Functional effects of enhancing or silencing adenosine A2b receptors in cardiac fibroblasts

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Chen, Yingshong, Sara Epperson, Lala Makhсудova, Bruce Ito, Jorge Suarez, Wolfgang Dillmann, and Francisco Villarreal. Functional effects of enhancing or silencing adenosine A2b receptors in cardiac fibroblasts. Am J Physiol Heart Circ Physiol 287: H2478–H2486, 2004. First published July 29, 2004; doi:10.1152/ajpheart.00217.2004.—Cardiac fibroblasts (CF) express adenosine (ADO) receptors, and pharmacological evidence suggests the possible involvement of the A2 (A2a and A2b) receptor (A2aR and A2bR) subtypes in inhibiting cell functions involved in fibrosis. The main objective of this study was to define the contributions derived from A2aR and/or A2bR in modulating ADO-induced decreases in CF functions. For this purpose, CF were either treated pharmacologically or had the A2bR or A2aR levels modified through the use of recombinant adenosine or siRNA. The assessment of mRNA expression in adult rat CF yielded evidence for A1R, A2aR, A2bR, and A3R. Endogenously or exogenously enhanced ADO significantly inhibits CF proliferation, collagen, and protein synthesis. A1R and A2aR agonists, although capable of inhibiting CF protein and collagen synthesis, were unable to define the contributions derived from A2aR or A2bR. Overexpression of A2bR in CF yielded significant decreases in basal levels of collagen and protein synthesis and correlated with increases in cAMP levels. However, at higher doses of ADO receptor agonists, significant increases in protein and collagen synthesis were observed. CF with underexpression of A2bR yielded increases in protein and collagen synthesis. In contrast, A2aR underexpression did not modify ADO-induced decreases in CF protein or collagen synthesis. In conclusion, results derived from the molecular manipulation of receptor levels indicate that A2bR are critically involved in ADO-mediated inhibition of CF functions.

Collagen deposition; extracellular matrix; remodeling

The increased incidence of congestive heart failure has stimulated therapeutic efforts to develop pharmacological strategies to prevent or treat the adverse processes of cardiac remodeling. Current therapies are mainly focused on blocking the actions of neurohormonal factors known to stimulate cardiac remodeling. However, the possibility of exploiting endogenously generated factors that are capable of inhibiting this process is only beginning to be assessed (25). An important component of the remodeling process observed with heart failure is the excess deposition of extracellular matrix (ECM) proteins (3). The main cell type responsible for the production of myocardial ECM proteins is the cardiac fibroblast (CF). Thus strategies intended to diminish the production of ECM proteins in the heart may specifically target surface receptors expressed by these cells.

Materials and Methods

CF isolation and culture. Primary adult rat CF cultures were generated from ventricular tissues of 6- to 8-wk-old male Sprague-Dawley rats (250–275 g), as previously described (33). Briefly, the

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rats were euthanized and the hearts were removed. The rats were treated according to the principles of the NIH “Guide for the Care and Use of Laboratory Animals” protocols.

Ventricular tissue was isolated, minced, and digested with the use of a solution containing collagenase (100 U/ml) and pancreatin (0.6 mg/ml). Isolated CF were seeded into 10-cm dishes containing growth media DMEM (GIBCO BRL), 10% FBS, and 1% penicillin-streptomycin-fungizone in a humidified atmosphere of 7% CO₂. All studies were performed with cells at passage 2, and characterized as previously published (33).

CF treatment. CFs were initially grown to the desired confluence in growth media and rendered quiescent by serum starvation for 24 h. For experiments involving the over- or underexpression of A₂bR, 70% confluent CFs were exposed to recombinant A₂bR adenovirus or small-interference RNAs (siRNAs). CF starvation was performed until adenovirus-infected or siRNA-transfected CF reached 100% confluency. Before treatment culture, the media were replaced with DMEM + 2.5% FBS. To test the effects of increasing endogenous or exogenous levels of ADO we used an adenosine kinase inhibitor (AKI), iodotubercidin (Itu), or an ADO analog, 2-chloroadenosine (Cl-Ad), respectively. Itu at the doses used has previously been shown to reduce ADO exogenous levels of ADO we used an adenosine kinase inhibitor (AKI), iodotubercidin (Itu), or an ADO analog, 2-chloroadenosine (Cl-Ad), respectively. Itu at the doses used has previously been shown to reduce ADO

The agonists 4-[

Preparation and transfaction of siRNA. Three pairs of rat A₂aR or A₂bR siRNA oligonucleotide templates were designed uniquely against rat A₂aR or A₂bR. The siRNA sequences targeting rat A₂aR corresponded to the coding regions from 795, 1566, and 1913, and rat A₂bR corresponded to the coding regions from 233, 539, and 718 plus the next 19 nucleotides as potential siRNAs. Sequences were blasted against genome databases to eliminate any sequences with homology to other genes. Selected target mRNA sequences for A₂aR were the following: siRNA 795: 5'-AATTATCATGTTTACTCAAC-3'; siRNA 1566: 5'-AAAGGGAAGAAACACCCATG-3'; and siRNA 1913: 5'-AAGTGCCATCTGTCTTTCT-3'. The selected target mRNA sequences for A₂bR are the following: siRNA 233: 5'-AACUAUCUCUCUGUGUGCCUG-3'; siRNA 539: 5'-AACAGUAAA-GACCUGCCACC-3'; siRNA 718: 5'-AACUUCUAUGUGGCGUGCA-3'; and control siRNA: 5'-AAGGCGACUACAU-GCGCUI-3'.

siRNA duplexes were synthesized with the use of a Silencer siRNA construction kit (Ambion; Austin, TX) and labeled with Cy3 to monitor transfection efficiency. The silencing capacity of the siRNAs was verified by RT-PCR and Western blot analysis. The three siRNAs designed, siRNA 1913 and siRNA 539 were the most effective in silencing A₂aR and A₂bR, respectively, in CF (Fig. 2B). For siRNA transfaction into CF, cells were grown in a 12-well plate to 70% confluency. CFs were divided into three groups for each A₂aR or A₂bR siRNA: transfaction reagent only, transfected with control siRNA, A₂aR siRNA1913, or A₂bR siRNA539. siRNAs were introduced into CF by GenePORTER transfection reagent (Gene Therapy System; San Diego, CA). A final concentration of 0.17 μmol/l siRNA was used to produce a 1.3-kb exogenous HA₂bR adenovirus at a MOI ratio of 200:1 demonstrated twofold A₂bR overexpression.
yielded 70% transfection efficiency with minimum cytotoxicity (Fig. 2A).

RT-PCR. Total RNA was extracted with the use of a RNeasy Kit (Qiagen), followed by DNase digestion. One microgram of total RNA was then reverse transcribed into cDNA using RT-PCR Kit (Invitrogen; Carlsbad, CA). Four microliters of cDNA were used for PCR. PCR was performed with an initial step of denaturation at 94°C 3 min, 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s or 60 s at 72°C (1 kb PCR product/min), followed by a final extension at 72°C for 10 min. To confirm CF overexpression of HA2bR after recombinant virus infection, a HA2bR-specific forward primer (5'- tcatcccagcgttcc-3') and a reverse primer from the multiple cloning site of SR(+) vector (5'- tccatcttcagccttctg-3') were used to produce a ~1.3-kb fragment (Fig. 1B). To detect the silencing of rat A2aR by siRNA, a pair of specific rat A2aR primers (forward: 5'-tcatccagcgttccgtctc-3'; reverse: 5'-tggtggtcatctgcttcagc-3') was used to amplify rat A2aR, resulting in a 371 bp fragment (Fig. 2B, top). To detect silencing of rat A2bR by siRNA, a pair of specific rat A2bR primers (forward: 5'-tcattgcagcgttccgtc-3'; reverse: 5'-ggtcatctgcttcagc-3') was used to amplify rat A2bR, resulting in a 243 bp fragment (Fig. 2B, bottom). To demonstrate the presence of adenosine receptor subtypes on CF, we used four pair of specific rat primers for A1R, A2aR, A2bR, and A3R. A1R (5'-accagaaacagggacttagc-3'), A2aR (5'- tcatcccagcgttcc-3'), A2bR (same as above), and A3R (5'- tcatcattgcagcgttccgtc-3') primer pairs yield fragments of 205, 371, 243, and 326 bp respectively.

Western blot analysis. Three days after HA2bR recombinant virus infection or siRNA transfection, CF lysates were prepared in lysis buffer. Protein samples were separated and transferred onto membranes. Membranes were exposed to 1 μg/ml of an anti-A2bR or anti-A2aR polyclonal antibody (Alpha Diagnostic; San Antonio, TX) and then a secondary horseradish peroxidase-labeled antibody. Protein bands were visualized with the use of ECL plus (Amersham) (Fig. 2B).

[3H] assay incorporation. [3H]thymidine, [3H]leucine, and [3H]proline incorporation assays were used as a means to measure CF proliferation, protein and collagen synthesis respectively. Subconfluent (70%) CFs were used for the CF proliferation assay. Confluent CFs were used for protein and collagen synthesis assays. CFs were pulsed with [3H]thymidine, leucine, and proline. Experiments were terminated according to Dubey’s protocol (6, 10). Radioactivity was counted in a liquid scintillation counter. CF proliferation was also assessed through cell counts.

cAMP assays. CF cAMP levels were determined utilizing a direct enzyme immunoassay kit according to manufacturer’s (Sigma-Aldrich; St. Louis, MO) instructions. CF were treated for a period of 10 min and cAMP precipitated with 0.1 N HCl. To prevent the breakdown of cAMP, the cells were pretreated with the phosphodiesterase inhibitor rolipram at 10 μmol/l for 15 min. To assess the functional

Fig. 2. Representative images of small interference RNA (siRNA)-mediated silencing of A2bR and A2aR in CF. A: CF transfected with siRNA under phase-contrast and fluorescence microscopy. Cy3-labeled siRNAs (A, bottom) were transfected into the cytoplasm of CF with 70% transfection efficiency. In all experiments (n = 3 in triplicate), the transfection efficiency of 70% was consistent. B: mRNA (RT-PCR) and protein (Western blot analysis) levels obtained from CF transfected with A2bR (top) or A2aR (bottom) siRNAs for 72 h. As derived from densitometric scans, bar graphs summarized mRNA and protein expression levels after siRNA transfection (n = 3). As can be observed, rat A2aR siRNA1913 (lane 3) was the most effective siRNA to silence A2aR. mRNA and protein levels were reduced to 13% and 30%, respectively. For rat A2bR siRNA539 (lane 4) was the most effective siRNA to silence A2bR. mRNA and protein levels were reduced to 58% and 35%, respectively. GAPDH and actin were used for normalizing mRNA and protein levels, respectively.
coupling of A1R and A3R to Gs, pertussis toxin pretreatment at 100 ng/ml was provided to the cells before agonist treatment.

Statistical analysis. Statistical analysis was performed with Student’s t-test or ANOVA. The results were considered statistically significant at \( P < 0.05 \).

RESULTS

ADO receptors in CF. RT-PCR was used to detect ADO A1R, A2aR, A2bR, and A3R mRNAs in CF. As shown in Fig. 3, ADO A1R, A2aR, A2bR, and A3R mRNAs were identified in CF. The approximate size of the bands was 205, 371, 243, and 326 base pairs for A1R, A2aR, A2bR, and A3R, respectively.

Proliferation. Results indicate that Cl-Ad or AKI treatment inhibited cell proliferation in a concentration-dependent manner, as assessed through \(^{[3]H}\)thyidine incorporation (Fig. 4A). In all cases, cells continued to proliferate, but the rates of proliferation were reduced with increases in either endogenous or exogenous adenosine levels. To determine the putative ADO receptor subtype-mediated CF proliferation, studies were performed using NECA, CGS-21680, or ATL-193. As one of the most potent A2bR agonists, NECA inhibited cell proliferation in micromolar concentrations (Fig. 4B). Treatment of CF with A2bR-specific agonists CGS-21680 and ATL-193 also resulted in inhibition of cell proliferation (Fig. 4C). However, compared with \( 10^{-5}\) mol/l NECA, which decreased the amount of incorporated \(^{[3]H}\)thyidine to 43.2 \pm 1.2\% of control values, the inhibition of cell proliferation induced by either \( 10^{-8}\) mol/l CGS-21680 or ATL-193 was not as pronounced as that induced by NECA (Fig. 4C). Similar trends were observed on cell counts with the use of CGS-21680 and ATL-193 in Fig. 4F. NECA induced a decrease in cell number to 71.6 \pm 2.6\% of control. This decrease was more prominent than those induced by either CGS-21680 or ATL-193 (\( P < 0.05 \).

Protein synthesis. Results indicate that Cl-Ad and AKI significantly and dose dependently inhibited leucine incorporation (Fig. 5A). NECA also induced a dose-dependent decrease in protein synthesis (Fig. 5B). At \( 10^{-7}\) mol/l NECA decreased the amount of incorporated \(^{[3]H}\)leucine to 62.9 \pm 3.4\% (\( P < 0.05 \)). Treatment with either \( 10^{-8}\) mol/l CGS-21680 or \( 10^{-8}\) mol/l ATL-193 inhibited \(^{[3]H}\)leucine incorporation to 71.4 \pm 5.1\% and 76.9 \pm 5.2\%, respectively (Fig. 5C).

The results of the effects of A2bR overexpression on CF protein synthesis are summarized in Fig. 6, A and B. Under basal (untreated) conditions, CF infected with HA2bR adenoviruses had a significant decrease in protein synthesis (\( P < 0.05 \) vs. control). However, when \( 10^{-4}\) mol/l Cl-Ad or \( 10^{-5}\) mol/l NECA were added to HA2bR overexpressing CF, increases in protein synthesis were observed (\( P < 0.05 \) vs. control). Control virus infection of CF did not affect protein synthesis compared with uninfected CF in the presence or absence of Cl-Ad or NECA.

Silencing of A2bR led to increases in protein synthesis in the absence or presence of treatment with \( 10^{-6}\) mol/l Cl-Ad (\( P < 0.05 \)) or \( 10^{-4}\) mol/l NECA (\( P < 0.001 \)) (Fig. 7A). In contrast, silencing of A2aR did not modify the capacity of \( 10^{-6}\) mol/l Cl-Ad (\( P < 0.05 \)) or \( 10^{-4}\) mol/l NECA to inhibit CF protein synthesis (Fig. 8A).

Collagen synthesis. As shown in Fig. 5D, Cl-Ad and AKI induced the inhibition of collagen synthesis in a concentration-dependent manner. NECA also induced a concentration-dependent inhibition of collagen synthesis (Fig. 5E). At \( 10^{-5}\) mol/l NECA suppressed collagen synthesis to 71.7 \pm 0.8\% (\( P < 0.05 \)). Similar inhibition of collagen synthesis was observed with the treatment of CF with either CGS-21680 or ATL-193 at \( 10^{-8}\) mol/l (Fig. 5F).

CF infected with HA2bR adenoviruses had a significant decrease in collagen synthesis in the absence of agonist treatment. A significant decrease in CF collagen synthesis was also observed in cells treated with \( 10^{-6}\) mol/l Cl-Ad (\( P < 0.001 \)) (Fig. 6C). However, when Cl-Ad or NECA, at \( 10^{-5}\) or \( 10^{-4}\) mol/l, respectively, was added to HA2bR overexpressing CF, a significant increase in collagen synthesis was observed (\( P < 0.001 \)) (Fig. 6, C and D). Control virus infection of CF did not affect collagen synthesis compared with uninfected CF in the presence or absence of Cl-Ad or NECA.

Silencing of A2bR led to an increase in CF collagen synthesis in the presence or absence of agonists (Fig. 7B). In contrast,
silencing of A2aR did not modify the capacity of 10^-6 mol/l Cl-Ad (P < 0.05) or 10^-4 mol/l NECA to inhibit CF collagen synthesis (Fig. 8B).

cAMP levels. The assessment of cAMP levels in CF was done in untreated cells and cells treated with 10^-5 mol/l Cl-Ad or 10^-4 mol/l NECA (Fig. 9). Results indicate that treatment of uninfected CF significantly increases cAMP levels. CF infected with a reverse-oriented HA2bR virus showed comparable results to those of uninfected cells. cAMP levels were also determined in sense HA2bR-overexpressing CF. Results indicate that overexpressing HA2bR in untreated CF yields enhanced levels of cAMP production (~10-fold vs. control or reverse-oriented control). The treatment of HA2bR overexpressing CF with Cl-Ad or NECA led to further increases in cAMP levels.

A1R and A3R are known to mainly couple to Gi signaling (17) ADO agonist-induced increases in CF cAMP levels in the presence of pertussis toxin was used as a means to detect functional A1R and A3R. The results indicate that pertussis toxin pretreatment of CF did not yield greater levels of cAMP, thus indicating that CF do not express functional A1R and A3R (data not shown).

DISCUSSION

Recent evidence indicates that ADO can act as an inhibitor of CF functions through its interaction with cell surface receptors. However, the role of ADO receptor subtypes, in particular A2bR, remains unclear. Through the use of molecular approaches (overexpressing or silencing A2aR and A2bR) we demonstrate important roles of A2bR in mediating CF functions. Furthermore, although CF express mRNA levels for all four types of ADO receptors, A1R and A3R do not appear functionally relevant as ascertained from the failure of these receptors to couple to Gi signaling.

The effects of ADO were explored in cultured CF by the exogenous addition of agents such as Cl-Ad or by endogenously enhancing its production through alterations in its metabolism. AKI treatment increases endogenous ADO levels by preventing the repolyphorylation of ADO into AMP (34).
Results from the present study demonstrate that both exogenously (Cl-Ad) and endogenously (AKI) enhanced ADO levels can inhibit serum-induced CF proliferation in a dose-dependent manner. The magnitude of the decreases for Cl-Ad and AKI are comparable to those reported by Dubey et al. (6, 9, 10). A pharmacological approach was initially utilized to gain insight into the ADO receptor subtypes that may be involved in modulating CF functions. The ADO analog NECA remains one of the most potent A2bR agonists known (6, 9, 10, 12, 14, 15) with an EC50 of $10^{-6}$ mol/l. In an attempt to ascertain the involvement of the A2bR we used NECA in the dose range of $10^{-6}$-10$^{-4}$ mol/l. Treatment of CF with NECA decreased proliferation in a concentration-dependent manner. However, NECA can also activate other ADO receptor subtypes in this dose range. Thus the responses elicited by NECA at this dose range are suggestive of the involvement of A2bR but are not conclusive.

To further establish the relative contributions derived from A2aR and A2bR on cell proliferation we utilized agents known to preferentially act as selective A2aR agonists. CGS-21680 and ATL-193 in the nanomolar dose range in principle do not activate A2bR but are as potent as NECA in activating A2aR (1, 14). The agonist ATL-193 was selected because it has been reported to be 50 times more potent than CGS-21680 for A2aR binding (31). Both agents were used at concentrations of $10^{-8}$ mol/l. Results indicate that CGS-21680 and ATL-193-induced significant inhibitions (70–80% of control values) in CF thymidine incorporation suggesting a role of A2aR in CF proliferation. A similar trend was observed in cell count experiments. However, when these results are contrasted to those obtained with 10$^{-5}$ mol/l concentrations of NECA (40% of control values), they suggest that A2bR may play a prominent role in inhibiting CF proliferation. These results differ from those reported by Dubey et al. (6, 10) that indicate only micromoles per liter concentrations of CGS-21680 were capable of inhibiting CF proliferation. Altogether, our pharmacological results suggest that both A2aR and A2bR may participate in the modulation of CF proliferation.

Experiments performed to evaluate the effects of ADO on total protein synthesis also indicate that both exogenous and endogenous ADO is capable of inhibiting serum-stimulated CF leucine incorporation in a dose-dependent manner. Similar
effects were observed with $10^{-5}$ mol/l NECA, which caused an inhibition of 62.9% of control values. Leucine incorporation experiments were also performed using the A$_{2a}$R agonists CGS-21680 and ATL-193. We observed an inhibition (~75%) of control values with $10^{-8}$ mol/l CGS-21680 but no significant difference with ATL-193. Thus results derived from pharmacological treatment of the cells cannot elucidate the role of A$_{2a}$R and A$_{2b}$R in CF protein synthesis.

The incorporation of proline was used as a means to assess collagen synthesis. Significant dose-dependent decreases in proline incorporation were observed with Cl-Ad, AKI, and NECA. However, when the A$_{2a}$R agonists were used, the magnitude of inhibition achieved was comparable to that generated by $10^{-6}$ mol/l NECA. These data suggest that A$_{2a}$R play important roles in regulating CF collagen synthesis.

As implied by the above discussion of results, the pharmacological characterization of A$_{2b}$R-mediated cell functions is far from ideal. Selective A$_{2a}$R agonists and antagonists are currently not readily available. A clearer understanding of the role that receptor subtypes may exercise in regulating cell functions can be gained by the use of molecular-based strategies. To elucidate how A$_{2b}$R affects CF functions, we generated recombinant adenoviruses to overexpress A$_{2b}$R. To control for any possible nonspecific effects of adenoviruses, control (reverse-oriented HA$_{2b}$R) and HA$_{2b}$R adenoviruses, respectively. *P < 0.05, ***P < 0.001, control vs. HA$_{2b}$R-infected cells.

These results provide evidence for A$_{2b}$R mediation of inhibitory actions on CF protein and collagen synthesis. Cl-Ad treatment at $10^{-6}$ mol/l decreased protein and collagen synthesis. However, when CF were treated with increasing doses of Cl-Ad or NECA, increases in collagen and protein synthesis were observed. These confounding responses could be due to abnormal receptor coupling (21) or secondary to the excessive production of cAMP (up to ~200-fold vs. controls). Alternatively, these responses may indicate that the regulation of protein and collagen synthesis by A$_{2b}$R is inherently complex.

siRNA-based technology was chosen to silence rat A$_{2b}$R or A$_{2b}$R gene expression. We successfully transfected siRNA into CF yielding a substantial degree of A$_{2b}$R mRNA and protein downregulation. CF with control siRNA transfection did not alter protein and collagen synthesis compared with CF with transfection reagent alone. The results indicate that the partial abolishment of A$_{2b}$R expression yields the enhancement of basal CF protein and collagen synthesis. The enhancement of these CF functions was also present when cells were treated with $10^{-6}$ mol/l Cl-Ad or $10^{-4}$ mol/l NECA. The stimulatory effects were not observed in CF transfected with control siRNA. The fact that the partial absence of A$_{2b}$R yields the stimulation of basal CF protein and collagen synthesis suggests that these receptors play key roles in the control of these functions. It also implies that an “imbalance” in ADO receptor subtype expression and activation (likely through endogenous ADO) alters basal cell functions. Altogether, given the absence of effects of A$_{2b}$R silencing on ADO-mediated inhibition of CF protein or collagen synthesis, these results support the concept that CF protein and collagen synthesis are critically mediated with A$_{2b}$R. It is worth noting that siRNA technology has proven to be minimally cytotoxic and highly specific for

Fig. 6. Effects of 2-chloroadenosine (Cl-Ad) and NECA on HA$_{2b}$R-overexpressing CF protein (A and B) and collagen (C and D) synthesis. Results were normalized to control uninfected values at the same dosage of Cl-Ad (A and C) or NECA (B and D) treatment. Data are means ± SE. Gray and black represent CF infected with control (reverse-oriented HA$_{2b}$R) and HA$_{2b}$R adenoviruses, respectively. *P < 0.05, ***P < 0.001, control vs. HA$_{2b}$R-infected cells.
reducing the expression of targeted genes compared with antisense-based methodologies (29).

The signaling mechanisms responsible for A2bR-mediated inhibition of protein and/or collagen synthesis in CF are not fully known. A2bR are known to couple to Gs proteins and when activated can upregulate cAMP production (10). Increases in cAMP levels have been associated with the inhibition of cellular functions such as proliferation, DNA, protein and collagen synthesis (7, 8, 10, 11). Our results indicate that stimulation of CF with adenosine or NECA does indeed elevate cAMP levels within the cells, thus indicating the possibility of cAMP-mediated regulation of protein and/or collagen synthesis. However, as documented in other cell types, A2bR activation can also increase intracellular levels of calcium (2, 13, 16, 26) that can lead to activation of other signaling pathways. Thus the effects of the A2bR may be mediated by the simultaneous activation of different signal transduction pathways. There is also the possibility that A2bR activation may also inhibit protein and/or collagen synthesis through indirect actions such as the suppression of cytokine production (4). Thus much work remains to be done so as to understand how adenosine inhibits CF functions associated with fibrosis.

In conclusion, by utilizing molecular approaches, we provide evidence that A2bR may be critically involved in the inhibition of CF proliferation, protein, and collagen synthesis.
The development of A2bR knockout animals should further clarify the role of A2bR in the modulation of these functions.

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DISCLOSURES

The authors do not have any business, commercial, or other proprietary support, relationships, or interests that relate directly or indirectly to the subject of the study.

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