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Adenosine inhibits cytosolic calcium signals and chemotaxis in hepatic stellate cells

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Abstract

Adenosine is produced during cellular hypoxia and apoptosis, resulting in elevated tissue levels at sites of injury. Adenosine is also known to regulate a number of cellular responses to injury, but its role in hepatic stellate cell (HSC) biology and liver fibrosis is poorly understood. We tested the effect of adenosine on the cytosolic Ca²⁺ concentration, chemotaxis, and upregulation of activation markers in HSCs. We showed that adenosine did not induce an increase in the cytosolic Ca²⁺ concentration in LX-2 cells and, in addition, inhibited increases in the cytosolic Ca²⁺ concentration in response to ATP and PDGF. Using a Transwell system, we showed that adenosine strongly inhibited PDGF-induced HSC chemotaxis in a dose-dependent manner. This inhibition was mediated via the A_{2a} receptor, was reversible, was reproduced by forskolin, and was blocked by the adenylate cyclase inhibitor 2,5-dideoxyadenosine. Adenosine also upregulated the production of TGF- β and collagen I mRNA. In conclusion, adenosine reversibly inhibits Ca²⁺ fluxes and chemotaxis of HSCs and upregulates TGF- β and collagen I mRNA. We propose that adenosine provides 1) a “stop” signal to HSCs when they reach sites of tissue injury with high adenosine concentrations and 2) stimulates transdifferentiation of HSCs by upregulating collagen and TGF- β production.

Keywords

platelet-derived growth factor; Ca²⁺; fibrosis

Adenosine is produced extracellularly and intracellularly from the dephosphorylation of adenosine tri-, di-, and monophosphates (27). A further source of adenosine is the degradation of nucleic acids, via the uric acid pathway, during cellular injury and apoptosis (12). These sources of adenosine result in elevated levels at sites of tissue ischemia and cellular apoptosis, with concentrations increasing ≥ 100 -fold from the 30- to 300-nM range present in health (16, 17). Adenosine is efficiently metabolized by deamination or phosphorylation to inosine and adenine, respectively. The tight association of adenosine levels with tissue injury provides a potential means to communicate tissue injury to adaptive cellular responses.

Hepatic stellate cells (HSCs) are central to the adaptive response of the liver to hepatocyte injury and apoptosis (2). Such injury results in the chemotaxis of HSCs to the sites of cellular damage and differentiation with upregulation of TGF- β and collagen in addition to

many other changes. PDGF induces HSC chemotaxis and is thought to be important in localizing HSCs to sites of liver injury (26). PDGF is produced by a variety of cells, including bile duct epithelia, and is upregulated during liver injury (3). In this model, HSCs move via chemotaxis up a gradient of PDGF until they reach the point of maximal concentration. There, they are subsequently retained at this point and may receive additional signals such as TGF- β to promote further differentiation.

It is clear that chronic liver injury results in excess liver fibrosis, which can progress to cirrhosis. However, the relative contribution of the injury stimulus and the subsequent hepatocyte apoptosis in the development of liver fibrosis remain unknown. The recent demonstration that hepatocyte apoptosis, in the absence of an injury stimulus, can result in liver fibrosis has made the identity of signals generated from apoptotic cells that regulate liver fibrosis of great interest (24). The known increase in tissue adenosine at sites of cellular injury and apoptosis stimulated us to test whether adenosine is involved in the regulation of HSCs. We studied the ability of adenosine to regulate HSC cytosolic Ca²⁺ fluxes, chemotaxis, and collagen production.

In this study, we demonstrated that adenosine inhibits the increase in HSC cytosolic Ca²⁺ concentration induced by ATP and PDGF. This inhibition was associated with an inhibition of PDGF-induced HSC chemotaxis, and the effect was dose dependent, reversible, and likely mediated via the A_{2a} receptor subtype. We further showed that adenosine upregulates TGF- β and collagen mRNA. This identifies a novel role for adenosine in providing a “stop” signal for HSCs when they have migrated to a site of cellular injury, with upregulation of collagen and TGF- β synthesis.

MATERIALS AND METHODS

Cell culture and reagents

LX-2 cells are immortalized human HSCs and were a gift from Dr. Scott Friedman (25). LX-2 cells were cultured in RPMI plus 5% FBS and 1% penicillin-streptomycin, and the media were changed every 3 days. Primary HSCs were cultured in medium 199, 10% FBS, 1% penicillin-streptomycin, 2% gentamycin, and fungizone at a 1:100 density, with a media change 24 h after cell isolation and then after every 3 days. Histodenz, DNase, collagenase, hematoxylin-eosin stain, forskolin, xanthine, inosine, adenine, hypo-xanthine, uric acid, MRS-1523 (an A₃ antagonist), 8-(*p*-sulfophenyl)-theophylline (8-SPT; a peripheral nonselective adenosine antagonist), and 5'-(*N*-ethylcarboxamido)adenosine (NECA; a nonselective adenosine receptor agonist) were obtained from Sigma (St. Louis, MO). Trypan blue, TRIzol reagent, fungizone, trypsin-EDTA, RNase-free water, PBS, medium 199, RPMI, DMEM, HBSS, and F-12 (HAM) were purchased from GIBCO-BRL Invitrogen (Carlsbad, CA). NaOH, HCl, and glutaraldehyde were bought from J. T. Baker (Mallinckrodt Baker, Phillipsburg, NJ). 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; an A₁ antagonist), ZM-241385 (an A_{2a} antagonist), and MRS-1706 (an A_{2b} antagonist) were obtained from Tocris (Ellisville, MI). Monocyte chemoattractant protein (MCP)-1 and PDGF were from Pepro-Tech (Rocky Hill, NJ). Triton X-100 was from Cole-Parmer (Vernon Hills, IL). Transwell inserts were from Corning Costar (Corning, NY). All reagents were of the highest quality grade commercially available. TGF- β concentrations were measured in the supernatant of LX-2 cells after the addition of adenosine by standard ELISA using primary BAF240 for detection and MAB1835 for capture (both from R&D Research).

Animals and HSC isolation

Mice of the C57BL/6 strain were used for HSC isolation. All experiments and animal handling were done according to Yale University Institutional Animal Care and Use

Committee guidelines. HSCs were isolated by in situ pronase-collagenase perfusion followed by density gradient centrifugation, as described previously (1). Primary cells were >95% pure. Cells were grown on standard tissue culture plastic dishes in medium 199 with 10% FCS and antibiotics. Primary cells were used at 7 days after isolation.

Semiquantitative real-time RT-PCR for expression of TGF- β and collagen I mRNA

LX-2 cells were incubated with the adenosine receptor agonist NECA at a concentration of 10 μ M for 24 and 48 h or incubated in media alone. After incubation, total RNA was extracted, and cDNA was prepared using murine Moloney leukemia virus reverse transcriptase (BD Biosciences, Clontech, Palo Alto, CA). Semiquantitative real-time PCR was performed for TGF- β and collagen I using commercial primer-probe sets (Applied Biosystems) and the Applied Biosystem 7500 real-time PCR system. The expression of GAPDH was used to standardize the samples, and the results were expressed as a ratio compared with untreated LX-2 cells. Nonquantitative RT-PCR was used to determine adenosine receptor mRNA expression in LX-2 cells. Specific oligonucleotide primers were made at the Yale Keck Facility, and the thermal cycles used were based on the sequences used in a previous study (10).

Measurement of changes in cytosolic Ca²⁺ concentration

LX-2 cells were grown on glass coverslips, loaded with the Ca²⁺-sensitive fluorophore fluo-4 AM (Molecular Probes), and put into a specially designed chamber for use on a confocal microscope as previously described (13). Cells were perfused initially with HEPES buffer and then with buffer containing ATP (100 μ M) or PDGF (10 ng/ml). Changes in fluo-4 fluorescence were monitored using a Zeiss confocal imaging system. Fluo-4 fluorescence was excited using a Kr/Ar laser at 488 nm; emitted fluorescence >515 nm was collected. Changes in fluorescence over time were expressed as peak fluorescence (f) divided by initial fluorescence (f_0).

Chemotaxis assay

The migration of LX-2 cells was studied using Transwell inserts equipped with 8- μ m-pore polycarbonate-free filters as previously described (6). Briefly, 2×10^4 LX-2 cells were plated per well. Cells were treated with the appropriate adenosine receptor antagonist (8-SPT or ZM-241385) 10 min before the addition of either adenosine, NECA, or forskolin. MCP-1 or PDGF was added to the lower chamber 2 h afterward. After 24 h, the lower surface of the membrane was stained using hematoxylin-eosin, photographed, and analyzed.

All experiments were repeated in triplicate. For each experimental group, a total of 40 high-power field images were taken. Cells per high-power field were counted, with results being expressed as average numbers of cells per high-power field. Statistical analysis in the form of a Student's *t*-test was performed with $P \leq 0.05$ as significant.

RESULTS

Adenosine does not induce an increase in cytosolic Ca²⁺ concentration

Confocal video microscopy was used to quantitate changes in cytosolic Ca²⁺ concentrations in response to adenosine. Adenosine did not induce an increase in the cytosolic Ca²⁺ concentration in LX-2 cells at concentrations between 10 and 100 μ M (Fig. 1, A and B).

Adenosine inhibits increases in cytosolic Ca²⁺ concentrations induced by ATP and PDGF

Confocal video microscopy was used to quantitate changes in cytosolic Ca²⁺ concentration in response to ATP and PDGF. ATP induced a rapid increase in cytosolic Ca²⁺

concentration in LX-2 cells (Fig. 1, *C* and *D*), and this was inhibited by pretreatment for 10 min by 10 μ M adenosine (Fig. 1, *E* and *F*). Figure 1, *G* and *H*, shows the typical time course for the increase in cytosolic Ca^{2+} concentration upon exposure to ATP or PDGF and the effects of pretreatment with adenosine. Figure 1*I* shows data from a number of cells, with significant ($P < 0.05$) inhibition by adenosine of ATP- and PDGF-induced increases in cytosolic Ca^{2+} concentration. The increase in cytosolic Ca^{2+} concentration by ATP is mostly due to the release of Ca^{2+} from endoplasmic reticulum stores by activation of inositol (1,4,5)-trisphosphate receptors. This mechanism is shared by a variety of extracellular signaling molecules that are active on HSCs, including PDGF (4).

Adenosine inhibits HSC chemotaxis by MCP-1 and PDGF

The recruitment of HSCs to sites of injury is thought to be an early step in their function in repair and matrix remodeling. PDGF induces HSC chemotaxis, and this is dependent on increases in cytosolic Ca^{2+} concentration. We tested whether adenosine can inhibit PDGF-induced HSC chemotaxis. Adenosine at 10 μ M almost completely blocked PDGF-induced chemotaxis of LX-2 cells (Fig. 2*A*) and primary mouse HSCs (Fig. 2*B*) across 8- μ m pores. The inhibition of chemotaxis was dose dependent (Fig. 2*A*), with adenosine concentrations below 1 μ M having no effect. We established that this is a receptor-mediated phenomenon by demonstrating that it could be blocked by preadministration of the pan-adenosine receptor antagonist 8-SPT (Fig. 3*A*). Adenosine is metabolized to a number of molecules that may be biologically active. To determine if the inhibition of chemotaxis could be mediated via adenosine receptors, the pan-adenosine receptor agonist NECA was also shown to inhibit HSC chemotaxis to PDGF efficiently (Fig. 3*B*).

Inhibition of chemotaxis is via the A_{2a} receptor subtype

Adenosine is known to signal via four receptor subtypes: A_1 , A_{2a} , A_{2b} , and A_3 . These are widely expressed including in the liver and mediate their effects via coupled G proteins. A number of antagonists are selective for the receptor subtypes. The following antagonists were used to antagonize adenosine receptor subtypes: DPCPX (10 nM), A_1 receptor subtype; ZM241385 (1 μ M), A_{2a} receptor subtype; MRS-1706 (10 nM), A_{2b} receptor subtype; and MRS-1523 (5 μ M), A_3 receptor subtype. As shown in Fig. 4, the ability of adenosine to inhibit HSC chemotaxis to PDGF was significantly antagonized by the A_{2a} receptor subtype antagonist but not by the A_1 , A_{2b} , and A_3 receptor-selective agents. The A_{2a} antagonist was as effective at blocking adenosine-mediated inhibition of PDGF chemotaxis as the combination of all four antagonists. This strongly implicates the A_{2a} receptor subtype as being responsible for the inhibition of HSC chemotaxis. The inability of A_1 , A_{2b} , and A_3 receptor antagonists to block the adenosine-induced inhibition of PDGF chemotaxis suggests that these receptor subtypes have minimal roles or no role at all. Caffeine is known to be a pan-adenosine receptor antagonist, and we found that it was also (at 10 μ M) able to block the adenosine-mediated inhibition of chemotaxis (data not shown, experiments repeated in triplicate).

Expression of adenosine receptors in HSCs

The presence of mRNA for adenosine receptor subtypes on LX-2 HSCs was assayed using RT-PCR. As shown in Fig. 5, LX-2 cells contained mRNA for A_{2a} , A_{2b} , and A_3 (but not A_1) receptor subtypes. Human peripheral blood lymphocytes were used as a positive control, and signals for all four receptor subtypes were detected.

The A_{2a} receptor effect is mediated via cAMP and is reversible

Signaling downstream of the A_{2a} receptor subtype is mediated predominantly via G_s -dependent adenylate cyclase activation, resulting in an increase in cAMP. An additional G_s -

independent, G_{12/13} pathway of Ras and ERK1/2 activation has also been documented (21, 22). Forskolin, an activator of adenylate cyclase, was used to test if elevations in cAMP could reproduce the adenosine-induced inhibition of HSC chemotaxis. As shown in Fig. 6A, forskolin also had potent inhibitory activity on HSC chemotaxis to PDGF. The importance of cAMP in the adenosine effect was further confirmed by the demonstration that pretreatment of cells with the adenylate cyclase inhibitor 2,5-DDA blocked the ability of adenosine to inhibit chemotaxis of HSCs in response to PDGF (Fig. 6B). Forskolin was found to completely block the ATP-induced increase in cytoplasmic Ca²⁺ concentration, further confirming the role of cAMP in this phenomenon (data not shown, experiments repeated in triplicate). It was of interest for us to know whether, at the cessation of cellular injury, when adenosine levels normalize, HSCs could be expected to recover the ability to undergo PDGF-mediated chemotaxis. As shown in Fig. 6C, 24 h after we replaced adenosine-containing with adenosine-free media, HSCs were again able to undergo chemotaxis in response to PDGF.

Adenosine upregulates collagen and TGF- β mRNA

HSCs are known to upregulate TGF- β (which subsequently functions in autocrine and paracrine loops) and also to upregulate a number of matrix constituents including collagen I. We tested whether adenosine upregulated the expression of TGF- β and collagen genes in HSCs by performing semiquantitative real-time PCR. As shown in Fig. 7, adenosine at 100 μ M resulted in an approximately threefold increase in TGF- β and collagen mRNA at 24 h. At 48 h, there was a decrease in the level of collagen mRNA to basal levels, but TGF- β levels were maintained. The assay of the TGF- β concentration in the supernatant by ELISA showed a significant increase between control and adenosine-treated plates at 3 and 24 h (3-h control: 3.6 ± 0.9 ng/ml, 3-h adenosine: 12.1 ± 2.0 ng/ml, 24-h control: 4.9 ± 1.0 ng/ml, 24-h adenosine: 15.1 ± 1.1 ng/ml, means \pm SE; this was significant at <0.01 for both time points). This demonstrates that at sites of high adenosine concentrations, adenosine induces HSC differentiation in addition to stopping HSC chemotaxis.

DISCUSSION

Developmental apoptosis is programmed at the level of the cell and organ, in contrast with necrosis, which is unpredictable and not programmed. Pathological apoptosis has features of developmental apoptosis, in that it uses much of the same cellular machinery but is not programmed at the level of the organ and is usually unpredictable. Pathological apoptosis, therefore, requires a complex adaptive response, which in the liver includes HSC migration, differentiation, and matrix remodeling (5). HSC numbers increase at the site of hepatocyte injury, and this local increase in HSCs is partly due to chemotaxis toward a gradient of a number of cytokines. For HSCs, PDGF is well characterized to induce chemotaxis and is produced by macrophages, activated HSCs, and injured bile duct segments (9). In such a model, HSCs are retained at the site of highest concentration of the chemotactic cytokine.

The role of hepatocyte apoptosis (as distinct from the injury stimulus and immune response) in the development of liver fibrosis has been of great interest. Clinical specimens from patients with hepatitis C infection have revealed a correlation between hepatocyte apoptosis, HSC activation, and increased stage of fibrosis (15). More convincing evidence that hepatocyte apoptosis is sufficient to stimulate liver fibrosis has been provided by a number of experimental models (23, 24). These studies bring to the forefront questions about the identity of the molecules that communicate hepatocyte apoptosis to the hepatic repair response. We chose to test whether adenosine might fulfill some of this function because it is produced during conditions of energy imbalance by the dephosphorylation of adenosine tri-, di-, and monophosphates and is also produced from the metabolism of purines during the degradation of nucleic acids of apoptosing cells. Adenosine is also rapidly metabolized

to inosine by adenosine deaminase. The production of adenosine therefore does not require any new protein synthesis, and extracellular concentrations rise rapidly in tissue injury from the 0.1- to 0.3- μM range to $>100 \mu\text{M}$. The rapid metabolism limits the half-life to a few minutes. There is a very large body of literature on the regulation by adenosine of adaptive responses to tissue injury in a number of organs, but very little is known about its role in liver injury and repair (8).

Our finding that adenosine inhibits increases in cytosolic Ca^{2+} concentration induced by ATP and PDGF is novel, and, as predicted, adenosine resulted in inhibition of HSC chemotaxis by PDGF. Since adenosine levels are highest in the immediate microenvironment of cellular injury and apoptosis, this new function of adenosine adds an important component to the model of migration of HSCs. The previously described chemotaxis of HSC toward an increasing gradient of PDGF is still valid, but our data suggest that upon arriving at a site of cellular injury with high adenosine levels, chemotaxis will be inhibited (11). This provides a stop signal to HSCs. A system in which there is a start signal (chemokine/cytokine) and a protein synthesis-independent stop signal (adenosine) has distinct advantages. First, it stops HSCs at the site where they are most needed and keeps them there until hepatocyte apoptosis is no longer occurring and adenosine levels drop. This is necessary as PDGF and other chemokines induce chemokinesis as well as chemotaxis; thus, even when HSCs have reached the peak of the chemokine gradient, they will be actively moving in the absence of a stop signal. Another advantage is that it may allow fine localization of HSCs to the site of cellular injury. In pathological states, chemokines are produced by many tissues and cells including Kupffer cells and bile segments. Such chemokine production may function to bring HSCs into a general area (i.e., around a bile duct). The adenosine-induced stop signal may function to localize HSCs into the exact vicinity of cellular injury. The demonstration that the adenosine stop signal is reversible suggests that after the local injury has resolved, HSCs are able to respond to new chemokine gradients. In addition to stopping HSCs, we demonstrated that adenosine increases HSC differentiation by an upregulation of TGF- β and collagen I mRNA. This is consistent with increasing HSC survival and stimulating matrix remodeling once HSCs have reached the site of tissue injury.

The important role of adenosine in liver fibrosis proposed by us is consistent with a study (14) in experimental lesions in the skin, where activation of the A_{2a} receptor accelerated wound healing. Elevated adenosine levels also result in pulmonary fibrosis. A number of studies have correlated coffee and tea consumption with a reduced risk of chronic liver disease. This was recently confirmed in a prospective study (19) of a population in the United States. Caffeine and its metabolites have a number of properties that may be responsible for this effect. These include antioxidant properties, inhibition of lipid peroxidation, and possibly an improvement of insulin sensitivity (18). However, the only known biological function of caffeine for which it has significant activity at concentrations achieved during normal human consumption (peak: $70 \mu\text{M}$) is antagonism of A_1 and A_2 adenosine receptors. Our demonstration of a role for the A_{2a} receptor in localizing HSCs to sites of tissue injury suggests that the effect of caffeine in decreasing liver fibrosis may be by A_{2a} receptor antagonism with a consequent interference with HSC trafficking and differentiation. This is further supported by our demonstration of the ability of caffeine to antagonize the effect of adenosine to stop HSC chemotaxis.

A previous study (7) on CCl_4 -induced liver fibrosis in rats demonstrated a decrease in the development of fibrosis and a more rapid resolution of fibrosis when adenosine was administered. The results of this study are very surprising because adenosine was administered 3 times/wk and was not in any sustained release form. Since adenosine is rapidly degraded, this would have resulted in elevated adenosine levels for a matter of

minutes for 3 times/wk. How such transient elevations in adenosine can have such significant effects is difficult to explain.

The adenosine A_{2a} receptor is G_{α_s} coupled and results in an increase in cAMP (20). Additional downstream pathways include PKA, CREB, and p38 activation. A separate G_{12/13}/Ras/MEK pathway has also been identified. Adenosine has been shown to increase cAMP levels in primary rat HSCs, and our replication of the antichemotactic effects by forskolin demonstrates that elevations in cAMP are sufficient for this effect. The inability of adenosine to inhibit chemotaxis in the presence of the adenylate cyclase inhibitor 2,5-DDA, moreover, demonstrates that elevations in cAMP are required for this effect.

In summary, we demonstrated that adenosine blocks cytosolic elevations in Ca²⁺ concentration in HSCs and is a potent stop signal for chemotaxis, and we propose that adenosine has an important role in liver fibrosis by localizing HSCs to the site of tissue injury and inducing HSC differentiation. This identifies adenosine receptors as potential targets for modulating HSC biology and regulating the development of liver fibrosis.

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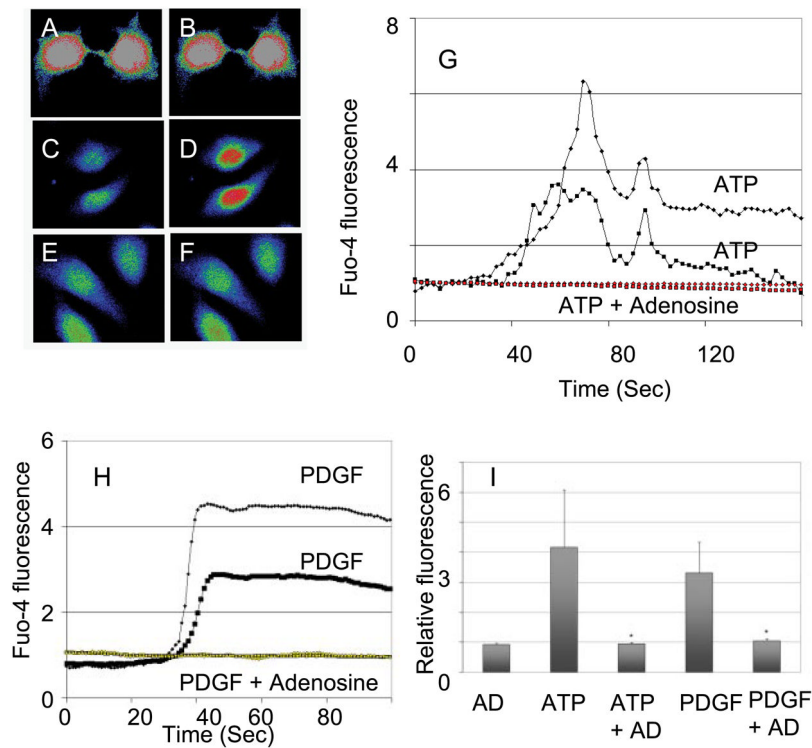


Fig. 1. Adenosine inhibits increase in cytosolic Ca²⁺ induced by ATP and PDGF. *A*: human LX-2 hepatic stellate cell (HSC) loaded with a Ca²⁺-sensing dye (fluorophore fluo-4 AM) before the addition of adenosine. *B*: same cell as in *A* at 60 s after the addition of adenosine (10 μM) with no change in cytosolic Ca²⁺. *C*: untreated LX-2 cell. *D*: same cell as in *C* at 60 s after the addition of ATP at 100 μM. *E*: LX-2 cell treated with adenosine (10 μM) for 10 min. *F*: same cell as in *E* at 60 s after the addition of ATP at 100 μM. *G*: representative plots of changes in cytosolic Ca²⁺ after treatment of the cells with ATP alone or ATP after cells had been exposed to adenosine (10 μM for 10 min). *H*: representative plots of changes in cytosolic Ca²⁺ after treatment of the cells with PDGF alone (10 ng/ml) or PDGF after cells had been exposed to adenosine (10 μM for 10 min). *I*: summary of relative changes in the fluorescence of fluorophore fluo-4 AM-loaded LX-2 cells after treatment with the listed agents. In all instances, when adenosine was used, it was at 10 μM and for 10 min before the addition of ATP or PDGF.

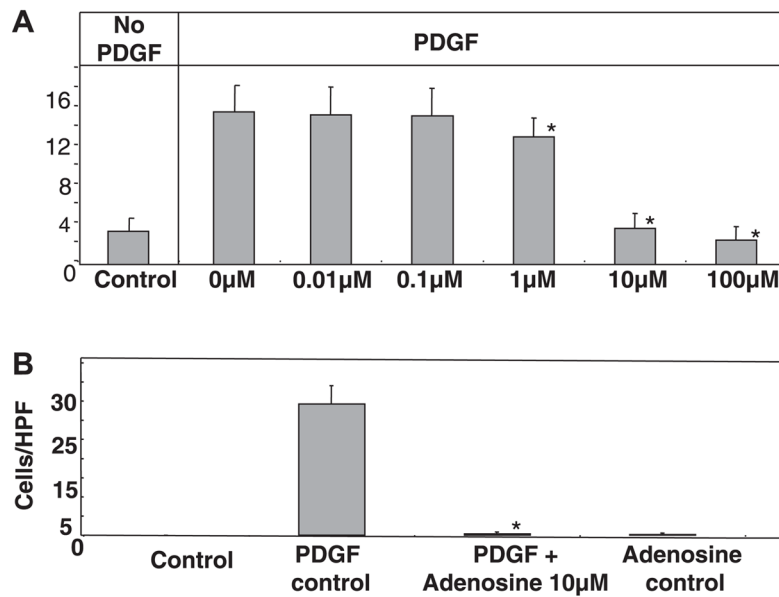


Fig. 2.

A: adenosine inhibited PDGF-induced chemotaxis in a dose-dependent manner. Human LX-2 HSCs (20,000 cells) were plated in the upper chamber of Transwell inserts with recombinant human PDGF-BB (10 ng/ml) as the chemoattractant in the lower chamber. Hematoxylin and eosin staining of the undersurface was done at the 24-h time point with migrating cells counted per high-power field (HPF). To test for the effect of adenosine on chemotaxis, cells were incubated with adenosine (at doses of 0.01, 0.1, 1, 10, and 100 μ M) for 120 min before the addition of PDGF, and adenosine was maintained in the culture medium until the end of the 24-h study period ($*P \leq 0.05$ vs. the PDGF control by Student's *t*-test). **B:** primary mouse HSC chemotaxis was inhibited by adenosine. Primary mouse HSCs were isolated from B6 mice and cultured on plastic. Cells at *day 5* were plated (20,000 cells) in the upper chamber of Transwell inserts, with PDGF-BB (10 ng/ml) as the chemoattractant in the lower chamber. Experiments were performed as in **A** with the addition of adenosine 120 min before the addition of PDGF ($*P \leq 0.05$ vs. the PDGF control by Student's *t*-test).

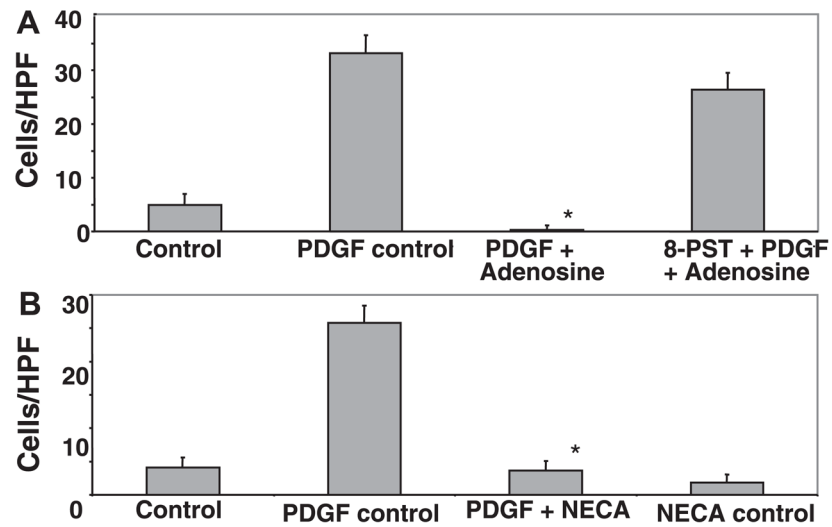


Fig. 3.

A: the pan-adenosine receptor antagonist 8-(*p*-sulfo-phenyl)-theophylline (8-SPT) blocked the ability of adenosine to inhibit PDGF-induced chemotaxis. Experiments were performed as in Fig. 2A with the addition of 8-SPT (10 μ M) 15 min before the addition of adenosine. *B:* the nonhydrolysable adenosine agonist 5'-(*N*-ethylcarboxamido)adenosine (NECA) inhibited chemotaxis of LX-2 cells induced by PDGF. Experiments were performed as in Fig. 2A except that NECA (10 μ M) was added 120 min before the addition of PDGF (* $P \leq 0.05$ vs. the PDGF control by Student's *t*-test).

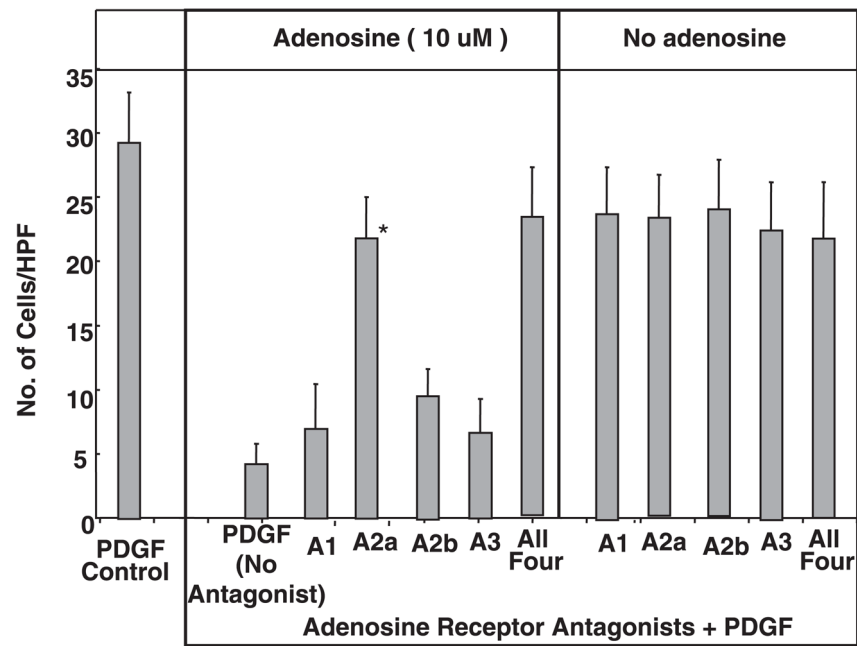


Fig. 4. Inhibition of PDGF (10 ng/ml)-mediated HSC chemotaxis by adenosine was reversed by preincubation with the A_{2a}-specific receptor antagonist ZM-241835 at 1 μ M [$*P \leq 0.05$ vs. the PDGF (no antagonist) + adenosine (10 μ M) control by Student's *t*-test], whereas the A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) at 10 nM, A_{2b} receptor antagonist MRS-1706 at 10 nM, and the A₃ receptor antagonist MRS-1523 at 5 μ M had no effect. A similar antagonism of adenosine inhibition was seen with all four inhibitors combined at the above concentrations. Experiments were performed as in Fig. 3A with the addition of the receptor antagonists 15 min before the addition of adenosine.

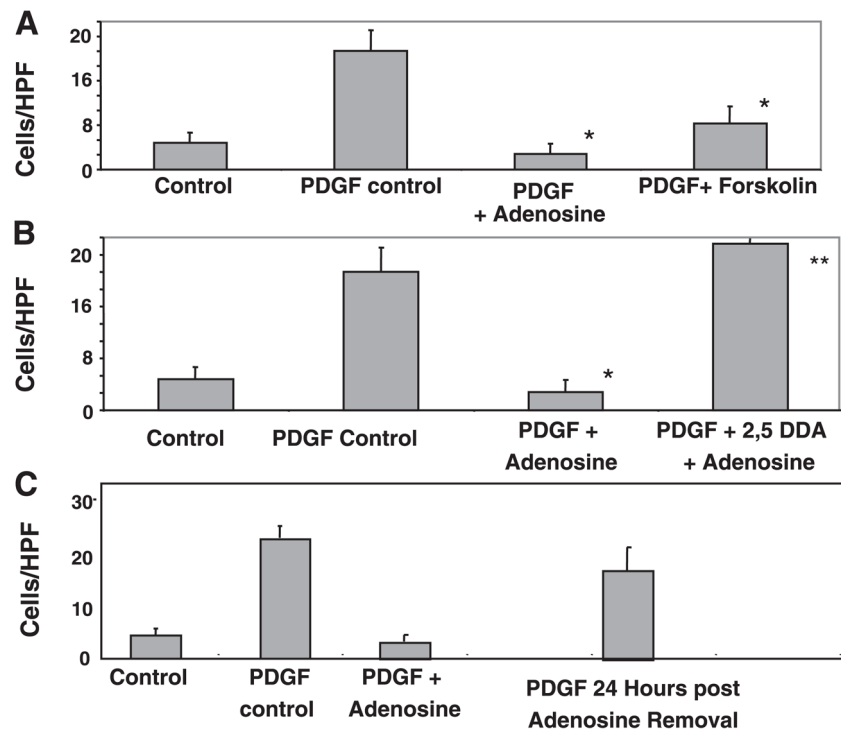


Fig. 6.

A: inhibition of HSC chemotaxis by adenosine ($*P \leq 0.05$ vs. the PDGF control by Student's *t*-test) was reproduced by the cAMP-inducing agent forskolin. Forskolin (100 μ M) was added 30 min before the addition of PDGF, and the rest of the assay was performed as previously described ($*P \leq 0.05$ vs. the PDGF control). *B:* inhibition of HSC chemotaxis by adenosine ($*P \leq 0.05$ vs. the PDGF control) was blocked by the adenylyl cyclase inhibitor 2,5-dideoxyadenosine (2,5-DDA). 2,5-DDA (100 μ M) was added 30 min before the addition of adenosine ($**P \leq 0.05$ vs. PDGF + adenosine). *C:* inhibition of PDGF-induced chemotaxis by adenosine was reversible. Adenosine inhibited PDGF-induced chemotaxis as shown in Fig. 2. Adenosine was removed from the media, and PDGF was added 24 h later. LX-2 cells were able to undergo chemotaxis in response to PDGF.

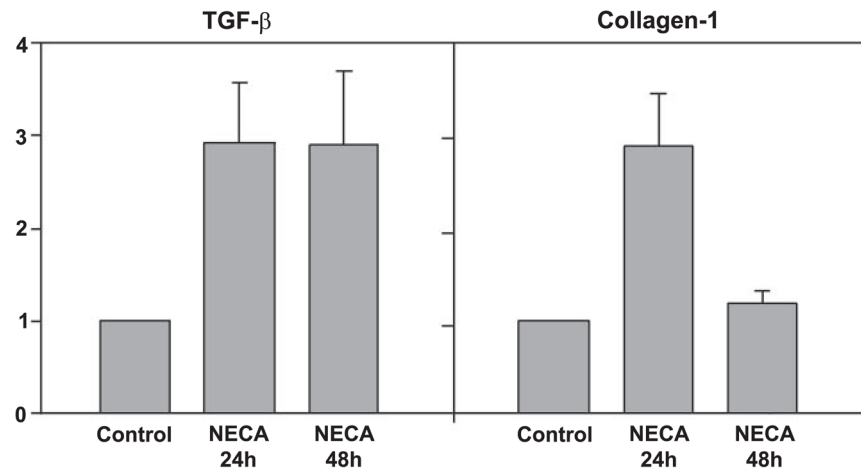


Fig. 7.

The adenosine receptor agonist NECA induced upregulation of TGF- β and collagen I mRNA. LX-2 cells were cultured in the presence or absence of NECA (10 μ M), and cDNA was isolated 24 and 48 h later. Semiquantitative real-time PCR was performed using commercially available primer probe combinations. Results were standardized to GAPDH and are shown relative to untreated LX-2 cells. NECA induced upregulation of TGF- β at the 24- and 48-h time points ($P \leq 0.05$ vs. the control) and induced upregulation of collagen I at 24 h ($P \leq 0.05$ vs. the control).