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Adenosine A_{2A} receptor-dependent proliferation of pulmonary endothelial cells is mediated through calcium mobilization, PI3-kinase and ERK1/2 pathways

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ABSTRACT

Hypoxia and HIF-2α-dependent A_{2A} receptor expression and activation increase proliferation of human lung microvascular endothelial cells (HLMVECs). This study was undertaken to investigate the signaling mechanisms that mediate the proliferative effects of A_{2A} receptor. A_{2A} receptor-mediated proliferation of HLMVECs was inhibited by intracellular calcium chelation, and by specific inhibitors of ERK1/2 and PI3-kinase (PI3K). The adenosine A_{2A} receptor agonist CGS21680 caused intracellular calcium mobilization in controls and, to a greater extent, in A_{2A} receptor-overexpressing HLMVECs. Adenoviral-mediated A_{2A} receptor overexpression as well as receptor activation by CGS21680 caused increased PI3K activity and Akt phosphorylation. Cells overexpressing A_{2A} receptor also manifested enhanced ERK1/2 phosphorylation upon CGS21680 treatment. A_{2A} receptor activation also caused enhanced cAMP production. Likewise, treatment with 8Br-cAMP increased PI3K activity. Hence A_{2A} receptor-mediated cAMP production and PI3K and Akt phosphorylation are potential mediators of the A_{2A}-mediated proliferative response of HLMVECs. Cytosolic calcium mobilization and ERK1/2 phosphorylation are other critical effectors of HLMVEC proliferation and growth. These studies underscore the importance of adenosine A_{2A} receptor in activation of survival and proliferative pathways in pulmonary endothelial cells that are mediated through PI3K/Akt and ERK1/2 pathways.

1. Introduction

Hypoxia may occur locally, regionally, or systemically in various lung diseases or in association with ischemic injury, causing activation of adaptive pathways. Hypoxia also increases intracellular and extracellular adenosine [1]. Several studies, including our own, have shown that adenosine and its analogs can promote endothelial cell proliferation and migration [2–8]. Hypoxic proliferation of endothelial cells is important in disease processes including pulmonary hypertension where the proliferative phenotype can result in vascular remodeling and subsequent narrowing of the blood vessel lumen. Factors that lead to vascular remodeling are incompletely understood, but likely the causes are multifactorial.

The biological activity of adenosine is mediated mainly through its binding to four receptors, namely A_1, A_{2A}, A_{2B} and A_3 [9]. These appear to have distinct function(s) in specific cell types. While A_1 and A_3 receptors inhibit adenylate cyclase activity, A_{2A} and A_{2B} stimulate it. Further, A_1 and A_{2A} have higher affinities for adenosine relative to A_{2B} and A_3. Therefore, depending on cell type, adenosine levels and adenosine receptor subtypes present, adenosine can differentially influence cell function and phenotype.

Extracellular adenosine, through its receptors, can modulate several signaling pathways including the PI3-kinase/Akt pathway and the MAPK pathways [10,11]. Depending on cell type, activation of A_{2A} receptor can either increase or decrease ERK1/2 phosphorylation [12–15]. Similarly activation of adenosine receptors can also increase or decrease PI3-kinase (PI3K) activity [16–18]. PI3K and Akt also can be involved in mediating protective effects of adenosine A_{2A} receptor against ischemia–reperfusion injury in heart and liver [16,19,20]. Further, A_{2A} receptor activation can normalize altered cell cycle signaling and promotes wound healing in diseased mice [21].

We have shown previously that hypoxia increases adenosine A_{2A} receptor expression by a HIF-2α-dependent pathway in pulmonary endothelial cells [8]. In addition, we found that adenosine A_{2A} receptor increases proliferation of pulmonary endothelial cells [8]. In the current report, we used primary cultures of human lung microvascular endothelial cells to determine mechanism(s) by which adenosine A_{2A} receptor causes proliferation.

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2. Materials and methods

2.1. Cell culture

HLMVECs were cultured in endothelial cell basal medium (EBM-2) supplemented with VEGF, human FGF, human EGF, hydrocortisone, ascorbic acid, insulin-like growth factor-1, GA-1000 (gentamicin/amphotericin-B), and 5% FBS per the supplier's protocol (Lonza).

2.2. Adenoviral transduction

Adenoviral A_{2A} (Ad.A_{2A}) was generated following the protocols outlined before [8]. Adenoviral transductions of HLMVECs were carried out at a multiplicity of infection of 10 plaque forming units per cell. For transduction controls Ad.LacZ was used.

2.3. RNA Isolation and RTPCR

For assessing mRNA levels, total RNA was isolated from cells, cDNA was synthesized and RTPCR was carried out with gene specific primers and Taqman probes as described by us previously (1).

2.4. Cell proliferation assay

HLMVECs were seeded on 6-well plates at a density of 1.3×10^5 cells per well in EBM2 complete medium. Medium was replaced with 1% FBS-containing medium 24 h later. Next day the cells were transduced with Ad.LacZ or Ad.A_{2A} in 2% FBS-containing medium. Twenty-four hours post transduction diluent or inhibitors were added and incubated for an additional 24 h after which cells were trypsinized and counted using a Bio Rad automated cell counter.

2.5. Calcium assay

A_{2A}- or ATP (positive control)-induced intracellular calcium mobilization was measured using a fluorescence technique as described previously [22]. Cells cultured on 96-well microtiter plates were loaded with 5 μM no wash (NW) Fluo-4-AM (Molecular Probes) dye for 90 min. Subsequent steps were performed with a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices). Test compounds were added, and cells were monitored for 20 min to detect agonist activity. Net peak calcium, expressed in arbitrary fluorescence units, was measured.

2.6. Western blot analysis of cellular extracts for phospho-ERK1/2 and phospho-Akt

Western blots were carried out as described in detail before [23]. Briefly, 20 μg of total protein lysate were subjected to SDS–PAGE (4–15%) and the separated proteins were transferred to nitrocellulose membranes using standard procedure. Phospho-ERK1/2 (Tyr 202/Thr 204) and phosphorylated Akt (Ser473) were detected using polyclonal antibodies (Cell Signaling Technology, Danvers, MA). Blots were probed with horseradish-peroxidase conjugated anti-rabbit antibody. Immunoreactive bands were detected using an enhanced chemiluminescence detection reagent (Pierce, Rockford, IL) followed by exposure to Hyperfilm (Amersham Pharmacia Biotech Inc.). For detection of the non-phosphorylated forms of ERK1/2 and Akt, respective membranes were stripped of the bound antibodies, blocked with 5% nonfat dry milk in TBS-T, and reprobed with rabbit polyclonal antibodies against total ERK1/2 and Akt (Cell Signaling Technology, Danvers, MA).

2.7. Measurement of intracellular cAMP levels

Using confluent HLMVECs in 24-well plates, cAMP levels were determined under basal conditions and in cells challenged for 10 min with CGS21680 (1 μM). After treatment cells were washed with HBSS containing PMSF and sodium orthovanadate and then scrapped in the same buffer and pelleted. Cells pellets were resuspended in 0.1 N HCl for 10 min after which the contents were centrifuged and the supernatant collected for cAMP assay using ELISA (ELISA Tech, Denver, CO).

2.8. Assay for PI3-kinase activity

PI3-kinase assay was carried out as described by us before [24]. PI3K activity was assessed by the incorporation of [^{32}P]ATP into exogenous phosphoinositide resulting in the production of PI3-phosphate (PI3-P). HLMVEC were lysed in buffer (20 mM Tris–HCl, pH 7.5, containing 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM Na_3VO_4, protease inhibitor cocktail (Calbiochem, La Jolla, CA), 1 mM PMSF, 10 mM sodium fluoride, 1% NP-40, and 1 mM EDTA) after treatments and the PI3K enzyme immunoprecipated with anti-phospho-Tyr agarose conjugate or anti-p85 agarose conjugate. The enzyme was then incubated with phosphotidylinositol and [^{32}P]ATP in kinase buffer for 15 min at 37 °C. The reaction was terminated by the addition of 1 M HCl, and lipids were then extracted using a mixture of chloroform and methanol (1:1). The lower chloroform phase containing the resulting PI3-P was separated by TLC and visualized by autoradiography. The product was identified by comparing its relative mobility value with the relative mobility value of the standard PI3-P, which was visualized by exposure of TLC plates to phospholipid-specific molybdenum blue spray reagent.

2.9. Statistical analysis

All statistical analyses were performed with JMP software (SAS Institute, Cary, NC). Means were compared by two-tailed *t* test for comparison between two groups and one-way ANOVA followed by the Tukey–Kramer test for multiple comparisons. A P value of <0.05 was considered significant.

3. Results

3.1. Adenosine A_{2A} receptor-dependent proliferation of HLMVECs is mediated by intracellular calcium mobilization and cell survival kinases

Hypoxia and HIF-2α have been shown to increase endothelial proliferation along with increase in A_{2A} receptor expression [8,25]. A_{2A} receptor overexpression and activation in HLMVECs causes enhanced proliferation, increased migration and angiogenesis [8]. Since kinases such as PI3K and ERK1/2 along with intracellular calcium mobilization play critical roles in promoting endothelial cell growth [26–28], and A_{2A} receptor has been implicated in activating these kinases in some cell types [11,16,19], we reasoned that these could be involved in A_{2A} receptor-mediated proliferation. To investigate the downstream pathways involved in A_{2A}-dependent proliferation, HLMVECs were transduced with Ad.LacZ or Ad.A_{2A} and treated with inhibitors of PI3K, ERK1/2 and intracellular calcium chelator BAPTA prior to assessing proliferation. As expected, A_{2A} overexpression (confirmed by RTPCR: data not shown) increased proliferation by HLMVECs (Fig. 1). We used the specific A_{2A} receptor antagonist SCH442416 to determine that the increased proliferation was indeed due to A_{2A} receptor. A_{2A} receptor-mediated proliferation in HLMVEC was attenuated by inhibition of ERK1/2, PI3K or by intracellular calcium inhibition (Fig. 1A and B).

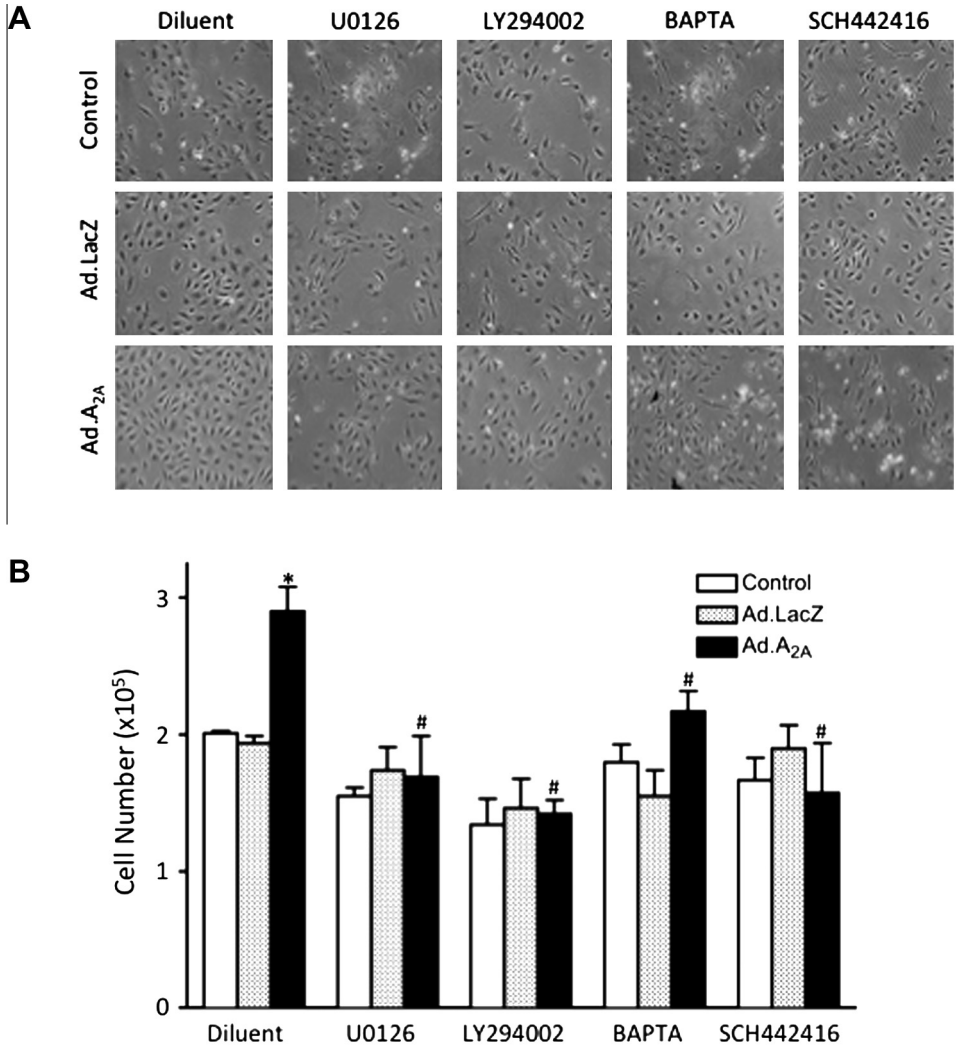

Fig. 1. Adenosine A_{2A} receptor overexpression-induced HLMVEC proliferation is mediated by cytosolic calcium mobilization, ERK1/2 and PI3K. 1.3×10^5 cells/well were cultured on 6-well plates. Cells were transduced with Ad.LacZ or Ad.A_{2A} in medium containing 2% FBS. Twenty-four hours post transduction diluent or inhibitors of ERK1/2 (U0126: 10 μM), PI3K (LY294002:10 μM), calcium mobilization (BAPTA:10 μM), or A_{2A} receptor antagonist (SCH442416: 100 nM) were added and cells were counted after an additional 24 h. Panel A shows representative images of cells prior to harvest for cell counts. Panel B shows cell counts in the presence or absence of inhibitors. Values are expressed as mean ± SEM ($n = 4$). $^*P < 0.05$ indicates significant difference from non-transduced and Ad.LacZ control. $^{\#}P < 0.05$ indicates significant difference from Ad.A_{2A} transduced cells.

3.2. Adenosine A_{2A} receptor activity mediates cellular calcium signaling response

Intracellular calcium ($[Ca^{2+}]_i$) mobilization plays an important role in pulmonary endothelial function [28,29]. Many endothelial cell primary messengers, such as ATP, activate specific receptors and increase $[Ca^{2+}]_i$ [28,29]. We evaluated whether A_{2A} receptor overexpression and activation by its specific agonist CGS21680 could mobilize $[Ca^{2+}]_i$ in HLMVECs. A_{2A} receptor activation increased $[Ca^{2+}]_i$ in HLMVECs similar to that due to extracellular ATP (Fig. 2) in control, Ad.LacZ- or Ad.A_{2A}-overexpressing HLMVECs. The magnitude of calcium mobilization appeared greater in Ad.A_{2A}-expressing cells. In these cells, however, basal cytosolic calcium levels were lower than those seen in control or Ad.LacZ-expressing cells (Fig. 2).

3.3. Protein tyrosine kinases mediate adenosine A_{2A} receptor-dependent PI3K and Akt activation

Protein tyrosine kinases are important for downstream signaling to effect PI3K and Akt activation in endothelial cells [30,31]. Adenosine is a natural ligand for adenosine receptors. We therefore investigated PI3K activity upon treatment of HLMVECs with adenosine or its stable analog, 2-chloro-adenosine. Both adenosine and 2-chloro-adenosine caused increased PI3K activity (Fig. 3A). Treatment with the specific A_{2A} receptor agonist CGS21680 also enhanced PI3K phosphorylation in HLMVECs (Fig. 3B). Adenosine and A_{2A} receptor agonist CGS21680 also increased Akt phosphorylation (Fig. 3C and D). Some studies have shown that adenosine A_{2A} receptor activates protein tyrosine kinases [32,33]. Therefore, we assessed the effect of genistein, a protein tyrosine kinase inhibitor, on Akt phosphorylation. Genistein inhibited adenosine A_{2A} receptor-induced Akt phosphorylation (Fig. 3E).

3.4. Adenosine A_{2A} receptor activation enhances cAMP production

The cellular effects of A_{2A} receptor activation are mediated by adenylate cyclase activation and cAMP production in endothelial cells [34]. Treatment of HLMVECs with A_{2A} receptor agonist CGS21680 increased cAMP (Fig. 3F). To understand whether A_{2A} receptor-mediated cAMP production caused PI3K activation, we treated HLMVECs with 8-Br-cAMP, a stable analog of cAMP. Treatment of HLMVECs with 8-Br-cAMP enhanced PI3K activity (Fig. 3G).

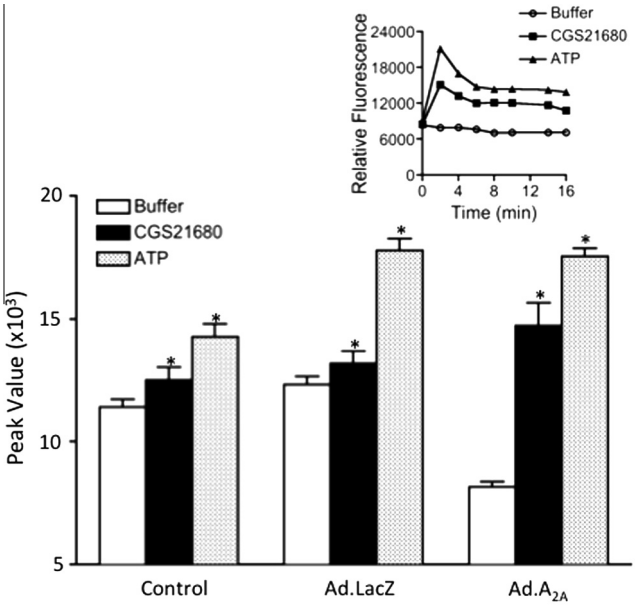

Fig. 2. Adenosine A_{2A} receptor activation mediates cytosolic calcium mobilization in HLMVECs. Cells were cultured on 96-well plates to 80% confluence and transduced. Forty-eight hours later intracellular calcium mobilization upon buffer (control), ATP (positive control) or CGS21860 was assayed in cells preloaded with no wash (NW) Fluo-4 calcium sensing dye as described in the methods. The graph shows the mean peak values ±SEM ($n = 8$). Inset shows the representative time-dependent calcium mobilization curves. $^{*}P < 0.05$ indicates significant difference from buffer control.

3.5. Adenosine A_{2A} receptor activation and overexpression promote ERK1/2 phosphorylation

ERK1/2 phosphorylation mediates hypoxia-induced proliferation in endothelial cells [35]. We sought to determine whether ERK1/2 contributed to proliferative effects of A_{2A} receptor activation. Treatment of HLMVEC with CGS21680 increased ERK1/2 phosphorylation within 5 min (Fig. 4A). This effect was transient and diminished at later time points. Similarly, CGS21680 treatment of cells overexpressing A_{2A} receptor also resulted in robust ERK1/2 phosphorylation (Fig. 4B).

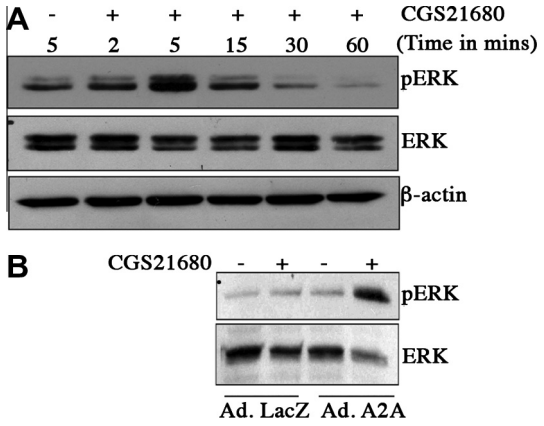

Fig. 4. Adenosine A_{2A} receptor activation enhances ERK1/2 phosphorylation. (A) Cells were serum starved for 48 h in EBM2 with 0.1% human serum albumin after which CGS21680 (1 μM) was added. After 10 min incubation, cell lysates were prepared and western blotting performed using phospho-ERK1/2 (Tyr 202/Thr 204) antibody. Blots were subsequently stripped and probed with ERK1/2 antibody. (B) Cells were transduced with Ad.A_{2A} or the control Ad.LacZ in complete EBM2 medium and serum starved for 24 h after which CGS21680 (1 μM) was added for 10 min. Cell lysates were then prepared and western blotting performed using phospho-ERK1/2 (Tyr 202/Thr 204) antibody. Blots were subsequently stripped and probed with ERK1/2 antibody.

4. Discussion

Among adenosine receptor subtypes in pulmonary endothelial cells, A_{2A} has a characteristic role in that it is induced by hypoxia and HIF-2α and it can promote endothelial proliferation [8]. This is in sharp contrast to its function in other cell types, including PC12 (pheochromocytoma) and smooth muscle cells, where adenosine A_{2A} receptor activation decreased proliferation, and receptor knockout caused increased proliferation [36–38]. Thus, the role of this receptor is dependent on cell and tissue type. ERK1/2 and PI3K are known mediators of growth and proliferation, and several studies suggest that these could have a role in A_{2A} receptor-mediated signaling. Endothelial dysfunction, as seen in pulmonary hypertension, can cause PI3K/Akt and ERK1/2 pathway activation [39–41]. However, mechanisms of such activation are unclear. It appears

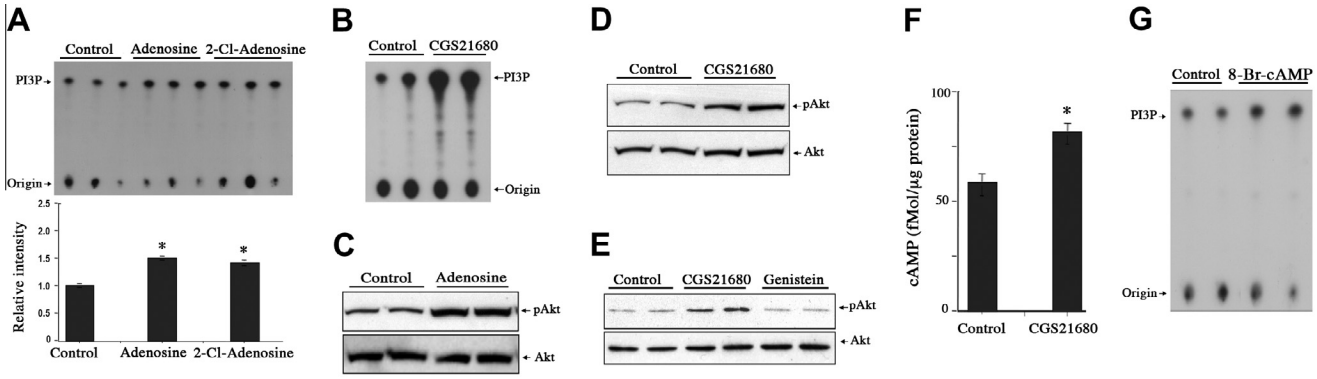

Fig. 3. Effect of adenosine and CGS21680 on Akt phosphorylation and PI3K activity. (A) Cells were serum-starved for 48 h in EBM2 with 0.1% human serum albumin after which adenosine (100 μM) or 2-Cl-Ado (1 μM) was added. After a 10 min incubation, cell lysates were prepared and 500 μg of protein was incubated with 20 μl (200 μg) of anti-p85–conjugated agarose, and the PI3K activity was measured in the immunoprecipitate as described in Methods. Upper panel shows a representative autoradiogram demonstrating PI3K-mediated phosphorylation of phosphoinositides. The lower panel shows a quantitative representation of PI3K activity as assessed by densitometry of the scans of spots obtained by autoradiogram. Results are arbitrary densitometric units. (B) Cells were similarly serum starved and treated with CGS21680 for 10 min after which PI3K activity was measured. (C) Cells were similarly serum starved and treated with adenosine (100 μM) for 10 min after which western blotting was performed using phospho-Akt (Ser473) antibody. (D) Cells were similarly serum starved and treated with CGS21680 (1 μM) for 10 min after which western blotting was performed using phospho-Akt (Ser473) antibody. Blots were subsequently stripped and probed with Akt antibody. (E) Serum-starved cells were treated with CGS21680 (1 μM) for 10 min in the presence or absence of Genistein (1 μM) after which lysates were made and western blotting was performed using phospho-Akt (Ser473) antibody. Blots were subsequently stripped and probed with Akt antibody. (F) Serum-starved cells were treated with the A_{2A} receptor agonist, CGS21680 (1 μM). cAMP was measured using ELISA. (G) Similarly serum-starved cells were treated with 8-Br-cAMP, a stable analog of cAMP, and PI3K activity measured.

that activation of A_{2A} receptor in HLMVECs can cause activation of the pro-proliferative PI3K/Akt and ERK1/2 pathways as inhibition of both ERK1/2 and PI3K can diminish the proliferative response to A_{2A} receptor activation.

Adenosine is a natural ligand for adenosine receptors and adenosine synthesis is induced by hypoxia [42]. Adenosine-activated signaling pathways are incompletely understood in pulmonary vascular cells. This point is relevant since significant differences exist in endothelial cells arising from different vascular beds [43–45]. Adenosine caused increased Akt phosphorylation as well as increased PI3K activity, possibly through A_{2A} receptor activation. The effect was mimicked by CGS21680, the specific agonist of A_{2A} receptor, and also by cAMP, which is formed upon activation of the receptor. These findings are consistent with another report in which A_{2A} receptor activated PI3K in hepatocytes through a cAMP/adenylyl cyclase-dependent pathway [46].

Adenosine A_{2A} receptor is also known to couple to tyrosine kinases [33,47], and tyrosine kinase inhibitors could influence PI3K/Akt signaling. Genistein, a tyrosine kinase inhibitor and inhibitor of PI3K/Akt [48], caused decreased A_{2A} receptor-mediated Akt phosphorylation. The fact that genistein can also reverse pulmonary hypertension [49] further highlights the significance of A_{2A} receptor in such diseases where endothelial proliferation is a hallmark. Additionally, the fact that A_{2A} receptor activation also increases ERK1/2 phosphorylation further supports its pro-proliferative role.

These studies underscore the importance of adenosine A_{2A} receptor in activation of survival and proliferative pathways in pulmonary endothelial cells. Additionally, these studies suggest that A_{2A} receptor could be targeted to decrease vascular remodeling in diseases such as pulmonary hypertension wherein endothelial cell proliferation is excessive.

Acknowledgments

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