ACCELERATED COMMUNICATION

Increase in Cellular Cyclic AMP Concentrations Reverses the Profibrogenic Phenotype of Cardiac Myofibroblasts: A Novel Therapeutic Approach for Cardiac Fibrosis

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ABSTRACT

Tissue fibrosis is characterized by excessive production, deposition, and contraction of the extracellular matrix (ECM). The second messenger cAMP has antifibrotic effects in fibroblasts from several tissues, including cardiac fibroblasts (CFs). Increased cellular cAMP levels can prevent the transformation of CFs into profibrogenic myofibroblasts, a critical step that precedes increased ECM deposition and tissue fibrosis. Here we tested two hypotheses: 1) myofibroblasts have a decreased ability to accumulate cAMP in response to G protein–coupled receptor (GPCR) agonists, and 2) increasing cAMP will not only prevent, but also reverse, the myofibroblast phenotype. We found that myofibroblasts produce less cAMP in response to GPCR agonists or forskolin and have decreased expression of several adenylyl cyclase (AC) isoforms and increased expression of multiple cyclic nucleotide phosphodiesterases (PDEs). Furthermore, we found that forskolin-

promoted increases in cAMP or N^6 -phenyladenosine-cAMP, a protein kinase A–selective analog, reverse the myofibroblast phenotype, as assessed by the expression of collagen $\alpha 1$, α -smooth muscle actin, plasminogen activator inhibitor-1, and cellular contractile abilities, all hallmarks of a fibrogenic state. These results indicate that: 1) altered expression of AC and PDE isoforms yield a decrease in cAMP concentrations of cardiac myofibroblasts (relative to CFs) that likely contributes to their profibrotic state, and 2) approaches to increase cAMP concentrations not only prevent fibroblast-to-myofibroblast transformation but also can reverse the profibrotic myofibroblastic phenotype. We conclude that therapeutic strategies designed to enhance cellular cAMP concentrations in CFs may provide a means to reverse excessive scar formation following injury and to treat cardiac fibrosis.

Introduction

The lack of drugs that can treat fibrosis of the heart (Fan et al., 2012), lung (van der Vliet and Bove, 2011), liver (Kisseleva et al., 2012), kidney (Kriz et al., 2011), and other tissues (Insel et al., 2012) represent an important unmet medical need. Fibrosis is characterized by the excessive deposition of collagens and extracellular matrix (ECM) proteins that lead to impaired

organ function (Goldsmith et al., 2013). Fibroblasts are the predominant cell type responsible for the homeostatic maintenance of tissue ECM, healing after injury, and age-associated remodeling (Tomasek et al., 2002; Wynn, 2008; van den Borne et al., 2010). In the heart, pathologic transformation of cardiac fibroblasts (CFs) to activated (profibrogenic) myofibroblasts leads to decreased myocardial compliance, diastolic dysfunction, and accompanying heart failure (Wynn, 2008; Creemers and Pinto, 2011; Fan et al., 2012). Myofibroblasts are characterized by increased protein synthesis, including of collagens (in particular, types I and III), other ECM proteins, certain cytokines, and α -smooth muscle actin (α -SMA), a contractile protein and marker of profibrogenic CF activation (Hinz et al., 2001; Swaney et al., 2005; Hinz, 2007).

Proper wound healing requires the resolution of inflammatory responses and a decrease in fibroblast activity (Tomasek

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ABBREVIATIONS: AC, adenylyl cyclase; ADM, adrenomedullin; α -SMA, α -smooth muscle actin; Bera, beraprost; CF, cardiac fibroblast; Coll $\alpha 1$, collagen $\alpha 1$; ECM, extracellular matrix; Iso, isoproterenol; Fsk, forskolin; GPCR, G protein–coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; N^6 -Phe-cAMP, N^6 -phenyladenosine-cAMP; PDE, phosphodiesterase; PKA, protein kinase A; TGF, transforming growth factor; qPCR, real-time polymerase chain reaction.

et al., 2002; Hinz, 2007; van den Borne et al., 2010; Kisseleva et al., 2012). The precise mechanisms responsible for such resolution are not well defined, especially in terms of the decrease in the activity of fibroblasts. While apoptosis of fibroblasts can contribute to the attenuation of profibrotic remodeling (Huang et al., 2009; Insel et al., 2012), other processes likely also contribute to the “reversal” of profibrotic phenotypes (Kisseleva et al., 2012). One potential mediator is the second messenger cyclic AMP. Activation of cAMP-dependent processes is known to inhibit CF-to-myofibroblast conversion (Swaney et al., 2005; Miller et al., 2011) and overexpression of adenylyl cyclase (AC) and activation of Gs-coupled G protein-coupled receptors (GPCRs), which stimulate cAMP synthesis and decrease collagen synthesis and α -SMA expression in CFs (Davaile et al., 2000; Heusinger-Ribeiro et al., 2001; Swaney et al., 2005; Yokoyama et al., 2008; Schiller et al., 2010). However, the contribution of cAMP to maintain, and potentially to reverse, the profibrotic state is not known. We thus undertook the current study using primary isolates of adult CFs to test the hypotheses that: 1) myofibroblasts have a decreased ability to generate cAMP in response to Gs-coupled GPCR agonists, and 2) increasing cAMP will not only prevent but also reverse the myofibroblast phenotype. The results shown here provide evidence in support of both of these hypotheses.

Materials and Methods

Cardiac Fibroblast Isolation and Culture. Approval for the ethical care and use of animals for this study was obtained from the University of California at San Diego Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. CFs were isolated from the hearts of 3-month-old male Sprague-Dawley rats, as previously described (Yokoyama et al., 2008). Briefly, hearts were removed and retrograde-perfused with collagenase type II (200 units/ml) (Worthington Biochemical Corp., Lakewood, NJ). Cardiac myocytes and CFs were separated via gravity sedimentation; CFs were then cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and incubated at 37°C, 10% CO₂. CFs were grown to confluency, split to appropriate-sized culture dishes, and serum-starved for 24 hours prior to use in experiments.

cAMP Radioimmunoassay. CFs were plated in 24-well plates (30,000 cells/well) and serum-starved for 48 hours prior to cAMP quantification by radioimmunoassay, as previously described (Swaney et al., 2005). After serum starvation, CFs were incubated with 200 μ M 3-isobutyl-1-methylxanthine (IBMX) for 20 minutes (when applicable) and then with GPCR agonists of interest, forskolin (Fsk) (Ascent, Cambridge, MA), and isoproterenol (Iso), beraprost (Bera), or adrenomedullin (ADM) (Sigma-Aldrich, St. Louis, MO), for 10 minutes. Incubations were terminated by addition of 7.5% trichloroacetic acid. Radioimmunoassay (Liu et al., 2004) was used to quantify cellular cAMP as compared with a standard curve and normalized to total protein content per sample, as determined by a Bradford assay (Bio-Rad, Hercules, CA).

Quantitative Real-Time Polymerase Chain Reaction. Total RNA was extracted from CFs using the RNeasy Mini kit (Qiagen, Alameda, CA) according to the manufacturer's instructions. cDNA was synthesized using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA), per the manufacturer's instructions. Real-time polymerase chain reaction (qPCR) was performed using 8 ng cDNA, 0.5 μ M forward and reverse primers, and qPCR Mastermix Plus enzyme kit (Eurogentec, San Diego, CA). Primers for polymerase chain reaction amplification (Table 1) were designed based on the nucleotide sequences of the respective gene targets using Primer3Plus

software. When possible, each forward and reverse primer set was designed between multiple exons. Primers for rat ACs were designed by QuantiTect Primer Assays (Qiagen). Amplification efficiency of each primer pair was tested prior to analysis, and relative gene expression levels were determined using the $\Delta\Delta$ CT method with 18S as the reference gene (Pfaffl, 2001).

Immunofluorescence Microscopy for α -SMA Protein. The abundance and organization of α -SMA into stress fibers was assessed by immunofluorescence microscopy. CFs were cultured in a three-dimensional gel consisting of rat tail collagen type I (BD Bioscience, San Jose, CA). After treatment, CFs were fixed in 10% buffered formalin for 15 minutes and permeabilized with 0.3% Triton X-100/phosphate-buffered saline. Primary antibodies for α -SMA (diluted 1:200; Sigma-Aldrich) and phalloidin (diluted 1:100; Life Technologies, Grand Island, NY) were used, followed by either ALEXA Fluor 488- or 555-conjugated secondary antibody (Life Technologies). Coverslips were mounted in gelvatol and images captured using a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY).

Immunoblotting. Whole cell lysates were prepared in 150 mM Na₂CO₃ buffer (pH 11) and homogenized by sonication. Equal amounts of protein (Bradford assay) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidenedifluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in phosphate-buffered saline Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody [α -SMA; Sigma-Aldrich; plasminogen activator inhibitor (PAI)-1; BD Bioscience; AC5/6, Santa Cruz; phosphodiesterase (PDE)-8A, Fabgennix; or glyceraldehyde-3-phosphate dehydrogenase, (Abcam)] overnight at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and enhanced chemiluminescence reagent (Amersham Pharmacia, Pittsburgh, PA). Bands were compared with molecular weight standards to confirm migration of proteins at the appropriate size.

Three-Dimensional Collagen Gel Contraction Assay. CFs were suspended at 100,000 cells/gel in a solution containing 2.5 mg/ml collagen type I (BD Biosciences) and cast into a 24-well plate. Collagen gel disks were detached from the sides of the well, and 0.5 ml of serum-free media was added to the well in the presence or absence of 10 ng/ml transforming growth factor (TGF)- β 1. Drug treatments were added at the concentrations and times indicated. Images of collagen gel contraction were taken at days 0, 2, 4, and 6 with a Sencam QE imager and analyzed by ImageJ (NIH, Bethesda, MD).

Statistical Analysis. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA). Numerical values are presented as mean $\pm$ S.E.M. Analysis of numerical data was done

TABLE 1
Primer sequences for qPCR

Gene	Forward 5'–3'	Reverse 5'–3'
PDE1A	tttgtatcggaagtcaacg	agctgcttccccatagtt
PDE1B	cagcccttgacagatgatga	caatggacatctggttggtg
PDE1C	ggcccttgaagtgggttaca	gcagccgagaagattattgc
PDE2A	gagagaaagtctgggagaaga	ccgtgctgtgtagtggaa
PDE3A	aagacatggggctcttgaa	gaatcgctgtgtgtgaga
PDE3B	cagtggcaagatgttcagga	aaggcttgggtcaatcagaa
PDE4A	gcaactctcactcttaaccaatgt	aggccaatccagctcct
PDE4B	caagcagaacgatgtggaaa	tgactccaagcgtgagatg
PDE4D	ggaggacaatcgtgagtgg	tcagtgtctgactgccatc
PDE5A	agttgtcttgacagcctat	ccaagccacagaagatgaca
PDE7B	ctcctaccgttcattgact	tgtcaaagtcacattcca
PDE8A	agttggcatttggagagggtg	ttgcatactggatgatgtgg
PDE8B	atcacaggcagaccgtaac	ctgcatggagaagaggaagg
PDE9A	cctttgatgtctggcttgg	gagtgatgtgggtgatgct
PDE10A	agcgatgaagtcctcaa	ccctcgaattacctctcc
PDE11A	aactgatgtcccaaaagtgc	tcgtgacattcacaggaag
Collagen I α 1	ctggcaagaacggagatgat	caccatccaaccactgaaa
18S	gtaaccggtgaaccctt	ccatccaatcggtatagtcg

using analysis of variance with Tukey's test or Student's *t* test, where appropriate. $P < 0.05$ was considered significant.

Results

cAMP Accumulation Is Decreased in Cardiac Myofibroblasts Compared with CFs. Treatment of adult rat ventricular CFs with several different GPCR agonists or with the AC activator Fsk increased cellular cAMP levels in a concentration-dependent manner (Fig. 1A). The rank order of the maximal responses generated by those stimulants of cAMP formation were as follows: Fsk > Iso (a β -adrenergic receptor agonist) > Bera (a prostaglandin I_2 receptor agonist) = ADM (a calcitonin/adrenomedullin receptor agonist). Butaprost, a prostaglandin E_2 receptor agonist, did not increase cAMP accumulation in CFs.

We next generated profibrogenic myofibroblasts to compare their cAMP accumulation with that of CFs. To generate

myofibroblasts, we incubated the adult rat ventricular CFs with 10-ng/ml TGF- β 1 for 48 hours, a treatment protocol that transforms CFs to myofibroblasts (Leask and Abraham, 2004; Bujak and Frangogiannis, 2007; Yokoyama et al., 2008). CFs and myofibroblasts were then incubated with 200 μ M IBMX, a nonselective cyclic nucleotide phosphodiesterase inhibitor, prior to stimulation with Fsk or the GPCR agonists (Iso, Bera, or ADM, each at 10 μ M, a saturating concentration for each agonist) (Fig. 1B). Fsk and Iso increased cAMP accumulation in CFs by ~55- and 70-fold, respectively, but had diminished responses in myofibroblasts, in which cAMP accumulation only increased by 22- and 15-fold, respectively. Decreased cAMP accumulation by myofibroblasts also occurred in response to Bera and ADM. The potential ability of IBMX to inhibit adenosine receptors did not contribute to these responses as rolipram, a PDE4-selective antagonist that does not inhibit adenosine receptors, elicited similar results (data not shown), indicating that the enhanced cAMP with addition of IBMX occurs via PDE inhibition. Thus, myofibroblasts accumulate substantially less cAMP in response to incubation with Fsk and Gs-coupled GPCR agonists.

Myofibroblasts Have Altered Profiles of Expression of AC and PDE Isoforms. To assess the basis for the decreased cAMP accumulation in myofibroblasts, we quantified the expression of AC and PDE isoforms (using real-time qPCR) by CFs and myofibroblasts. The rank-order of expression of AC isoforms was as follows: AC6 > AC5 > AC3 > AC4 > AC7 > AC9 > AC8, with AC1 and AC2 not detected. Compared with CFs, myofibroblasts had a statistically significant decrease in expression of AC6 and AC5, the two highest expressed AC isoforms, and also of AC9 (decreases of 28%, $P < 0.01$; 27%, $P < 0.05$; and 57%, $P < 0.05$, respectively) (Fig. 2A).

The cyclic nucleotide PDE profile of CFs revealed the following rank-order of expression of the six highest expressed PDE isoforms: 8A, 1A, 4D, 3B, 4A, and 10A, with mRNA for several other PDEs (PDEs 2A, 3A, 5A, and 7A) expressed at lower levels (Fig. 2B). Compared with CFs, myofibroblasts had increased expression of multiple PDE isoforms. PDE8A, the most abundant PDE isoform in adult rat CFs, increased by 13% ($P < 0.01$) in myofibroblasts. Expression of other abundant PDE isoforms: 4D, 4A, and 10A, was increased relative to CFs by 22%, 38%, and 45%, respectively ($P < 0.05$). One exception was PDE1A, whose expression was decreased by 45% ($P < 0.01$) in myofibroblasts. PDEs 1B, 1C, 7B, 9A, and 11A were detected at very low levels and their expression was unchanged in myofibroblasts, while expression of PDE8B was not detected.

Protein expression of AC5/6 and PDE8A, the most abundant of their respective isoforms, was assessed via immunoblotting (Fig. 2C). Upon TGF- β stimulation, AC5/6 protein expression decreased by 90% ($P < 0.05$), while PDE8A protein expression increased by 2.7-fold ($P < 0.05$). TGF- β treatment also increased CF α -SMA protein expression by 2.5-fold ($P < 0.01$). Overall, the decrease in expression of the most abundant ACs (AC5/6) in myofibroblasts, accompanied by an increase in expression of several highly abundant PDEs, likely accounts for the decreased cAMP accumulation of myofibroblasts in response to Fsk or Gs-coupled GPCR stimulation (Fig. 1). Since increased cellular concentrations of cAMP can blunt the transformation of CFs to myofibroblasts (Swaney et al., 2005; Yokoyama et al., 2008; Miller et al., 2011), we infer that the decrease in the ability of myofibroblasts to generate cAMP and

Fig. 1. cAMP accumulation is decreased in myofibroblasts compared with CFs. (A) CFs were incubated with increasing concentrations of Fsk or GPCR agonists (Iso, Bera, ADM, and butaprost). Cellular concentrations of cAMP increased in a concentration-dependent manner in response to Fsk, Iso, Bera, and ADM. (B) CFs (open bars) or myofibroblasts (CFs stimulated with 10 ng/ml TGF- β 1 for 48 hours; solid bars) were incubated with saturating concentrations of GPCR agonists or Fsk (10 μ M) in the presence of 200 μ M IBMX. Myofibroblasts generated significantly less cAMP compared with CFs: Fsk increased cAMP concentrations 22-fold in myofibroblasts versus 55-fold in CFs, while Iso increased cAMP concentrations 15-fold in myofibroblasts versus 70-fold in CFs. Data are presented as mean $\pm$ S.E.M. of four independent experiments. ** $P < 0.01$. CTRL, control.

Fig. 2. TGF- β 1-induced myofibroblasts have altered profiles of AC and PDE isoform expression. (A) Myofibroblasts (CFs incubated with 10 ng/ml TGF- β 1 for 48 hours; solid bars) decreased their expression of several AC isoforms. AC6, AC5, and AC9 mRNA expression were decreased in myofibroblasts compared with CFs (open bars) by 28, 27, and 57%, respectively. (B) Four of the six most highly expressed PDE isoforms in CFs increase expression following myofibroblast transformation: PDE 8A, 4D, 4A, and 10A by 13, 22, 38, and 45%, respectively. In contrast, PDE1A decreased in expression by 45% in myofibroblasts. (C) AC5/6 protein expression decreased by 90%, while PDE8A and α -SMA expression increased by 2.7- and 2.5-fold, respectively, in myofibroblasts incubated with 10 ng/ml TGF- β for 72 hours. Data are presented as mean $\pm$ S.E.M. of at least three independent experiments. * P < 0.05; ** P < 0.01. CTRL, control.

increased ability to hydrolyze cAMP contribute to the myofibroblastic phenotype.

Incubation with Forskolin Can Reverse the Enhanced Synthesis of Collagen, Collagen Gel Contraction, and Expression of α -SMA and Plasminogen Activator Inhibitor-1 of Cardiac Myofibroblasts. Previous data have indicated that incubation of CFs with agents that increase cellular cAMP levels when cells are also incubated with TGF- β 1 blocks the conversion of CFs to myofibroblasts (Swaney et al., 2005; Yokoyama et al., 2008; Miller et al., 2011). To test whether raising cAMP levels in myofibroblasts can reverse an already-developed profibrotic state, we treated myofibroblasts (CFs that were incubated with TGF- β 1 for 4 or 6 days) (Fig. 3A) with Fsk (10 μ M) for 48 hours prior to quantifying the expression of collagen I α 1 (Coll α 1) mRNA expression. Incubation with Fsk reversed the increases in Coll α 1 expression in cells that had been incubated with TGF- β 1 for either 4 or 6 days (Fig. 3B). The most dramatic effect of the ability of Fsk to reverse Coll α 1 expression occurred in cells incubated with TGF- β 1 for 6 days: Fsk reduced expression of Coll α 1 by 44% (P < 0.001), i.e., to a level akin to that of untreated CFs.

To test whether this reversal in Coll α 1 expression could be promoted by activation of protein kinase A (PKA), we used a similar protocol and treated the myofibroblasts with the selective PKA agonist *N*⁶-phenyladenosine-cAMP (*N*6-Phe-cAMP) (from Biolog/Axxora, Farmingdale, NY). Treatment with *N*6-Phe-cAMP inhibited Coll α 1 mRNA expression (P < 0.001; Fig. 3C). Together, these findings imply that the antifibrotic effects of cAMP can occur via PKA activation.

As a second approach to assess reversal of the myofibroblastic phenotype, we seeded CFs into a deformable three-dimensional collagen gel to quantify their contractile abilities. Within 2 days of incubation with TGF- β 1, the cells had a statistically significant increase in gel contraction (Fig. 4A). This increase progressed over the 6-day period of the experiment. By day 6, gels containing TGF- β 1-treated CFs had a 20% (P < 0.01) smaller diameter than did CFs in gels without TGF- β 1. Incubation with Fsk for the last 48 hours of the 6-day incubation with treatment with TGF- β 1 significantly (P < 0.01) reversed the extent of collagen gel contraction (Fig. 4A).

The expression of α -SMA confers the contractile phenotype to CFs (Hinz et al., 2001). We used immunostaining to assess the expression of α -SMA in CFs cultured in the three-dimensional

Fig. 3. cAMP, acting via PKA, reverses TGF- β 1-induced myofibroblast differentiation. (A) Treatment regimen showing the duration of TGF- β 1 (10 ng/ml), Fsk (10 μ M), or N6-Phe-cAMP (N6; 50 μ M) treatment. (B) Incubation of CF with TGF- β 1 for 6 days increased collagen 1 α 1 mRNA expression by 38%. Coll α 1 expression decreased 44% following Fsk treatment for the last 48 hours of the 6-day treatment regimen. (C) N6-Phe-cAMP, a PKA-selective analog, reversed Coll α 1 mRNA expression stimulated by both 4- and 6-day incubation with TGF- β 1. Data are presented as mean $\pm$ S.E.M. of four independent experiments. * P < 0.05; *** P < 0.001. CTRL, control; mRNA, messenger RNA; pre, pretreatment.

collagen gels. The 6-day incubation with TGF- β 1 stimulated α -SMA expression and converted the CFs to a myofibroblastic morphology (Fig. 4B). Treatment with Fsk during the final 2 days of the 6-day incubation with TGF- β 1 reversed this morphologic appearance and reduced the content of α -SMA-positive stress fibers. Consistent with these findings, Fsk treatment decreased the TGF- β 1-stimulated expression of α -SMA protein and that of plasminogen activator inhibitor-1, a profibrotic marker that inhibits the activation of plasmin and matrix metalloproteinases (Ghosh and Vaughan, 2012) (Fig. 4C).

Discussion

The data presented here provide an explanation for the lower cAMP accumulation of CFs that undergo conversion to cardiac myofibroblasts: altered expression of AC isoforms (with decreased expression of major AC isoforms) and of PDE isoforms (with increased expression of several isoforms known to hydrolyze cAMP, i.e., PDE 8A, 4A, 4D, and 10A) (Bender and Beavo, 2006). We found that several GPCR agonists and Fsk have a reduced ability to increase cellular cAMP concentrations in myofibroblasts compared with CFs. Consistent with the role of cAMP in attenuating the myofibroblastic phenotype, decreased AC5/6 expression has been noted in rat CFs following myocardial infarction (Swaney et al., 2007), and overexpression of these isoforms can prevent the conversion of CFs (Swaney et al., 2005) and pulmonary fibroblasts (Kolodnick et al., 2003; Liu et al., 2004; Dunkern et al., 2007) to myofibroblasts. Antifibrotic roles for cAMP have also been noted in studies of hepatic (Windmeier and Gressner, 1997; Davaille et al., 2000), renal (Heusinger-Ribeiro et al., 2001), and dermal (Parekh et al., 2007) fibroblasts. Thus, in addition to the heart, the antifibrotic action of cAMP may occur in fibroblasts of other organs prone to fibrosis. We and others have reported that antifibrotic effects of cAMP can occur via actions of both PKA and the exchange protein activated by cAMP, and have observed an inhibition of expression of the exchange protein activated by cAMP by incubation of cardiac fibroblasts with profibrotic agonists (Huang et al., 2008; Yokoyama et al., 2008). In contrast to the data shown here, Miller et al. (2011) have reported that PDE1A is the major isoform increased in cardiac myofibroblasts and that its inhibition can limit fibrosis following myocardial infarction. However, the analysis by those authors of expression of PDE isoforms was conducted in neonatal rat CFs stimulated with angiotensin II, while our studies were conducted with adult rat CFs. Explanations for the different results that we and Miller et al. find may reflect differences in PDE isoform expression between neonatal and adult CFs or perhaps differences in myofibroblasts that are generated by CFs incubated with angiotensin II rather than TGF- β 1. Regardless of the explanation, our data suggest that multiple PDE isoforms are potentially attractive therapeutic targets to blunt cardiac fibrosis.

Important results of the current study are that treatment of TGF- β 1-generated cardiac myofibroblasts with Fsk reverses multiple features of the myofibroblastic phenotype and that treatment with N6-Phe-cAMP has similar effects to Fsk to decrease Coll α 1 expression. Such phenotypic reversal is thus a potential mechanism for attenuation of abnormal wound healing responses and tissue fibrosis and occurs independent of (or in parallel with) myofibroblast apoptosis (Huang et al., 2009; Insel et al., 2012). While other reports describe the ability of cAMP to inhibit the conversion of fibroblasts to myofibroblasts (Schiller et al., 2010), the current findings indicate that cAMP-dependent signaling can reverse fibrotic activity by decreasing the expression of collagen, α -SMA, and plasminogen activator inhibitor-1, and by reducing contractility of myofibroblasts stimulated by TGF- β 1. These results thus identify a cAMP-dependent action to reverse fibrosis and are consistent with recent observations noted in pulmonary myofibroblasts treated with prostaglandin E2, which likely acts via Gs-linked GPCRs (Garrison et al., 2013). Therefore, agents targeted to GPCRs, ACs, and PDEs preferentially expressed by CFs and myofibroblasts

Fig. 4. cAMP reverses α -SMA and PAI-1 expression and contractility in three-dimensional collagen gels of TGF- β 1-induced myofibroblasts. (A) TGF- β 1 incubation of CF for 2–6 days increased the contraction of collagen gels. Gels at day 6 had 20% less surface diameter with TGF- β treatment. Addition of Fsk for the final 48 hours significantly reversed the contractile phenotype. (B) Incubation with TGF- β 1 for 6 days increased expression of α -SMA–positive stress fibers of CFs grown in three-dimensional culture. Incubation with Fsk for the last 48 hours diminished α -SMA expression (red: F-actin, green: α -SMA, blue: DAPI; 40 $\times$ magnification). (C) Incubation with Fsk reduced expression of α -SMA and PAI-1 protein in CFs incubated in the absence (control/Fsk) or presence (TGF- β 1, TGF- β 1 + Fsk) for 4 days and are shown for triplicate samples. Data are presented as mean $\pm$ S.E.M. of three independent experiments. * P < 0.05; ** P < 0.01. CTRL, control; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PAI-1, plasminogen activator inhibitor-1.

(Snead and Insel, 2012) and to the activation of post-cAMP-dependent signaling pathways (Yokoyama et al., 2008; Schiller et al., 2010) have the potential to be novel treatment strategies to prevent and potentially reverse cardiac fibrosis, and perhaps fibrosis in other tissues.

Authorship Contributions

Participated in research design: Yokoyama, Patel, Insel.

Conducted experiments: Aroonsakool, Yokoyama, Lu.

Performed data analysis: Yokoyama, Lu, Aroonsakool.

Wrote or contributed to the writing of the manuscript: Lu, Aroonsakool, Yokoyama, Patel, Insel.

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