Adenosine A2A receptors promote adenosine-stimulated wound healing in bronchial epithelial cells

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Adenosine A2A 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 (A1, A2A, A2B, A3). Adenosine, acting particularly at the A2A adenosine receptor (A2AAR), 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 response to injury. Recent studies on epidermal wounds have identified the A2AAR as the main adenosine receptor responsible for altering the kinetics of wound closure. We hypothesized that A2AAR 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 A1, A2A, A2B, and A3 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 A2AAR. 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 A2AAR agonist 5′-(N-cyclopropyl)carboxamidoadenosine (CPCA) augmented wound closure, with a maximal closure rate occurring between 4 and 6 h. Inhibition of A2AAR with ZM-241385, a known A2AAR 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 ligating A2AAR. Collectively, the data suggest that the A2AAR is involved in BEC adenosine-stimulated wound healing and may prove useful in understanding purinergic-mediated actions on airway epithelial repair.

Airway injury; airway repair

Adenosine, the metabolic breakdown product of ATP, is recognized as an important modulator of hypoxic, ischemic, and inflammatory processes critical to both tissue homeostasis and injury. As a purine nucleoside, adenosine produces a wide variety of physiological effects through the activation of the four adenosine cell surface receptors, A1, A2A, A2B, and A3. This receptor complexity reflects the multidimensional role adenosine plays in health and disease. Through these multiple receptors, adenosine can either protect or damage tissues depending on the receptor(s) activated. As an example of protection, adenosine is critical for skin wound repair in a murine model of diabetes (22, 23, 34). In contrast, defective lung development occurs when adenosine deaminase, the principal adenosine-degrading enzyme, is knocked out in mice (2, 3). Despite our evolving recognition of adenosine as a key tissue regulatory molecule, little is known about the role adenosine plays in maintaining and repairing the airways.

Recent studies indicate that adenosine is emerging as a critical molecule in the pathophysiology of inflammatory airway diseases. For example, adenosine responsiveness is now being viewed as a marker of airway hyperreactivity (10, 25). As a potent regulator of inflammation, adenosine also initiates the first stage of the wound healing process. In studies using a dermal wound repair model, Montesinos et al. (23) demonstrated that adenosine receptor agonists, applied topically, might enhance dermal excisional wound healing both in vitro and in vivo. Previous studies showed that adenosine, acting at the A2 receptor, inhibits neutrophil accumulation and function and promotes endothelial cell proliferation, migration, and growth factor secretion (5, 7, 9, 20, 27). Adenosine is reported to be angiogenic (22), possibly contributing to its wound healing effect. Similarly, Victor-Vega et al. (34) used a murine model to demonstrate that topical application of adenosine A2A receptor agonists promote more rapid dermal wound healing than human platelet-derived growth factor. In contrast to the established role that adenosine plays in dermal wound repair, little is known about the role adenosine plays in airway injury and repair. If repair responses restore normal tissue architecture, function will be preserved. In light of this, we hypothesized that adenosine promotes airway wound repair by activation of the A2A receptor. The objective of these studies was to provide an understanding of the mechanisms that control these adenosine-driven inflammation and repair processes as well as to demonstrate the multidimensional properties of adenosine in the airway, particularly in adenosine-stimulated airway epithelial repair.

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-N6-cyclopentyladenosine (CPA; A1 receptor agonist), ZM-241385 (A2A receptor antagonist), 1-[2-chloro-6-[[3-iodophenyl]methyl]amino]-9H-purin-
leupeptin, aprotinin, PMSF, and chymostatin). Cell lysates were flash-frozen in PBS containing protease inhibitors (1 g/ml each of leupeptin, aprotinin, PMSF, and chymostatin), 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 (BBECs) were obtained from bovine lungs by a modification (1, 33) of a method described by Wu and Smith (36). BBECs 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. BBECs were grown to confluence in 96-well flat-bottomed or 60-mm tissue culture dishes. Cell monolayers were “wounded” with a small, sterile scraper 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. 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 of the wound correspondingly is reduced (11, 17, 41). The 96-well dish setup allows simultaneous assay of many different treatment conditions in triplicate. For signaling experiments, BBECs 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 BBECs 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, 0.2% nonfat milk, and 0.2% Tween (Tris-buffered saline-Tween-Blotto; pH 7.4). Transferred proteins were probed with rabbit anti-canine adenosine A2A receptor antisera antibody (A2A; 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).

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>15 ml</td>
</tr>
<tr>
<td>100 mm</td>
<td>60</td>
<td>30</td>
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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.
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 \(P \leq 0.05\).

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 \(\mu M\) 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 maintained, 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. Data are means \(\pm SE\) 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.

Fig. 2. Effects of adenosine on wound repair in BBECs. Cells were incubated in serum-free medium in the presence or absence of various concentrations of adenosine until wounds approached closure as determined by wound area digital imaging. Data are means \(\pm SE\) 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.
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-
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 via occupancy of the A2A receptor 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 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 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 epithelial repair in airway disease will be important for our understanding of 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 (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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