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

Raghvendra K. Dubey, Jürgen Fingerle, Delbert G. Gillespie, Zaichuan Mi, Marinella Rosselli, Bruno Imthurn, Edwin K. Jackson

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 N6-cyclopentyladenosine, CGS21680, or N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide, inhibited HCASMC proliferation (A2B 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 p27Kip1) and upregulated levels of p27Kip1 (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 A2B receptors prevented the effects of 2-chloroadenosine on ERK1/2, Akt, Skp2, p27Kip1, cyclin D1, cyclin A, and proliferation. Likewise, inhibition of adenyl cyclase and protein kinase A abrogated 2-chloroadenosine’s inhibitory effects on Skp2 and stimulatory effects on p27Kip1 and rescued HCASMCs from 2-chloroadenosine–mediated inhibition. Knockdown of p27Kip1 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 p27Kip1, and reduced neointima hyperplasia by 71% (P<0.05; neointimal thickness: control, 37424±18371 pixels; treated, 10352±2824 pixels). In conclusion, the adenosine/A2B 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.)

Key Words: adenosine ■ A2B receptor ■ cyclin D1 ■ p27Kip1 ■ 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 prepelomeral VSMCs,2,3 rat aortic VSMCs, rat atrial and human10 atrioventricular nodal VSMCs,11 rat trabecular and human11 ventricular myocytes,12 rat cardiac fibroblasts,12–16 yet, adenosine stimulates the proliferation of rat aortic17,18 and porcine coronary17 vascular endothelial cells, as well as human19 renal epithelial cells. In addition, adenosine has several other desirable tissue-protecting actions, such as promoting neovascularization 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...
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, 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 p27Kip1 and thus accelerates p27Kip1 degradation,30 thereby decreasing levels of p27Kip1. Normally, p27Kip1 binds to complexes of cyclins with their respective cyclin-dependent kinases (Cdk), thus preventing cyclin–Cdk complexes from phosphorylating their substrates.31 Importantly, p27Kip1 impairs the function of cyclin D–Cdk4/6 complexes3 that are primarily responsible for promoting cell-cycle progression in G1 phase of the cell cycle.32 Therefore, a reduction of p27Kip1 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 A2B receptors.35,36 Stimulation of A2B receptors activates adenyl 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, which in turn increases the levels of p27Kip1. In addition, PKA can interfere with signaling cascades that phosphorylate (activate) ERK1/2 and Akt,41 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 A2B receptor/adenyl 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), N6-cyclopentyladenosine (CPA; selective A1 receptor agonist), CGS21680 (selective A2a receptor agonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; selective A1 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-[(3-iodophenyl)methyl]amino-[9H-purin-9-yl]-N-methylβ-D-ribofururanosamide (IB-MECA; selective A2a receptor agonist), SCH442416 (selective A2a receptor antagonist), MRS1754 (selective A2a receptor antagonist), and VUF5574 (selective A2a receptor antagonist) were purchased from Tocris (Minneapolis, MN). 1H-thymidine (specific activity, 11.8 Ci/mmol) was purchased from PerkinElmer NEN (Waltham, 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 comparable (<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.

**1H-Thymidine Incorporation**

To assess DNA synthesis by HCASMCs, we used 1H-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 1H-thymidine, and the incorporation of 1H-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 Boyden chamber as described previously 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 secondary 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).

**A2B and p27 Silencing Studies**

Smart pool on target plus siRNA kit from Dharmacon was used according to the instructions to silence CDKN1B (p27Kip1) or ADORA2B (human A2B adenosine receptors) in HCASMCs. Control smart pool siRNA from Dharmacon was used as control.

**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.6

**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.5,46 Briefly, animals were anesthetized with ketamine plus xylazine (intraperitoneal injection). To induce arterial injury, the left common carotid artery was completely denuded of the endothelium, 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-Chloroadenosine (20 μmol/L) was added to 25% (wt/vol) pluronic gel solution (F127, BASF Corp, Parsippany, 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 stitches of silk suture. After 7 days, the animals 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 p27Kip1 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 densitometric 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; p27Kip1 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 A1 and A2B receptors, but only weak mRNA expression for A3 and A2A receptors (Figure 2A). Likewise, Western blotting detected strong bands for A1 and A2B receptors, a faint band for A2A receptors.
Hypertension December 2015

receptors, and no signal for A3 receptors (Figure 2A). These findings suggest that A1 or A2B 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 (A1, A2A, A2B, and A3) in mediating the anti-mitogenic effects in HCASMCs. The highest (1 μmol/L) concentrations of CPA (A1 receptor–selective agonist), CGS21680 (A2A receptor–selective agonist), and IB-MECA (A3 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 (A2B receptor–selective antagonist), but not DPCPX (A1 receptor–selective antagonist), SCH442416 (A2A receptor–selective antagonist), or VUF5574 (A3 antagonist), similar to Cl-Ad, the effects of MECA were blocked by MRS1754. Similar to Cl-Ad, the effects of MECA were mimicked by MECA, but not by N6-cyclopentyladenosine (CPA; A1 agonist), CGS21680 (CGS; A2A agonist), or 1-deoxy-1-[6-[(3-iodophenyl) methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA; IB-M: A3 adenosine receptor agonist). Moreover, the effects of Cl-Ad and MECA were reversed by MRS1754 (MRS; A2B receptor antagonist). 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.

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; A2B receptor antagonist), but not by SCH442416 (SCH; A1 receptor antagonist), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; A1 antagonist), or VUF5574 (VUF; A3 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 N6-cyclopentyladenosine (CPA; A1 agonist), CGS21680 (CGS; A2A agonist), or 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA; IB-M: A3 adenosine receptor agonist). Moreover, the effects of Cl-Ad and MECA were reversed by MRS1754 (MRS; A2B receptor antagonist). 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.

agents (adenosine receptor subtype selective and nonsselective agonists and antagonists), we further assessed the role of all adenosine receptor subtypes (A1, A2A, A2B, and A3) in mediating the anti-mitogenic effects in HCASMCs. The highest (1 μmol/L) concentrations of CPA (A1 receptor–selective agonist), CGS21680 (A2A receptor–selective agonist), and IB-MECA (A3 receptor–selective agonist) failed to inhibit DNA synthesis (Figure 2B). MECA was slightly more potent than NECA (both are nonsensitive adenosine receptor agonists; Figure 2B). MRS1754 (A2B receptor–selective antagonist), but not DPCPX (A1 receptor–selective antagonist), SCH442416 (A2A receptor–selective antagonist), or VUF5574 (A3 receptor–selective antagonist), blocked 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 SCFSkp2 ubiquitin ligase responsible for polyubiquitination of and subsequent proteolysis of p27Kip1) and upregulated levels of p27Kip1 (p27Kip1 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 A2B 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 A2B 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 A2B receptor siRNA (Figure 5A). 2-Chloroadenosine inhibited DNA synthesis in HCASMCs treated with negative-control siRNA but not in cells treated with A2B siRNA (Figure 5B). Also the inhibitory effects of Meca, NECA, and EHNA+IDO were blocked by A2B siRNA (Figure 5B). Downregulation of A2B 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 A2B receptors mediate the antimitogenic effects of adenosine.

In HCASMCs in which A2B 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 A2B receptors. Additionally, the inhibitory effects of 2-chloroadenosine on Skp2 and stimulatory effects on p27Kip1 were lost in HCASMCs lacking A2B receptors (Figure 6).

cAMP may inhibit HCASMC proliferation via Skp2 downregulation and p27Kip1 upregulation. Because 2-chloroadenosine–stimulated cAMP production was inhibited in HCASMCs with silenced A2B 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 p27Kip1 expression (Figure 7A). The stimulatory effects of 2-chloroadenosine on p27Kip1 expression in HCASMCs was abolished in cells in which adenosine A2B receptors were silenced with siRNA (Figure 7B). Moreover, the inhibitory effects of 2-chloroadenosine on DNA synthesis were abrogated in HCASMCs in which adenosine A2B receptors were silenced (Figure 7B). The observations that the stimulatory effects of 2-chloroadenosine on p27Kip1 and inhibitory effects on DNA synthesis are abolished in HCASMCs lacking adenosine A2B receptors suggest a role
for p27kip1 in A2B receptor-mediated regulation of HCASMC proliferation.

Next, we tested whether adenylyl cyclase and PKA mediate the effects of 2-chloroadenosine on p27kip1 and HCASMC growth via A2B receptors. 2-Chloroadenosine inhibited Skp2 and upregulated p27kip1 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 p27kip1 in mediating the inhibitory effects of 2-chloroadenosine via A2B receptors, we assessed the effects of 2-chloroadenosine on DNA synthesis in HCASMCs, where p27kip1 expression was silenced. Treatment of HCASMCs with p27kip1 siRNA silenced p27kip1 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 p27kip1 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 p27kip1 was silenced (Figure 8C).

In serum-starved HCASMCs, silencing of A2B receptors with siRNA resulted in a significant decrease in DNA synthesis, and these effects were further enhanced by the A1 adenosine receptor agonist CPA (Figure 9). Pretreatment with DPCPX, an A1 receptor antagonist, blocked the stimulatory effect of A2B silencing under basal conditions and in response to CPA. These finding indicate that in the absence of A2B receptors, endogenous adenosine induces HCASMC growth via A1 receptors.

Morphometric 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 37.4±24.1 pixels), the neo-intima formation was reduced by 71% in rats receiving periarterial 2-chloroadenosine (n=7; 10.35±2.84 pixels; P<0.05 versus placebo group (n=7; 371±424 pixels).
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 A2B 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 A2B 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 p27Kip1 and downregulation of Skp2, we assessed adenosine’s effect on the expression of both p27Kip1 and Skp2. As shown in Figure 11C, treatment of HCASMCs with adenosine upregulated p27Kip1 and downregulated Skp2. As compared with the placebo, A2B receptor antagonist MRS1754, and Skp2. As compared with the placebo, A2B receptor antagonist MRS1754, and Skp2, we observed a significant decrease in Ki67-positive VSMCs (Table S2). In carotid arteries obtained from animals receiving placebo, Ki67-positive cells (indicating proliferating VSMCs) were observed (Figure 11B). 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 11B). 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 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 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 p27Kip1 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 p27Kip1.

In the studies described earlier, we used 2-chloroadenosine rather than adenosine because adenosine is rapidly
of 2-chloroadenosine on HCASMC proliferation and the fact that MRS1754 attenuates 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 p27kip1. Western blots show Skp2 and p27kip1 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 cyclase inhibitor (2′,5′ dideoxyadenosine; 1 μmol/L; AC-I) for 48 hours. Treatment of HCASMCs with Cl-Ad upregulated p27kip1 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.

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 p27kip1. Western blots show Skp2 and p27kip1 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 cyclase inhibitor (2′,5′ dideoxyadenosine; 1 μmol/L; AC-I) for 48 hours. Treatment of HCASMCs with Cl-Ad upregulated p27kip1 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 p27kip1 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 p27kip1 siRNA (+Si RNA or p27-siRNA) blocked Cl-Ad–induced expression of p27kip1 (Western blots). The inhibitory effects of Cl-Ad on DNA synthesis were abrogated in HCASMCs with silenced p27kip1.

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 p27kip1 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 p27kip1 to β-actin ratio.
on p27Kip1, we also observe that via A2B receptors (pharmaco-
our contention that the anti-mitogenic effects of adenosine are
with the hypothesis that A2B-mediated cAMP production par-
gated by inhibition of adenylyl cyclase and PKA are consistent
adenosine on HCASMC proliferation are significantly abro-
and Skp2. Our finding that the inhibitory effects of 2-chloro-
in mediating the effects of 2-chloroadenosine on ERK1/2, Akt,
(Figure
proliferation involves the adenylyl cyclase/cAMP/PKA axis
N6-cyclopentyladenosine (CPA; 100 nmol/L). Pretreatment with
0.4% BSA; §significant reversal of the proliferative effects of A2B
P<0.05, vs no siRNA
by the observation that CPA further stimulated DNA synthesis,
are further enhanced by the A1 adenosine receptor agonist
silencing of A2B receptors with siRNA (+A2B-siRNA) resulted
silencing under basal conditions and in response to CPA. These
finding indicate that downregulation of A2B receptors increases
DNA synthesis under basal conditions, suggesting that in the
absence of A2B receptors, endogenous adenosine induces
HCASMC growth via A1 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 A1
siRNA with or without CPA. Values represent mean±SEM from 3
separate experiments using separate cultures, each conducted
in triplicates.

notion, our results show that treatment with 2-chloroadenosine
reduces Skp2 expression, and this is accompanied by a simulta-
aneous increase in p27Kip1 levels. Using pharmacological ago-
nists and antagonists and molecular silencing of A1 receptors,
we demonstrate that the modulatory effects of adenosine on
Skp2 and p27Kip1 are A2B 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 p27Kip1, we also observe that via A2 receptors (pharma-
cological 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, p27Kip1, 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 A2B receptors inhibit prolif-
eration involves the adenyl cyclase/cAMP/PKA axis (Figure 1). A2B receptors induce cAMP formation via activa-
tion of adenyl cyclase.30 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-chloro-
adenosine on HCASMC proliferation are significantly abro-
gated by inhibition of adenyl cyclase and PKA are consistent
with the hypothesis that A2B-mediated cAMP production par-
ticipates in the anti-mitogenic effects of 2-chloroadenosine.
We also observe that in HCASMCs with siRNA-silenced
A2B receptors, 2-chloroadenosine–induced cAMP produc-
tion is abrogated and the anti-mitogenic effects of 2-chloro-
adenosine 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 A2B
receptor activation. The involvement of cAMP in mediating
the effects of 2-chloroadenosine on Skp2 and p27Kip1 via A2B
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 p27Kip1 levels are
attenuated by inhibition of adenyl cyclase, PKA, and A2B
receptors and abrogated in HCASMCs lacking A2B recep-
tors. Taken together and as shown in Figure 1, our findings suggest
that the anti-mitogenic effects of adenosine are mediated via

Figure 9. Bar graphs depict the balanced regulation of human
coronary artery smooth muscle cell (HCASMC) proliferation by
A2B and A1 adenosine receptors. In serum-starved HCASMCs,
silencing of A2B receptors with siRNA (+A2B-siRNA) resulted
in a significant increase in DNA synthesis, and these effects
were further enhanced by the A1 adenosine receptor agonist
N6-cyclopentyladenosine (CPA; 100 nmol/L). Pretreatment with
8-cyclopentyl-1,3-dipropoxanthine (DPCPX; 10 nmol/L), an
A1 receptor antagonist, blocked the stimulatory effect of A1
silencing under basal conditions and in response to CPA. These
finding indicate that downregulation of A2B receptors increases
DNA synthesis under basal conditions, suggesting that in the
absence of A2B receptors, endogenous adenosine induces
HCASMC growth via A1 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 A1
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 periarterially 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 means±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 means±SEM. C, Effects of
Cl-Ad on neointimal expression of S-phase kinase–associated
protein-2 (Skp2) and p27Kip1 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 p27Kip1 normalized to β-actin.
*P<0.05 vs placebo.
A2B 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 p27Kip1 in rat aortic VSMCs. Recent studies provide strong evidence for a major role of p27Kip1 upregulation in mediating anti-mitogenic actions in many cell types. Decreased or defective expression of p27Kip1 is linked to proliferative disorders, including atherosclerosis, restenosis after balloon injury, and cancer. In animal models, molecular approaches for targeted upregulation of p27Kip1 prevent injury-induced intimal thickening, as well as cancer cell growth. Our finding that 2-chloroadenosine induces p27Kip1 expression via A2B receptors suggests that p27Kip1 mediates in part the anti-mitogenic effects of A2B receptors. Consistent with this notion, our experiments show that the stimulatory effects of 2-chloroadenosine on p27Kip1 expression and inhibitory effects on HCASMC proliferation are blocked by A2B receptor antagonism or knockdown of A2B receptors. Moreover, silencing of p27Kip1 in HCASMCs abrogates the inhibitory effects of 2-chloroadenosine on cell proliferation and the stimulatory effects of 2-chloroadenosine on p27Kip1 expression. Similar to 2-chloroadenosine, the inhibitory effects of MECA and cAMP are abrogated in HCASMCs with silenced p27Kip1, suggesting that p27Kip1 is a key mediator for the anti-mitogenic actions of 2-chloroadenosine, which requires the sequential involvement of A2B receptors and cAMP generation.

To confirm that the observed anti-mitogenic effects of adenosine in HCASMCs in vitro would also translate to prevent 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 p27Kip1. Taken together, these findings suggest that 2-chloroadenosine prevents intimal thickening in part by downregulating the expression of Skp2 and upregulating p27Kip1 levels. These findings are consistent with recent reports that injury-induced intimal thickening and high-lipid diet–induced atherosclerosis are increased in mice lacking A2B receptors, suggesting that the anti-vasoocclusive effects of adenosine are A2B 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 and cardiac fibroblasts, A2B 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. 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 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 p27Kip1 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-chloroadenosine. 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. 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 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 findings 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 proinflammatory and proinflammatory effects. 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. 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 adenyl 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/adenyl 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.

**Acknowledgments**

We thank Doris Müller (University Hospital Zurich, Department of Reproductive Endocrinology, Zurich, Switzerland) for cell growth and protein analysis.

**Sources of Funding**

This work was financially supported by the Olten Heart Foundation (to R.K. Dubey); the Swiss National Science Foundation Grant No. IZZERO-142213/1 and Grant No. 31003A-138067 to R.K. Dubey; and the National Institutes of Health grants NS087978, HL109002, DK091190, HL069846, DK068575, and DK079307 to E.K. Jackson.

**Disclosures**

None.

**References**

10. Dubey RK, Rosselli M, Gillespie DG, Mi Z, Jackson EK. Extracellular 3’,5’-cAMP-adenosine pathway inhibits glomerular mesangial cell...


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.

$A_{2B}$ 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, p27kip1, cyclin D, Rb, or cyclin A may have therapeutic efficacy in cardiovascular medicine.

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

Raghvendra K. Dubey*, Juergen Fingerle†, Delbert G. Gillespie#, Zaichuan Mi #, Marinella Rosselli*, Bruno Imthurn* and Edwin K. Jackson#

Short Title: Mechanism of Adenosine on VSMC Proliferation

*Department of Obstetrics and Gynecology, Clinic for Reproductive Endocrinology, University Hospital Zurich; ¶Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland; #Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, USA; †Preclinical Pharma Research 68/209 (J.F.), F. Hoffmann-La-Roche, Basel, Switzerland

Address for Correspondence:

Dr. Raghvendra K. Dubey
Department of Obstetrics and Gynecology
Clinic for Endocrinology
D215, NORD-1; Frauenklinik
University Hospital Zurich
8091 Zurich, SWITZERLAND
Telephone: (41)-44-556-3070
Fax: (41)-1-255-4439

e-mail: Raghvendra.dubey@usz.ch
SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLES

Table S1: Details of the primary antibodies used.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution of Primary Antibody (Time and Temperature of Incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rb hypo/hyperphosphorylated</td>
<td>1:1000 (overnight at 4°C)</td>
</tr>
<tr>
<td>(BD Biosciences)</td>
<td></td>
</tr>
<tr>
<td>Anti-cyclin D1</td>
<td>1:1000 (1 hour at room temperature; RT)</td>
</tr>
<tr>
<td>(Upstate Biotechnology)</td>
<td></td>
</tr>
<tr>
<td>Anti-β actin</td>
<td>1:10000 (40 min at RT)</td>
</tr>
<tr>
<td>(Sigma)</td>
<td></td>
</tr>
<tr>
<td>Anti-ERK1/2</td>
<td>1:1000 (1 hour at RT)</td>
</tr>
<tr>
<td>(Upstate Biotechnology)</td>
<td></td>
</tr>
<tr>
<td>Anti-ERK1/2 phosphorylated</td>
<td>1:1000 (1 hour at RT)</td>
</tr>
<tr>
<td>(Calbiochem)</td>
<td></td>
</tr>
<tr>
<td>Anti-Akt</td>
<td>1:1000 (1 hour at RT)</td>
</tr>
<tr>
<td>(Cell Signaling Technology)</td>
<td></td>
</tr>
<tr>
<td>Anti-Akt phosphorylated</td>
<td>1:1000 (1 hour at RT)</td>
</tr>
<tr>
<td>(Cell Signaling Technology)</td>
<td></td>
</tr>
<tr>
<td>Anti-cyclin A1</td>
<td>1:1000 (1 hour at RT)</td>
</tr>
<tr>
<td>(Upstate Biotechnology)</td>
<td></td>
</tr>
<tr>
<td>Anti-Skp2</td>
<td>1:1000 (2 hours at RT)</td>
</tr>
<tr>
<td>(Cell Signalling)</td>
<td></td>
</tr>
<tr>
<td>Anti-p27</td>
<td>1:250 (1 hour at RT)</td>
</tr>
<tr>
<td>(Pharmigen)</td>
<td></td>
</tr>
<tr>
<td>Anti-Adenosine Receptor A₁</td>
<td>0.5-5 ug/ml (1 hour at RT)</td>
</tr>
<tr>
<td>(Santa Cruz)</td>
<td></td>
</tr>
<tr>
<td>Anti A₂A Adenosine receptor</td>
<td>1:200 (1 hour at RT)</td>
</tr>
<tr>
<td>(Chemicon)</td>
<td></td>
</tr>
<tr>
<td>Parameters</td>
<td>Vehicle</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Sample size</td>
<td>(n=7)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
</tr>
<tr>
<td>Balloon Day</td>
<td>363 ± 3</td>
</tr>
<tr>
<td>Perfusion Day</td>
<td>366 ± 4</td>
</tr>
<tr>
<td>Area of Media, pixels</td>
<td>25809 ± 1613</td>
</tr>
<tr>
<td>Area of Intima, pixels</td>
<td>37424 ± 18371</td>
</tr>
<tr>
<td>Area of Lumen, pixels</td>
<td>94528 ± 12582</td>
</tr>
<tr>
<td>I/M ratios</td>
<td>1.45 ± 0.02</td>
</tr>
<tr>
<td>WBC, count x10^3/mm^3</td>
<td>4.461 ± 0.51</td>
</tr>
<tr>
<td>RBC, count x 10^3/mm^3</td>
<td>6.77 ± 0.336</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>38.90 ± 1.45</td>
</tr>
</tbody>
</table>

* p<0.05 vs placebo treated animals.
Adenosine Attenuates Human Coronary Artery Smooth Muscle Cell Proliferation by Inhibiting Multiple Signaling Pathways That Converge on Cyclin D
Raghvendra K. Dubey, Jürgen Fingerle, Delbert G. Gillespie, Zaichuan Mi, Marinella Rosselli, Bruno Imthurn and Edwin K. Jackson

Hypertension. published online September 28, 2015;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2015/09/28/HYPERTENSIONAHA.115.05912

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/09/28/HYPERTENSIONAHA.115.05912.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/