Epac is required for exogenous and endogenous stimulation of adenosine A_{2B} receptor for inhibition of angiotensin II-induced collagen synthesis and myofibroblast differentiation

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Abstract

Angiotensin II (Ang II) plays an important role on the pathogenesis of cardiac fibrosis. Prolong and overstimulation of angiotensin II type 1 receptor with Ang II-induced collagen synthesis and myofibroblast differentiation in cardiac fibroblasts, leading to cardiac fibrosis. Although adenosine and its analogues are known to have cardioprotective effects, the mechanistic by which adenosine A₂ receptors (A₂Rs) inhibit Ang II-induced cardiac fibrosis is not clearly understood. In the present study, we examined the effects of exogenous adenosine and endogenous adenosine on Ang II-induced collagen and myofibroblast differentiation determined by α -smooth muscle action (α -SMA) overexpression and their underlying signal transduction. Elevation of endogenous adenosine levels resulted in the inhibition of Ang II-induced collagen type I and III and α -SMA synthesis in cardiac fibroblasts. Moreover, treatment with exogenous adenosine which selectively stimulated A₂Rs also suppressed Ang II-induced collagen synthesis and α -SMA production. These antifibrotic effects of both endogenous and exogenous adenosines are mediated through the A_{2B} receptor (A_{2B}R) subtype. Stimulation of A_{2B}R exhibited antifibrotic effects via the cAMP-dependent and Epac-dependent pathways. Our results provide new mechanistic insights regarding the role for cAMP and Epac on A_{2B}R-mediated antifibrotic effects. Thus, A_{2B}R is one of the potential therapeutic targets against cardiac fibrosis.

Keywords Adenosine A_{2B} receptor · Angiotensin II · Cardiac fibrosis · Epac · cAMP · Collagen · α -SMA

Abbreviations

α -SMA	α -Smooth muscle actin
AC	Adenylate cyclase
A ₂ R	Adenosine A ₂ receptor
Ang II	Angiotensin II
cAMP	Cyclic adenosine monophosphate
DDA	2',5'-Dideoxyadenosine
ECM	Extracellular matrix
EHNA	Erythro-9-(2-hydroxy-3-nonyl) adenine
Epac	Exchange protein directly activated by cAMP
ET-1	Endothelin-1
FCS	Fetal calf serum

IDO	5-Iodotubercidin
PKA	Protein kinase A
PKI	Myristoylated protein kinase A inhibitor amide 14–22
siRNA	Small interfering RNA
TGF- β	Transforming growth factor beta

Introduction

Cardiac fibrosis is a pathophysiological condition of cardiac remodeling after myocardial infarction (MI), cardiac injury, and other heart diseases [1]. Progression of fibrosis in the heart leads to cardiac dysfunctions, distortion of cardiac structure, and induction of heart failure [2]. After cardiac injury, a variety of mediators, including angiotensin II (Ang II), endothelin-1 (ET-1), and transforming growth factor beta (TGF- β) accelerate cardiac fibroblast activation, resulting in fibroblast proliferation, fibroblast migration, extracellular matrix (ECM) protein overproduction, and myofibroblast

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differentiation [3]. Differentiation of cardiac fibroblasts into myofibroblasts is predominantly characterized by the α -SMA overexpression, and this profibrotic phenotype enhanced secretory, migratory, and proliferative properties [4]. These pathological and detrimental effects cause cardiac fibrosis, leading to heart diseases.

Ang II is a small peptide produced from the renin-angiotensin system cascade and considered as a potential profibrotic stimulus in the heart [5]. Ang II plays a critical role on cardiovascular diseases (e.g., myocardial infarction, heart failure, and hypertension) and also promotes the activation of cardiac fibroblast, leading to cardiac fibrosis through angiotensin II type 1 receptor (AT₁R) [5, 6]. Overstimulation of AT₁R induced collagen production and secretion in rat adult cardiac fibroblasts [5] and induced myofibroblast differentiation [7]. Ang II also promoted fibroblast hyperplasia and interstitial cardiac fibrosis [8], resulting in myocardial wall stiffness and ultimately impaired heart functions. In addition, Ang II induced fibronectin, integrin, and alpha-actinin productions in the heart which associated with cardiac fibrosis [9, 10]. Hence, Ang II contributes to the pathogenesis of cardiac fibrosis. In our study, we used Ang II as a profibrotic agent for activation of cardiac fibroblasts.

Inhibition of profibrotic effects of Ang II might be the potential target for treatment of cardiac remodeling and cardiac fibrosis. Endogenous adenosine can regulate the functions of cardiac fibroblasts. Previous studies have been shown that stimulation of adenosine receptor with adenosine exhibited the antifibrotic effects in the heart, including cardiac fibroblasts [11, 12]. For instance, treatment with adenosine receptor agonists inhibited cell proliferation [13], collagen overproduction [14], and α -SMA synthesis [12] in adult and neonatal rat cardiac fibroblasts. Antiproliferative and antifibrotic effects of endogenous adenosine and exogenous adenosine (e.g., adenosine receptor agonists) mediated through adenosine A₂ receptor subtypes [13, 15].

In rat model of myocardial infarction, agents that elevate endogenous adenosine levels are able to diminish tissue scar formation [11]. Moreover, both exogenous and endogenous adenosines attenuate serum-induced cell proliferation, protein synthesis, and collagen synthesis in cardiac fibroblasts [16, 17]. Adenosine can be synthesized in the heart, including cardiac fibroblasts [18] and exert numerous cardioprotective effects. Adenosine is a purine nucleoside that is phosphorylated to 5'-AMP by enzyme adenosine kinase or changed to inosine by enzyme adenosine deaminase [19]. In cardiac fibroblasts, adenosine was found in the media of cultured cells, providing for basal synthesis of adenosine in these cells [17]. Blockade of adenosine kinase activity by using 5-iodotubercidin (IDO) and blockade of adenosine deaminase activity using erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) caused the elevation

of cellular adenosine levels [17]. Inhibitory effects of EHNA and IDO on fetal calf serum (FCS)-induced fibroblast growth were significantly augmented when treated with ENHA plus IDO combination in cardiac fibroblasts [17]. Therefore, elevation of endogenous adenosine levels in cardiac fibroblasts may play a major role on inhibition of cardiac fibrosis.

Currently, there are at least four subtypes of adenosine receptors (ARs), A₁R, A_{2A}R, A_{2B}R, and A₃R. All four subtypes are the member of G protein-coupled receptor (GPCR). A₁R and A₃R couple to G α_i proteins whereas A_{2A}R and A_{2B}R couple to G α_s proteins [20]. As all four subtypes are found in cardiac fibroblasts [15], elevation of adenosine levels may activate all receptor subtypes. The cardioprotective effects, including antifibrotic effects of adenosine are mediated through A₂Rs, but not through A₁R and A₃R signaling. For instance, adenosine-mediated inhibition of collagen and protein synthesis were blunted by the selective A₂R antagonist, but not by the selective A₁R antagonist [16]. Stimulation of A_{2B}R has been shown to decrease mRNA expression of profibrotic gene markers, including collagen I and connective tissue growth factor (CTGF) [21]. Consistent with these in vitro studies, chronic administration of 2-chloroadenosine attenuated cardiac fibrosis and cardiac remodeling after MI [22]. The effects of 2-chloroadenosine were inhibited in the presence of the selective A_{2B}R antagonist, indicating that stimulation of A_{2B}R exhibits antifibrotic actions. Although several studies have been reported antifibrotic effects of adenosine and AR agonist, it remains unclear which receptor subtypes are responsible for the inhibition of Ang II-induced collagen synthesis and myofibroblast differentiation.

Stimulation of A₂ receptors, including A_{2A}R and A_{2B}R, increases adenylate cyclase (AC) activity through G α_s protein activity and subsequently elevates cAMP levels. In cardiac fibroblasts, cAMP binds to and activates PKA and Epac activities [12, 23]. Interestingly, cAMP plays an essential role on inhibition of fibroblast activation and also myofibroblast activity [24, 25]. Stimulation of A₂Rs exhibited antifibrotic effects through cAMP-dependent pathway [12]. Moreover, stimulation of A₂Rs significantly inhibited Ang II-induced collagen production through Epac/PI3K signaling in cardiac fibroblasts [14]. Our recent study also demonstrated stimulation of A_{2B}R with agonist attenuates ET-1-induced fibroblast proliferation and myofibroblast differentiation through cAMP/Epac/PI3K/Akt signaling pathway [12]. However, the molecular mechanism by which adenosine inhibited Ang II-induced collagen synthesis and myofibroblast differentiation remains unknown. Therefore, we investigated the effects of endogenous and exogenous adenosines on the inhibition of Ang II-induced collagen type I and III synthesis and α -SMA synthesis in cardiac fibroblasts.

Materials and methods

Reagents and plasmid construction

Angiotensin II, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261), 5-iodotubercidin (IDO), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), 2-phenylaminoadenosine (CV1808), and ESI-09 were purchased from Sigma-Aldrich. PKA inhibitor 14–22 amide (PKI), 2',5'-dideoxyadenosine (DDA), forskolin, and 8-[4-[(4-cyanophenyl)carbamoylmethyl]oxy]phenyl]-1,3-di(n-propyl)xanthine hydrate (MRS1754) were purchased from Calbiochem. Dulbecco's modified Eagle medium (DMEM), Opti-MEM, fetal bovine serum (FBS), penicillin/streptomycin (P/S) solution, and 0.25% trypsin-EDTA solution were purchased from Gibco. For preparing stock solutions, DDA, SCH58261, MRS1754, IDO, and ESI-09 were dissolved in DMSO. Ang II, forskolin, PKI, and EHNA were dissolved in H₂O. CV1808 was dissolved in 50% ethanol.

Neonatal rat cardiac fibroblast isolation and culture

Cardiac fibroblasts were isolated as previously described [12]. Briefly, neonatal Sprague-Dawley rats were euthanized by decapitation and hearts were removed under aseptic condition. The atria tissue was removed, and the ventricle was cut into 2- to 3-mm² pieces and digested by collagenase A. The digested cells were plated on 10-cm dishes and incubated for 2–3 h at 37 °C. Cardiac fibroblasts attached to the bottom of the culture dishes during 2–3 h and other non-adherent cells were removed by changing the medium. Cardiac fibroblasts were cultured in DMEM containing 10% FBS and 1% P/S and used during the first and second passages for all experiments.

The thin and triangular cells were identified as cardiac fibroblast. The purity of fibroblasts was also confirmed by immunostaining. All animals received care according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and this study was approved by the Committee for Animal Care and Use of the Faculty of Pharmacy, Mahidol University (Protocol Nos. PYR005/2557 and PYR002/2560).

mRNA expression analysis by quantitative real-time PCR

Cardiac fibroblasts (5×10^5 cells/well) were plated in six-well plates. Cells were starved of serum for overnight before stimulation. Cells were pretreated without or with specific inhibitors/antagonists for 1 h before treatment with CV1808 for 1 h and further stimulated with 200 nM Ang II for 6 h.

Cells were washed with phosphate buffer saline (PBS) and then performed the isolation of RNA using GeneJET RNA purification kits (Thermo scientific). Quantitative mRNA levels were performed using the KAPA SYBR FAST One-step RT-qPCR kits (KAPA biosystems) and the Mx 3005p Real Time PCR system (Stratagene) as described previously [12]. All sequences of primers are shown in Supplemental Table 1. Relative mRNA levels were calculated by the comparative cycle threshold (C_T) method and normalized to the endogenous reference, GAPDH relative to a calibrator.

Western blotting

After treatment, cardiac fibroblast lysates were prepared in lysis buffer containing 20 mM Tris (pH 7.4), 0.8% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 100 μM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich) [12]. The protein concentration of lysates was measured using a Bio-Rad protein assay kit with BSA as the standard. Samples were separated and transferred onto polyvinylidene fluoride (PVDF) membrane. Membranes were subjected to immunoblotting with primary antibodies to collagen I (1:1000; Abcam), α-SMA (1:1000; Sigma-Aldrich), and GAPDH (1:1000; Cell Signaling). The signal was developed using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (GE Healthcare).

Detection of α-SMA by immunofluorescence microscopy

Cardiac fibroblasts (1×10^4 cells/well) were plated in 35-mm glass dishes as previously described [26]. Cells were starved of serum for overnight before stimulation. Cells were pretreated without or with specific inhibitors/antagonists for 1 h before treatment with CV1808 for 1 h and further stimulated with 200 nM Ang II for 24 h. Cells were washed with PBS, fixed with 4% paraformaldehyde, and stored for overnight at 4 °C. Cells were permeabilized by adding 0.1% Triton-X100 for 5 min, and then washed again with PBS. After that, cells were added 1% bovine serum albumin (BSA) for 30 min, and then added anti-α-SMA antibody (1:250 dilution) and incubated for 1 h. Cells were washed with PBS and incubated with secondary antibody (Alexa Fluor 488) for 1 h, and further incubated with 4',6-diamidino-2-phenylindole (DAPI; 5 μg/mL) for 10 min and washed with PBS. The α-SMA was visualized by fluorescent inverted microscope (Olympus IX81).

siRNA experiments targeting A_{2B} receptor

Stealth siRNA duplex oligoribonucleotides against rat A_{2B} receptor (NCBI Reference Sequence: NM_017161.1) were synthesized by Invitrogen (Grand Island, NY). The siRNA sequences are shown in Supplemental Table 2. For siRNA transfection, cardiac fibroblasts were plated in six-well plates to 70% confluency. Cells were transfected with 100 nM Epac1 siRNA or control siRNA using Lipofectamine 2000 reagent (Invitrogen) as previously described [27]. siRNA duplex was mixed with Opti-MEM (Gibco), whereas Lipofectamine 2000 reagent was mixed with Opti-MEM and incubated for 5–10 min. After that, both solutions were mixed together, then incubated for 20 min, and added into the cells. After 5 h, transfection media was replaced with the original conditioned medium. Experiments were performed at least 3 days after siRNA transfection.

cAMP assays

Cardiac fibroblasts cAMP levels were determined using cAMP ELISA kit (Cayman) according to manufacturer's instructions. Cardiac fibroblasts (2×10^5 cells) were plated in six-well plates in DMEM containing 10% FBS and 1% P/S solution, and then starved in serum-free DMEM for 2 days. To prevent the degradation of cAMP, cells were pretreated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor) for 1 h as previously described [12]. Cells were pretreated without or with 10 μ M DDA (AC inhibitor) for 1 h. After 1 h, cells were treated with vehicle (DMSO), 10 μ M forskolin, or 10 μ M CV1808 for 30 min. After stimulation, cells were washed with PBS and then lysed with 0.1 M HCl containing 0.15% Triton X-100. Cyclic AMP levels were measured using cAMP ELISA kit and shown as pmol/mg protein.

Statistical analysis

Data are presented as mean $\pm$ SEM and were obtained from four different experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. $P < 0.05$ was considered significant.

Results

Elevation of endogenous adenosine levels attenuates Ang II-induced collagen synthesis and α -SMA expression

We first evaluated the effects of modulators of adenosine levels by using EHNA (inhibitor of adenosine deaminase)

and IDO (inhibitor of adenosine kinase) on inhibition of Ang II-induced collagen synthesis and myofibroblast differentiation detected by α -SMA mRNA and protein expressions. Fibroblasts were pretreated with either EHNA or IDO alone at concentrations of 1 and 10 μ M before treatment with 200 nM Ang II (AT₁R agonist). Stimulation of AT₁Rs with Ang II leads to a significant increase in the collagen type I and III and α -SMA mRNA expression compared to the control group (Supplemental Fig. 1a). Pretreatment with either EHNA or IDO alone inhibited Ang II-induced collagen type I and III mRNA expressions in a dose-dependent manner that exhibited the maximal effects at 10 μ M (Supplemental Fig. 1a). Thus, we used EHNA and IDO at a concentration of 10 μ M for further studies.

Inhibition of adenosine kinase activity by using IDO and inhibition of adenosine deaminase activity by using EHNA at 10 μ M significantly inhibited Ang II-induced collagen type I and III synthesis, but not completely blocked (Fig. 1a, b). In addition, pretreatment with either IDO or EHNA alone (10 μ M) tended to inhibit Ang II-induced α -SMA synthesis (Fig. 1a, b). Due to combination of EHNA plus IDO resulted in an increase of adenosine levels approximately 650-fold in cardiac fibroblasts [17]. We next determined the synergistic effects of IDO and EHNA combination in inhibition of Ang II-mediated upregulation of collagens and α -SMA synthesis. As shown in Fig. 1a, b, pretreatment with EHNA together with IDO significantly attenuated Ang II-induced mRNA and protein expressions of collagen types I and III and α -SMA in an additive manner. Moreover, combination of EHNA and IDO is able to inhibit Ang II-induced α -SMA protein expression as assessed by immunohistochemistry (Fig. 1c). These findings support our hypothesis that elevation of endogenous adenosine levels exhibits an antifibrotic effect through activation of adenosine receptors in cardiac fibroblasts.

Stimulation of A₂Rs inhibits Ang II-induced collagen synthesis and α -SMA production

As shown in Fig. 1, treatment of cardiac fibroblasts with agents that increase endogenous adenosine levels (e.g., EHNA and IDO) attenuated the profibrotic effects of Ang II. Our previous study also demonstrated that stimulation of A_{2B}R inhibited ET-1-induced fibroblast proliferation and α -SMA synthesis [12]. We next investigated the effects of CV1808 (A₂R agonist) which acts as exogenous adenosine, on inhibition of Ang II-induced collagen I and III, and α -SMA synthesis. We found that stimulation of A₂Rs with CV1808 significantly inhibited Ang II-induced collagen type I and III mRNA and protein expressions (Fig. 2a, b), and also attenuated Ang II-induced α -SMA mRNA and protein levels (Fig. 2a–c). The inhibitory effects of CV1808

Fig. 1 Effects of endogenous adenosine on Ang II-induced collagen and α -SMA synthesis. Cardiac fibroblasts were either pretreated with EHNA (10 μ M), IDO (10 μ M), or EHNA plus IDO for 1 h and then stimulated with 200 nM Ang II for 6 h (**a**) or 24 h (**b**, **c**) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n = 4$). **b** Relative collagen I and α -SMA protein levels were quantified and shown as the mean $\pm$ SEM ($n = 3$). *Significant

difference ($P < 0.05$) versus vehicle (control); #significant difference ($P < 0.05$) versus Ang II; *significant difference ($P < 0.05$) versus EHNA alone. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m

on Ang II-induced collagens and α -SMA mRNA expression are slightly greater than combination of EHNA plus IDO. However, these inhibitory effects of CV1808 on Ang II-induced collagen I and α -SMA protein expression are not statistically significant difference compared with EHNA/IDO combination (Fig. 2a, b).

A_{2B}R-mediated the inhibition of Ang II-induced collagen synthesis and α -SMA production

We next determined the subtypes of adenosine A₂ receptor which are responsible for antifibrotic effects by using a selective A_{2A}R antagonist (SCH58261) and a selective A_{2B}R

Fig. 2 Effects of CV1808 (A_2R agonist) on Ang II-induced collagen and α -SMA synthesis. Cardiac fibroblasts were pretreated with either CV1808 or EHNA/IDO combination for 1 h and then stimulated with 200 nM Ang II for 6 h (**a**) or 24 h (**b**, **c**) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n=4$). **b** Relative collagen I and α -SMA protein levels were quantified and shown as the mean $\pm$ SEM ($n=3$). *Significant

difference ($P<0.05$) versus vehicle (control); #significant difference ($P<0.05$) versus Ang II. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m

antagonist (MRS1754). Our previous study has been demonstrated that both SCH58261 and MRS1754 antagonize receptor-mediated cAMP elevation, indicating both antagonists effectively block the receptor signaling through $G\alpha_s$ protein [12]. As shown in Fig. 3, pretreatment with MRS1754, but not SCH58261, blocked the inhibitory effect of CV1808 (A_2R agonist; exogenous adenosine) on Ang II-induced collagen synthesis and myofibroblast differentiation assessed by

α -SMA mRNA and protein expressions (Fig. 3). Furthermore, MRS1754 also attenuated the inhibitory effect of EHNA/IDO combination (increased endogenous adenosine levels) on Ang II-induced collagen synthesis, and α -SMA mRNA and protein expressions, whereas SCH58261 had no effect (Fig. 3a, b, respectively). These results demonstrated that stimulation of $A_{2B}R$ with either exogenous or endogenous adenosine resulted in the inhibition of Ang II-induced

collagen synthesis, and a reduction of Ang II-induced α -SMA production.

We next used antisense oligonucleotide $A_{2B}R$ silencing to confirm the role of $A_{2B}R$ in adenosine-mediated antifibrotic effects. As shown in Fig. 4a, siRNA targeting $A_{2B}R$ significantly suppressed the expression of endogenous $A_{2B}R$ compared to nonsilencing control siRNA-transfected cells. In addition, this $A_{2B}R$ siRNA did not affect on endogenous $A_{2A}R$ expression (Fig. 4a). These results showed that $A_{2B}R$ siRNA efficiently and specifically suppressed $A_{2B}R$ expression in cardiac fibroblasts. We found that treatment with either CV1808 or EHNA/IDO combination significantly inhibited Ang II-induced collagen type I and III and α -SMA synthesis in control siRNA-transfected cells (Fig. 4b, c). In contrast, the inhibitory actions of both CV1808 and EHNA/IDO on Ang II-induced these fibrotic effects were completely abolished in the presence of siRNA targeting $A_{2B}R$ (Fig. 4b, c). Collectively, these data demonstrated the important role of the $A_{2B}R$ in adenosine-mediated antifibrotic signal transduction.

A_2 receptor-mediated antifibrotic effects is cAMP dependent

Stimulation of A_2R leads to cAMP elevation via the activation of AC activity that signals through $G\alpha_s$ protein. Because A_2R agonists (e.g., adenosine, NECA) were also shown to elevate intracellular cAMP levels in the heart including cardiac fibroblasts [12, 28] and cAMP is a key modulator of fibroblast functions, we next determined whether the inhibition of Ang II-mediated fibrotic effects by CV1808 is required cAMP. We first demonstrated that treatment of cardiac fibroblasts with either CV1808 (A_2R agonist) or forskolin (AC activator) significantly increased intracellular cAMP levels (Fig. 5d). The stimulatory effect of CV1808 on cAMP elevation was completely abolished in cardiac fibroblasts pretreated with DDA (AC inhibitor) (Fig. 5d).

As shown in Fig. 5a–c, the inhibitory effects of CV1808 on Ang II-induced collagen type I and III and α -SMA mRNA and protein expressions were blunted by DDA. In addition, cAMP elevation induced by forskolin (10 μ M) also inhibited these fibrotic effects of Ang II as shown by the similar results to those of CV1808 (A_2R agonist) (Fig. 5a–c). Taken together, stimulation of A_2R elicits strong antifibrotic actions through cAMP-dependent pathway by inhibiting collagen synthesis and attenuating myofibroblast differentiation induced by Ang II.

Stimulation of A_2Rs inhibits Ang II-induced collagen synthesis and myofibroblast differentiation through Epac-dependent pathway

After A_2R stimulation, $G\alpha_s$ mediates activation of AC, leading to cAMP elevation. cAMP then binds to and stimulates

both PKA and Epac, which are important downstream effectors. As cAMP is responsible for antifibrotic effects following A_2R stimulation (Fig. 5), we next determined whether PKA and/or Epac play an important role on A_2R -mediated antifibrotic effects in cardiac fibroblasts. Blockade of PKA activity using PKI did not inhibit the effects of CV1808 on inhibition of Ang II-induced collagen type I and III and α -SMA mRNA expression (Fig. 6a), and PKI did not alter the inhibitory effects of CV1808 on Ang II-induced collagen I and α -SMA protein expression (Fig. 6b, c). Taken together, these results demonstrated that A_2R -mediated antifibrotic actions are independent of PKA.

Interestingly, pretreatment with ESI-09 (specific Epac inhibitors) significantly blocked the antifibrotic effects of CV1808 on Ang II-induced collagens and α -SMA synthesis (Fig. 6). Thus, Epac is necessary for A_2R -mediated inhibition of fibrotic effects of Ang II.

Elevation of endogenous adenosine levels elicits antifibrotic effects through cAMP/Epac-signaling pathway

As shown in Figs. 1 and 2, combination of EHNA and IDO which robustly increased the levels of endogenous adenosine can inhibit Ang II-induced collagens and α -SMA synthesis in cardiac fibroblasts. We next investigated whether elevation of endogenous adenosine levels by treatment with EHNA/IDO combination also activates $A_{2B}R$ -mediated antifibrotic actions through cAMP/Epac signaling pathway. Pretreatment with DDA (an AC inhibitor) significantly antagonized the effects of EHNA/IDO combination on inhibition of Ang II-induced collagen type I and III and α -SMA synthesis (Fig. 7a–c), suggesting that cAMP is necessary for antifibrotic actions of endogenous adenosine. Moreover, the inhibitory effects of EHNA/IDO combination on Ang II-induced collagen synthesis and α -SMA expression were blunted by ESI-09 (an Epac inhibitor) (Fig. 7). Taken together, elevation of adenosine levels stimulates $A_{2B}R$ -cAMP-Epac signaling pathway that is necessary for antifibrotic actions in cardiac fibroblasts.

Discussion

Our findings indicate the essential role for Epac signaling on A_2 receptor-mediated antifibrotic actions in cardiac fibroblasts. We demonstrated that elevation of endogenous adenosine levels attenuated Ang II-induced collagen type I and III and α -SMA synthesis. Moreover, treatment with exogenous adenosine which selectively stimulated A_2Rs (CV1808) also inhibited Ang II-induced collagen synthesis and α -SMA production. These antifibrotic effects of both endogenous and exogenous adenosines are mediated through the $A_{2B}R$ subtype.

c α -SMA protein expression

Ang II regulates several functions of cardiac fibroblasts such as cell proliferation, induction of collagens, and myofibroblast differentiation [8, 29]. Stimulation of AT₁R

with Ang II also promoted interstitial fibrosis that contributes to myocardial wall stiffness and impaired cardiac functions [8]. In addition, inhibition of Ang II actions using

Fig. 3 Stimulation of A_{2B}R inhibits Ang II-induced collagen and α -SMA synthesis. Cardiac fibroblasts were pretreated without or with either 0.5 μ M SCH58261 (SCH; A_{2A}R antagonist) or 0.5 μ M MRS1754 (MRS; A_{2B}R antagonist) 1 h before treatment with vehicle (control), 10 μ M CV1808, or EHNA/IDO combination for 1 h and further stimulated with 200 nM Ang II for 6 h (a) or 24 h (b, c) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n=4$). **b** Relative collagen I and α -SMA protein levels were quantified and shown as the mean $\pm$ SEM ($n=3$). *Significant difference ($P<0.05$) versus vehicle (control); #significant difference ($P<0.05$) versus Ang II; †significant difference ($P<0.05$) versus Ang II plus CV1808; §significant difference ($P<0.05$) versus Ang II plus EHNA/IDO. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m

angiotensin-converting enzyme inhibitors (ACEIs) or AT₁ receptor blockers (ARBs) resulted in the reduction of cardiac fibrosis in pressure overload and MI rat models [30, 31]. Thus, inhibition of Ang II-induced cardiac fibrosis might represent the therapeutic targets for attenuation the progression of cardiac fibrosis. In this study, we utilized Ang II as a profibrotic agent to stimulate collagen synthesis and myofibroblast formation to investigate the molecular mechanistic by which A₂R stimulation could counteract Ang II-induced cardiac fibrosis.

The actions of endogenous adenosine and exogenous adenosine (AR agonists) are receptor-dependent manner [32]. Adenosine receptors are member of GPCR with four subtypes, A₁R, A_{2A}R, A_{2B}R, and A₃R. These receptors are expressed throughout the body including cardiac fibroblasts [15]. Adenosine receptors exhibit distinct biological effects through differential coupling with heterotrimeric G α subunits. The A₁R and A₃R preferentially couple and activate G $\alpha_{i/o}$ proteins to inhibit AC activity and subsequently decrease cAMP levels, while the A_{2A}R and A_{2B}R signal through G α_s protein to activate AC activity and increase cAMP level in the cells [33]. Elevation of cAMP leads to stimulate several downstream effectors such as PKA and Epac [12, 15, 28]. Administration of 2-chloroadenosine (AR agonist) led to prevention of cardiac remodeling and reduction of the markers of fibrosis, including collagen and matrix metalloproteinase, in rat model of post-myocardial infarction [22]. In addition the inhibition of adenosine uptake using dipyrindamole also reduced cardiac fibrosis, which had effects similar to those of 2-chloroadenosine [22]. Termination of adenosine signaling involves the catabolism of adenosine with various enzymes and also dependent on the action of adenosine transporters which transport adenosine across the membrane [34, 35]. Within the cells, degradation of adenosine to 5'-AMP by adenosine kinase and to inosine by adenosine deaminase play an important role in removing of active adenosine [35, 36]. Thus, blockade of adenosine kinase activity by IDO and inhibition of adenosine deaminase enzyme activity by EHNA increase

endogenous adenosine levels [17]. Inhibition of endogenous adenosine metabolism by using EHNA (adenosine deaminase inhibitor) and IDO (adenosine kinase inhibitor) which results in an elevation of adenosine levels attenuated fetal calf serum-induced fibroblast proliferation [17]. Moreover, treatment with 2-chloroadenosine and with agents that increase endogenous adenosine levels (e.g., EHNA plus IDO) significantly attenuated PDGF-BB-mediated collagen synthesis, DNA synthesis, and fibroblast proliferation in cardiac fibroblasts [13]. Consistent with these previous studies, our present data also reported the combination of EHNA and IDO which robustly increased the levels of endogenous adenosine significantly inhibited Ang II-induced collagen type I and III and α -SMA synthesis. These findings support our hypothesis that elevation of endogenous adenosine levels exhibits an antifibrotic effect through stimulation of adenosine receptors in fibroblast cells. Adenosine has been widely reported to attenuate the renin-angiotensin system and elevation of adenosine, thus, modulation of adenosine signaling might contribute to the beneficial impact on treatment of heart failure [37, 38]. The increased adenosine levels in animal model of chronic heart failure may be compensatory against heart failure [22], leading to the idea that elevation of adenosine levels or prolonged stimulation of A_{2B}R might be the novel promising therapeutic approach for the treatment of chronic heart failure.

All four subtypes of ARs are found in cardiac fibroblasts and synchronous stimulation of multiple receptor subtypes results in both synergistic and opposing signal transduction for regulation of cardiac fibroblasts. However, which adenosine receptor subtype is responsible for endogenous adenosine-mediated antifibrotic effects in cardiac fibroblasts remains unsolved. In our present study, demonstrating antifibrotic effects of CV1808 (A₂R agonist) was mediated through A_{2B}R subtype. In concordance with our present study, exogenous and endogenous adenosine inhibited FCS-induced cardiac fibroblast proliferation via stimulation of A_{2B}R [17]. In addition, antifibrotic effects of 2-chloroadenosine and combination of EHNA plus IDO were antagonized by A₂R antagonist, but not by A₁R antagonist [13]. Overexpression of A_{2B}R significantly inhibited fibroblast proliferation and collagen production [39]. Furthermore, the role for A_{2B}R in adenosine-mediated antifibrotic effects was confirmed via silencing of A_{2B}R using siRNA, which resulted in collagen overproduction and activation of fibroblast proliferation [13]. Consistent with this previous study, we demonstrated that antifibrotic effects of both CV1808 (exogenous adenosine) and EHNA/IDO combination (endogenous adenosine) were abolished when A_{2B}Rs were knocked down in cardiac fibroblasts. However, further studies using genetically engineered animals are needed to confirm the exact role of adenosine receptor subtype which plays a role on inhibition of Ang II-induced cardiac fibrosis. Interestingly, the antifibrotic effects of A_{2B}R might be lost upon the

Fig. 4 Depletion of A_{2B}R by siRNA abolishes antifibrotic effects of CV1808. **a** Cardiac fibroblasts were transfected with 100 nM A_{2B}R siRNA or control siRNA. A_{2A}R and A_{2B}R mRNA levels were quantified and shown as the mean ± SEM (*n* = 3). *Significant difference (*P* < 0.05) versus control siRNA. **b, c** Cardiac fibroblasts were transfected with siRNAs targeting A_{2B}R or control siRNA. After 3 days, cells were treated with vehicle (control), 10 μM CV1808, or EHNA/IDO combination for 1 h and further stimulated with 200 nM

Ang II for 6 h (**b**) or 24 h (**c**) at 37 °C. **b** Relative collagen I, collagen III, and α-SMA mRNA levels were quantified and shown as the mean ± SEM (*n* = 4). **c** Relative collagen I and α-SMA protein levels were quantified and shown as the mean ± SEM (*n* = 3). *Significant difference (*P* < 0.05) versus vehicle-treated control siRNA; #significant difference (*P* < 0.05) versus Ang II-stimulated control siRNA; *significant difference (*P* < 0.05) versus vehicle-treated A_{2B}R siRNA

downregulation of A_{2B}R as observed in hearts from patients with chronic heart failure [40]. Thus, the A_{2B}R is considered as the major subtype which plays a role on cardioprotection.

Both A_{2A}R and A_{2B}R are expressed in the heart and found to have antifibrotic effects in the heart [33]. Although A_{2A}R and A_{2B}R can couple with Gα_s proteins, considerable

differences exist in their antifibrotic effects in the heart. For example, activation of A_{2B}R, but not A_{2A}R, resulted in the suppression of ET-1-induced fibroblast proliferation and α-SMA expression in cardiac fibroblasts [12]. The antifibrotic effects of adenosine on collagen production are most likely mediated through A_{2B}R rather than A₁R or A_{2A}R [16].

Fig. 5 CV1808-mediated inhibition of Ang II-induced collagen and α -SMA synthesis is cAMP dependent. Cardiac fibroblasts were pretreated without or with 10 μ M DDA (AC inhibitor) for 1 h. After 1 h, cells were treated with vehicle (control), 10 μ M CV1808, or 10 μ M forskolin (Forsk; AC activator) for 1 h and further stimulated with 200 nM Ang II for 6 h (**a**) or 24 h (**b**, **c**) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n = 4$). **b** Relative collagen I and α -SMA protein levels were quantified and shown as the mean $\pm$ SEM ($n = 3$). *Significant difference ($P < 0.05$) versus control; #significant difference ($P < 0.05$) versus Ang II;

*significant difference ($P < 0.05$) versus Ang II plus CV1808. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m. **d** Cells were pretreated without or with 10 μ M DDA for 1 h. After 1 h, cells were treated with vehicle (DMSO), 10 μ M forskolin, or 10 μ M CV1808 for 30 min. The cAMP levels were measured and shown as pmol/mg protein ($n = 3$). * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. CV1808

Consistent with these studies, we found that stimulation of $A_{2B}R$ with endogenous and exogenous adenosine inhibited Ang II-induced collagen synthesis and α -SMA production. Upon agonist binding, $A_{2B}R$ couples to G_{α_s} proteins, and subsequently activates AC activity, leading to cAMP elevation [41]. Cyclic AMP has been shown to have a central role in

inhibition of cardiac fibrosis and cAMP-elevating agents (e.g., adenosine receptor agonists) might be useful as prevention and/or treatment of cardiac fibrosis. For example, treatment with forskolin (AC activator) and cAMP analogs inhibited TGF- β -induced collagen synthesis and myofibroblast differentiation [24]. Furthermore, agents which elevate cAMP

Fig. 6 Stimulation of A_2R inhibits Ang II-induced collagen and α -SMA synthesis in an Epac-dependent pathway. Cardiac fibroblasts were pretreated with 10 μ M ESI-09 (Epac inhibitor), PKI, or ESI plus PKI for 1 h. After 1 h, cells were treated with vehicle (control) or 10 μ M CV1808 for 1 h and further stimulated with 200 nM Ang II for 6 h (a) or 24 h (b, c) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n = 4$). **b** Relative collagen I and α -SMA protein levels were quantified and shown

as the mean $\pm$ SEM ($n = 3$). *Significant difference ($P < 0.05$) versus control; #significant difference ($P < 0.05$) versus Ang II; †significant difference ($P < 0.05$) versus Ang II plus CV1808. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m

levels significantly inhibited fetal calf serum-induced fibroblast proliferation [17] and attenuated ET-1-induced fibroblast proliferation and myofibroblast differentiation in cardiac fibroblasts [12]. Our data are consistent with these previous studies reporting that stimulation of A_2R which leads to an increase of cAMP levels exerts antifibrotic effects by

inhibiting Ang II-induced collagen type I and III synthesis and myofibroblast differentiation, and these effects were blunted by DDA (an AC inhibitor). Thus, data from our study and previous studies demonstrate an important role for cAMP that appears to be the second messenger of $A_{2B}R$ signaling on inhibition of cardiac fibrosis.

Fig. 7 Endogenous adenosine-mediated inhibition of Ang II-induced collagen and α -SMA synthesis through cAMP/Epac signaling pathway. Cardiac fibroblasts were pretreated without or with either 10 μ M DDA (AC inhibitor) or ESI-09 (Epac inhibitor) for 1 h. After 1 h, cells were treated with vehicle (control), or 10 μ M EHNA plus IDO for 1 h and further stimulated with 200 nM Ang II for 6 h (**a**) or 24 h (**b**, **c**) at 37 °C. **a** Relative collagen I, collagen III, and α -SMA mRNA levels were quantified and shown as the mean $\pm$ SEM ($n = 4$). **b** Relative collagen I

and α -SMA protein levels were quantified and shown as the mean $\pm$ SEM ($n = 3$). *Significant difference ($P < 0.05$) versus control; #significant difference ($P < 0.05$) versus Ang II; *significant difference ($P < 0.05$) versus Ang II plus EHNA/IDO. **c** Cells were incubated with anti- α -SMA antibody followed by goat anti-mouse antibody (Alexa Fluor 488). The α -SMA was visualized by fluorescent microscope. Cells were stained for α -SMA (green) and nuclear staining of nucleus with DAPI (blue). Bar, 10 μ m

The second messenger cAMP interacts with and stimulates PKA and Epac activities and their signaling pathways. The compartmentalization of cAMP-signaling components such as $A_{2B}R$, AC, PKA, and Epac allows the establishment of multiple cAMP compartments and different signal transductions [42]. Stimulation of $A_{2B}R$ s resulted in an inhibition of collagen production through the PKA-independent, Epac-dependent pathway in rat cardiac fibroblasts [14]. In addition, $A_{2B}R$ exhibits antifibrotic effects on suppression of ET-1-induced cell proliferation and myofibroblast differentiation

via Epac-dependent signaling in neonatal rat cardiac fibroblasts [12]. Moreover, stimulation of $A_{2B}R$ activated ERK1/2 phosphorylation via cAMP-dependent, PKA-independent manner in HUVEC [43]. Our present study also demonstrated that $A_{2B}R$ -mediated inhibitions of Ang II-induced collagen synthesis and α -SMA expression were not affected by blockade of PKA activity, whereas blockade of Epac activity significantly antagonized this antifibrotic effect of $A_{2B}R$, suggesting that Epac is necessary for $A_{2B}R$ -mediated antifibrotic actions in the heart. Additionally, Ang II is the

Fig. 8 Schematic diagram representing the essential role of cAMP/Epac for A_{2B}R-mediated antifibrotic action. In cardiac fibroblasts, agonists (endogenous adenosine and AR agonist) binding to A_{2B} receptors lead to cAMP elevation through Gα_s activation. Cyclic AMP then binds to

and activates Epac activity, leading to stimulation of Epac downstream effector and subsequently inhibits Ang II-induced collagen synthesis and α-SMA production, eliciting antifibrotic effects

profibrotic factor that plays an essential role in cardiac fibrosis. In adult rat cardiac fibroblasts, inhibition of Epac activity, but not PKA, antagonized the effects of exogenous adenosine on inhibition of Ang II-induced collagen production [14]. In addition, our present study also demonstrated that Epac is necessary for exogenous and endogenous stimulation of A_{2B}R for inhibition of Ang II-induced collagen synthesis and α-SMA synthesis. Thus, stimulation of A_{2B}R through Epac-dependent fashion can inhibit cardiac fibrosis induced by Ang II. The A_{2B}R/cAMP/Epac signaling pathway might be the potential target for prevention and/or inhibition of cardiac fibrosis induced by upregulation of RAS.

Based on our present findings, we have identified the important of both endogenous and exogenous adenosine for an inhibition of Ang II-induced collagen type I and III synthesis and α-SMA production in cardiac fibroblasts signaling through the A_{2B}R (Fig. 8). Agonists (endogenous adenosine and CV1808) binding to A_{2B}R lead to the elevation of cAMP levels through Gα_s activation. cAMP interacts with Epac and then subsequently activates Epac-dependent signaling pathway which leads to the inhibition of Ang II-induced cardiac fibrosis.

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Compliance with ethical standards

Conflicts of interest Sarawuth Phosri declares that he has no conflict of interest.

Kwanchai Bunrukchai declares that he has no conflict of interest. Warisara Parichatikanond declares that she has no conflict of interest. Vilasinee H. Sato declares that she has no conflict of interest. Supachoke Mangmool declares that he has no conflict of interest.

Ethical approval This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, Mahidol University (Protocol Nos. PYR005/2557 and PYR002/2560).

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