Adenosine Inhibits Collagen and Total Protein Synthesis in Vascular Smooth Muscle Cells

Raghvendra K. Dubey, Delbert G. Gillespie, Edwin K. Jackson

Abstract—The objective of this study was to characterize the effects of exogenous, drug-induced and cAMP-adenosine pathway–derived adenosine on collagen synthesis by and hypertrophy of vascular smooth muscle cells (SMCs). Confluent vascular SMCs were stimulated with 2.5% fetal calf serum in the presence and absence of adenosine receptor agonists [adenosine, 2-chloroadenosine, N6-cyclopentyladenosine, 5'-N-ethylcarboxamidoadenosine, 5'-N-methylcarboxamidoadenosine, and 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamino adenosine], drugs that increase levels of endogenous adenosine [erythro-9-(2-hydroxy-3-nonyl) adenine, dipyridamole, and iodotubericidin], and cAMP (increases adenosine by conversion to AMP and hence to adenosine via the cAMP-adenosine pathway). Adenosine receptor agonists inhibited fetal calf serum-induced collagen and total protein synthesis (as assessed by [3 H]proline and [3 H]leucine incorporation, respectively) with a relative potency profile consistent with the effects being mediated by adenosine A2B receptors. Erythro-9-(2-hydroxy-3-nonyl) adenine, dipyridamole, iodotubericidin, and cAMP also inhibited collagen and total protein synthesis. The effects of 2-chloroadenosine, erythro-9-(2-hydroxy-3-nonyl) adenine, iodotubericidin, and cAMP on collagen and total protein synthesis were attenuated by KF17837 and 1,3-dipropyl-8-p-sulfophenylxanthine (selective and nonselective A2 receptor antagonists, respectively) but not by 8-cyclopentyl-1,3-dipropylxanthine (selective A1 receptor antagonist). These studies indicate that exogenous, drug-induced and cAMP-adenosine pathway–derived adenosine inhibit vascular SMC collagen synthesis and hypertrophy via A2B receptors. Thus, exogenous A2B receptor agonists and drugs that modulate endogenous adenosine levels may protect against vasoocclusive disorders by attenuating extracellular matrix synthesis by and cellular hypertrophy of vascular SMCs. Moreover, the cAMP-adenosine pathway may protect against vascular hypertrophy. (Hypertension. 1999;33[part II]:190-194.)

Key Words: adenosine ■ muscle, smooth ■ extracellular matrix ■ collagen ■ hypertrophy

We recently showed that exogenous and endogenous adenosine inhibits fetal calf serum-induced proliferation of vascular smooth muscle cells (SMCs).1,2 Because many factors that induce or inhibit vascular SMC proliferation also affect vascular SMC hypertrophy and extracellular matrix synthesis, we hypothesized that adenosine receptor agonists and drugs that increase endogenous levels of adenosine may have pharmacotherapeutic potential as inhibitors of vascular SMC hypertrophy and extracellular matrix synthesis. In support of this hypothesis, we reported that exogenous and endogenous adenosine inhibits collagen biosynthesis in human vascular SMCs.3 However, whether adenosine receptor agonists and drugs that increase endogenous adenosine alter vascular SMC hypertrophy and, if so, what adenosine receptor subtypes are involved are unanswered questions that are addressed in the present study.

Stimulation of adenylyl cyclase leads to cAMP egress which can be metabolized to AMP and hence to adenosine on the cell surface by the enzymes ecto-5'-nucleotidase and ectophosphodiesterase, respectively (ie, the cAMP-adenosine pathway), thus providing high local concentrations of adenosine that importantly might contribute to the regulation of vascular SMC growth4 and nitric oxide production.5 However, whether the cAMP-adenosine pathway might contribute to the regulation of vascular SMC collagen synthesis and hypertrophy is unknown, and this hypothesis is also addressed in the current study.

Methods

Adenosine, 2-chloroadenosine (Cl-Ad), cAMP, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), and dipyridamole (DIP) were purchased from Sigma Chemical Co. N6-Cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamino adenosine were synthesized in our laboratory.
phenethylamino-5'-N-ethylcarboxamino adenosine hydrochloride (CGS21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), iodotubericidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), 5'-N-ethylcarboxamidoadenosine (NECA), and 5'-N-methylcarboxamidoadenosine (MECA) were purchased from Research Biochemicals, Inc. and all tissue culture ware were purchased from Gibco Laboratories. Fetal calf serum (FCS) was purchased from HyClone Laboratories Inc. KF17837 was a generous gift from Dr. F. Suzuki, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co, Ltd, Suto, Shizuoka, Japan. L-[3H]Proline (specific activity, 23 Ci/mmol) and L-[4,5-3H(N)]leucine (specific activity, 50 Ci/mmol) were purchased from NEN. All other chemicals used were of tissue culture or best grade available.

Aortic SMCs were cultured as explants from the abdominal aortas obtained from ether-anesthetized male Sprague-Dawley rats (Charles River, Wilmington, Mass), after a midline abdominal incision including the diaphragm and as described previously by us. Vascular SMC purity was characterized by immunofluorescence staining with smooth muscle specific anti-smooth muscle α-actin monoclonal antibodies and by morphological criteria specific for smooth muscle as described in detail previously. Vascular SMCs were passaged by trypsinization, and cells in 3rd passage were used for growth studies.

[LH]Proline and [1H]leucine incorporation studies were performed to investigate the effects of agents on FCS-induced collagen and total protein synthesis, respectively. Vascular SMCs were plated in 24-well tissue culture dishes and allowed to grow to confluence in DMEM/F12 containing 10% FCS under standard tissue culture conditions. Vascular SMCs were made quiescent by incubating in DMEM containing 0.4% bovine serum albumin for 48 hours. Collagen and protein synthesis were initiated by incubating growth-arrested vascular SMCs for 36 and 24 hours, respectively, with DMEM supplemented with 2.5% FCS and with or without adenosine receptor agonists, modulators of adenosine levels, cAMP, adenosine receptor antagonists, and/or enzyme inhibitors. For collagen synthesis the cells were treated for 36 hours in the presence of L-[3H]proline (1 μCi/mL); whereas, for total protein synthesis after 20 hours of treatment, the cells were pulsed for 5 hours with L-[3H]leucine (1 μCi/mL). The experiments were terminated by washing the cells twice with Dulbecco's PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3 N NaOH and 0.1% SDS after incubation at 50°C for 2 hours. Aliquots from 4 wells for each treatment with 10 mL scintillation fluid were counted in a liquid scintillation counter.

All experiments were conducted in confluent monolayers of cells in which changes in cell number were precluded. Additionally, cell counting was performed in cells treated in parallel to the cells used for the collagen and total protein synthesis studies, and the data were normalized to cell number. Experiments were performed in quadruplicate with 3 to 6 separate cultures, and the data are presented as mean±SEM. Statistical analysis was performed by ANOVA, paired Students' t test, and Fisher's least significant difference test as appropriate. A value of P<0.05 was considered statistically significant.

Results

Adenosine and CI-Ad (a metabolically stable adenosine analog) inhibited proline and leucine incorporation in a concentration-dependent manner (Figure 1; P<0.005). The threshold concentration for inhibition of proline and leucine incorporation was 10 nmol/L for adenosine and 0.1 nmol/L for CI-Ad. CI-Ad (IC50 values of 10 and 4 μmol/L for inhibition of proline and leucine incorporation, respectively) was more potent than adenosine (IC50 of 100 μmol/L for inhibition of both proline and leucine incorporation). CGS21680, an adenosine agonist selective for A2A receptors, had little effect on proline or leucine incorporation (Figure 1). High (>10^-6 to 10^-5 mol/L), but not low, concentrations of CPA, a selective A1 adenosine receptor agonist, inhibited proline and leucine incorporation (Figure 1). NECA, an adenosine agonist which has equal affinity for both A1 and A2 receptors, was more potent than CPA, but less potent than CI-Ad in inhibiting proline and leucine incorporation (Figure 1). MECA, which has greater affinity for A2 than A1 receptors, was more potent than NECA, CPA, and CGS (Figure 1) and as potent as CI-Ad in inhibiting proline and leucine incorporation (Figure 1). Thus, the order of potency for inhibition of proline and leucine incorporation was CI-Ad > MECA > NECA > adenosine > CPA > CGS21680 (Figure 1).

EHNA, DIP, and IDO, which elevate endogenous levels of adenosine by inhibiting adenosine deaminase, blocking nucleoside transport, and inhibiting adenosine kinase, respectively, inhibited proline and leucine incorporation in a concentration-dependent manner (P<0.001; Figure 2). Threshold concentrations for inhibition of both proline and leucine incorporation were 10, 0.01, and 0.01 μmol/L for EHNA, DIP, and IDO, respectively. IC50 values for inhibition of proline incorporation by EHNA, DIP, and IDO were 50, 5, and 5 μmol/L, respectively, and for inhibition of leucine incorporation were 50, 1, and 1 μmol/L, respectively.

To assess whether metabolism of adenosine was responsible for the decreased potency of adenosine relative to CI-Ad, we studied the effects of adenosine on proline and leucine incorporation in the presence and absence of EHNA, IDO, and EHNA plus IDO. As shown in Figure 3, the inhibitory effects of adenosine+EHNA, adenosine+IDO, and adenosine+IDO+EHNA on proline and leucine incorporation were significantly greater than adenosine per se. Moreover, in vascular SMCs treated with adenosine+EHNA+IDO, proline, and leucine incorporation were reduced to almost basal levels.
To further evaluate the role of adenosine receptors in mediating the inhibitory effects of adenosine, experiments were conducted with use of the adenosine receptor antagonists DPCPX, DPSPX, and KF17837, which inhibit the effects of adenosine by blocking A1, A1 plus A2, and A2 receptors, respectively. DPSPX (10^{-8} mol/L) and KF17837 (10^{-9} mol/L), but not DPCPX (10^{-8} mol/L), significantly attenuated the inhibitory effects of Cl-Ad (10^{-8} mol/L) on proline and leucine incorporation (Figure 4). Like Cl-AD, cAMP, a putative precursor of adenosine, also attenuated proline and leucine incorporation and this effect was antagonized by DPSPX and KF17837, but not DPCPX (Figure 4). The inhibitory effects of EHNA (10 μmol/L) and IDO (0.1 μmol/L) on proline and leucine incorporation were also significantly attenuated by DPSPX and KF17837, but not by DPCPX (Figure 5).

Trypan blue exclusion tests were conducted in parallel to confirm that cell death did not contribute to the observed inhibitory effects of the various treatments. Moreover cells in the supernatant were counted to confirm that loss of cells by detachment did not occur during these treatments. At the concentrations used in this study, no loss in cell viability was observed in cells treated with adenosine, CPA, CGS21680, MECA, NECA, DPSPX, KF17837, or DPCPX. The highest concentrations of Cl-Ad (1 mmol/L), EHNA (100 μmol/L), DIP (100 μmol/L), and IDO (100 μmol/L) did decrease cell viability by approximately 7%, but not cell number. No floating cells were present in the supernatant, and the cell number in the well surface was not significantly different in controls and treated wells.

**Discussion**

The results of the present study demonstrate that treatment of rat aortic SMCs with adenosine or a stable adenosine analog...
(Cl-Ad) inhibited FCS-induced collagen as well as total protein synthesis. The inhibitory effects of adenosine were fully mimicked by MECA, an adenosine agonist with high affinity for A2 receptors, and partially by NECA, an adenosine agonist with equal affinity for both A1 and A2 receptors, but not by the adenosine agonists CPA and CGS21680, which are selective A1 and A2A receptor agonists, respectively. Thus, the inhibitory effects of adenosine are likely mediated via A2B receptors and not via A1 or A2A receptors.

Our conclusion that the inhibitory effects of adenosine are mediated via A2B receptors is supported further by the recently proposed subclassification of A2A and A2B receptors. Gurden et al demonstrated that the relative potencies of CGS21680 and NECA can be used as a reference to differentiate A2A from A2B. When the effects of CGS21680 are as potent as NECA, this implicates the A2A receptor. However, when CGS21680 is less potent than NECA, this indicates that the observed effects are mediated via activation of the A2B receptor. In the present study, compared with CGS21680, NECA was more effective in mimicking the inhibitory effects of adenosine, which further substantiates our conclusion that the inhibitory effects of adenosine are mediated via A2B receptors. Also consistent with this conclusion are the observations that the inhibitory effects of Cl-Ad were significantly reversed by KF17837, a selective A2 receptor antagonist, and by DPSPX, a selective A1 receptor antagonist, but not by DPCPX, a selective A2 receptor antagonist.

The above-mentioned findings provide the first evidence that exogenous adenosine inhibits FCS-induced collagen and protein synthesis in rat vascular SMCs and that the inhibitory effects of adenosine are mediated via activation of A2B receptors. However, whether endogenous adenosine has similar inhibitory effects cannot be inferred from studies with agonists. Hence, we examined the inhibitory effects of agents that elevate cellular adenosine levels via different mechanisms to assess the role of endogenous, ie, vascular SMC-derived adenosine on FCS-induced synthesis of collagen and protein in vascular SMCs.

The physiological effects of adenosine are governed partly by the rapid rate of elimination of adenosine from the extracellular space. Elimination of adenosine from the interstitial space is mediated by facilitated transport of adenosine into cells and also by the metabolism of adenosine to inosine by adenosine deaminase and to adenosine monophosphate by adenosine kinase. Inhibition of the enzyme adenosine deaminase by EHNA and the enzyme adenosine kinase by IDO, as well as the inhibition of adenosine transport and metabolism by DIP, has been shown to increase endogenous levels of adenosine. Hence, these three compounds were used in the present study to increase endogenous levels of adenosine to evaluate the effects of endogenously generated adenosine on FCS-induced collagen and protein synthesis.

EHNA and IDO inhibited collagen and protein synthesis, and KF17837, a selective A2 adenosine receptor antagonist, and DPSPX, a nonselective A1 receptor antagonist, significantly reversed the inhibitory effects of endogenous adenosine and IDO. The inhibitory effects of EHNA, IDO, and DIP also were significantly enhanced when vascular SMCs were treated with a combination of these agents. These findings support our contention that the inhibitory effects of these agents on collagen and protein synthesis in vascular SMCs are mediated via generation of adenosine. Moreover, the finding that DPCPX, a selective A1 receptor antagonist, did not reverse the inhibitory effects of EHNA and IDO on vascular SMCs strongly suggests that the inhibitory effects of endogenous adenosine are mediated via A2 receptors.

An important pathway by which adenosine is formed at the vascular SMC surface and within and/or near the blood vessel wall is the cAMP-adenosine pathway. Adenosine production in vascular SMCs via this pathway would be more amenable to physiological modulation by hormones. In this regard, stimulation of adenyl cyclase results in egress of cAMP and relatively modest increases in cAMP production could give rise to significant concentrations of adenosine at the cell surface, because adenosine would be synthesized by a series of spatially linked enzymatic reactions. We recently showed that the inhibitory effects of cAMP on vascular SMC proliferation are blocked by A2 adenosine receptor antagonists, this implying that the cAMP-adenosine pathway regulates vascular SMC growth. Because cAMP is a precursor for adenosine and adenosine inhibits mitogen-induced cell growth, we hypothesized that the cAMP-adenosine pathway may be a potential pathway regulating collagen and total protein synthesis in vascular SMCs. To test this hypothesis, we evaluated the effects of cAMP on FCS-induced collagen and protein synthesis in the presence and absence of the A1-specific and nonspecific receptor antagonists, KF17837 and DPSPX, respectively, and the A2 adenosine receptor antagonist, DPCPX. Exogenous cAMP inhibited FCS-induced collagen and protein synthesis in vascular SMCs, and the inhibitory effects of cAMP on collagen and protein synthesis were significantly attenuated by the adenosine receptor antagonists KF17837 and DPSPX, but not by DPCPX, which suggests the involvement of A2 receptors. This conclusion is consistent with our observations that the inhibitory effects of exogenous and endogenous adenosine on collagen and protein synthesis in vascular SMCs are mediated via A2 receptors and not via A1 or A2A receptors.

In conclusion we provide evidence that both exogenous and vascular SMC-derived adenosine inhibits FCS-induced collagen and total protein synthesis by vascular SMCs. Thus, our findings suggest that adenosine produced by vascular SMCs may play a vital role as a local inhibitory agent regulating vascular hypertrophy. Moreover, decreased synthesis of adenosine by vascular SMCs or increased catabolism of adenosine by adenosine deaminase or adenosine kinase may contribute importantly to the abnormal synthesis and deposition of collagen and hypertrophy of vascular SMCs observed in vasoocclusive disorders associated with hypertension, atherosclerosis, and restenosis. Agents that elevate endogenous adenosine could be clinically important in preventing neointima...
formation by inhibiting extracellular matrix synthesis and deposition by vascular SMCs, thus exerting beneficial effects on the vascular structure.

Acknowledgments
This work was supported by grants from the National Institutes of Health (HL 55314 and HL 35909) and the Swiss National Science Foundation (32-54172.98).

References
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Hypertension. 1999;33:190-194
doi: 10.1161/01.HYP.33.1.190

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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