-MECA (10 μM), an A3 receptor agonist (Fig. 3C). To verify A2A receptor involvement in promoting adenosine-stimulated wound closure, cells were pretreated with or without 0.1 μM ZM-241385, a known A2A receptor antagonist, for 60 min, wounded, and then stimulated with 10 μM adenosine. ZM-241385 blocked adenosine’s wound closure stimulatory effect (Fig. 3D). None of the concentrations of adenosine agonist or antagonist analogs used in these studies were associated with any significant cell toxicity as determined by cellular LDH release (data not shown). These findings suggest that adenosine stimulates wound repair through the activation of the A2A receptor.

Adenosine stimulates airway wound repair in BBECs via activation of PKA. We previously established (38) that mechanical wounding of epithelial monolayers modulates cAMP production/PKA activation and plays an active role in accelerating wound closure. To evaluate whether adenosine-stimulated wound repair activates PKA, wounded BBEC monolayers were stimulated with adenosine (10 μM) at various times (30–120 min). Cells treated with adenosine for 30 min activated PKA, and this activation was sustained when cells were pretreated with adenosine up to 120 min (Fig. 4). To further evaluate A2A receptor involvement, wounded cell monolayers were stimulated with A2A agonist (CPCA; 10 μM), and within 30 min there was a significant increase in PKA activity. This increase in PKA activity was sustained by 60 min and returned...
to baseline by 2 h (Fig. 5). These data suggest that adenosine occupancy of A2A receptor promotes wound closure in a manner coincident with PKA activation.

**KT-5720, a specific PKA inhibitor, blocks A2A-mediated wound closure in BBECs.** We previously showed (39) that inhibition of PKA activity with a specific PKA inhibitor, KT-5720, blocked ciliary beating in BBECs. To investigate the role of PKA in A2A-mediated wound closure, BBECs were pretreated for 1 h with a specific PKA inhibitor (KT-5720; 1 μM), wounded, and then treated with or without adenosine (10−5 M) were monitored. Data are means ± SE of triplicate wells within a single experiment. The experiment was repeated twice with different preparations of BBECs with similar results (n = 3). *P < 0.05 for comparison to control at same time point, by ANOVA.

**Adenosine A1 and A3 receptors activate PKC and decrease airway wound repair.** We have shown that PKC activation decreases wound repair in bronchial epithelial cells (37). Our data suggest that both A1 and A3 receptors inhibit wound closure (Fig. 3, A and C). On the basis of these findings we conducted kinase assays to determine whether occupancy of A1 or A3 receptor activates PKC. Wounded cell monolayers stimulated with either A1 agonist (CCPA; 1 μM) or A3 agonist (2Cl-IB-MECA; 1 μM) at various time points (15−60 min) resulted in activation of PKC (Fig. 7). To confirm that occu-
pency of A1 or A3 receptor activates PKC, selective and potent antagonists of A1 receptor [8-cyclopentyl-1,3-dipropylxanthine (DPCPX); 10 μM] or A3 receptor (MRS-1220; 10 μM) were used. Cells pretreated with A1 or A3 receptor antagonist for 30 min showed inhibited adenosine activation of PKC (Fig. 8). The presence of inhibitors for 30 min before and during the assay suppressed PKC activation. None of the concentrations of adenosine agonist or antagonist analogs used in these studies was associated with any significant cell toxicity as determined by cellular LDH release (data not shown). On the basis of these findings, we correlated PKC activation via A1 and A3 receptors with retarding wound closure.

DISCUSSION

The present study provides evidence that adenosine evokes wound closure in bronchial epithelial cells by activation of A2A adenosine receptor(s). To our knowledge, this is the first demonstration that a small nonpeptide agent, such as a purine nucleoside, promotes wound healing in airway injuries. Our studies also reveal that adenosine receptor-mediated activation of wound closure involves stimulation of a PKA pathway. Previous studies have suggested that adenosine acting at the A2A receptor stimulates endothelial cell migration, proliferation, and secretion of vascular endothelial growth factor in vitro (9, 12, 20, 27). In addition, adenosine acts as a potent regulator of inflammation, the first stage of the wound healing process (21). The capacity of adenosine to suppress inflammation and wound closure was first described for neutrophils, and subsequently adenosine was shown to regulate the inflammatory function of many other cell types including macrophages, endothelial cells, lymphocytes, and mast cells (6, 31, 35). Therefore, the A2A receptor is most relevant to human pulmonary disease, i.e., asthma, bronchitis, and chronic obstructive pulmonary disease, where inflammation and damage to airway epithelium occur. On the basis of these earlier findings, we hypothesized that A2A adenosine receptor(s) serve to enhance wound healing. Our data strongly suggest that adenosine acting on the A2A receptor may have the ability to promote airway epithelial wound, an important step in the resolution of airway inflammation and disease.

Utilizing our mechanical wounding model, we demonstrated that adenosine stimulates wound repair in cultured bronchial epithelial cells. Treatment with adenosine rapidly accelerated wound closure, and this was significantly observed by 6 h. Because adenosine facilitated wound closure in our studies, we demonstrated that this effect is mediated through subsequent activation of adenosine A2A receptor(s). Pharmacological characterization with selective adenosine receptor agonists revealed that neither A1 nor A3 agonists promote wound closure. However, the A2A agonist CPCA demonstrated that these effects are mediated predominantly by activation of the adenosine A2A receptor(s). This is further supported by inhibition of adenosine-mediated wound closure by the A2A-selective antagonist ZM-241385. The observation that adenosine stimulates airway wound closure by activation of A2A receptor(s) suggests a role for the involvement of intracellular cAMP and subsequent activation of PKA. Our data confirmed that inactivation of PKA with KT-5720 blocks A2A-mediated wound closure in bronchial epithelial cells. Therefore, the activation of PKA is crucial to enhancing the rate of wound healing when bronchial epithelial cells are occupied and stimulated by A2A receptor(s).

It has been well established that signal transduction at A2A receptor(s) proceeds via activation of G protein leading to cAMP signaling events and activation of PKA (7, 24). We previously (29, 38) characterized that PKA activation acceler-
ates wound closure during the first 6 h, and all of the wounds were nearly closed by 20–24 h after wounding. Subsequently, all our experiments were focused on wound closure during the first 6 h. We first examined PKA activity in wounded bronchial epithelial cells treated with adenosine. Increased PKA activity resulted after 30 min. Similarly, PKA activity increased in wounded cell monolayers stimulated with A2A agonist (CPCA; 10 μM). In addition, we have shown that activation of PKC by either A1 or A3 receptor retarded wound repair in bronchial epithelial cells. Our studies demonstrate that adenosine receptor occupancy via A2A receptor promotes airway repair and further support our view that PKA is critical for the protective effect of A2A-mediated wound repair. Furthermore, these data support a bidirectional control model whereby PKC regulates the decrease in cell migration into a wound.

Much of what is known about epithelial repair in airways comes from studies in which the epithelium is injured and repair is followed histologically. Early events in the repair process include cell spreading, migration, and proliferation (15, 16). The effects of adenosine were apparent as early as 2 h after wounding, well before proliferation would be significant. Previous studies of wound closure in the airway epithelium have also demonstrated that, depending on the size of the wound, the cells will flatten and migrate to cover the open area long before proliferation becomes important (8, 40, 41). Thus for wound closure to be accelerated early in the process, adenosine and its analogs, acting at A2A receptor(s), must stimulate either cell spreading or cell migration. On the basis of our data, our wounding model proposes that occupancy of A2A receptor promotes early wound closure by stimulation of cell migration and cell spreading. Further studies are under way to determine the involvement of adenosine-mediated cell migration and cell spreading in early wound closure. However, supporting results suggest that topical application of A2A receptor agonists promotes more rapid dermal wound healing than human platelet-derived growth factor (34). It is unlikely that an adenosine receptor-mediated stimulation is solely responsible for accelerating wound closure. Several studies have demonstrated that adenosine and its analogs, acting at A2A receptor(s), increase secretion of vascular endothelial growth factor as well as promoting endothelial cell proliferation and migration (5, 9, 20, 27).

There are a number of adenosine-mediated effects that may contribute to the accelerated rate of wound closure that are mediated by activation of adenosine receptors, i.e., cell proliferation, migration, and synthesis of message for angiogenic growth factor (30, 32). Enhanced understanding of the roles and mechanisms underlying adenosine receptor activation and signaling in airway injury and wound repair processes could facilitate future exploitation of the potential of targeting adenosine receptor subtypes. In summary, the present studies demonstrate that adenosine-mediated wound closure in bronchial epithelial cells occurs via occupancy of A2A receptor(s) and activation of A2A receptor induces the integrated activation of PKA. It is this integrated activation of PKA that determines adenosine effects on promoting wound closure. Ongoing studies to more clearly define adenosine-stimulated airway epithelial repair in airway disease will be important for our understanding of the use of adenosine therapeutically in airway diseases.

REFERENCES

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