Adenosine Inhibits PDGF-Induced Growth of Human Glomerular Mesangial Cells Via A_{2B} Receptors

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

Abstract—The objectives of the present study were to determine whether adenosine attenuates proliferation of glomerular mesangial cells (GMCs), which adenosine receptor (AR) mediates the antimitogeneic actions of adenosine, and the cellular mechanisms by which adenosine inhibits growth of GMCs. Studies were conducted in both human and rat GMCs. Platelet-derived growth factor (PDGF)-BB (25 ng/mL) increased DNA synthesis ([^{3}H]thymidine incorporation), cellular proliferation (cell number), collagen synthesis ([^{3}H]proline incorporation), and mitogen-activated protein kinase (MAPK) activity, and these effects were attenuated by 2-chloroadenosine (nonselective AR agonist) and 5′-*N*-methylcarboxamidoadenosine (MECA; nonselective AR agonist), but not by N^6-cyclopentyladenosine (selective A_1 AR agonist), AB-*N*-MECA (selective A_3 AR agonist), or CGS21680 (selective A_{2A} AR agonist). KF17837 (selective $A_{2A/B}$ AR antagonist) and 1,3-dipropyl-8-*p*-sulfophenylxanthine (nonselective AR antagonist), but not 8-cyclopentyl-1,3-dipropylxanthine (selective A_1 AR antagonist), blocked the growth-inhibitory effects of 2-chloroadenosine and 5′-*N*-MECA. Antisense, but not sense or scrambled, oligonucleotides to the A_{2B} receptor increased both basal and PDGF-induced DNA synthesis, cell proliferation, and collagen synthesis, and the growth-inhibitory effects of 2-chloroadenosine, 5′-*N*-MECA, and *erythro*-9-(2-hydroxy-3-nonyl)adenine (inhibitor of adenosine deaminase) plus iodotubercidin (inhibitor of adenosine kinase) were abolished by antisense, but not scrambled or sense, oligonucleotides to the A_{2B} receptor. We conclude that adenosine causes inhibition of GMC growth by activating A_{2B} receptors coupled to inhibition of MAPK activity. A_{2B} receptors may play an important role in regulating glomerular remodeling associated with GMC proliferation. Pharmacological or molecular biologic activation of A_{2B} receptors may prevent glomerular remodeling associated with glomerulosclerosis, renal disease, and abnormal growth associated with hypertension and diabetes. **(*Hypertension*. 2005;46:628-634.)**

Key Words: adenosine ■ mesangium ■ receptors, adenine ■ remodeling ■ glomerulosclerosis ■ renal disease ■ kidney

Adenosine, a nucleoside long known as a “retaliatory” metabolite within the heart, has several physiologically significant effects on renal function.[1] The biologic effects of adenosine are mediated by adenosine receptors (ARs), which exist in multiple subtypes (A_1, A_{2A}, A_{2B}, and A_3).[1,2] However, participation of A_1 and A_2 ARs appears to be more important in renal biology, particularly with regard to reducing the risks and consequences of glomerular remodeling events associated with hypertension and diabetes.[1] For example, activation of A_1 receptors by adenosine attenuates the sympathetic nervous system by inhibiting the release of norepinephrine and attenuates the renin-angiotensin system by inhibiting renin release from juxtaglomerular cells.[1] Both of these pathways are involved in the vascular and glomerular remodeling processes associated with hypertension, diabetes, and renal dysfunction. Through activation of A_2 receptors, adenosine causes renal vasodilation, inhibits platelet aggregation, diminishes neutrophil adhesion to vascular endothelial cells, attenuates neutrophil-induced endothelial cell damage, and stimulates nitric oxide release from endothelial cells.[3,4] These A_2 receptor–mediated effects may increase renal medullary blood flow, resulting in decreased NaCl reabsorption, and may attenuate inflammation. Finally, adenosine induces apoptosis in glomerular mesangial cells (GMCs), an effect that may play an important role in regulating the glomerular remodeling process.[5]

Adenosine is a potent regulator of growth in both vascular and nonvascular cells.[6–8] We have previously shown that adenosine inhibits growth of renal arteriolar[9] and aortic smooth muscle [6] cells, which play a key role in vascular remodeling leading to vaso-occlusive disorders. Moreover, using receptor-specific agonists and antagonists as well as antisense oligonucleotides, we demonstrated that the antimitogenic effects of adenosine are mediated by activation of A_{2B} receptors.[6] The role of A_{2B} receptors in mediating the antigrowth effects of adenosine is further supported by our recent

Received May 3, 2005; first decision May 28, 2005; revision accepted July 11, 2005.
From the Center for Clinical Pharmacology, Departments of Medicine (R.K.D., D.G.G., Z.M., E.K.J.) and Pharmacology (E.K.J.), University of Pittsburgh Medical Center, Pittsburgh, Pa, and the Clinic for Endocrinology (R.K.D.), Department of Obstetrics and Gynecology, University Hospital Zurich, Zurich, Switzerland.

Correspondence to Dr Raghvendra K. Dubey, Department of Obstetrics and Gynecology Clinic for Endocrinology, D215 NORD-1, Frauenklinik University Hospital Zurich, 8091 Zurich, Switzerland. E-mail Raghvendra.dubey@usz.ch

Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000178464.63393.88

Agents Used in the Study

Pharmacological Agent	Pharmacological Characteristics
2-Chloroadenosine (Cl-Ad)	Nonselective but stable adenosine analogue
5′-*N*-methylcarboxamidoadenosine (MECA)	Nonselective adenosine receptor agonist
4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine (AB-MECA)	Selective A_3 receptor agonist
5′-*N*-ethylcarboxamidoadenosine (NECA)	Nonselective adenosine receptor agonist
N^6-Cyclopentyladenosine (CPA)	Selective A_1 receptor agonist
2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxaminoadenosine (CGS21680)	Selective A_{2A} receptor agonist
KF17837 (KF)	Selective A_2 receptor antagonist
1,3-Dipropyl-8-*p*-sulfophenylxanthine (DPSPX)	Nonselective adenosine receptor antagonist
8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)	Selective A_1 receptor antagonist
Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA)	Adenosine deaminase inhibitor
Iodotubericidin (IDO)	Adenosine kinase inhibitor

finding that this AR inhibits mitogen-induced growth of cardiac fibroblasts.[7] The same relation between abnormal vascular smooth muscle cell (VSMC) proliferation and vascular disease may hold for abnormal growth of GMCs and glomerular remodeling associated with glomerulosclerosis, renal disease, hypertension, and diabetes.[10] Therefore, it is important to determine the effects and mechanism of ARs on the growth of GMCs.

Because VSMCs are phenotypically similar to GMCs[10] and because A_{2B} receptors are expressed in the kidney,[11] we hypothesized that A_{2B} receptors mediate antimitogenic actions in GMCs. Accordingly, the overall aim of the present study was to determine whether A_{2B} receptors affect 4 major growth processes (DNA synthesis, collagen synthesis, cell proliferation, and mitogen-activated protein kinase [MAPK] activity) in GMCs. To accomplish this goal, we studied the effects of adenosine and its receptor-specific analogues and antagonists (the Table) on the growth of human and rat GMCs. Moreover, to provide direct evidence of a role for A_{2B} receptors in inhibiting GMC growth, by using the sequence of the recently cloned A_{2B} receptor in rats, we designed antisense oligonucleotides to block A_{2B} receptor synthesis in rat GMCs.

Methods

Materials

Adenosine, 2-chloroadenosine (Cl-Ad), *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), and PDGF-BB were purchased from Sigma Chemical Co. N^6-cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), iodotubercidin (IDO), 1,3-dipropyl-8-*p*-sulfophenylxanthine (DPSPX), 5′-*N*-ethylcarboxamidoadenosine (NECA), 5′-*N*-methylcarboxamidoadenosine (MECA), and 4-aminobenzyl-5′-*N*-MECA (AB-MECA) were purchased from Research Biochemicals International. KF17837 was obtained from Kyowa Hakko Kogyo Co. Ltd (Sunto). [^{3}H]thymidine (specific activity, 11.8 Ci/mmol) and L-[^{3}H]proline (23 Ci/mmol) were purchased from NEN. All other reagents were of tissue culture or the best grade available.

GMC Cultures

Human GMCs were obtained from Clonetics Corp. Rat glomerular GMCs were grown as explants from glomeruli isolated from Sprague-Dawley rats and as described previously.[12] GMCs in the fourth passage were used for all experiments.

Antisense Oligonucleotides for A_{2B} Receptors

The antisense oligonucleotide 5′-CTCGTGTTCCAGTGACCAA-3′ was used for all experiments with rat GMCs. In a previous study, we showed that this antisense oligonucleotide is effective in modulating the effects of adenosine on rat VSMC growth.[6] Sense (5′-TTGGTCACTGGAACACGAG-3′) and scrambled (5′-GCAGCTC-TATACTGCATG-3′) oligonucleotides were used as controls.

Growth Studies

[^{3}H]thymidine incorporation (index of DNA synthesis), [^{3}H]proline incorporation (index of collagen synthesis), and cell proliferation were conducted as previously described.[6] GMCs were exposed to various treatments for 24 hours (thymidine incorporation studies), 48 hours (proline incorporation studies), or 5 days (cell proliferation studies).

MAPK Activity

MAPK activity in the cytosolic extracts of GMCs was quantified by our previously described radioactive method with myelin basic protein as the substrate and [γ-^{32}P]ATP.[13] GMCs were pretreated with oligonucleotides for 72 hours and pretreated with other test agents for 24 hours before MAPK activity was measured.

Expression Studies With Western Blots

The expression of A_{2B} ARs in lysates from GMCs was analyzed by Western blotting and was probed with antibodies against A_{2B} receptors (rabbit anti-adenosine A_{2B} receptor polyclonal antibodies; Chemicon International Inc). The expression of phosphorylated MAPK (p42MAPK/p44MAPK) in lysates from GMCs was analyzed by Western blotting and was probed with antibodies against phosphorylated MAPK (extracellular signal–regulated kinase [ERK]1/ERK2; anti-MAPK ERK1/ERK2, phosphospecific, human; Calbiochem). Antibodies against nonphosphorylated ERK1/ERK2 were used to measure total ERK1/ERK2.

cAMP Synthesis

Extracellular (supernatant) and intracellular (cellular fraction) portions of cAMP were pooled, and total cAMP levels were analyzed by high-performance liquid chromatography according to our previously described method.[7] In GMCs grown in parallel and treated similarly, the monolayers were inspected microscopically for intactness, the protein content was measured, and the data were normalized to protein content.

Statistics

All growth experiments were performed in triplicate or quadruplicate with 3 or 4 separate cultures. Data are presented as mean±SEM. Statistical analysis was performed with ANOVA, paired Student *t*

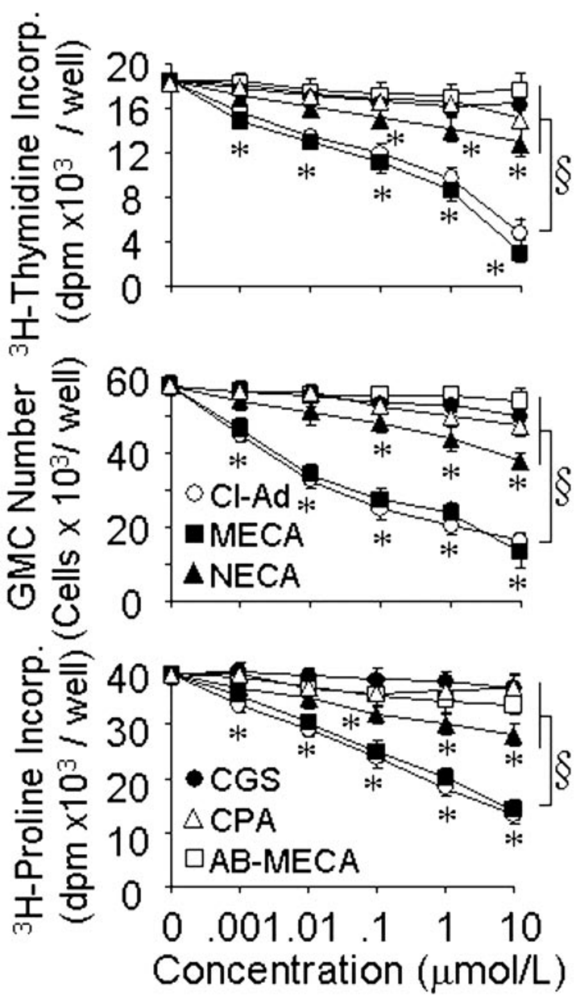

Figure 1. Concentration-response relations for the inhibition of PDGF-BB–induced [³H]thymidine incorporation (DNA synthesis; top panel), cell number (middle panel), and [³H]proline incorporation (collagen synthesis; bottom panel) by Cl-Ad, MECA, AB-MECA, NECA, CPA, or CGS21680 (CGS) in human GMCs. Values represent mean±SEM from 3 separate experiments, each conducted in quadruplicate. *$P<0.05$, compared with control; §$P<0.05$, vs Cl-Ad or MECA. Abbreviations are as defined in text.

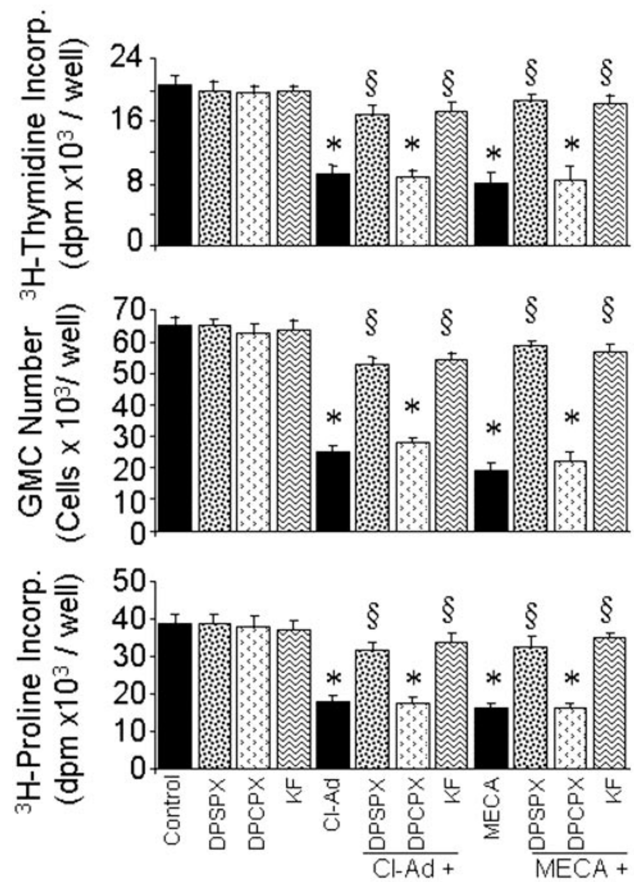

Figure 2. Effects of Cl-Ad (1 μmol/L) and MECA (1 μmol/L) on PDGF-BB–induced [³H]thymidine incorporation (DNA synthesis; top panel), cell number (middle panel), and [³H]proline incorporation (collagen synthesis; bottom panel) in the presence or absence of KF17837 (KF; 10^{-8} mol/L), DPSPX (10^{-8} mol/L), or DPCPX (10^{-8} mol/L) in human GMCs. Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. *$P<0.05$, compared with control; §$P<0.05$, significant reversal of Cl-Ad effects. Abbreviations are as defined in text.

test, or Fisher least significant difference test, as appropriate. A value of $P<0.05$ was considered statistically significant.

Results

In human GMCs, PDGF-BB increased by severalfold all indices of cellular growth, including DNA synthesis, collagen synthesis, and cell proliferation. In PDGF-BB–treated cells, Cl-Ad concentration-dependently attenuated all indices of cell growth (Figure 1). With the various pharmacological agents (AR subtype–selective and nonselective agonists and antagonists) listed in the Table, we further assessed the role of various AR subtypes (A_1, A_{2A}, A_{2B}, and A_3) in mediating the antimitogenic actions in GMCs. Only very high (10^{-6} mol/L) concentrations of CPA and CGS21680 decreased PDGF-BB–induced cell growth (Figure 1). MECA was more potent than NECA, and MECA and NECA were more potent than AB-MECA, CPA, and CGS21680 in reducing PDGF-BB–induced increases in cell growth (Figure 1). KF17837 and DPSPX, but not DPCPX, blocked the effects of Cl-Ad on all indices of cell growth (Figure 2). Also, the inhibitory effects of MECA were antagonized by KF17837 and DPSPX, but not by DPCPX (Figure 2). EHNA and IDO, administered separately, inhibited PDGF-BB–induced DNA synthesis, collagen synthesis, and cell proliferation, and these effects were enhanced in cells treated with EHNA plus IDO (Figure 3). KF17837 and DPSPX, but not DPCPX, reduced the inhibitory effects of EHNA plus IDO on PDGF-BB–induced cell growth (Figure 3). Trypan blue exclusion tests demonstrated that none of the aforementioned treatments altered cell viability. Western blots of human GMC lysates showed the presence of A_{2B} ARs (Figure 3).

Similar to human GMCs, the PDGF-BB–induced growth of rat GMCs was inhibited by Cl-Ad, MECA, and NECA, but not by CPA, CGS21680, or AB-MECA (Figure 4A). Compared with NECA, both MECA and Cl-Ad were more potent in inhibiting GMC growth. Moreover, the inhibitory actions of both MECA and NECA were significantly reversed by KF17837 and DPSPX, but not by DPCPX (Figure 4B). Rat

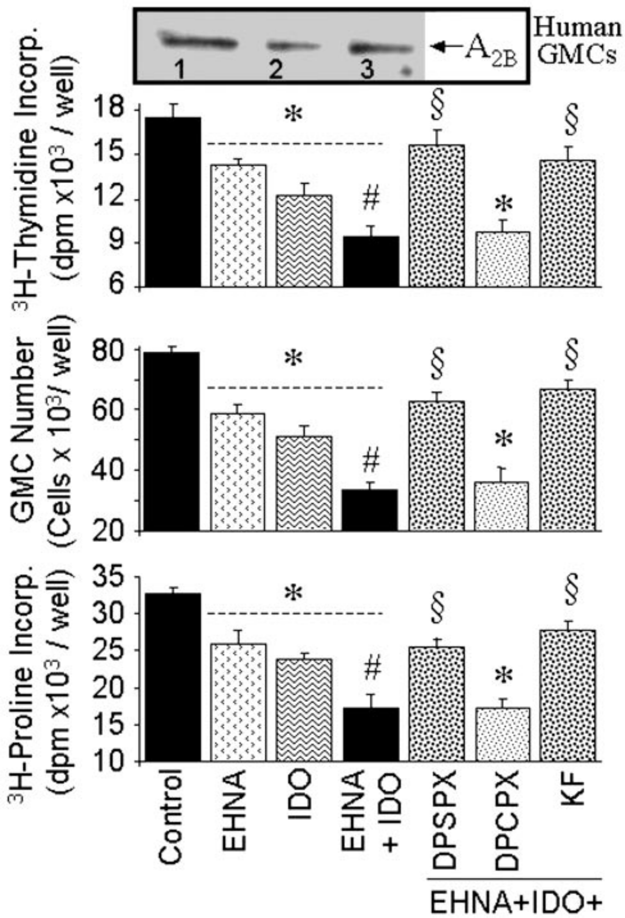

Figure 3. Western blot depicting expression of the A$_{2B}$ AR in human GMCs used for the growth study (lanes 1, 2, and 3 specify cell lysates from 3 separate GMCs) and the effects of EHNA (10 μmol/L), IDO (0.1 μmol/L), or EHNA (10 μmol/L) plus IDO (0.1 μmol/L) on PDGF-BB–induced [^{3}H]thymidine incorporation (DNA synthesis; top panel), cell number (middle panel), and [^{3}H]proline incorporation (collagen synthesis; bottom panel) in the presence or absence of KF17837 (KF; 10^{-8} mol/L), DPSPX (10^{-8} mol/L), or DPCPX (10^{-8} mol/L) in human GMCs. Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. **P*<0.05, vs control; #*P*<0.05, vs EHNA or IDO alone; §*P*<0.05, vs EHNA plus IDO (significant reversal of the inhibitory effect). Abbreviations are as defined in text.

GMC growth was also inhibited by both EHNA and IDO, and this effect was antagonized by KF17837 and DPSPX, but not by DPCPX (Figure 4B).

In rat GMCs, antisense oligonucleotide increased basal and PDGF-BB–induced DNA synthesis by 65% and 69%, respectively; however, neither sense nor scrambled oligonucleotide altered basal or PDGF-BB–induced DNA synthesis (Figure 4C). The effect of antisense oligonucleotide on DNA synthesis was biphasic, with maximal stimulatory effects observed at 0.2 to 0.5 μmol/L (Figure 4D). At 0.5 μmol/L, antisense oligonucleotide did not cause cell toxicity, as assessed by trypan blue exclusion; however, at concentrations $\geq$1 μmol/L, cell shrinkage and cell death were evident. The induction of growth by antisense oligonucleotide alone may have been caused by downregulation of the A$_{2B}$ receptor, which would attenuate the growth-inhibitory effects of endogenously produced adenosine. The lack of stimulatory effects at high concentrations may have been caused by cell toxicity. Antisense oligonucleotide, but not sense or scrambled oligonucleotide, attenuated the inhibitory effects of Cl-Ad, MECA, and EHNA plus IDO on PDGF-BB–induced cell proliferation, DNA synthesis, and collagen synthesis (Figure 5).

In human GMCs, PDGF-BB increased MAPK activity, from 0.625±0.07 to 10.87±0.9 pmol·min^{-1}·mg protein^{-1}. The MAPK inhibitor PD98059 (10 μmol/L) attenuated the stimulatory effects of PDGF-BB to 1.3±0.04 pmol·min^{-1}·mg protein^{-1}. PDGF-BB–mediated stimulation of MAPK activity was reduced in GMCs pretreated for 24 hours with 1 μmol/L Cl-Ad, MECA, NECA, or EHNA plus IDO but not pretreated with CGS21680, AB-MECA, or CPA (Figure 6A). DPSPX, but not by DPCPX, attenuated the inhibitory effects of Cl-Ad and EHNA plus IDO (Figure 6A).

Similar modulatory effects of adenosine analogues were observed in rat GMCs (data not shown). Moreover, in rat GMCs, antisense, but not sense or scrambled, oligonucleotide abolished the inhibitory effects of Cl-Ad or EHNA plus IDO on PDGF-BB–induced MAPK activity (Figure 6B). Western blot analysis of lysates collected from human GMCs treated with Cl-Ad, MECA, or EHNA plus IDO showed a significant decrease in the expression of phosphorylated MAPK (ERK1/ERK2; Figure 6C).

In control rat GMCs, Cl-Ad increased cAMP levels by 5.5-fold (Figure 7). The stimulatory effects of Cl-Ad on cAMP synthesis activity were completely abolished in GMCs pretreated with antisense, but not sense or scrambled, oligonucleotide (Figure 7). Rat GMCs expressed A$_{2B}$ ARs. In GMCs treated with antisense, but not sense or scrambled, oligonucleotide, the expression of A$_{2B}$ ARs was abolished, as analyzed by Western blot analysis (Figure 7).

Discussion

These experiments provide evidence that exogenous as well as GMC-derived adenosine inhibits PDGF-BB–induced growth and MAPK activity via the A$_{2B}$ receptor. In support of this conclusion, we found that treatment of both human and rat GMCs with a metabolically stable adenosine analogue (Cl-Ad) or with agents that increase endogenous adenosine (EHNA plus IDO) inhibited PDGF-BB–induced GMC DNA synthesis, collagen synthesis, cell proliferation, and MAPK activity. MECA, an adenosine agonist with high affinity for A$_2$ receptors, expressed potency similar to that of Cl-Ad; whereas CPA and CGS21680, which are selective A$_1$ and A$_{2A}$ receptor agonists, respectively, were only mildly inhibitory and then only at high concentrations. AB-MECA, an A$_3$ AR agonist, did not alter PDGF-BB–induced GMC growth. KF17837, a selective A$_2$ receptor antagonist,[3] and DPSPX, a nonselective A$_2$ receptor antagonist,[3] but not DPCPX, a selective A$_1$ receptor antagonist, attenuated the effects of Cl-Ad, MECA, or EHNA plus IDO. Also, in rat GMCs, the inhibitory effects of Cl-Ad or EHNA plus IDO on growth were blocked by antisense oligonucleotide, but not by sense or scrambled oligonucleotide, to A$_{2B}$ receptors. Taken to-

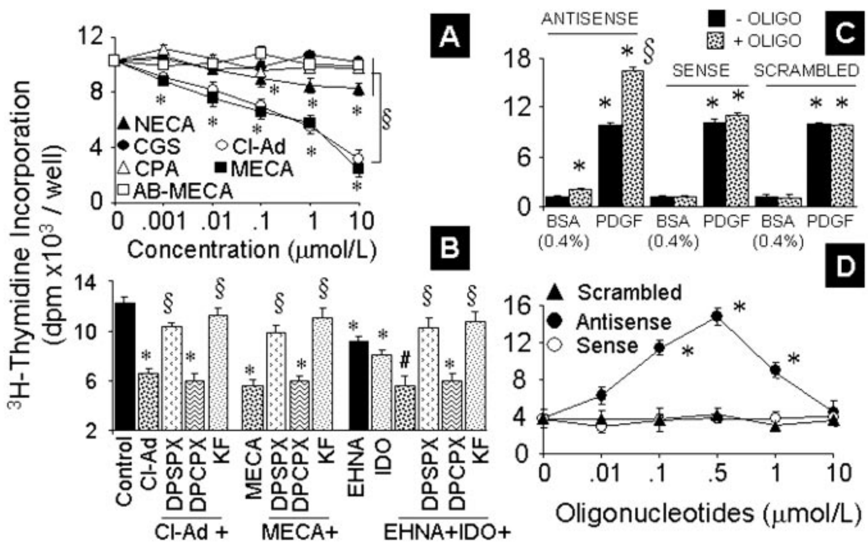

Figure 4. A, Concentration-response relations for the inhibition of PDGF-BB–induced [^{3}H]thymidine incorporation (DNA synthesis) by Cl-Ad, MECA, AB-MECA, NECA, CPA, or CGS21680 (CGS) in rat GMCs. Values represent mean±SEM from 3 separate experiments, each conducted in quadruplicate. *$P<0.05$, compared with control; §$P<0.05$, vs Cl-Ad or MECA. B, Bar graph showing the effects of Cl-Ad (1 μmol/L), MECA (1 μmol/L), EHNA (10 μmol/L), IDO (0.1 μmol/L), or EHNA (10 μmol/L) plus IDO (0.1 μmol/L) on PDGF-BB–induced [^{3}H]thymidine incorporation (DNA synthesis) in the presence or absence of KF17837 (KF; 10^{-8} mol/L), DPSPX (10^{-8} mol/L), or DPCPX (10^{-8} mol/L) in rat GMCs. Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. *$P<0.05$, vs control; #$P<0.05$, vs EHNA or IDO alone; §$P<0.05$, significant reversal of the inhibitory effects of agonist. C, Effects of antisense, sense, and scrambled oligonucleotides (OLIGO; 0.5 μmol/L) to A_{2B} receptors on basal and PDGF-BB–induced [^{3}H]thymidine incorporation (DNA synthesis). Values represent mean±SEM from 3 separate experiments. *$P<0.05$, vs bovine serum albumin (BSA) without (−OLIGO) oligonucleotides; §$P<0.05$, PDGF with oligonucleotides (+OLIGO) vs PDGF without oligonucleotides. D, Concentration response of antisense, sense, and scrambled oligonucleotides to A_{2B} receptors on basal [^{3}H]thymidine incorporation (DNA synthesis). Values represent mean±SEM from 3 separate experiments. *$P<0.05$, vs no oligonucleotides. Abbreviations are as defined in text.

gether, these results strongly support the conclusion that A_{2B} receptors inhibit the growth of human and rat GMCs.

CPA and DPCPX are highly selective and potent A_1 receptor agonists and antagonists, respectively. Because low concentrations of CPA do not inhibit PDGF-BB–induced growth and because DPCPX does not block the inhibitory effects of Cl-Ad on GMC growth, it is highly unlikely that A_1 receptors mediate inhibition of GMC growth. AB-MECA is a selective A_3 AR agonist. Because AB-MECA does not attenuate PDGF-BB–induced GMC growth, it is also unlikely that A_3 receptors mediate inhibition of GMC growth.

Both MECA and NECA are AR agonists that activate multiple AR subtypes, including A_{2B} receptors, and KF17837 and DPSPX are AR antagonists that block A_{2A} and A_{2B} receptors. The fact that MECA and NECA mimic the effects of Cl-Ad on GMC growth and the fact that KF17837 and DPSPX attenuate the inhibitory effects of Cl-Ad on GMC growth strongly support the conclusion that A_{2B} receptors mediate the inhibitory effects on GMC growth. The conclusion that A_{2B} receptors mediate inhibitory effects on GMC growth is supported further by the finding that the inhibitory effects of MECA and Cl-Ad on GMC growth are blocked by antisense oligonucleotide against A_{2B} receptors, yet neither sense nor scrambled oligonucleotide affects the inhibitory actions of MECA and Cl-Ad.

Metabolism of adenosine to inosine and AMP by adenosine deaminase and adenosine kinase, respectively,[14,15] importantly regulates cellular levels of adenosine, and blockade of adenosine deaminase with EHNA and adenosine kinase with IDO increase endogenous levels of adenosine.[8] Treatment of GMCs with EHNA plus IDO inhibits PDGF-BB–induced GMC growth, and the inhibitory effects of EHNA plus IDO on GMC growth are attenuated by KF17837 and DPSPX, but not by DPCPX. These findings provide strong evidence that the inhibitory effects of EHNA plus IDO are mediated via generation of adenosine and that these effects are A_2 AR mediated. The observation that the growth-inhibitory effects of EHNA plus IDO are abolished in GMCs pretreated with antisense oligonucleotide to A_{2B} receptors provides strong evidence that the inhibitory effects of endogenous GMC-derived adenosine are A_{2B} receptor mediated. The aforementioned findings provide the first evidence that endogenous adenosine also inhibits GMC growth via A_{2B} receptors.

The MAPK pathway is activated by multiple growth factors[10] and is involved in pathologic conditions associated with glomerular remodeling and abnormal growth of GMCs.[11] MECA and Cl-Ad, but not CPA, AB-MECA, or CGS21680, attenuate PDGF-BB–induced MAPK activity, and these effects are antagonized by KF17837 and DPSPX, but not by DPCPX. In additional, the inhibitory actions of MECA and Cl-Ad on PDGF-BB–induced MAPK activity are abolished by antisense oligonucleotide to A_{2B} receptors. Also, treatment of GMCs with EHNA plus IDO inhibits MAPK activity, and this inhibition is antagonized by DPSPX, but not by DPCPX. The inhibitory effects of EHNA plus IDO are also abolished by antisense to A_{2B} receptors. Taken together, these findings suggest that A_{2B} ARs inhibit GMC growth in part by reducing activation of the MAPK pathway.

In the present study, we measured MAPK activity in the presence of a protein kinase inhibitor, a calmodulin antago-

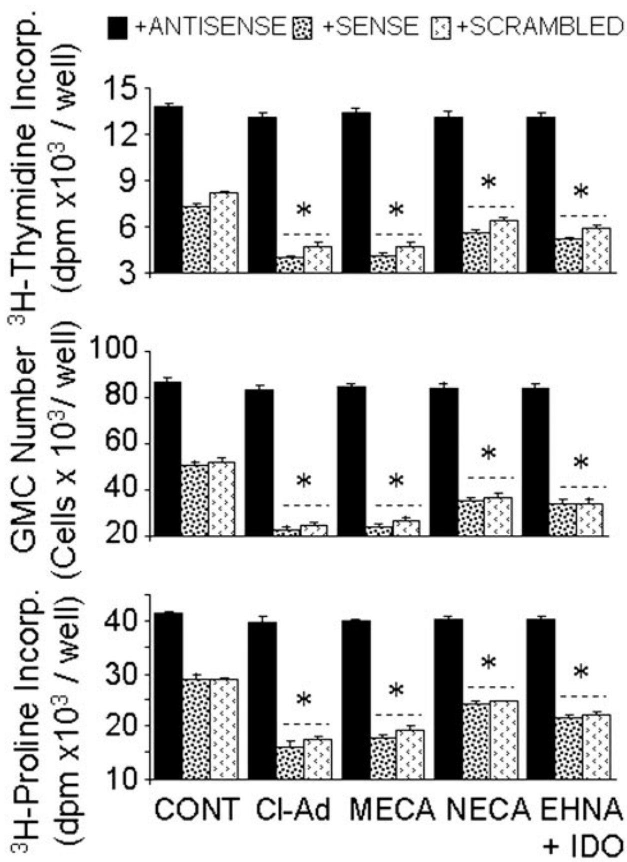

Figure 5. Bar graph showing the inhibitory effects of Cl-Ad (1 μmol/L), MECA (1 μmol/L), NECA (1 μmol/L), or EHNA (10 μmol/L) plus IDO (0.1 μmol/L) on PDGF-BB–induced [^{3}H]thymidine incorporation (DNA synthesis; top panel), cell number (middle panel), and [^{3}H]proline incorporation (collagen synthesis; bottom panel) in rat GMCs treated with 0.5 μmol/L antisense, sense, or scrambled oligonucleotides to adenosine A$_{2B}$ receptor. Also, CPA, CGS21680, and AB-MECA did not influence PDGF-BB–induced GMC growth in the presence of the oligonucleotides (data not shown). Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. *$P<0.05$ vs control.

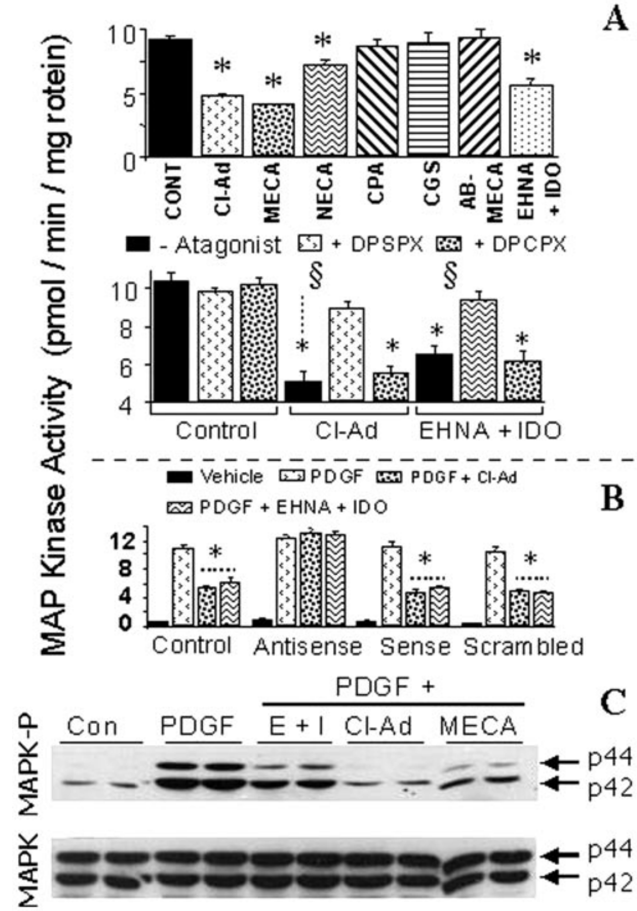

Figure 6. A, Inhibitory effects of 1 μmol/L Cl-Ad, MECA, NECA, CPA, CGS21680, or AB-MECA or 10 μmol/L EHNA plus 0.1 μmol/L IDO on PDGF-BB–induced MAPK activity. Also shown is the effect of Cl-Ad or EHNA plus IDO in the absence or presence of DPSPX (10^{-8} mol/L) or DPCPX (10^{-8} mol/L) in human GMCs. *$P<0.05$, vs control; §$P<0.05$, vs Cl-Ad plus DPSPX or EHNA plus IDO plus DPSPX. B, Inhibitory effects of Cl-Ad (1 μmol/L) or EHNA (10 μmol/L) plus IDO (0.1μmol/L) on PDGF-BB–induced MAPK activity in the absence or presence of 0.5 μmol/L antisense, sense, or scrambled oligonucleotides to adenosine A$_{2B}$ receptor in rat GMCs. *$P<0.05$, vs PDGF. C, Inhibitory effects of Cl-Ad (1 μmol/L), MECA (1 μmol/L), or EHNA (10 μmol/L) plus IDO (0.1 μmol/L) on PDGF-BB (25 ng/mL)–induced expression of phosphorylated MAPK (ERK1/ERK2) in human GMCs. Abbreviations are as defined in text.

nist, and a calcium chelator. Thus, kinase activity from protein kinase A, calmodulin-dependent kinases, and calcium-dependent forms of protein kinase C were excluded. In addition, stimulation of kinase activity, as measured by our assay, was abolished by PD98059, a selective MAPK kinase (MEK) inhibitor. Because MEK selectively activates p42^{MAPK} (ERK1)and p44^{MAPK} (ERK2), the results with PD98059 suggest that the kinase activity measured with our assay was caused by p42^{MAPK} and/or p44^{MAPK}. This conclusion is supported by our observation that the expression of phosphorylated p42^{MAPK} and p44^{MAPK} was significantly inhibited in GMCs treated with Cl-Ad, MECA, or EHNA plus IDO.

In the present study, the expression of A$_{2B}$ receptors was abolished in GMCs treated with antisense oligonucleotide, but not in cells treated with sense or scrambled oligonucleotide. Also, the effect of Cl-Ad on cAMP production, a second messenger activated by A$_{2B}$ receptors, was abolished in GMCs treated with antisense, but not sense or scrambled, oligonucleotide. These findings confirm the conclusion that antisense oligonucleotide to the A$_{2B}$ receptor downregulates A$_{2B}$ receptor expression in GMCs.

Our finding that adenosine inhibits GMC growth, together with our previous observation that adenosine inhibits growth of renal arteriolar SMCs, suggests that adenosine may play an important role in regulating the remodeling process within the glomeruli as well as in preglomerular arterioles. Because abnormal growth of renal arteriolar SMCs and glomerulosclerosis are observed in hypertension, it is possible that suppressed adenosine levels or decreased expression of A$_{2B}$ receptors may contribute to hypertension-induced renal injury. This idea is supported by our previous finding that adenosine levels are decreased in renal arteriolar SMCs obtained from spontaneously hypertensive rats. However, detailed studies are required to elucidate the association between adenosine levels, A$_{2B}$ receptors, and the glomerular

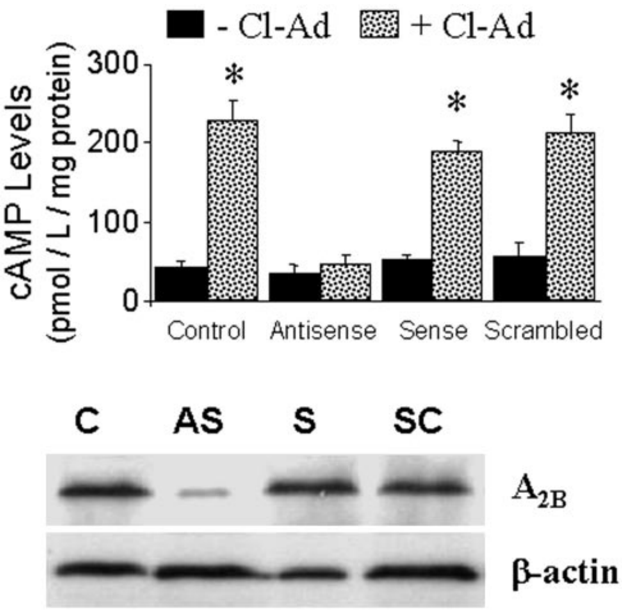

Figure 7. Top panel, Stimulatory effects of Cl-Ad (1 μmol/L) on cAMP synthesis in rat GMCs treated with or without 0.5 μmol/L antisense, sense, or scrambled oligonucleotides to adenosine A_{2B} receptor. **P*<0.05, vs no Cl-Ad. Bottom panel, Western blot showing the expression of A_{2B} ARs in rat GMCs treated with or without 0.5 μmol/L antisense (AS), sense (S), or scrambled (SC) oligonucleotides to adenosine A_{2B} receptor. Abbreviations are as defined in text.

remodeling process in hypertension, diabetes, and renal disease.

Recent studies by Shen et al[16] have demonstrated that adenosine stimulates, rather than inhibits, porcine coronary artery SMC number, cellular DNA content, protein synthesis, and proliferating cell nuclear antigen staining. Also, in these cells, the mitogenic effects of adenosine were mediated by the A_1 AR. The findings of Shen et al illustrate that the overall effect of adenosine on growth is dependent on the specific cell type and species. This underscores the need to perform growth studies in human cells wherever possible. Because the present study was conducted with human GMCs, the results are applicable to human kidneys. We are currently investigating the effects of adenosine and adenosine analogues on the growth of human coronary artery SMCs, and our results to date indicate that adenosine inhibits growth of human coronary artery SMCs via the A_{2B} receptor (manuscript in preparation).

Perspectives

Our results provide evidence that adenosine inhibits GMC growth and that the inhibitory effects of adenosine are mediated via A_{2B} ARs. Because abnormal growth of GMCs contributes importantly to the glomerular remodeling process associated with glomerulosclerosis, hypertension, and diabetes, our results suggest that abnormal and/or decreased expression of A_{2B} receptors or decreased synthesis of adenosine may contribute to the abnormal growth of GMCs in various renal pathologies. Moreover, development of pharmacological agents that activate A_{2B} receptors, as well as molecular targeting of the A_{2B} receptor to increase its expression, may be of therapeutic importance in protecting against glomerular remodeling associated with glomerulosclerosis, renal disease, and abnormal GMC growth associated with hypertension and diabetes.

Acknowledgments

This study was supported by Swiss National Science Foundation grant 32–64040-00, an Oncosuisse grant (OCS-01551–08-2004) and National Institutes of Health (Bethesda, Md) grant DK68575.

References

1. Jackson EK, Dubey RK. Role of the extracellular cAMP-adenosine pathway in renal physiology. *Am J Physiol Renal Physiol*. 2001;28: F597–F612.
2. Olah SN, Stiles GL. The role of receptor structure in determining adenosine receptor activity. *Pharmacol Ther*. 2000;85:55–75.
3. Feoktistov I, Biaggioni I. Adenosine A_{2B} receptors. *Pharmacol Rev*. 1997;49:381–402.
4. Dubey RK, Gillespie DG, Jackson EK. Cyclic AMP-adenosine pathway induces nitric oxide synthesis in aortic smooth muscle cells. *Hypertension*. 1998;31:296–302.
5. Zhao Z, Kapoian T, Shepard M, Lianos EA. Adenosine-induced apoptosis in glomerular mesangial cells. *Kidney Int*. 2002;61:1276–1285.
6. Dubey RK, Gillespie DG, Shue H, Jackson EK. A_{2B} receptors mediate antimitogenesis in vascular smooth muscle cells. *Hypertension*. 2000;35(pt 2):267–272.
7. Dubey RK, Gillespie DG, Zacharia LC, Mi Z, Jackson EK. A_{2B} receptors mediate antimitogenic effects of adenosine in cardiac fibroblasts. *Hypertension*. 2001;37(pt 2):716–721.
8. Dubey RK, Gillespie DG, Mi Z, Jackson EK. Exogenous and endogenous adenosine inhibits fetal calf serum–induced growth of rat cardiac fibroblasts: role of A_{2B} receptors. *Circulation*. 1997;96:2656–2666.
9. Dubey RK, Mi Z, Gillespie DG, Jackson EK. Dysregulation of extracellular adenosine levels by vascular smooth muscle cells from spontaneously hypertensive rats. *Arterioscler Thromb Vasc Biol*. 2001;21: 249–254.
10. Dubey RK, Jackson EK, Rupprecht HD, Sterzel RB. Factors controlling growth and matrix production in vascular smooth muscle and glomerular mesangial cells. *Curr Opin Nephrol Hypertens*. 1997;6:88–105.
11. Jackson EK, Zhu C, Tofovic SP. Expression of adenosine receptors in the preglomerular microcirculation. *Am J Physiol Renal Physiol*. 2002;283: F41–F51.
12. Kreisberg JI, Karnovsky MJ. Glomerular cells in culture. *Kidney Int*. 1983;23:439–447.
13. Dubey RK, Jackson EK, Gillespie DG, Zacharia LC, Imthurn B, Keller PJ. Clinically used estrogens differentially inhibit human aortic smooth muscle cell growth and mitogen-activated protein kinase activity. *Arterioscler Thromb Vasc Biol*. 2000;20:964–972.
14. Schrader, J. Formation and metabolism of adenosine and adenine nucleotides in cardiac tissue. In: Phillis JW, ed. *Adenosine and Adenine Nucleotides as Regulators of Cellular Function.* Boca Raton/Ann Arbor/ Boston/London: CRC Press; 1991:55–69.
15. Jackson EK, Kohler M, Mi Z, Dubey RK, Tofovic SP, Carcillo JA, Jones GS. Possible role of adenosine deaminase in vaso-occlusive diseases *J Hypertens.* 1996;14:19–29.
16. Shen J, Halenda SP, Sturek M, Wilden PA. Novel mitogenic effect of adenosine on coronary artery smooth muscle cells: role for the A_1 adenosine receptor. *Circ Res*. 2005;96:982–990.

Hypertension
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

Adenosine Inhibits PDGF-Induced Growth of Human Glomerular Mesangial Cells Via A_{2B} Receptors

Raghvendra K. Dubey, Delbert G. Gillespie, Zaichuan Mi and Edwin K. Jackson

Hypertension. 2005;46:628-634; originally published online August 15, 2005;
doi: 10.1161/01.HYP.0000178464.63393.88
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563