Original Article

Adenosine Attenuates Human Coronary Artery Smooth Muscle Cell Proliferation by Inhibiting Multiple Signaling Pathways That Converge on Cyclin D

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Abstract—The goal of this study was to determine whether and how adenosine affects the proliferation of human coronary artery smooth muscle cells (HCASMCs). In HCASMCs, 2-chloroadenosine (stable adenosine analogue), but not N^6-cyclopentyladenosine, CGS21680, or N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide, inhibited HCASMC proliferation (A_{2B} receptor profile). 2-Chloroadenosine increased cAMP, reduced phosphorylation (activation) of ERK and Akt (protein kinases known to increase cyclin D expression and activity, respectively), and reduced levels of cyclin D1 (cyclin that promotes cell-cycle progression in G1). Moreover, 2-chloroadenosine inhibited expression of S-phase kinase–associated protein-2 (Skp2; promotes proteolysis of $p27^{Kip1}$) and upregulated levels of $p27^{Kip1}$ (cell-cycle regulator that impairs cyclin D function). 2-Chloroadenosine also inhibited signaling downstream of cyclin D, including hyperphosphorylation of retinoblastoma protein and expression of cyclin A (S phase cyclin). Knockdown of A_{2B} receptors prevented the effects of 2-chloroadenosine on ERK1/2, Akt, Skp2, $p27^{Kip1}$, cyclin D1, cyclin A, and proliferation. Likewise, inhibition of adenylyl cyclase and protein kinase A abrogated 2-chloroadenosine's inhibitory effects on Skp2 and stimulatory effects on $p27^{Kip1}$ and rescued HCASMCs from 2-chloroadenosine–mediated inhibition. Knockdown of $p27^{Kip1}$ also reversed the inhibitory effects of 2-chloroadenosine on HCASMC proliferation. In vivo, peri-arterial (rat carotid artery) 2-chloroadenosine (20 µmol/L for 7 days) downregulated vascular expression of Skp2, upregulated vascular expression of $p27^{Kip1}$, and reduced neointima hyperplasia by 71% ($P<0.05$; neointimal thickness: control, 37 424±18 371 pixels; treated, 10 352±2824 pixels). In conclusion, the adenosine/A_{2B} receptor/cAMP/protein kinase A axis inhibits HCASMC proliferation by blocking multiple signaling pathways (ERK1/2, Akt, and Skp2) that converge at cyclin D, a key G1 cyclin that controls cell-cycle progression. **(*Hypertension*. 2015;66:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05912.)** • Online Data Supplement

Key Words: adenosine ■ A_{2B} receptor ■ cyclin D1 ■ $p27^{Kip1}$ ■ Skp2 ■ vascular smooth muscle cells

Excessive proliferation of some cell types (eg, vascular smooth muscle cells [VSMCs], glomerular mesangial cells [cells phenotypically similar to VSMCs], and cardiac fibroblasts) and deficient proliferation of other cell types (eg, vascular endothelial cells and renal epithelial cells) can trigger hypertension-induced pathological vascular, cardiac, and renal remodeling, leading to cardiovascular and renal diseases.[1] Thus, endogenous factors that inhibit proliferation of VSMCs, glomerular mesangial cells, and cardiac fibroblasts and that stimulate the proliferation of vascular endothelial cells and renal epithelial cells may provide protection against cardiovascular and renal diseases. Adenosine seems to be one such factor. Adenosine potently inhibits the proliferation of rat renal preglomerular VSMCs,[2,3] rat[4–8] and human[9] aortic VSMCs, rat[3,10] and human[11] glomerular mesangial cells, and rat cardiac fibroblasts[12–16]; yet, adenosine stimulates the proliferation of rat aortic,[17] rat renal microvascular,[18] and porcine coronary[17] vascular endothelial cells, as well as human[18] renal epithelial cells. In addition, adenosine has several other desirable tissue-protecting actions, such as promoting neovascularization[19–21] and preventing and reducing inflammation and hypoxia.[22–27] Thus, adenosine per se, adenosine receptor agonists, or adenosine-modulating drugs (ie, the broad class of adenosinergic drugs) may be useful for preventing and treating several cardiovascular

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and renal diseases induced by hypertension, particularly those associated with excessive proliferation of VSMCs. However, whether adenosine inhibits human coronary artery smooth muscle cell (HCASMC) proliferation is unclear, and one objective of the current study was to determine the effects of adenosine on this critically important cell type.

Although adenosine is well known to inhibit proliferation of some types of VSMCs, the underlying mechanism by which adenosine inhibits mitogen-induced cell proliferation is unknown. There is increasing evidence that mitogens promote cell proliferation by engaging ERK1/2 and Akt signaling pathways that converge at cyclin D (Figure 1), a G1 phase cyclin with 3 isoforms (D1, D2, and D3, with D1 being the most widely expressed). ERK1/2 phosphorylates transcription factors that increase the expression of cyclin D[28], whereas Akt increases the activity of cyclin D via phosphorylating ezrin–radixin–moesin–binding phosphoprotein 50. In this regard, ezrin–radixin–moesin–binding phosphoprotein 50 stabilizes S-phase kinase–associated protein-2 (Skp2) and optimizes its cellular location.[29] Skp2 promotes the polyubiquitination of $p27^{Kip1}$ and thus accelerates $p27^{Kip1}$ degradation,[30] thereby decreasing levels of $p27^{Kip1}$. Normally, $p27^{Kip1}$ binds to complexes of cyclins with their respective cyclin-dependent kinases (Cdk), thus preventing cyclin–Cdk complexes from phosphorylating their substrates.[31] Importantly, $p27^{Kip1}$ impairs the function of cyclin D–Cdk4/6 complexes[31] that are primarily responsible for promoting cell-cycle progression in G1 phase of the cell cycle.[32,33] Therefore, a reduction of $p27^{Kip1}$ augments cyclin D activity. Cyclin D promotes, via activation of Cdk4/6, hyperphosphorylation of retinoblastoma protein (Rb), causing Rb to release the protein elongation 2 factor.[34] Elongation 2 factor then serves as a transcription factor to increase the expression of genes for G1/S and S phase cyclins,[34] thus driving the cell cycle through S and G2 phases and finally mitosis and cytokinesis (Figure 1).

How could adenosine interfere with mitogen-induced cell proliferation? Accumulating evidence suggests that in some cell types, adenosine mediates antiproliferative effects via A_{2B} receptors.[7,9,35,36] Stimulation of A_{2B} receptors activates adenylyl cyclase, resulting in increased cAMP production,[37] and studies by Wu et al demonstrate that cAMP, via protein kinase A (PKA), may downregulate the expression of Skp2,[38,39] which in turn increases the levels of $p27^{Kip1}$. In addition, PKA can interfere with signaling cascades that phosphorylate (activate) ERK1/2[40,41] and Akt,[42] thus providing additional mechanisms for inhibiting cyclin D signaling. Together, this information suggests the hypothesis shown in Figure 1 that adenosine could inhibit HCASMC proliferation by engaging the A_{2B} receptor/adenylyl cyclase/cAMP/PKA pathway, which is followed by PKA-mediated inhibition of multiple signaling pathways that converge at cyclin D. The net result is the reduced expression and function of cyclin D, which arrests cells in G1. Another goal of the present study was to test this hypothesis.

Methods

Materials

Adenosine, 2-chloroadenosine (stable adenosine analogue), and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; increases endogenous adenosine by inhibiting adenosine deaminase and thus reducing the metabolism of adenosine to inosine) were purchased from Sigma-Aldrich (St. Louis, MO). N^6-cyclopentyladenosine (CPA; selective A_1 receptor agonist), CGS21680 (selective A_{2A} receptor agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; selective A_1 receptor antagonist), 5-iodotubercidin (IDO; increases endogenous adenosine by inhibiting adenosine kinase and thus reducing the metabolism of adenosine to 5′-AMP), 5′-*N*-ethylcarboxamidoadenosine (NECA; nonselective adenosine receptor agonist), 5′-*N*-methylcarboxamidoadenosine (MECA; nonselective adenosine receptor agonist), 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA; selective A_3 receptor agonist), SCH442416 (selective A_{2A} receptor antagonist), MRS1754 (selective A_{2B} receptor antagonist), and VUF5574 (selective A_3 receptor antagonist) were purchased from Tocris (Minneapolis, MN). ^{3}H-thymidine (specific activity, 11.8 Ci/mmol) was purchased from PerkinElmer NEN (Walthan, MA). All other reagents were of tissue culture or best grade available.

Cell Cultures

HCASMCs from 3 donors were procured from GIBCO (Life Technologies, CA) and LONZA (Walkersville, MD). The cells were precharacterized for smooth muscle cell–specific markers and for their compatible (<5% variation) growth response to fetal calf serum (FCS; 2.5%). Cells were cultured in M231 culture medium containing smooth muscle growth supplement (Life Technologies, CA) and under standard tissue culture conditions as described previously.[43] HCASMCs in third to fifth passage were used for the growth and molecular assays.

^{3}H-Thymidine Incorporation

To assess DNA synthesis by HCASMCs, we used ^{3}H-thymidine incorporation as described previously.[43] HCASMCs grown to subconfluence and serum-starved for 24 hours were treated for 48 hours with 2.5% FCS in medium with or without test agents. Four hours before the termination of the experiment, cells were pulsed with ^{3}H-thymidine, and the incorporation of ^{3}H-thymidine into the DNA was analyzed by measuring radioactivity in the acid-insoluble fraction using a β-scintillation counter.

Cell Number

After serum starvation, cultures were treated with 2.5% FCS with or without test agents. After 4 days, cells were dislodged by trypsinization and counted in a Coulter Counter.

Cell Cycle Analysis

HCASMCs at 60% confluence were serum-starved for 24 hours and then grown in 2.5% FCS for 3 days. Cells were stained with propidium iodide, and DNA content was analyzed by flow cytometry.

Cell Migration Studies

2.5% FCS-induced HCASMC migration was assessed using the modified Boydens chamber and as previously described in detail by us.[43]

Assays for Intracellular Mechanisms

Changes in the phosphorylation state of signal transduction proteins and changes in the expression of cell cycle regulatory proteins were analyzed by Western blotting as previously described.[44] Briefly, cells were grown and treated in 60 mm culture dishes and were washed once with PBS and then lysed in 70 µL of lysis buffer (Cell Signaling Technology, Beverly, MA). The samples were sonicated, and the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were denatured by boiling the samples at 95°C for 5 minutes. Equal amounts of protein (10–20 µg/lane) were diluted in 5× loading buffer (Fermentas, Hanover, MD) plus 0.1 mol/L dithiothreitol and 2.5% 2-mercaptoethanol, and proteins were resolved using a 10% sodium dodecyl sulfate–polyacrylamide gel and then transferred to a nitrocellulose membrane. Subsequently, for specific protein expression, the membranes were blocked in 5% nonfat dry milk in PBS/0.2% Tween 20 (overnight at 4°C) and incubated with the primary antibody for specific times at room temperature or 4°C (Table S1 in the online-only Data Supplement). Primary antibodies (Table S1) were diluted in washing buffer (1% nonfat dry milk in PBS/0.2% Tween 20) and were specific for the proteins investigated and had cross-reactivity for both human and rat proteins. Following incubation with the primary antibodies, the membranes were incubated for 1 hour with the second antibody (goat anti-mouse IgG-peroxidase conjugated [Pierce 31430, diluted 1:25 000] or goat anti-rabbit IgG-peroxidase conjugated [Pierce 31460, diluted 1:25 000]). Peroxidase activity was detected using ECL (Pierce), and the membranes were exposed to Hyperfilm ECL (Amersham, Dübendorf, CH).

A_{2B} and p27 Silencing Studies

Smart pool on target plus siRNA kit from Dharmacon was used according to the instructions to silence CDKN1B ($p27^{kip1}$) or ADORA2B (human A_{2B} adenosine receptors) in HCASMCs. Control smart pool siRNA from Dharmacon was used as control.

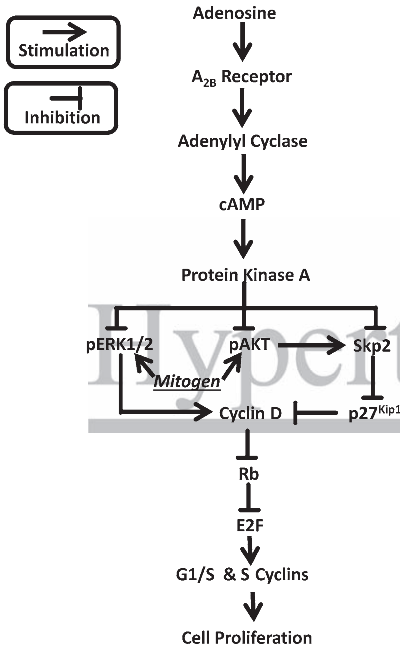

Figure 1. Signaling schematic depicting our hypothesis of how adenosine regulates human coronary artery smooth muscle cell cell-cycle progression. Extracellular mitogens activate classical signal transduction pathways that ultimately phosphorylate (and thus activate) ERK1/2 and Akt. ERK1/2 is well known to increase expression of cyclin D (G1 phase cyclin). Phosphorylated Akt activates a signal transduction pathway that stabilizes S-phase kinase–associated protein-2 (Skp2), which is the F-box protein of SCF^{Skp2} ubiquitin ligase that polyubiquitinates $p27^{Kip1}$ and thus accelerates the degradation of $p27^{Kip1}$. Removal of $p27^{Kip1}$ deinhibits cyclin D activity. Cyclin D activates cyclin-dependent kinases 4 and 6 to hyper-phosphorylate retinoblastoma protein (Rb), thus releasing the transcription factor elongation 2 factor (E2F) and allowing increased expression of G1/S and S phase cyclins. G1/S and S phase cyclins then drive the cell cycle forward to complete mitogenesis and cytokinesis (cell proliferation). Adenosine stimulates A_{2B} receptors that are positively coupled to adenylyl cyclase, which increases the formation cAMP and activates protein kinase A. Protein kinase A decreases expression of Skp2 and inhibits phosphorylation of Akt, resulting in increased levels of $p27^{Kip1}$ which reduce cyclin D activity. Protein kinase A also inhibits phosphorylation of ERK1/2, which reduces cyclin D expression. This protein kinase A (PKA)–induced signaling converges at cyclin D. This results in inhibition of Rb hyperphosphorylation, and hypophosphorylated Rb can now bind E2F and prevent this transcription factor from increasing the expression of G1/S and S phase cyclins.

cAMP Levels

Extracellular (supernatant) and intracellular (cellular fraction) cAMPs were pooled, and total cAMP levels were analyzed by high-performance liquid chromatography using our previously described method.[9]

Carotid Artery Injury Studies

Balloon injury–induced neointima formation was assessed in animals (male Wistar–Kyoto rats; 350–400 g; Harlan, Fullinsdorf, Switzerland), as described previously.[45,46] Briefly, animals were anesthetized with ketamine plus xylazine (intraperitoneal injection). To induce arterial injury, the left common carotid artery was exposed at the bifurcation, and a 2F Fogarty embolectomy catheter was inserted. The inflated balloon was pulled through the common carotid artery 3× to completely denude the endothelium, and the external carotid artery was permanently ligated. 2-Chlororadenosine (20 µmol/L) was added to 25% (wt/vol) pluronic gel solution (F127, BASF Corp, Parsipanny, NJ) and kept in nongelled form at 4°C. The neck muscles adjacent to the carotid artery were separated to expose the artery and to provide a space for the gel by lifting the artery slightly from the muscle with forceps. The liquid solution (100 µL) was then topically applied with an Eppendorf pipet on the exposed carotid artery. At 37°C, the solution rapidly gelled, and the vessel was thus covered by a translucent layer enveloping the treated area (≈1 cm length of artery). Because no muscles were cut, all tissues returned to their original position, and the carotid artery was covered again by muscle. The skin was subsequently sutured into place with 3 to 4 stiches of silk suture. After 7 days, the animals were euthanized and perfusion-fixed for morphometric analysis. To assess the impact of 2-chloroadenosine on proliferation of intimal carotid artery VSMCs after balloon injury, animals (placebo n=7, treated n=7) receiving the vehicle or 2-chloroadenosine were euthanized and perfusion-fixed 7 days after balloon injury and sections immunostained for Ki67 to assess proliferating carotid artery VSMCs. The 7-day period was selected because it is well documented that the proliferative activity of carotid artery VSMCs peaks at day 7 after injury.[47,48] To assess whether 2-chloroadenosine affects expression of Skp2 and $p27^{Kip1}$ in vivo, rats (placebo n=5 and treated n=5) were euthanized on day 8 and the carotid arteries snap-frozen in liquid nitrogen. Subsequently, segments from placebo or 2-chloroadenosine–treated animals were homogenized and lysed, and proteins of interest were analyzed using Western blotting.

Statistics

Treatment effects on cross-sectional areas were analyzed by using analysis of variance or the nonparametric Kruskal–Wallis test. Expression and growth data were analyzed using analysis of variance, and statistical significance ($P<0.05$) was calculated using Fisher's least significant difference test. All growth experiments were performed in triplicates or quadruplicates using 3 separate HCASMC cultures. For Western blotting experiments, each treatment was conducted in triplicate or quadruplicates and with 3 separate HCASMC cultures. The densitometeric analysis of protein expression is presented as a ratio against the appropriate control (phosphorylated ERK1/2 to ERK1/2; phosphorylated Akt to Akt; cyclin D1 to β-actin; cyclin A to β-actin; $p27^{Kip1}$ to β-actin; Skp2 to β-actin).

Results

Because the expression profile of adenosine receptor subtypes may determine the overall pharmacology of adenosine, we first probed for the presence of adenosine receptor subtypes in the HCASMCs used in the present study. cDNA size fractionation showed strong expression of mRNA for A_1 and A_{2B} receptors, but only weak mRNA expression for A_{2A} and A_3 receptors (Figure 2A). Likewise, Western blotting detected strong bands for A_1 and A_{2B} receptors, a faint band for A_{2A}

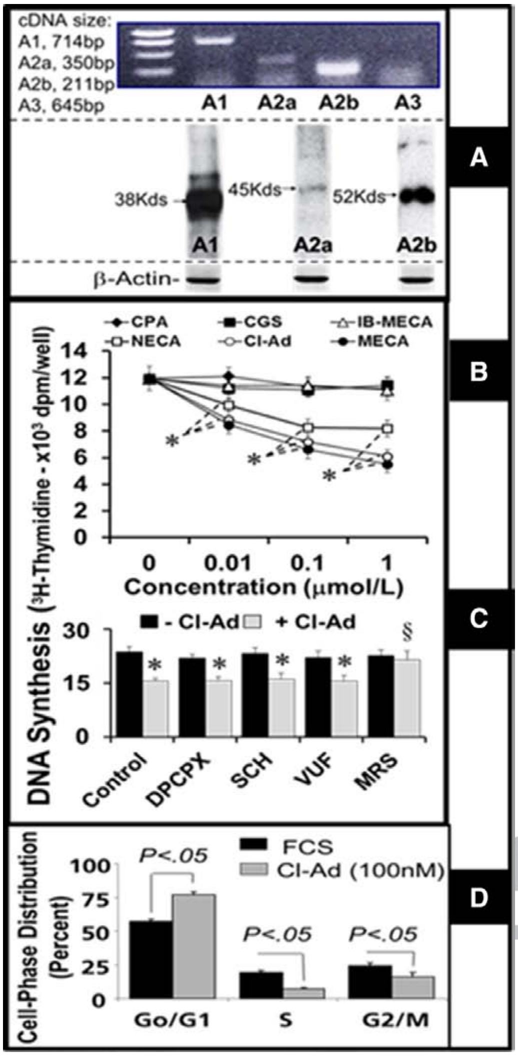

Figure 2. Adenosine A_{2B} receptor mediated inhibition of human coronary artery smooth muscle cell growth. **A**, Representative northern blots (**upper**) and western blots (**lower**) showing the presence of A_1 and A_{2B} receptors, with low expression of A_{2A} receptors and minimal expression of A_3 receptors. **B**, Concentration–response relationships for the inhibition of ^{3}H-thymidine incorporation (DNA synthesis) by 2-chloroadenosine (Cl-Ad), 5′-*N*-methylcarboxamidoadenosine (MECA), 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA), 5′-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), and CGS21680 (CGS). **C**, The effects of Cl-Ad (1 μmol/L) on DNA synthesis in the presence and absence of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; selective A_1 antagonist, 100 nmol/L); SCH442416 (SCH; selective A_{2A} antagonist, 100 nmol/L); VUF5574 (VUF; selective A_3 antagonist, 100 nmol/L); and MRS1754 (MRS; selective A_{2B} antagonist, 100 nmol/L). **D**, The inhibitory effects of Cl-Ad (100 nmol/L) on cell-cycle distribution. *$P<0.05$ vs no treatment; §$P<0.05$ significant reversal of Cl-Ad effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates or quadruplicates.

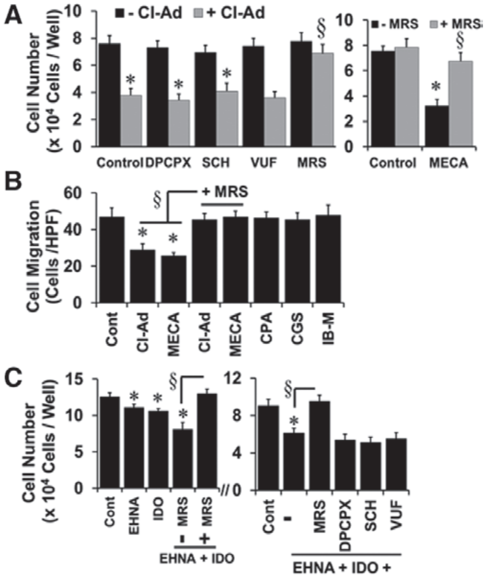

Figure 3. A, Bar graphs show the effects of 2-chloroadenosine (Cl-Ad; 1 μmol/L) and 5′-*N*-methylcarboxamidoadenosine (MECA; 1 μmol/L) on cell number in human coronary artery smooth muscle cells (HCASMCs). The inhibitory effects of Cl-Ad were reversed by MRS1754 (MRS; A_{2B} receptor antagonist), but not by SCH442416 (SCH; A_{2A} receptor antagonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A_1 antagonist), or VUF5574 (VUF; A_3 antagonist). Similar to Cl-Ad, the effects of MECA were blocked by MRS1754. *$P<0.05$ vs control; §significant reversal of the inhibitory effects. **B**, Bar graph demonstrates the effects of Cl-Ad (1 μmol/L) and MECA (1 μmol/L) on cell migration in HCASMCs. The inhibitory effects of Cl-Ad were mimicked by MECA, but not by N^6-cyclopentyladenosine (CPA; A_1 agonist), CGS21680 (CGS; A_{2A} agonist), or 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA; IB-M: A_3 adenosine receptor agonist). Moreover, the effects of Cl-Ad and MECA were reversed by MRS1754 (MRS; A_{2B} receptor antagonist). *$P<0.05$ vs control; §significant reversal of the inhibitory effects. **C**, Effects of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 5 μmol/L; adenosine deaminase inhibitor) and 5-iodotubercidin (IDO; 0.1 μmol/L; adenosine kinase inhibitor) on cell number in HCASMCs. The inhibitory effects were significantly enhanced when the adenosine catabolism inhibitors EHNA+IDO were combined. Moreover, the effects of EHNA+IDO were reversed by MRS1754 (MRS; A_{2B} receptor antagonist), but not by SCH442416 (SCH; A_{2A} receptor antagonist), DPCPX (A_1 antagonist), or VUF5574 (VUF; A_3 antagonist), suggesting that endogenous adenosine inhibits HCASMC growth via A_{2B} receptors. *$P<0.05$ vs control; §significant reversal of the inhibitory effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates or quadruplicates.

receptors, and no signal for A_3 receptors (Figure 2A). These findings suggest that A_1 or A_{2B} receptors would likely dominate the pharmacology of adenosine in these HCASMCs.

Treatment of HCASMCs with 2-chloroadenosine (stable adenosine analogue) concentration-dependently attenuated DNA synthesis (Figure 2B). Using various pharmacological agents (adenosine receptor subtype selective and nonselective agonists and antagonists), we further assessed the role of all adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) in mediating the anti-mitogenic effects in HCASMCs. The highest (1 μmol/L) concentrations of CPA (A_1 receptor–selective agonist), CGS21680 (A_{2A} receptor–selective agonist), and IB-MECA (A_3 receptor–selective agonist) failed to inhibit DNA synthesis (Figure 2B). MECA was slightly more potent than NECA (both are nonselective adenosine receptor agonists; Figure 2B). MRS1754 (A_{2B} receptor–selective antagonist), but not DPCPX (A_1 receptor–selective antagonist), SCH442416 (A_{2A} receptor–selective antagonist), or VUF5574 (A_3 receptor–selective antagonist), blocked the

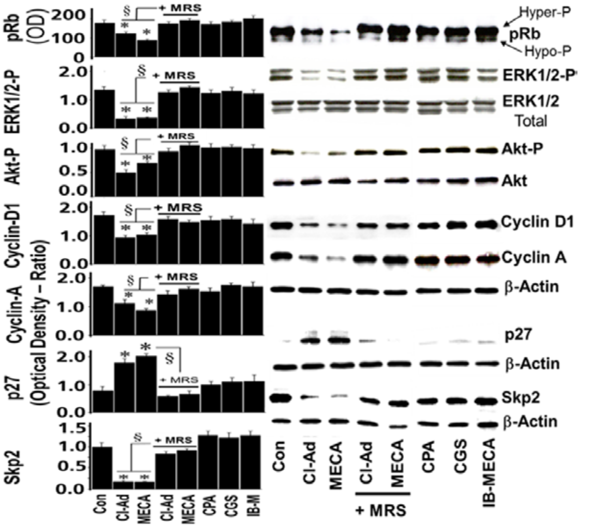

Figure 4. Western blots show effects of 0.5 μmol/L of 2-chloroadenosine (Cl-Ad), 5′-*N*-methylcarboxamidoadenosine (MECA), N^6-cyclopentyladenosine (CPA), CGS21680 (CGS), 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA; IB-M), Cl-Ad+MRS1754 (MRS; 100 nmol/L), or MECA+MRS1754 on levels of hyperphosphorylated retinoblastoma protein (pRb), phosphorylated ERK1/2 (ERK1/2-P), total ERK1/2, phosphorylated Akt (Akt-P), total Akt, cyclin D1, cyclin A, p27^{Kip1} (p27), and S-phase kinase–associated protein-2 (Skp2) expression in human coronary artery smooth muscle cells (HCASMCs). Sub-confluent monolayers of HCASMCs growth arrested for 36 hours in 0.4% BSA were treated and stimulated with 2.5% fetal calf serum (FCS) for 48 hours. Cell lysates were subsequently analyzed by western blotting. The densitometric analysis of all the protein expression is presented as a ratio against the appropriate control (phosphorylated ERK1/2 to ERK1/2; phosphorylated Akt to Akt; cyclin D1 to β-actin; cyclin A to β-actin; p27^{Kip1} to β-actin; Skp2 to β-actin). *P<0.05 versus control; §significant reversal of the inhibitory effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates or quadruplicates.

effects of 2-chloroadenosine on DNA synthesis (all antagonists at 100 nmol/L; Figure 2C). Moreover, the inhibitory effects of 2-chloroadenosine on cell number were reversed by MRS1754, but not by DPCPX, SCH442416, or VUF5574 (Figure 3A). Similar to 2-chloroadenosine, the inhibitory effects of MECA on cell number were antagonized by MRS1754 (Figure 3A). Treatment with 2-chloroadenosine and MECA, but not CPA, CGS21680, or IB-MECA, inhibited HCASMC migration, and the inhibitory effects of 2-chloroadenosine and MECA on cell migration were blocked by MRS1754 (Figure 3B). EHNA (blocks adenosine deaminase) and IDO (blocks adenosine kinase), administered separately, inhibited cell proliferation, and these effects were enhanced in cells treated with EHNA plus IDO (Figure 3C). MRS1754, but not DPCPX, SCH442416, or VUF5574, abrogated the inhibitory effects of EHNA plus IDO on cell proliferation (Figure 3C, right panel). Cell cycle distribution experiments using flow cytometry demonstrated that 2-chloroadenosine increased the percentage of cells in G0/G1 while reducing the percentage of cells in S phase and G2/M phase (Figure 2D). Trypan blue exclusion tests demonstrated that none of the aforementioned treatments altered cell viability. These findings indicate that in HCASMCs, A$_{2B}$ receptors dominate the pharmacology of adenosine, leading to inhibition of cell proliferation, DNA synthesis, and cell migration and arrest of cells in the G0/G1 phase of the cell cycle.

Treatment of HCASMCs with 2-chloroadenosine inhibited hyperphosphorylation of Rb and phosphorylation of ERK1/2 and Akt (Figure 4). Moreover, treatment with 2-chloroadenosine decreased levels of Skp2 (F-box protein of SCF^{Skp2} ubiquitin ligase responsible for polyubiquitination of and subsequent proteolysis of p27^{Kip1}) and upregulated levels of p27^{Kip1} (p27^{Kip1} inhibits cell cycle progression by blocking function of cyclins). These effects were accompanied by inhibition of cyclin A and cyclin D1 expression (Figure 4). The modulatory effects of 2-chloroadenosine on signal transduction proteins were mimicked by MECA, but not by CPA, CGS21680, or IB-MECA (Figure 4). The modulatory effects of 2-chloroadenosine and MECA on signal transduction pathways were blocked by MRS1754 (Figure 4), implying a role for A$_{2B}$ receptors in mediating the inhibitory effects of adenosine on cell cycle progression in HCASMCs.

Western blotting confirmed that treatment with siRNA silenced the expression of A$_{2B}$ receptors (Figure 5A). At the functional level, 2-chloroadenosine increased cAMP production in control cells and cells treated with negative-control siRNA, but not in cells treated with A$_{2B}$ receptor siRNA (Figure 5A). 2-Chloroadenosine inhibited DNA synthesis in HCASMCs treated with negative-control siRNA but not in cells treated with A$_{2B}$ siRNA (Figure 5B). Also the inhibitory effects of MECA, NECA, and EHNA+IDO were blocked by A$_{2B}$ siRNA (Figure 5B). Downregulation of A$_{2B}$ receptors by siRNA did not abrogate the inhibitory effects of 8-bromo-cAMP on DNA synthesis (Figure 5B**).** These results further support the conclusion that A$_{2B}$ receptors mediate the antimitogenic effects of adenosine.

In HCASMCs in which A$_{2B}$ receptors were silenced, treatment with 2-chloroadenosine failed to abrogate phosphorylation of key signal transduction proteins (pRb, ERK1/2, and Akt) associated with cell proliferation (Figure 6). The inhibitory effects of 2-chloroadenosine on cell cycle regulatory proteins cyclin A and cyclin D1 were also abrogated in HCASMCs with silenced A$_{2B}$ receptors. Additionally, the inhibitory effects of 2-chloroadenosine on Skp2 and stimulatory effects on p27^{Kip1} were lost in HCASMCs lacking A$_{2B}$ receptors (Figure 6).

cAMP may inhibit HCASMC proliferation via Skp2 downregulation and p27^{Kip1} upregulation.[38] Because 2-chloroadenosine–stimulated cAMP production was inhibited in HCASMCs with silenced A$_{2B}$ receptors, we further elucidated the role of this pathway in mediating the inhibitory effects of 2-chloroadenosine on cell proliferation. Treatment of HCASMCs with 2-chloroadenosine concentration–dependently decreased Skp2 and increased p27^{Kip1} expression (Figure 7A). The stimulatory effects of 2-chloroadenosine on p27^{Kip1} expression in HCASMCs was abolished in cells in which adenosine A$_{2B}$ receptors were silenced with siRNA (Figure 7B). Moreover, the inhibitory effects of 2-chloroadenosine on DNA synthesis were abrogated in HCASMCs in which adenosine A$_{2B}$ receptors were silenced (Figure 7B). The observations that the stimulatory effects of 2-chloroadenosine on p27^{Kip1} and inhibitory effects on DNA synthesis are abolished in HCASMCs lacking adenosine A$_{2B}$ receptors suggest a role

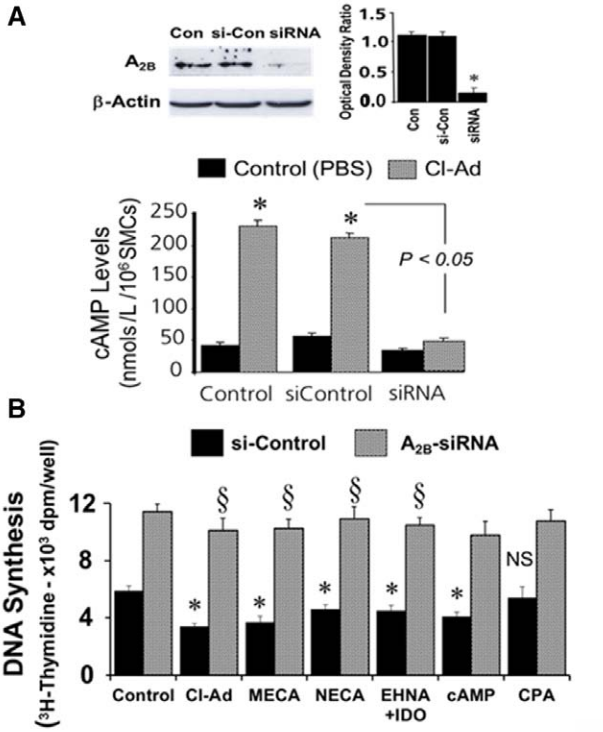

Figure 5. A (**Top**), Western blot depicting the downregulation of the expression of A_{2B} receptors in human coronary artery smooth muscle cells (HCASMCs) by siRNA against A_{2B} receptors. No treatment with siRNA (Con); treated with negative-control siRNA (si-Con); treated with siRNA against A_{2B} receptor (siRNA). Bar graph for the western blot represents change in optical density ratio of A_{2B} to β-actin. (**Bottom**), Depicts the effects of siRNA against A_{2B} receptors on the stimulatory effects of 2-chloroadenosine (Cl-Ad; 1 μmol/L) on cAMP levels in HCASMCs. No treatment with siRNA (Control); treated with negative-control siRNA (siControl); treated with siRNA against A_{2B} receptor (siRNA). **P*<0.05 vs no Cl-Ad. **B**, Inhibitory effects of Cl-Ad, 5′-*N*-methylcarboxamidoadenosine (MECA), 5′-*N*-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), 8-bromo-cAMP (cAMP), and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 10 μmol/L) plus 5-iodotubercidin (IDO; 0.1 μmol/L) on DNA synthesis in the absence and presence of A_{2B} receptor siRNA in HCASMCs. No treatment with agonists (Control); treated with negative-control siRNA (si-Control); treated with siRNA against A_{2B} receptor (A_{2B}-siRNA)]. **P*<0.05 vs no agonist; §significant reversal of the inhibitory effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates.

for p27^{Kip1} in A_{2B} receptor-mediated regulation of HCASMC proliferation.

Next, we tested whether adenylyl cyclase and PKA mediate the effects of 2-chloroadenosine on p27^{Kip1} and HCASMC growth via A_{2B} receptors. 2-Chloroadenosine inhibited Skp2 and upregulated p27^{Kip1} in the absence (Figure 8A) but not in the presence of the adenylyl cyclase inhibitor myristoylated trifluoroacetate or the PKA inhibitor 2′,5′-dideoxyadenosine (Figure 8A). Also, the inhibitory effects of 2-chloroadenosine on HCASMC DNA synthesis were blocked by PKA and adenylyl cyclase inhibitors (Figure 8A). To further confirm the link between cAMP and p27^{Kip1} in mediating the inhibitory effects of 2-chloroadenosine via A_{2B} receptors, we assessed the effects of 2-chloroadenosine on DNA synthesis in HCASMCs, where p27^{Kip1} expression was silenced. Treatment

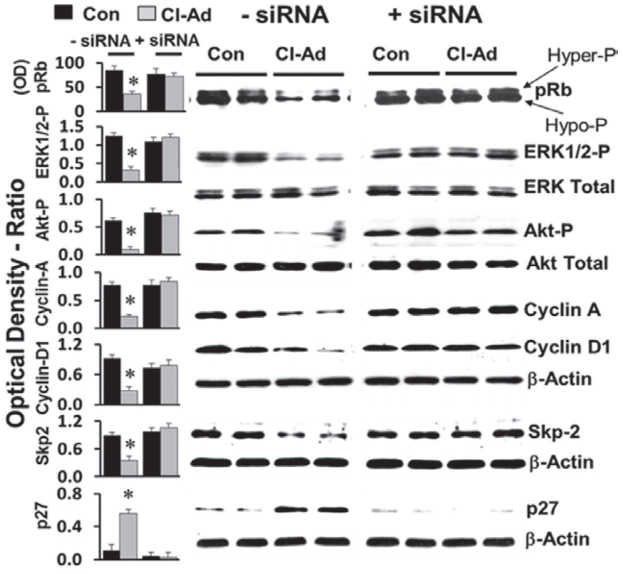

Figure 6. Inhibitory effects of 0.5 μmol/L of 2-chloroadenosine (Cl-Ad) on levels of hyper-phosphorylated (Hyper-P) and hypo-phosphorylated (Hypo-P) pRb, phosphorylated ERK1/2 (ERK1/2-P), total ERK1/2, phosphorylated Akt (Akt-P), total Akt, cyclin A, cyclin D1, S-phase kinase–associated protein-2 (Skp2), and p27^{Kip1} (p27) expression in human coronary artery smooth muscle cells (HCASMCs) in the absence (–siRNA) and presence (+siRNA) of A_{2B} receptor siRNA. Sub-confluent monolayers of HCASMCs were growth-arrested for 36 hours in 0.4% BSA and were treated and stimulated with 2.5% fetal calf serum (FCS) for 48 hours. Cell lysates were subsequently analyzed by Western blotting. The modulatory effects of Cl-Ad on the signaling and cell cycle–regulating proteins were lost in the absence of A_{2B} receptors. Bar graphs depict the optical density (OD) or OD ratio (phosphorylated ERK1/2 to ERK1/2; phosphorylated Akt to Akt; cyclin D1 to β-actin; cyclin A to β-actin; p27^{Kip1} to β-actin; Skp2 to β-actin) for the Western blots. **P*<0.05 versus control. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates.

of HCASMCs with p27^{Kip1} siRNA silenced p27^{Kip1} expression compared with cells treated with negative-control siRNA (Figure 8B). 2-Chloroadenosine inhibited DNA synthesis in HCASMCs treated with negative-control siRNA, but not in HCASMCs where p27^{Kip1} was silenced. Similar to 2-chloroadenosine, MECA and 8-bromo-cAMP inhibited DNA synthesis in HCASMCs treated with negative-control siRNA, but not in cells in which p27^{Kip1} was silenced (Figure 8C).

In serum-starved HCASMCs, silencing of A_{2B} receptors with siRNA resulted in a significant increase in DNA synthesis, and these effects were further enhanced by the A_1 adenosine receptor agonist CPA (Figure 9). Pretreatment with DPCPX, an A_1 receptor antagonist, blocked the stimulatory effect of A_{2B} silencing under basal conditions and in response to CPA. These finding indicate that in the absence of A_{2B} receptors, endogenous adenosine induces HCASMC growth via A_1 receptors.

Morphometeric analysis of carotid arteries showed significant intimal thickening after balloon injury, and this was significantly inhibited in rats receiving 2-chloroadenosine for 7 days. As shown in Figure 10A, compared with the placebo group (n=7; intima 37 424±18 371 pixels), the neointima formation was reduced by 71% in rats receiving periarterial 2-chloroadenosine (n=7; 10 352±2824; *P*<0.05 versus

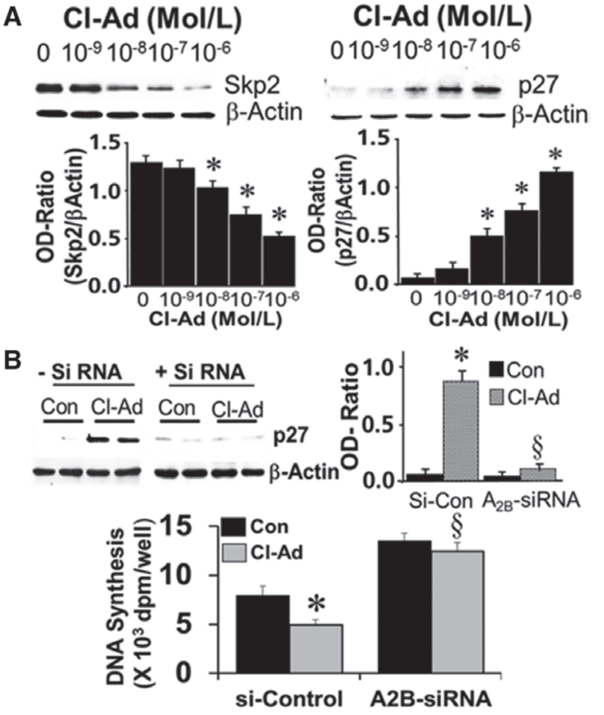

Figure 7. A, Concentration-dependent modulatory effects of 2-chloroadenosine (Cl-Ad) on S-phase kinase–associated protein-2 (Skp2) and p27^{Kip1} (p27) levels in human coronary artery smooth muscle cells (HCASMCs). Treatment of HCASMCs with Cl-Ad for 48 hours downregulated the expression of Skp2 and upregulated the expression of p27^{Kip1} (p27). **B,** Effects of Cl-Ad on p27^{Kip1} expression and DNA synthesis in HCASMCs with downregulated A_{2B} receptors. Compared with HCASMCs treated with negative-control siRNA (-Si RNA or siControl), Cl-Ad –induced expression of p27^{Kip1} and inhibition of DNA synthesis was abrogated in HCASMCs treated with siRNA against A_{2B} receptors (+SiRNA or A2B-siRNA). The optical density (OD) ratio in the bar graphs represents ratio of Skp2, p27^{Kip1}, or A_{2B} receptor to β-actin. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates.

placebo). The media and lumen areas did not significantly differ between the placebo and the 2-chloroadenosine-treated group (Table S2); however, the intimal/medial ratio was significantly reduced in animals receiving 2-chloroadenosine (Table S2). In carotid arteries obtained from animals receiving placebo, Ki67-positive cells (indicating proliferating of VSMCs) were observed (Figure 10B). As compared with the placebo group, a significant decrease in Ki67-positive VSMCs was observed in arteries obtained from animals treated with 2-chloroadenosine (Figure 10B). Treatment with 2-chloroadenosine was not associated with any toxic adverse effects. In this regard, the WBC count, RBC count, and hematocrit did not differ between placebo and 2-chloroadenosine-treated groups (Table S2). To assess whether Skp2 and p27^{Kip1} are involved in mediating the inhibitory effects of 2-chloroadenosine on intimal formation after balloon injury, we analyzed their levels in carotid lysates. As shown in Figure 10C, compared with placebo (n=5), treatment with 2-chloroadenosine (n=5) downregulated the expression of Skp2 and upregulated the expression of p27^{Kip1}.

In the studies described earlier, we used 2-chloroadenosine rather than adenosine because adenosine is rapidly metabolized (necessitating frequent treatments), whereas 2-chloroadenosine is resistant to metabolism (allowing once daily treatments). To make sure that adenosine per se qualitatively has the same effects as 2-chloroadenosine, we assessed the effects of adenosine on HCASMC proliferation. As shown in Figure 11A, adenosine inhibited DNA synthesis in a concentration-dependent manner. As expected, when cells were treated daily, adenosine was ≈10-fold less potent than 2-chloroadenosine in inhibiting HCASMC proliferation. Moreover, similar to 2-chloroadenosine, the inhibitory effects of adenosine were blocked by the A_{2B} receptor antagonist MRS1754 (Figure 11B). To assess whether decreased potency of adenosine is caused by its catabolism by adenosine deaminase and adenosine kinase, we assessed the growth inhibitory effects of adenosine in the presence and absence of adenosine deaminase and adenosine kinase inhibitors EHNA and IDO. The inhibitory effects of adenosine on cell number were significantly enhanced by EHNA+IDO (Figure 11B), and this effect was reversed by the A_{2B} receptor antagonist MRS1754, suggesting that adenosine catabolism is responsible for its reduced inhibitory potency in HCASMCs. To assess whether adenosine, like 2-chloroadenosine, inhibits HCASMC growth via upregulation of p27^{Kip1} and downregulation of Skp2, we assessed adenosine's effect on the expression of both p27^{Kip1} and Skp2. As shown in Figure 11C, treatment of HCASMCs with adenosine upregulated p27^{Kip1} and downregulated Skp2 expression. Taken together, these observations suggest that adenosine, although less potent than 2-chloroadenosine, inhibits HCASMC growth via similar mechanisms.

Discussion

Our experiments demonstrate that exogenous, as well as endogenous, adenosine inhibits mitogen-induced proliferation and migration of HCASMCs. In support of this conclusion, we observe that treatment of HCASMCs with a metabolically stable adenosine analog (2-chloroadenosine) or with agents that increase endogenous adenosine (EHNA plus IDO) inhibits HCASMC DNA synthesis, cell proliferation, and cell migration.

Our results also support the conclusion that adenosine inhibits proliferation of HCASMCs via activation of A_{2B} receptors. CPA, CGS21680, and IB-MECA are selective A_1 receptor, A_{2A} receptor, and A_3 receptor agonists, respectively; and DPCPX, SCH442416, and VUF5574 are selective A_1 receptor, A_{2A} receptor, and A_3 receptor antagonists, respectively. Because neither low concentrations of CPA, CGS21680, nor IB-MECA inhibit HCASMC proliferation and because neither DPCPX, SCH442416, nor VUF5574 blocks the inhibitory effects of 2-chloroadenosine on HCASMC proliferation, it is highly unlikely that A_1, A_{2A}, or A_3 receptors mediate the anti-mitogenic effects of adenosine on HCASMCs. Because there are only 4 known adenosine receptor subtypes and 3 of the 4 are ruled out, by the process of elimination, the A_{2B} receptor most likely is the receptor mediating the effects of adenosine on HCASMC growth. MECA and NECA are adenosine receptor agonists that activate multiple adenosine receptor subtypes, including A_{2B} receptors, and MRS1754 is an adenosine receptor antagonist that blocks selectively A_{2B} receptors. The fact that MECA and NECA mimic the effects

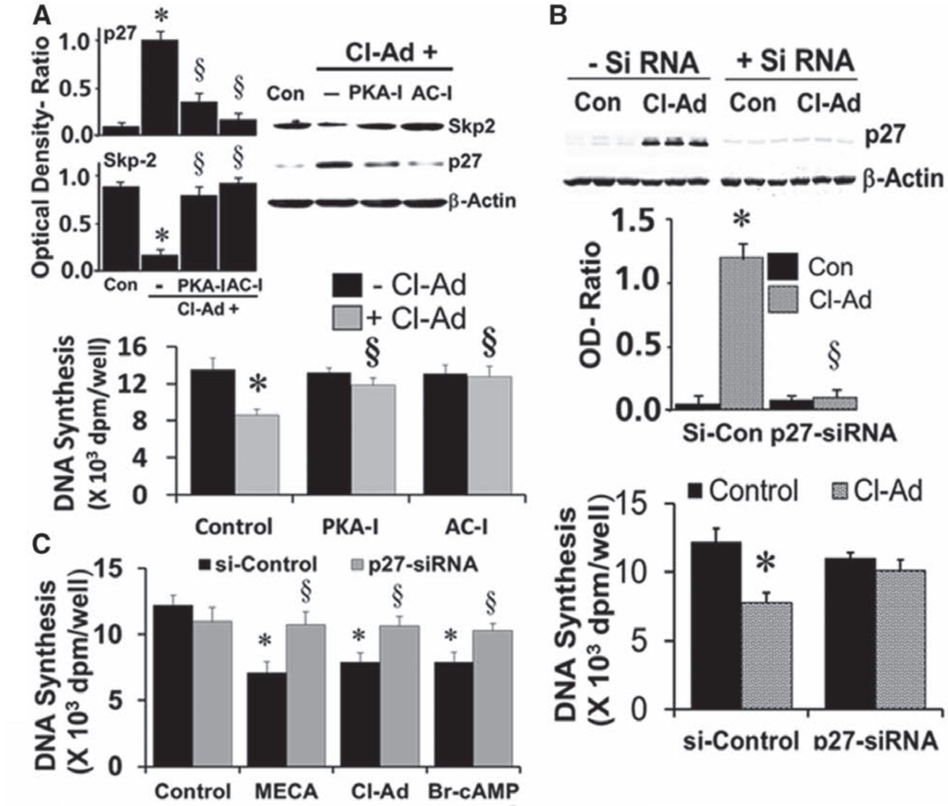

Figure 8. A, Protein kinase A (PKA) and adenylyl cyclase (AC) pathways mediate the inhibitory effects of 2-chloroadenosine (Cl-Ad) on human coronary artery smooth muscle cell (HCASMC) proliferation via modulation of S-phase kinase–associated protein-2 (Skp2) and $p27^{Kip1}$. Western blots show Skp2 and $p27^{Kip1}$ expression in HCASMCs treated with Cl-Ad (0.5 µmol/L) in presence or absence of a protein kinase A inhibitor (myristoylated trifluoroacetate, frag 14–22; 10 µmol/L; PKA-I) or adenylyl cycliase inhibitor (2′,5′ dideoxyadenosine; 1 µmol/L; AC-I) for 48 hours. Treatment of HCASMCs with Cl-Ad upregulated $p27^{Kip1}$ and downregulated Skp2, and these effects were abrogated by both PKA-I and AC-I. Both PKA-I and AC-I also reversed the inhibitory effects of Cl-Ad on DNA synthesis in HCASMCs. *$P<0.05$, no Cl-Ad (–Cl-Ad) vs Cl-Ad (+Cl-Ad); §significant reversal of the inhibitory effects. **B**, Role of $p27^{Kip1}$ in mediating the growth inhibitory actions of Cl-Ad in HCASMCs. In contrast to HCASMCs treated with control siRNA (-Si RNA or Si-Con), treatment of HCASMCs with $p27^{Kip1}$ siRNA (+Si RNA or p27-siRNA) blocked Cl-Ad –induced expression of $p27^{Kip1}$ (Western blots). The inhibitory effects of Cl-Ad on DNA synthesis were abrogated in HCASMCs with silenced $p27^{Kip1}$. **C**, Similar to Cl-Ad, the inhibitory effects of 5′-N-methylcarboxamidoadenosine (MECA) and 8-bromo-cAMP (Br-cAMP) were abrogated in HCASMCs treated with $p27^{Kip1}$ siRNA (p27-siRNA) but not in HCASMCs treated with control siRNA (si-Control). *$P<0.05$ vs control; §significant reversal of the inhibitory effects. Values represent mean±SEM from 3 separate experiments, each conducted in triplicates. The optical density (OD) ratio in the bar graphs represents Skp2 or $p27^{Kip1}$ to β-actin ratio.

of 2-chloroadenosine on HCASMC proliferation and the fact that MRS1754 attenuates the inhibitory effects of 2-chloroadenosine and MECA on HCASMC proliferation corroborate the conclusion that A_{2B} receptors mediate the inhibitory effects of adenosine on HCASMC proliferation. This conclusion is confirmed by our findings that the inhibitory effects of MECA, NECA, and 2-chloroadenosne on HCASMC proliferation are blocked by siRNA against A_{2B} receptors.

Multiple pro-mitogenic pathways—including ERK1/2 and Akt—are involved in triggering the proliferative response of mitogens generated at sites of vascular dysfunction or injury. These early signaling pathways trigger proliferation of HCASMCs by upregulating cell cycle regulatory proteins—such as cyclin D and cyclin A—that promote cell-cycle progress or by downregulating regulatory proteins—such as $p27^{Kip1}$—that retard cell-cycle progression.[45,47] The present study shows that treatment with 2-chloroadenosine or MECA, but not CPA, CGS21680, or IB-MECA, inhibits phosphorylation of ERK1/2 and Akt, decreases expression of Skp2, increases levels of $p27^{Kip1}$, decreases expression of cyclin D1, inhibits hyper-phosphorylation of Rb, and downregulates expression of cyclin A. These results are entirely consistent with the proposed mechanism of adenosine's antiproliferative action outlined in Figure 1. The role of A_{2B} receptors in modulating these key signaling mechanisms to negatively influence cell proliferation is further supported by our observation that the effects of 2-chloroadenosine and MECA on these signaling pathways are blocked by the A_{2B} receptor antagonist MRS1754 and by silencing of A_{2B} receptors using siRNA. Therefore, these findings corroborate the concept that A_{2B} receptor activation causes a realignment of signaling pathways to inhibit HCASMC proliferation by the mechanism shown in Figure 1.

Skp2 is an F-box protein of SCF^{Skp2} ubiquitin ligase and therefore promotes polyubiquitination of and subsequent proteolysis of $p27^{Kip1}$.[30,49] Because $p27^{Kip1}$ binds to and inhibits the function of cyclin–Cdk complexes (such as cyclin D/Cdk4/6), an increase in $p27^{Kip1}$ levels would inhibit the function of cyclin D. Thus, we hypothesize that via A_{2B} receptors, adenosine inhibits HCASMC proliferation in part by downregulating Skp2 and upregulating $p27^{Kip1}$. Consistent with this

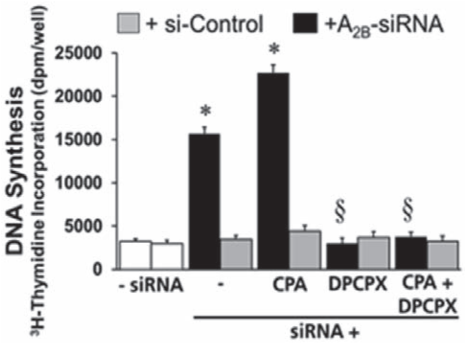

Figure 9. Bar graphs depict the balanced regulation of human coronary artery smooth muscle cell (HCASMC) proliferation by A_{2B} and A_1 adenosine receptors. In serum-starved HCASMCs, silencing of A_{2B} receptors with siRNA (+A_{2B}-siRNA) resulted in a significant increase in DNA synthesis, and these effects were further enhanced by the A_1 adenosine receptor agonist N^6-cyclopentyladenosine (CPA; 100 nmol/L). Pretreatment with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10 nmol/L), an A_1 receptor antagonist, blocked the stimulatory effect of A_{2B} silencing under basal conditions and in response to CPA. These finding indicate that downregulation of A_{2B} receptors increases DNA synthesis under basal conditions, suggesting that in the absence of A_{2B} receptors, endogenous adenosine induces HCASMC growth via A_1 receptors. This is further supported by the observation that CPA further stimulated DNA synthesis, and this effect was blocked by DPCPX. *$P<0.05$, vs no siRNA (–siRNA) and vs control siRNA (+si-Control) in medium with 0.4% BSA; §significant reversal of the proliferative effects of A_{2B} siRNA with or without CPA. Values represent mean±SEM from 3 separate experiments using separate cultures, each conducted in triplicates.

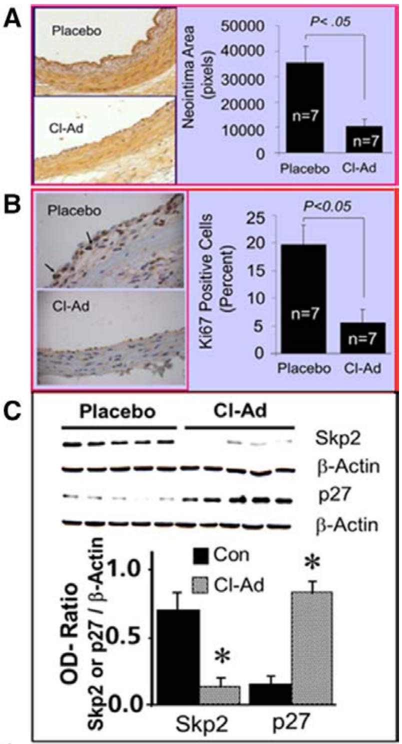

Figure 10. A, Inhibitory effects of 2-chloroadenosine (Cl-Ad) on intimal thickening after balloon injury. Image shows representative photomicrographs (40× magnification) of the cross sections of rat carotid arteries 7 days after balloon injury. Compared with rats receiving vehicle (placebo), intimal thickening was significantly reduced in rats exposed peri-arterially with Cl-Ad (20 µmol/L in 25% pluronic gel). Bar graph compares the intimal area in rats receiving vehicle (n=7) versus Cl-Ad (n=7) after injury. Data are mean±SEM. **B**, Inhibitory effects of Cl-Ad on proliferation of vascular smooth muscle cells (VSMCs) in the intima 7 days after balloon injury. Image shows representative photomicrographs (40× magnification) of cross sections of carotid arteries stained for Ki67-positive proliferating VSMCs. Bar graph compares the number of Ki67-positive cells in placebo versus Cl-Ad–treated groups. Data are mean±SEM. **C**, Effects of Cl-Ad on neointimal expression of S-phase kinase–associated protein-2 (Skp2) and $p27^{Kip1}$ proteins in vivo. Rats were treated with placebo (n=5) or Cl-Ad (20 µmol/L in 25% pluronic gel, n=5) and were euthanized on day 8. Carotid arteries were snap-frozen in liquid nitrogen. Subsequently, segments from placebo or Cl-Ad–treated animals were homogenized, lysed, and proteins analyzed using Western blotting. Bar graph depicts the changes in optical density (OD) of Skp2 or $p27^{Kip1}$ normalized to β-actin. *$P<0.05$ vs placebo.

notion, our results show that treatment with 2-chloroadenosine reduces Skp2 expression, and this is accompanied by a simultaneous increase in $p27^{Kip1}$ levels. Using pharmacological agonists and antagonists and molecular silencing of A_{2B} receptors, we demonstrate that the modulatory effects of adenosine on Skp2 and $p27^{Kip1}$ are A_{2B} receptor–mediated. Consistent with our contention that the anti-mitogenic effects of adenosine are mediated in part by inhibiting the proteolytic actions of Skp2 on $p27^{Kip1}$, we also observe that via A_{2B} receptors (pharmacological and molecular approaches), 2-chloroadenosine as well as MECA inhibit cyclin D–dependent downstream signaling, that is, hyper-phosphorylation of Rb and expression of cyclin A (Figure 1). These modulatory actions of 2-chloroadenosine on ERK1/2, Akt, Skp2, $p27^{Kip1}$, cyclin D, Rb, and cyclin A are also consistent with our observation that 2-chloroadenosine increases the percentage of cells in the G0/G1 phase of the cell cycle, although decreasing the percentage of cells in the S and G2/M phases of the cell cycle.

Our studies are consistent with the concept that the proximal signaling mechanism by which A_{2B} receptors inhibit proliferation involves the adenylyl cyclase/cAMP/PKA axis (Figure 1). A_{2B} receptors induce cAMP formation via activation of adenylyl cyclase.[50] Therefore, cAMP may be involved in mediating the effects of 2-chloroadenosine on ERK1/2, Akt, and Skp2. Our finding that the inhibitory effects of 2-chloroadenosine on HCASMC proliferation are significantly abrogated by inhibition of adenylyl cyclase and PKA are consistent with the hypothesis that A_{2B}-mediated cAMP production participates in the anti-mitogenic effects of 2-chloroadenosine. We also observe that in HCASMCs with siRNA-silenced A_{2B} receptors, 2-chloroadenosine–induced cAMP production is abrogated and the anti-mitogenic effects of 2-chloroadenosine and MECA, but not 8-bromo-cAMP, are prevented. Together, these data suggest that the antiproliferative effects of 2-chloroadenosine are mediated by cAMP produced via A_{2B} receptor activation. The involvement of cAMP in mediating the effects of 2-chloroadenosine on Skp2 and $p27^{Kip1}$ via A_{2B} receptors is supported by the fact that the inhibitory effects of 2-chloroadenosine on Skp2 expression and the concomitant stimulatory effects of 2-chloroadenosine on $p27^{Kip1}$ levels are attenuated by inhibition of adenylyl cyclase, PKA, and A_{2B} receptors and abrogated in HCASMCs lacking A_{2B} receptors. Taken together and as shown in Figure 1, our findings suggest that the anti-mitogenic effects of adenosine are mediated via

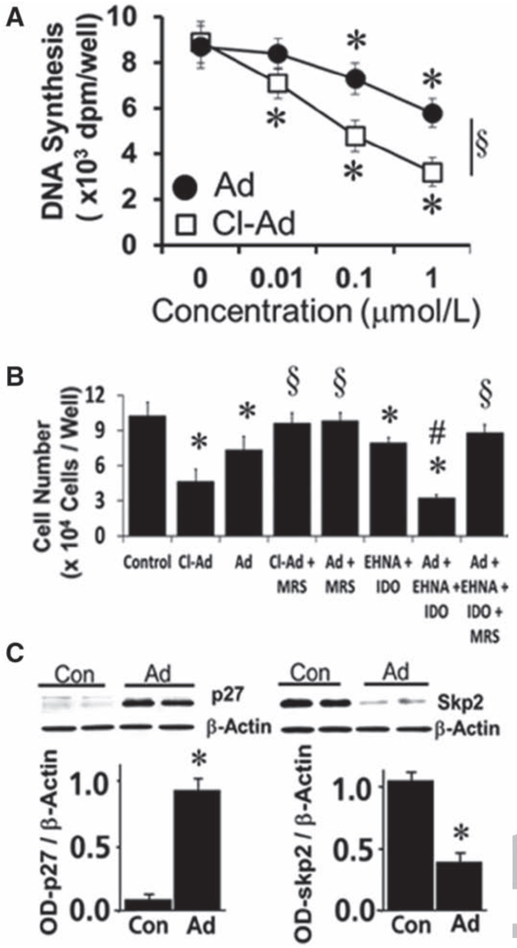

Figure 11. A, Concentration (0, 0.01, 0.1, 1 μmol/L)–response relationships for the inhibition of DNA synthesis (^{3}H-thymidine incorporation) by 2-chloroadenosine (Cl-Ad) and adenosine (Ad) in human coronary artery smooth muscle cells (HCASMCs). **B**, The effects of 100 nmol/L of 2-chloroadenosine (Cl-Ad) and adenosine (Ad) on cell number in the presence and absence of MRS1754 (MRS; selective A_{2B} antagonist, 100 nmol/L) or inhibitory effects of adenosine in presence or absence of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 1 μmol/L) plus 5-iodotubercidin (IDO; 0.1 μmol/L) and with or without MRS1754 (100nmol/L). **C**, The effects of adenosine on p27^{Kip1} and S-phase kinase–associated protein-2 (Skp2) expression in HCASMCs treated with or without adenosine (Ad; 100 nmol/L) for 48 hours. *$P<0.05$ vs no treatment; §$P<0.05$ significant reversal of inhibitory effects; #$P<0.05$ significant increase of inhibitory effects. Values represent mean±SEM from 3 separate experiments using separate HCASMCs, each conducted in triplicate. The optical density (OD) ratio in the bar graphs represents Skp2 or p27^{Kip1} to β-actin ratio.

A_{2B} receptor stimulation of cAMP production and sequential activation of PKA. This concept is supported by a previous report that cAMP inhibits neointima formation via PKA activation and by downregulating Skp2 and upregulating p27^{Kip1} in rat aortic VSMCs.[38,39,51]

Recent studies provide strong evidence for a major role of p27^{Kip1} upregulation in mediating anti-mitogenic actions in many cell types.[38,51–53] Decreased or defective expression of p27^{Kip1} is linked to proliferative disorders, including atherosclerosis, restenosis after balloon injury, and cancer. In animal models, molecular approaches for targeted upregulation of p27^{Kip1} prevent injury-induced intimal thickening, as well as cancer cell growth.[53] Our finding that 2-chloroadenosine induces p27^{Kip1} expression via A_{2B} receptors suggests that p27^{Kip1} mediates in part the anti-mitogenic effects of A_{2B} receptors. Consistent with this notion, our experiments show that the stimulatory effects of 2-chloroadenosine on p27^{Kip1} expression and inhibitory effects on HCASMC proliferation are blocked by A_{2B} receptor antagonism or knockdown of A_{2B} receptors. Moreover, silencing of p27^{Kip1} in HCASMCs abrogates the inhibitory effects of 2-chloroadenosine on cell proliferation and the stimulatory effects of 2-chloroadenosine on p27^{Kip1} expression. Similar to 2-chloroadenosine, the inhibitory effects of MECA and cAMP are abrogated in HCASMCs with silenced p27^{Kip1}, suggesting that p27^{Kip1} is a key mediator for the anti-mitogenic actions of 2-chloroadenosine, which requires the sequential involvement of A_{2B} receptors and cAMP generation.

To confirm that the observed anti-mitogenic effects of adenosine in HCASMCs in vitro would also translate to preventing vascular remodeling in vivo, we investigated the effects of 2-chloroadenosine on injury-induced neointima formation. In this regard, we used the rat carotid artery injury model. The present study showed that treatment of rats peri-arterially with 2-chloroadenosine significantly inhibited intimal thickening. Moreover, the inhibitory effects of 2-chloroadenosine on neointima formation were associated with downregulation of Skp2 and upregulation of p27^{Kip1}. Taken together, these findings suggest that 2-chloroadenosine prevents intimal thickening in part by downregulating the expression of Skp2 and upregulating p27^{Kip1} levels. These findings are consistent with recent reports that injury-induced intimal thickening[54,55] and high-lipid diet–induced atherosclerosis[56] are increased in mice lacking A_{2B} receptors, suggesting that the anti-vasoocclusive effects of adenosine are A_{2B} receptor–mediated. Interestingly, in the present study, 2-chloroadenosine reduced neointimal area without altering lumen area. This suggests that 2-chloroadenosine blocked both neointimal formation and remodeling such that lumen area remained constant with a more normal intimal lining.

In mast cells[57] and cardiac fibroblasts,[58] A_{2B} receptors couple to protein kinase C, and it is conceivable that this also occurs in HCASMCs. However, if so, this would probably not contribute to inhibition of HCASMC proliferation because our previous studies suggest that PKC is involved in stimulating, rather than inhibiting, VSMC proliferation.[59]

Our finding that application of 2-chloroadenosine peri-arterially inhibits injury-induced intimal thickening has potential therapeutic significance. Restenosis after balloon angioplasty is a major post-angioplasty–associated clinical problem. Because abnormal growth of HCASMCs occurs mainly during the first 7 days after angioplasty[47] and peri-arterial application of 2-chloroadenosine inhibits intimal thickening, its peri-arterial application may prevent restenosis after balloon angioplasty in humans. Peri-arterial application may also resolve the limitations associated with the rapid clearance and short half-life of adenosine or its analogs.

Our data provide evidence that 2-chloroadenosine is effective in inhibiting HCASMC growth and injury-induced neointima formation. Likely, adenosine would mimic the antiproliferative/anti-vasoocclusive effects of 2-chloroadenosine. Indeed, our findings that adenosine inhibits HCASMC growth, inhibits Skp2 expression, and induces p27^{Kip1} expression suggest that adenosine would also mediate vascular protective actions. However, because of rapid catabolism of adenosine by adenosine kinase and adenosine deaminase, adenosine likely would be less potent than 2-choroadenosine. Although adenosine's effect on neointima formation in vivo was not assessed in the present study, experiments using A_{2B} receptor knockout mice provide evidence for enhanced proliferation of VSMCs following endothelial denudation.[53,54] This suggests that endogenous adenosine indeed is capable of suppressing intimal growth and vascular remodeling, which lead to vascular occlusion. Future studies using adenosine in pluronic acid gels are required to confirm whether adenosine has a physiological role in regulating growth of VSMCs.

Experiments by Shen et al[60,61] demonstrate that A_1 receptors, rather than A_{2B} receptors, are dominant in porcine coronary artery smooth muscle cells and that, in this setting, adenosine stimulates proliferation via A_1 receptor activation. Because A_1 receptors inhibit, rather than stimulate, adenylyl cyclase, the findings of Shen et al are highly consistent with the mechanism proposed in Figure 1. Indeed we find that silencing of A_{2B} receptors augments HCASMC proliferation via activation of A_1 receptors by endogenous adenosine. Taken together, our finding and the findings of Shen et al suggest the possibility that the ratio of A_1 to A_{2B} receptors in HCASMCs in individual patients contributes significantly to the risk of coronary artery disease. If true, this would be an extremely important concept because this novel idea would suggest that administration of A_1 receptor antagonists would be protective in patients with a high A_1 to A_{2B} ratio, whereas an A_{2B} receptor agonist would be preferred in patients with a high A_{2B} to A_1 ratio (ie, personalized medicine). However, a caveat is that A_{2B} receptors when activated chronically can induce profibrotic and proinflammatory effects.[62,63] Therefore, it may be important to limit the duration of treatment with A_{2B} receptor agonists to just the critical time period in which HCASMC proliferation occurs in response to injury.

It is interesting that in the absence of A_{2B} receptors, A_1 receptor activation leads to HCASMC proliferation. Given that A_1 receptors have much higher affinity for adenosine, why would their effect not predominate? There are reports that A_1 receptors form heterodimers with A_{2A} receptors and β-adrenoceptors and that heterodimer formation blocks A_1 receptor signaling.[62,63] Therefore, one possibility is that in HCASMCs, A_{2B} receptors directly block A_1 receptor signaling via heterodimerization. Another possibility is that A_{2B}/Gs-mediated stimulation of adenylyl cyclase overrides A_1/Gi-mediated signaling, despite higher agonist binding to A_1 receptors.

In conclusion, we provide strong evidence that (1) adenosine inhibits HCASMC proliferation and migration; (2) the inhibitory effects of adenosine on HCASMC proliferation are mediated via A_{2B} receptor activation of adenylyl cyclase, leading to the accumulation of cAMP and stimulation of PKA; (3) PKA inhibits HCASMC proliferation by blocking multiple signaling pathways (ERK1/2, Akt, and Skp2) that converge at cyclin D—the net result being a reduced expression and function of this key G1 cyclin that governs cell-cycle progression; (4) this mechanism is operative in vivo; and (5) if the A_{2B} receptor system is deficient, A_1 receptors become dominant and increase HCASMC proliferation.

Perspective

Activation of A_{2B} receptors by adenosine inhibits HCASMC proliferation. This effect is profoundly efficacious because the A_{2B} receptor/adenylyl cyclase/cAMP/PKA pathway blocks cell cycle progression by inhibiting multiple downstream signaling events that are required for cyclin D production and function. Because A_{2B} and A_1 receptors have opposing effects on HCASMC proliferation, pharmacological activation of A_{2B} receptors or inhibition of A_1 receptors or both may prevent vascular remodeling associated with coronary artery disease, hypertension, atherosclerosis, and restenosis.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Endogenous and exogenous adenosine inhibits human coronary artery smooth muscle cell (HCASMC) proliferation and migration.
- The inhibitory effect of adenosine on HCASMC proliferation is mediated via A_{2B} receptor activation of adenylyl cyclase, leading to the accumulation of cAMP and stimulation of protein kinase A.
- Protein kinase A inhibits HCASMC proliferation by blocking multiple signaling pathways (ERK1/2, Akt, and Skp2) that converge at cyclin D—the net result being a reduced expression and function of this key G1 cyclin that governs cell-cycle progression.
- Adenosine analogues can be applied peri-arterially in a slow release gel formulation to inhibit vascular injury–induced neointimal hyperplasia.
- If the A_{2B} receptor system becomes deficient, A_1 receptor signaling becomes dominant and increases HCASMC proliferation.

What Is Relevant?

- A_{2B} receptor activation is a straightforward approach to inhibit HCASMC proliferation and migration.
- It is possible to apply A_{2B} receptor agonists peri-arterially to block neointimal hyperplasia while obviating unwanted systemic adverse effects.
- The ratio of A_1 to A_{2B} receptor expression may determine risk of coronary artery disease and the response to adenosine receptor agonists.
- Other agents that modulate the actions of ERK1/2, Akt, Skp2, $p27^{Kip1}$, cyclin D, Rb, or cyclin A may have therapeutic efficacy in cardiovascular medicine.

Summary

The adenosine/A_{2B} receptor/cAMP/protein kinase A axis inhibits HCASMC proliferation by blocking multiple signaling pathways (ERK1/2, Akt, and Skp2) that converge at cyclin D; the net result being a reduced expression and function of this key G1 cyclin that governs cell-cycle progression.

ONLINE SUPPLEMENT

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Short Title: **Mechanism of Adenosine on VSMC Proliferation**

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLES

Table S1: Details of the primary antibodies used.

Primary Antibody (Source)	**Dilution of Primary Antibody (Time and Temperature of Incubation)**
Anti-Rb hypo/hyperphosphorylated (BD Biosciences)	1:1000 (overnight at 4°C)
Anti-cyclin D1 (Upstate Biotechnology)	1:1000 (1 hour at room temperature; RT)
Anti-β actin (Sigma)	1:10000 (40 min at RT)
Anti-ERK1/2 (Upstate Biotechnology)	1:1000 (1 hour at RT)
Anti-ERK1/2 phosphorylated (Calbiochem)	1:1000 (1 hour at RT)
Anti-Akt (Cell Signaling Technology)	1:1000 (1 hour at RT)
Anti-Akt phosphorylated (Cell Signaling Technology)	1:1000 (1 hour at RT)
Anti-cyclin A1 (Upstate Biotechnology)	1:1000 (1 hour at RT)
Anti-Skp2 (Cell Signalling)	1:1000 (2 hours at RT)
Anti-p27 (Pharmigen)	1:250 (1 hour at RT)
Anti-Adenosine Receptor A_1 (Santa Cruz)	0.5-5 ug/ml (1 hour at RT)
Anti A_{2A} Adenosine receptor (Chemicon)	1:200 (1 hour at RT)

Anti A_{2B} Adenosine receptor (Santa Cruz)	1:200 (1 hour at RT)
Anti A_3 Adenosine receptor (Santa Cruz)	1:200 (overnight at 4°C)

Table S2: Effects of administration of 2-chloroadenosine (20 µmol/L in 25% pluronic acid) peri-arterially for 7 days on myointimal proliferation after balloon injury of carotid arteries of intact male Wistar-Kyoto rats.

Parameters	**Vehicle**	**2-Chloroadenosine**
Sample size	(n=7)	(n=7)
Body weight, g		
Balloon Day	363 ± 3	360 ± 2
Perfusion Day	366 ± 4	364 ± 3
Area of Media, pixels	25809 ± 1613	23228 ± 645
Area of Intima, pixels	37424 ± 18371	10352 ± 2824 *
Area of Lumen, pixels	94528 ± 12582	88914 ± 7291
I/M ratios	1.45 ± 0.02	0.445 ± 0.012 *
WBC , count x10^3/mm^3	4.461 ± 0.51	4.6 ± 0.55
RBC, count x 10^3/mm^3	6.77 ± 0.336	6.8 ± 0.44
HCT (%)	38.90 ± 1.45	40.05 ± 1.5

* $p<0.05$ vs placebo treated animals.

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Adenosine Attenuates Human Coronary Artery Smooth Muscle Cell Proliferation by Inhibiting Multiple Signaling Pathways That Converge on Cyclin D

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