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Authors

Swaney, J S
Roth, D M
Olson, E R
et al.

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Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase

James S. Swaney*†, David M. Roth‡, Erik R. Olson§, Jennifer E. Naugle§, J. Gary Meszaros§, and Paul A. Insel*¶

*Departments of Pharmacology and Medicine, †Graduate Program in Molecular Pathology, and ‡Veterans Affairs Medical Center of San Diego and Department of Anesthesiology, University of California at San Diego, La Jolla, CA 92093; and §Department of Physiology and Pharmacology, Northeastern Ohio Universities College of Medicine, Rootstown, OH 44272

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Transformation of fibroblasts to myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA) and production of extracellular matrix (ECM) components, is a key event in connective tissue remodeling. Approaches to inhibit this transformation are needed in tissues, such as the heart, where excessive ECM production by cardiac fibroblasts (CFs) causes fibrosis, myocardial stiffening, and cardiac dysfunction. We tested whether adenylyl cyclase (AC) activation (increased cAMP levels) modulates the transformation of adult rat CF to myofibroblasts, as assessed by immunofluorescent microscopy, immunoblotting, and collagen synthesis. A 24-h incubation of CF with TGF-β or angiotensin II increased α-SMA expression, which was inhibited by the AC agonist forskolin and a cAMP analog that activates protein kinase A. Treatment with forskolin blunted serum-, TGF-β-, and angiotensin II-stimulated collagen synthesis. CFs engineered to overexpress type 6 AC had enhanced forskolin-promoted cAMP formation, greater inhibition by forskolin of TGF-β-stimulated α-SMA expression, and a decrease in the EC_{50} of forskolin to reduce serum-stimulated collagen synthesis. The AC stimulatory agonist adrenomedullin inhibited collagen synthesis in CF that overexpressed AC6 but not in controls. Thus, AC stimulation blunts collagen synthesis and, in parallel, the transformation of adult rat CF to myofibroblasts. AC overexpression enhances these effects, ''uncovering'' an inhibition by adrenomedullin. These findings implicate cAMP as an inhibitor of ECM formation by means of blockade of the transformation of CF to myofibroblasts and suggest that increasing AC expression, thereby enhancing cAMP generation through stimulation of receptors expressed on CF, could provide a means to attenuate and prevent cardiac fibrosis and its sequelae.

cardiac fibroblast | cyclic AMP | extracellular matrix | fibrosis | heart failure

Fibroblasts are widely recognized as a critical cell type involved in wound healing and tissue repair. Less generally appreciated is the notion that the transformation of fibroblasts to myofibroblasts is a key, perhaps essential, event for the cells to perform those functions (1–4). Myofibroblasts are smooth muscle-like fibroblasts that express α-smooth muscle actin (α-SMA) and contain a contractile apparatus composed of actin filaments and associated proteins organized into prominent stress fibers (3, 5). In addition to their normal role in tissue homeostasis and repair, altered number and function of myofibroblasts have been implicated in diseases with increased extracellular matrix (ECM) deposition and resultant fibrosis, such as those involving liver, skin, lung, and kidney (6–8).

Myofibroblast formation is controlled by a variety of growth factors, cytokines, and mechanical stimuli (1, 3, 9). TGF-β_1 is one such factor that stimulates both myofibroblast formation and collagen production (3, 10). Such results imply that agents able to inhibit fibroblast-to-myofibroblast transformation may provide a means to inhibit maladaptive tissue remodeling in response to profibrotic stimuli.

Cardiac fibroblasts (CFs), the most abundant cell type in the heart (constituting two-thirds of the total cell population), are responsible for ECM deposition and create the scaffold for cardiac myocytes (11). Cardiac fibrosis is characterized by overproduction of ECM, predominantly collagen types I and III, into the interstitial and perivascular space (12). Excessive collagen deposition leads to myocardial stiffening, impaired cardiac relaxation and filling (diastolic dysfunction), and overload of the heart, perhaps as a consequence of transformation of quiescent fibroblasts, responsible for basal ECM homeostasis, to activated myofibroblasts (4).

cAMP, a ubiquitous second messenger produced in response to activation of adenylyl cyclase (AC), influences growth, death, and differentiated functions of many cell types, primarily by promoting protein phosphorylation via cAMP-dependent protein kinase (PKA). G protein-coupled receptor (GPCR) agonists that signal through G_s to activate AC and stimulate cAMP production can inhibit collagen synthesis (13–15). Forskolin and GPCR agonists that stimulate AC were recently shown to inhibit serum or TGF-β-stimulated α-SMA expression and collagen formation by human lung fibroblasts (16, 17). Although such data suggest that increased cAMP production inhibits collagen synthesis via an inhibition of fibroblast-to-myofibroblast transformation, no direct evidence has linked the two events and no studies have examined the effects of increased cAMP on myofibroblast formation in the heart.

We hypothesized that the inhibition of collagen synthesis by cAMP results from its ability to blunt myofibroblast formation. Here, we use both pharmacological activators and gene transfer of AC to show that AC activation inhibits myofibroblast formation and, in parallel, reduces collagen synthesis by adult rat CF, effects that are strikingly enhanced in cells that overexpress an AC isoform. These results document a key antifibrotic action of cAMP in adult CF and suggest that overexpression of AC6 may provide a means to blunt cardiac fibrosis.

Materials and Methods

Materials. Antibodies for α-SMA and vimentin were obtained from Sigma. Antibodies for collagen type I was obtained from Rockland (Gilbertsville, PA). Alexa Fluor 647 Phalloidin probe for F-actin was obtained from Molecular Probes. Radiolabeled chemicals were obtained from PerkinElmer. All other drugs and reagents were obtained from Sigma.

Abbreviations: α-SMA, α-smooth muscle actin; ECM, extracellular matrix; CF, cardiac fibroblast; AC, adenylyl cyclase; GPCR, G protein-coupled receptor; IBMX, isobutylmethylxanthine; Ang II, angiotensin II.

¶To whom correspondence should be addressed. E-mail: pinsel@ucsd.edu.

Isolation and Culture of Adult Rat CFs. CFs were isolated from adult Sprague–Dawley rats (250–300 g, male) by using a modification of published methods (18). CFs were separated from cardiac myocytes by gravity separation and grown to confluency on 10-cm cell culture dishes at 37°C with 90% air/10% CO_2 in growth media (DMEM/10% FBS/1% penicillin/1% streptomycin). For overexpression studies, CFs were incubated with ≈100 viral particles per cell of an adenovirus containing either *lacZ* (control) or the murine AC6 gene as described (17, 19). Cells were incubated with adenovirus for 18–24 h before stimulation with serum or agonists of interest. Experiments using LacZ adenovirus and β-galactosidase staining indicated that the efficiency of the adenoviral gene transfer was >80% (data not shown).

Collagen Synthesis Assay. We assessed collagenase-sensitive [^{3}H]proline incorporation (using a modification of established methods; ref. 20) to measure collagen synthesis. CFs cultured on 12-well plates were serum-starved for 48 h and then assayed by adding 1 μCi/ml [^{3}H]proline (PerkinElmer Life Sciences, 1 Ci = 37 GBq) along with drugs of interest and, where indicated, 0% or 2.5% FBS for 48 h. Cells were removed from tissue culture dishes with trypsin and protein was precipitated overnight with 20% trichloroacetic acid (TCA). After centrifugation, pellets were washed three times with 1.0 ml 5% TCA plus 0.01% proline, then dissolved with 0.2 M NaOH, and the solutions were titrated to neutral pH with 0.2 M HCl. Collagenase II (100 μl, 2 mg/ml) in Tris-$CaCl_2$-*N*-ethylmaleimide buffer was added to each tube, and samples were incubated for 1 h at 37°C. Samples were then placed on ice, proteins were precipitated with 10% TCA for 1 h and centrifuged at 18 × *g* for 10 min, and radioactivity of the supernatant fraction was determined by liquid scintillation counting.

Immunoblot Analysis. Whole cell lysates were prepared in cell lysis buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Igepal CA-630, protease inhibitor mixture) and homogenized by sonication. Equal protein (assayed by using a dye-binding reagent, Bio-Rad) amounts of the lysates were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane by electroblotting. Membranes were blocked in 20 mM PBS Tween (1%) containing 1.5% nonfat dry milk and incubated with primary antibody overnight at 4°C. Bound antibodies were visualized by using secondary antibodies with conjugated horseradish peroxidase (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia). All displayed bands were compared to molecular weight standards to confirm that the proteins migrated at the appropriate size. Antibody specificity was confirmed by positive and negative expression of α-SMA by rat aortic smooth muscle cell and rat neuroepithelial cell lysates, respectively (data not shown).

Immunohistochemistry. CFs cultured on 12-mm glass coverslips were serum-starved for 48 h and then stimulated for 24 h in media alone (control) or with drugs of interest. CF were then fixed in 10% buffered formalin for 10 min, washed twice with PBS, and permeabilized in 0.3% Triton X-100/PBS for 10 min. CFs were washed twice with PBS/Tween 20 (0.1% Tween), incubated with 1% BSA/PBS/Tween for 10 min and then with an FITC-conjugated α-SMA antibody (1:1,000) for 1–2 h. After two washes with PBS/Tween and incubation with DAPI (1:5,000) for 20 min, CFs were washed for 10 min with PBS and mounted in gelvatol for microscopic imaging. All incubation and wash steps were conducted at room temperature.

Image Deconvolution Analysis. Deconvolution images were obtained as described (21). Images were captured with a DeltaVision deconvolution microscope system (Applied Precision, Issaquah, WA.) The system includes a Photometrics charge-coupled device mounted on a Nikon TE-200 inverted epifluorescence microscope. In general, between 30 and 80 optical sections spaced by ≈0.1–0.3 μm were taken. Exposure times were set such that the camera response was in the linear range for each fluorophore. Lenses included ×100 (numerical aperture 1.4), ×60 (numerical aperture 1.4), and ×40 (numerical aperture 1.3) magnifications. The data sets were deconvolved and analyzed by using SOFTWORX software (Applied Precision) on a Silicon Graphics Octane workstation.

cAMP Production. CFs grown on 24-well plates were serum-starved for 48 h and assayed for cAMP production by 10-min incubation with 0.2 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, followed by addition of drugs of interest for an additional 10 min. Assays were terminated by aspiration of media and addition of 250 μl of ice-cold trichloroacetic acid (7.5%) to each well. RIA was used to quantitate cAMP (14, 17); results were normalized to the amount of protein per sample, as determined by a colorimetric protein assay (Bio-Rad).

Data Analysis. Statistical comparisons and graphical representation were performed by using PRISM 3.0 (GraphPad, San Diego). Statistical significance was set at $P < 0.05$.

Results

Adenylyl Cyclase Activation Inhibits Total Collagen Synthesis and Expression of Collagen I Protein. We used a collagenase-sensitive [^{3}H]proline incorporation assay as a measure of collagen synthesis to investigate the effects of forskolin or adrenomedullin (two activators of AC in CF; refs. 14 and 15), CPT-cAMP (a cAMP analog that is selective for PKA activation; ref. 22), or CPT-Me-cAMP (a PKA-independent cAMP analog; ref. 23) on serum-stimulated collagen synthesis by adult rat CF. Forskolin (10 μM) or CPT-cAMP (100 μM), but not adrenomedullin (1 μM) or CPT-Me-cAMP, prominently inhibited 2.5% FBS-stimulated collagen synthesis (Fig. 1*A*). Because serum contains multiple hormones and growth factors that might promote collagen synthesis, we examined the effects of forskolin in response to two key profibrotic agents: angiotensin II (Ang II) and TGF-β, both tested under serum-free conditions (2, 24). Forskolin significantly ($P < 0.05$) inhibited collagen synthesis in response to both these profibrotic agents, as well as to aldosterone (data not shown), thus showing that an increase in cAMP inhibits collagen formation in response to multiple agents that promote fibrosis (Fig. 1 *A* and *B*). The results with CPT-cAMP vs. those with CPT-Me-cAMP imply that this effect occurs via PKA activation. To determine the effects of AC activation on formation of a specific collagen found in the heart, we measured expression of collagen type I. Similar to results of collagenase-sensitive [^{3}H]proline incorporation (Fig. 1 *A* and *B*), forskolin inhibited Ang II- and TGF-β-stimulated collagen type I expression (Fig. 1*C*). Taken together, these data demonstrate that increases in cAMP act via PKA to inhibit collagen production of adult rat CFs stimulated by serum or profibrotic agents.

Phosphodiesterase Inhibition Blunts Spontaneous Myofibroblast Differentiation and Forskolin Attenuates TGF-β-Stimulated Myofibroblast Transformation of Adult Rat CFs. Cell culture conditions have been shown to be critical when conducting *in vitro* studies of fibroblast-to-myofibroblast differentiation. Previous studies have shown that fibroblasts rapidly differentiate into myofibroblasts (as indicated by increased α-SMA expression) when cultured on rigid substrates and/or in culture media containing serum and when plated at low density (25–27). We found that untreated CFs spontaneously undergo this differentiation under

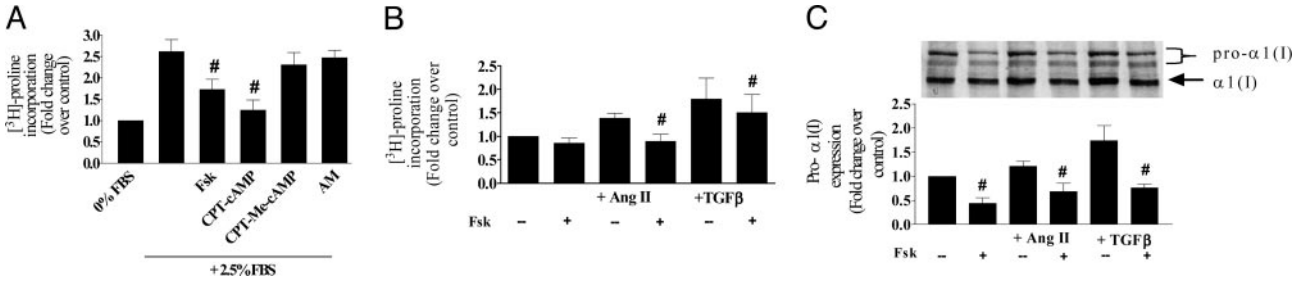

Fig. 1. Forskolin and CPT-cAMP blunt collagen synthesis of adult rat cardiac fibroblasts. Shown are collagenase-sensitive [^{3}H]proline incorporation by fibroblasts grown for 48 h in serum-free media and then stimulated for 48 h with serum-free media (control) or 2.5% in the absence or presence of forskolin (Fsk, 10 μM), CPT-cAMP (100 μM), CPT-cAMP (100 μM), or adrenomedullin (AM, 1 μM) (*A*) or serum-free media alone or with Ang II (100 nM) or TGF-β (10 ng/ml) in the presence or absence of Fsk (10 μM) (*B*). (*C*) Cardiac fibroblasts were cultured and serum-starved as noted above and subsequently treated with the indicated agents for 48 h before protein isolation (see *Materials and Methods* for lysis buffer composition). Densitometry and quantitation for *C* was performed on the pro-α1 (I) band (top band). Values represent mean ± SEM of at least four experiments performed in triplicate and compared by using a paired *t* test. #, $P < 0.05$ in response to forskolin or CPT-cAMP.

normal culture conditions (see *Materials and Methods*), as shown by enhanced α-SMA expression in CF between passages 2 and 5 (Fig. 2*A*). Thus, adult rat CFs convert to myofibroblasts during early passage when plated on plastic tissue culture dishes in serum-containing media, emphasizing the importance culture conditions when conducting studies of the effects of exogenous agents on myofibroblast formation. For this reason, all differentiation studies were conducted under serum-free conditions using low-passage CFs (passage $\leq$2) that were plated at relatively high density ($\approx$200 cells per mm^2).

We hypothesized that increases in cellular cAMP would inhibit myofibroblast differentiation. Cellular cAMP levels are determined in part by the expression and activity of phosphodiesterases (PDEs), which catalyze the hydrolysis of cAMP to AMP. To determine whether endogenous levels of cAMP are sufficient to prevent myofibroblast transformation, we examined α-SMA expression by CFs maintained under normal culture conditions for up to 72 h in the absence or presence of IBMX (200 μM), a nonspecific PDE inhibitor. We found that α-SMA expression increases in a time-dependent manner under normal culture conditions and that this increase is blunted by addition of IBMX (Fig. 2*B*).

To determine the impact of AC activation on myofibroblast formation, we investigated the effects of forskolin on basal (0% FBS), TGF-β-, or Ang II-stimulated α-SMA expression (Fig. 2 *C* and *D*). Immunohistochemical analysis revealed enhanced

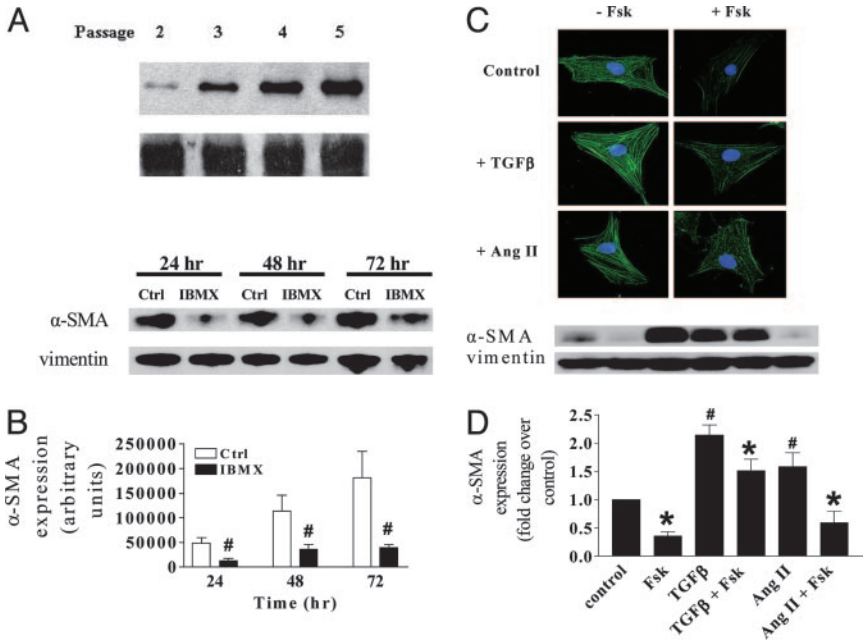

Fig. 2. α-SMA expression in CF of different passage number and in response to TGF-β and Ang II. (*A*) α-SMA expression was measured by immunoblot analysis using adult rat CF at passages 2–5. CFs at passage 1 were split from confluent monolayers at a ratio of 1:3 and maintained in the presence of 10% FBS for 48 h before collection of whole cell lysates. After immunoblotting for α-SMA (*Upper*), the blot was stripped and reprobed with GAPDH as a loading control (*Lower*). (*B*) α-SMA expression was measured by using CF maintained in the presence of 10% FBS, with and without isobutylmethylxanthine (IBMX; 200 μM). After immunoblotting for α-SMA, the blot was stripped and reprobed with vimentin as a loading control, and quantitation of α-SMA immunoreactive bands was performed. (*C*) Immunohistochemistry was performed by using CF (passage 2) grown for 48 h in serum-free media and then for 24 h in serum free media alone (control), Ang II (100 nM), or TGF-β (10 ng/ml) in the presence or absence of Fsk (10 μM). Cells were stained for α-SMA (green) and nuclear staining of DNA with DAPI (blue). (*D*) α-SMA protein expression with these same treatments was verified by immunoblot. Data are expressed as fold change relative to control. Values represent mean ± SEM of at least three experiments and compared by using a paired *t* test and ANOVA with post hoc multiple comparison tests. #, $P < 0.05$ as compared to control; *, $P < 0.05$ in response to forskolin.

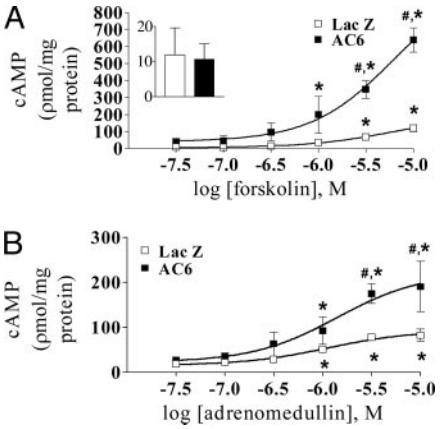

Fig. 3. Overexpression of AC6 enhances forskolin- or adrenomedullin-stimulated cAMP production. cAMP production was measured by RIA using CF grown for 48 h in serum-free media and then stimulated for 10 min with 2.5% FBS alone (*A Inset*) or in the presence of the indicated concentrations of forskolin (*A*) or adrenomedullin (*B*). CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 h before stimulation. Values represent mean ± SEM of at least three experiments and were compared by using a paired *t* test and ANOVA. *, $P < 0.05$ compared to 2.5% FBS; #, $P < 0.05$ compared to LacZ cells.

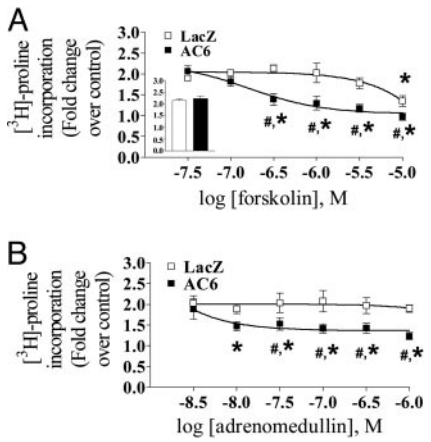

Fig. 4. Overexpression of AC6 enhances forskolin- and adrenomedullin-promoted inhibition of collagen synthesis. Collagenase-sensitive [^{3}H]proline incorporation was measured by using adult rat CF grown for 48 h in serum-free media and then stimulated for 48 h with 2.5% FBS in the absence (*A Inset*) or presence of the indicated concentrations of forskolin (*A*) or adrenomedullin (*B*). CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 h before stimulation. Data are normalized for [^{3}H]proline incorporation into cells grown under serum-free conditions. Values represent mean ± SEM of at least three experiments and were compared by using a paired *t* test and one-way ANOVA. *, $P < 0.05$ compared to 2.5% FBS; #, $P < 0.05$ compared to LacZ cells.

α-SMA staining and formation of prominent stress fibers by CFs treated for 24 h with TGF-β (10 ng/ml) or Ang II (100 nM). Forskolin (10 μM) reduced basal, TGF-β-, or Ang II- stimulated α-SMA expression, as assessed by immunohistochemistry (Fig. 2*C*) or immunoblot analysis (Fig. 2*D*). Treatment of CFs with TGF-β or Ang II significantly ($P < 0.05$) enhanced α-SMA expression (114 ± 18% and 58 ± 24%, respectively), increases that were reduced by addition of forskolin. Thus, treatment with forskolin for 24 h inhibits α-SMA protein expression in response to profibrotic agents that induce myofibroblast transformation. Forskolin also produced a nearly 70% reduction in basal α-SMA protein expression, indicating that increases in cAMP can inhibit "spontaneous" myofibroblast transformation of CF.

AC6 Overexpression Enhances Forskolin- and Adrenomedullin-Stimulated cAMP Production and Inhibition of Serum-Stimulated Collagen Synthesis by Forskolin and Adrenomedullin. We used an adenoviral vector to enhance expression in CF of AC6, an AC isoform expressed natively at relatively high levels (14). Basal cAMP levels did not differ between LacZ- and AC6-overexpressing CF (Fig. 3*A Inset*), but cAMP production was substantially increased in response to both forskolin and adrenomedullin (Fig. 3). Compared to LacZ controls, CFs that overexpressed AC6 had enhanced cAMP production in response to forskolin with >5-fold enhancement at 3 and 10 μM; maximal response to adrenomedullin was enhanced, but its EC_{50} was similar in LacZ- and AC6-overexpressing CFs (Fig. 3). Thus, AC6-overexpression does not change basal cAMP levels in CF, but enhances maximal cAMP formation by agents that stimulate AC, with no differences in cell viability at any agonist concentration.

AC6 overexpression in the absence of AC stimulants did not alter serum-stimulated collagen synthesis (Fig. 4*A Inset*) but enhanced forskolin- and adrenomedullin-mediated inhibition of collagen synthesis (Fig. 4). This enhanced inhibition resulted from a lowering (>10-fold) of the EC_{50} concentration for forskolin and an "uncovering" of inhibition of collagen synthesis by adrenomedullin at concentrations ≥10 nM (compare the latter results with those in Figs. 1*A* and 4*B*). Overexpression of AC6 thus substantially sensitizes adult rat CF to inhibition of collagen synthesis by both forskolin and adrenomedullin.

TGF-β treatment is known to enhance the profibrotic state by altering expression of proteins such as plasminogen activator inhibitor (PAI-1) and IL-6 (28, 29). Consistent with the ability of increased AC expression to blunt collagen synthesis, overexpression of AC6 decreased basal and TGF-β-regulated expression of PAI-1 by 89 ± 3% and 59 ± 8%, respectively, and IL-6 by 81 ± 2% and 92 ± 3%, respectively.

Overexpression of AC6 Enhances Forskolin- or Adrenomedullin-Mediated Inhibition of Myofibroblast Formation. To determine whether AC overexpression alters CF-to-myofibroblast transformation, we examined the effects of forskolin and adrenomedullin on TGF-β-stimulated α-SMA expression. Submaximal concentrations of forskolin and adrenomedullin were used because, as shown in Fig. 4, these concentrations produced the most profound differences in collagen synthesis between LacZ- and AC6-overexpressing CF. LacZ-overexpressing CFs showed moderate α-SMA expression that was enhanced by treatment with TGF-β (10 ng/ml); this enhancement was inhibited by treatment with forskolin (1 μM) but not adrenomedullin (0.1 μM) (Fig. 5 *A* and *C*). AC6 overexpression inhibited basal α-SMA expression and enhanced the ability of both forskolin and adrenomedullin to blunt TGF-β-stimulated α-SMA expression (Fig. 5 *B* and *C*). Consistent with the effects observed on collagen synthesis (Figs. 1*A* and 4*B*), overexpression of AC6 "uncovered" a prominent inhibition of α-SMA expression by adrenomedullin. Similar trends in the intensity of F-actin staining with phalloidin were observed in response to forskolin and adrenomedullin (Fig. 5 *A* and *B*), suggesting that actin stress fiber formation is blunted in parallel with the decrease in α-SMA expression. Expression of vimentin, a marker for both fibroblasts and myofibroblasts (30), did not change during myofibroblast transformation. Overall, these data show that AC6-overexpression blunts basal α-SMA expression and enhances the ability of both forskolin and a G_S-coupled receptor agonist (adrenomedullin) to inhibit TGF-β-stimulated α-SMA expression, thus attenuating the fibroblast-to-myofibroblast transformation in parallel with a decrease in collagen synthesis.

Discussion

Gabbianni *et al.* (31) first identified a modified form of fibroblasts from granulation tissue that exhibited smooth muscle-like

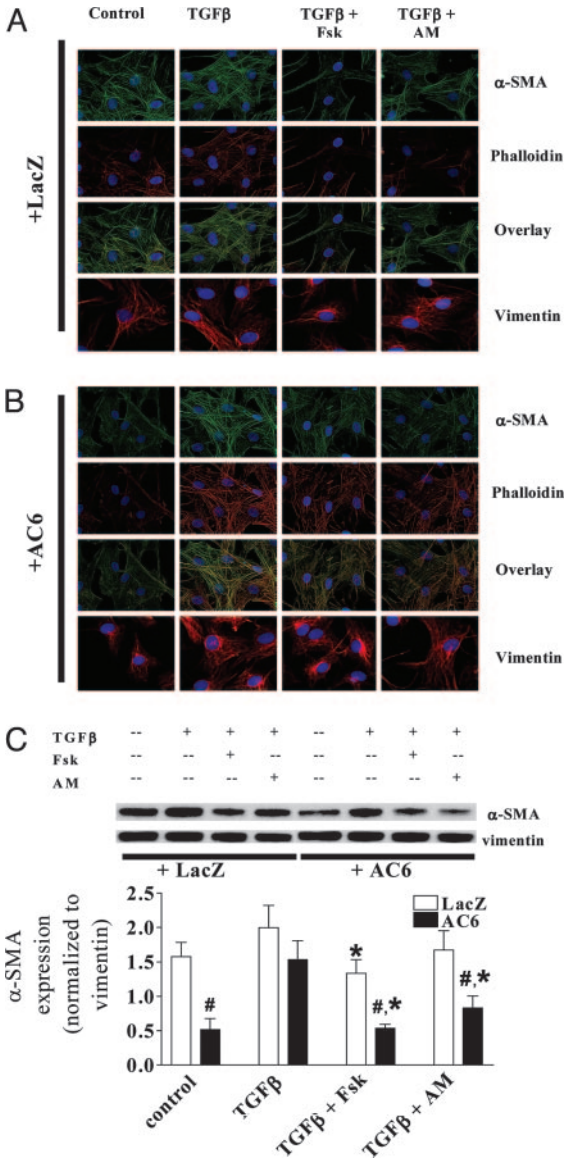

Fig. 5. AC6 overexpression enhances forskolin- and adrenomedullin-mediated inhibition of TGF-β-stimulated α-SMA expression. Immunohistochemistry was performed by using adult rat CF that overexpressed either LacZ (*A*) or AC6 (*B*). CF (passage 2) were grown for 48 h in serum-free media and then for 24 h in serum-free media alone (control) or with TGF-β (10 ng/ml) in the presence or absence of forskolin (Fsk, 1 μM) or adrenomedullin (AM, 0.1 μM). CF were incubated with an adenovirus expressing either LacZ (control) or AC6 for 24 h before stimulation. For immunohistochemical studies, CF were stained for α-SMA (green), F-actin (phalloidin, red), vimentin (red), and DNA (DAPI, blue). Overlap between α-SMA and F-actin staining is also represented (yellow). (*C*) α-SMA and vimentin protein expression in these same treatments were verified by immunoblot analysis. Quantitation of immunoreactive bands was performed; α-SMA expression was normalized to that of vimentin. Values represent mean $\pm$ SEM of at least four experiments and compared by using a paired *t* test. *, $P < 0.05$ compared to TGF-β; #, $P < 0.05$ compared to LacZ cells.

properties, suggesting a role for these cells in wound contraction during tissue repair. These fibroblasts, termed myofibroblasts, contribute to wound repair and connective tissue remodeling, in particular to establishment of tension and enhanced synthesis of ECM (1). Although a number of hormones and growth factors are known to stimulate formation of myofibroblasts from fibroblasts, much less is known regarding approaches to blunt this transformation and the increased tissue fibrosis that results. Glucocorticoids are one class of agents that sometimes, but not always, inhibits myofibroblast formation and proliferation (32, 33), perhaps by blockade of TGF-β production (34). Other therapies to blunt fibrosis are under study, but none has been shown to be efficacious and nontoxic (35).

Here, we demonstrate the ability of AC activation to inhibit myofibroblast transformation of adult rat CF in a manner that correlates with a reduction in collagen synthesis. Our findings also show the importance of levels of AC expression in determining the ability of a GPCR agonist or forskolin to regulate myofibroblast formation and collagen production. Taken together, these data suggest that blockade of myofibroblast formation, by means of increases in cAMP, blunts fibrosis by inhibiting generation of the cellular phenotype responsible for exaggerated ECM production.

Previous studies show that AC is the component in the GPCR–G-protein–AC pathway that limits agonist-mediated increases in maximal cAMP production, including in cardiac myocytes (19, 36, 37). AC6, one of nine membrane isoforms of AC, is expressed in many tissues, including CF, and is subject to inhibition by many factors, including G$\beta\gamma$, Gα_i, Ca^{2+}, protein kinase C, and nitric oxide (38, 39). We hypothesized that AC6-overexpression might sensitize CF to GPCR agonists that stimulate AC (without altering basal cAMP levels; ref. 19) and thereby enhance the effects of AC activation on myofibroblast formation and collagen production. Consistent with our hypothesis, we found that cAMP production in response to a GPCR agonist (adrenomedullin) or direct activation of AC (forskolin) was enhanced by AC6-overexpression, and that this enhanced cAMP production correlated with increased inhibition of collagen synthesis and decreased formation of myofibroblasts. In this regard, the data concerning adrenomedullin are of particular interest.

Adrenomedullin is an endogenous vasodilator and natriuretic peptide that was originally discovered in human pheochromocytoma (40) and has since been shown to exhibit a wide range of cardioprotective effects. *In vitro* studies have shown that adrenomedullin inhibits neonatal rat CF proliferation and collagen production in a cAMP-dependent manner and, in addition, Ang II receptor expression and function (15, 41). Ang II is profibrotic for CFs from multiple species, including those isolated from explanted human hearts (2, 42). Gene delivery of adrenomedullin attenuates cardiac and renal fibrosis in deoxycorticosterone acetate–salt-hypertensive rats (43), and adrenomedullin-knockout mice exhibit increased fibrosis and elevated expression of TGF-β in both heart and kidney (44). Unlike previous studies (15) that used CFs isolated from neonatal rats, in our studies with adult rats, we did not observe an inhibition of collagen synthesis unless we overexpressed AC6 (compare Figs. 1*A* and 4*B*). Consistent with previous findings, our findings emphasize the role of increased cAMP in the antifibrotic effects of adrenomedullin, but our data further show the enhancement of these effects by AC6 overexpression and that the antifibrotic effects of adrenomedullin likely result from cAMP-mediated regulation of myofibroblast formation. These findings thus define a potential means to attenuate cardiac fibrosis by strategies that increase expression of AC6 in CF and sensitize cells to both endogenous and exogenous agonists, such as adrenomedullin, that increase cAMP formation.

The precise mechanisms by which AC activation inhibits myofibroblast formation require further study. A primary component of the fibroblast-to-myofibroblast transformation is the formation of a contractile apparatus containing actin stress fibers, which are a defining characteristic of myofibroblasts (Figs. 2*B* and 5 *A* and *B*; ref. 3). Activation of the low-molecular-weight G protein RhoA plays a key role in proper formation and assembly of actin cytoskeleton (45, 46). cAMP, through the activation PKA, can inactivate RhoA and promote loss of stress

fibers (47, 48). The inhibition of fibroblast-to-myofibroblast transformation by AC activation may result from an inhibition of RhoA and the contractile machinery that defines a myofibroblast. It is possible that other effects are involved, such as blockade of α-SMA production at the level of gene expression: a TGF-β response element in the α-SMA promoter is required for α-SMA gene expression (49, 50). TGF-β signaling occurs via Smad proteins, and a requirement for Smad proteins, specifically Smad3, in TGF-β regulation of α-SMA gene expression has been suggested during myofibroblast transformation of rat lung fibroblasts (51). Recent data indicate that cAMP acts in a PKA-dependent manner to inhibit TGF-β/Smad signaling and gene activation by disruption of transcriptional cofactor binding (52).

The phenomenon of myofibroblast formation from fibroblasts and its profibrogenic role in connective tissue production is well conserved regardless of the tissue of residence (1, 3). In the heart, one confronts this problem in the setting of cell injury and associated cardiac fibrosis, especially after myocardial infarction (4, 12, 53). A major dilemma is how to limit continued ECM production in regions remote from the site of injury that can eventually lead to diminished contractile function. Agents that inhibit the fibroblast-to-myofibroblast transformation may provide a therapeutic means to inhibit maladaptive remodeling in the heart and other organs. The data shown here demonstrate that pharmacological activation of AC, in particular after overexpression of AC6, inhibits collagen synthesis by adult rat CF, in parallel with an inhibition of myofibroblast formation. The results provide a rationale for strategies to increase cAMP levels in cardiac fibroblasts, perhaps by pharmacological agents or gene transfer techniques, the latter as a means to enhance the efficacy of circulating or local factors that stimulate production of cAMP and thereby attenuate and prevent fibrosis in the heart.

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