G Protein-coupled Receptor Kinase-2 Is a Novel Regulator of Collagen Synthesis in Adult Human Cardiac Fibroblasts*

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Cardiac fibroblasts (CF)2 are key components of the myocardial extracellular matrix (ECM) because of their ability to synthesize and secrete fibrillar collagen types I and III (1). Under normal conditions, heart collagen deposition is low, but it is markedly increased in disease states, including hypertrophy, post-myocardial infarction (MI), and heart failure (HF) (2). Although ECM degradation because of increased matrix metalloproteinase activity dominates the early, adaptive wound healing response after MI, enhanced collagen synthesis is a feature of the later stages of healing and results in increased ECM deposition (3). Early after an injury such as MI, a series of cellular responses are activated to promote tissue repair and scar formation in the infarct zone. However, in some cases the repair process involves myocardial tissue remote from the infarct, resulting in superfluous ECM deposition in non-infarcted myocardium that progresses to pathological fibrosis (4). Fibrosis appears to underlie most cardiac pathologies where overproduction of ECM compromises the structure and architecture of the heart and affects cardiac function (5). The expression of interstitial collagen results in a phenotype that account for significant morbidity and mortality (6).

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This work was supported, in whole or in part, by National Institutes of Health Grant HL081472 (to S. A. A.). This work was also supported by the Thoracic Surgery Foundation for Research and Education (to S. A. A.).

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2 The abbreviations used are: CF, cardiac fibroblast(s); ECM, extracellular matrix; MI, myocardial infarction; HF, heart failure; β-AR, β-adrenergic receptor; GRK, G protein-coupled receptor kinase; sc, scrambled; α-SMA, α smooth muscle actin; ISO, isoproterenol; Ad, adenosine.

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plays an important role in regulating CF β-AR signaling, myo-fibroblast formation, and collagen synthesis.

EXPERIMENTAL PROCEDURES

All cell culture reagents were purchased from Invitrogen except FBS, which was obtained from Atlanta Biologicals (Lawrenceville, GA). Unless stated otherwise, all additional chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) except α-SMA and vimentin, which were obtained from Sigma; collagen types I and III, which were obtained from Calbiochem; and collagen type VI, which was obtained from Fitzgerald Industries International (Acton, MA).

Isolation and Culture of Adult Human Cardiac Fibroblasts—All procedures for tissue procurement in this study were performed in compliance with institutional guidelines for human research and an approved Institutional Review Board protocol at the University of Chicago Medical Center. Left ventricular tissue was taken from patients with severe left ventricular dysfunction undergoing heart transplantation. The indication for transplant in all patients (n = 10) was end-stage ischemic heart failure. Tissue was taken from a region remote from the territory of infarction, most commonly the left circumflex/postero-basal segment. Failing cardiac fibroblasts were isolated by a modified method of Turner et al. (10). Biopsy specimens were minced and digested in DMEM containing 0.05% collagenase 2 (Worthington) and 0.003% trypsin at 37 °C with continuous shaking for 90 min. Cells were plated for 1 h to allow fibroblast removal of non-adherent cells in fresh growth medium. Passage cells (≤2) from human left ventricular cardiomyocytes purchased from Cell Applications, Inc. (San Diego, CA) were assayed for cAMP content by direct ELISA kit (Assay Designs, Ann Arbor, MI).

Protein Immunoblotting—Cells were lysed in buffer containing 25 mM HEPES, 1 mM EDTA, 0.5 mM NaF, 0.25% Nonidet P-40, 5% glycerol (pH 6.8), and protease inhibitor mixture (Calbiochem). Equal amounts of protein for each sample were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted. Bands were visualized with ECL Western blotting substrate (Thermo Scientific, Rockford, IL). The band intensity was quantitated using BioRad Quantity One software. GAPDH was used as a loading control.

Quantitative Real-Time RT-PCR—RNA was isolated from confluent 60-mm dishes of CF using TRIzol reagent followed by RNA cleanup with the PureLink RNA mini kit, both from Invitrogen. The following oligonucleotide primers were used: Collagen Iα: forward, 5′-TCA CCT ACA GCA CGG TTG-3′; and reverse, 5′-GTT CTG TTT CCA GGG TTG-3′. β2-AR: forward, 5′-AAG CCA TGC GCC GGA CGA CCA GCA C-3′; and reverse, 5′-ATG ATC ACC CGG GCC TTA TTC TTG-3′.
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GAPDH: forward, 5’-ACC ACA GTC CAT GCC ATC AC-3’; and reverse, 5’-TCC ACC ACC CTG TTG CTG TA-3’.

Real time PCR was performed in a 20-μl reaction, 96-well format (0.2 μl cDNA, 250 nm forward and reverse primers, 1× DyNAmo HS SYBR Green Master Mix-Finnzymes) using an Opticon 2 real-time PCR machine (Bio-Rad). Three samples were measured in triplicate for each experimental group. Real-time PCR data analysis was carried out using the ΔΔ ct (threshold cycle) method. GAPDH was used as an internal control.

Confocal Microscopy—CF were grown to 60% confluence on 12-mm coverslips, washed with PBS, fixed with 3.7% formaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min. Following blocking in 3% BSA in PBS, the cells were treated with primary antibody (α-SMA and Vimentin 1:400 dilution; GRK2, collagen types I, III, and VI; and fibronectin 1:50 dilution) overnight at 4 °C and thereafter with either Alexa Fluor 594 goat anti-mouse IgG (1:100; Invitrogen) or goat anti-rabbit IgG-FITC (1:100) secondary antibody for 1 h. After extensive washing in PBS, cells were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen). Cells were visualized using a Leica SP2 laser scanning microscope.

siRNA Transfection of Cardiac Fibroblasts—Target-specific siRNA duplexes were designed using the sequence from the open reading frame of human GRK2 mRNA to knock down mRNA and protein expression of GRK2. The target sequence from 16 to 36 bases downstream of the start codon of GRK2 mRNA (5’-GCUCGCAUCCCUUUCUUUG-3’) was utilized, and the selected sequence was then subjected to a BLAST search to ensure that GRK2 mRNA was targeted. A scrambled oligo-ribonucleotide homologous to any mammalian mRNA was utilized as control. Both siRNAs were obtained from Dharmacon Research (Lafayette, CO). Transfection of siRNA to human cardiac fibroblasts was accomplished with DharmaFECT 1 transfection reagent. Briefly, 60–70% confluent cardiac fibroblasts were transfected with 15 nmol of siRNA to human GRK2. siRNA-scrambled (scr) control in RNase-free medium. Protein expression of GRK2 was examined after 48–72 h for transfection.

Statistical Analysis—All data are expressed as mean ± S.E. and were analyzed using Student’s t test. cAMP quantification was done using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Values of p < 0.05 were considered significant.

RESULTS

Fibroblasts Isolated from Failing Human Left Ventricles Display a Myofibroblast-predominant Phenotype—CF isolated from failing human left ventricles had a 2.2-fold increase in α-SMA expression by protein immunoblotting, indicative of transformation to a myofibroblast phenotype compared with the normal control CF (Fig. 1A). In addition, they displayed a robust (7-fold) increase in the extracellular matrix glycoprotein fibronectin typical of the failing phenotype. Confocal imaging again demonstrated the up-regulation of α-SMA expression in the failing CF as well as increased expression of fibronectin. Vimentin is a fibroblast marker protein that was studied to verify the purity of the CF cultures (Fig. 1B). Collagen type I is the most abundant isoform in the myocardial ECM. Collagen type 1α mRNA expression was up-regulated 3.5-fold in the failing CF relative to the control (Fig. 1C). Protein expression of collagen types Iα and III were also significantly increased by 2-fold, whereas collagen type VI was increased by 10-fold in CF isolated from the failing left ventricles (Fig. 1D). Increased collagen types I, III, and VI expression in failing CF is also clearly illustrated in the confocal imaging studies relative to normal control ventricular fibroblasts (Fig. 1E).

β-Adrenergic Receptor Signaling Is Uncoupled, and GRK2 Activity Is Up-regulated in Failing Cardiac Fibroblasts—Increasing intracellular levels of cAMP have been shown previously to inhibit collagen synthesis in adult rat CF (7). We first examined the effects of β-agonist stimulation on collagen synthesis in normal and failing CF. Control adult human CF demonstrated a dose-dependent decline in β-adrenergic (isoproterenol)-stimulated collagen synthesis as measured by [3H]proline incorporation (Fig. 2A). In contrast, failing CF had a significantly higher base-line level of collagen synthesis, which remained unchanged following β-adrenergic stimulation (Fig. 2A). To investigate the potential mechanism for this finding, we studied CF β-AR mRNA and total protein expression between groups (Fig. 2B). We noted that the adenyl cyclase moiety is intact in both groups, although the mechanism of impaired β-agonist-stimulated cAMP production occurs upstream in the signaling pathway. There was no difference in β2-AR mRNA or total protein expression between failing and normal CF (Fig. 2C). β2-ARs are the predominant adrenergic receptors expressed in CF that couple to adenyl cyclase. To determine whether β-AR desensitization may be the mechanism of impaired cAMP production in the failing CF, we studied expression of GRK2, the highest expressed GRK in the heart (11). There was an ~4-fold increase in GRK2 expression in the failing CF compared with normal controls (Fig. 2D). Confocal imaging also demonstrated increased GRK2 expression in the failing CF and robust membrane localization (Fig. 2E), which is consistent with the greater than 2-fold increase in GRK2 activity relative to the control as assessed by rhodopsin phosphorylation (Fig. 2F). As both GRK2 and GRK5 are expressed in the myocardium (12), monoclonal antibodies for GRK2 and GRK5 were added to the rhodopsin phosphorylation reactions and revealed that both GRK activity in the failing CF is attributable to GRK2 (Fig. 2F).

GRK2 Regulates Collagen Synthesis in Normal Human Cardiac Fibroblasts—To directly investigate the role of GRK2 in regulating collagen synthesis in adult human CF, adenoviral-mediated overexpression of GRK2 was performed in normal CF. The level of increased GRK2 expression was similar to the failing CF, ~3.5-fold, which was also demonstrated by confocal imaging (Fig. 3A). This led to severely blunted isoproterenol-stimulated cAMP production (Fig. 3B) and the relative loss of β-adrenergic-mediated inhibition of collagen synthesis compared with normal CF infected with an identical titer of a null adenov-
viral construct (Fig. 3C). Increased intracellular cAMP has been shown previously to inhibit myofibroblast formation and collagen synthesis in adult rat CF by TGFβ, a potent profibrotic growth factor (13). GRK2 overexpression did not alter collagen synthesis in response to TGFβ stimulation; however, there was loss of inhibition of TGFβ-stimulated collagen synthesis following β-agonist stimulation in Ad-GRK2 infected CF compared with those infected with Ad-Null (Fig. 3D). To more specifically investigate regulation of CF transformation and collagen synthesis by GRK2, GRK2 expression was knocked down using a siRNA approach in normal adult human ventricular CF. Expression of the GRK2 siRNA construct led to a 50% decrease in CF GRK2 expression compared with the scrambled siRNA control (Fig. 4A). Knockdown of GRK2 significantly increased β-agonist-stimulated cAMP production compared with CF treated with the scrambled siRNA (88.9 ± 1.5 versus 53.9 ± 6.2 pmol cAMP/ml, p < 0.04) (Fig. 4B). There was no difference between groups in baseline collagen synthesis.
(2417 ± 78 versus 2528 ± 230 cpm/mg protein, p > 0.05), but there was much greater isoproterenol-stimulated inhibition of collagen synthesis following GRK2 knockdown versus the control (802 ± 32 versus 1434 ± 113 cpm/mg protein, p < 0.04) (Fig. 4C). Decreased GRK2 expression in normal CF did not alter collagen synthesis following TGFβ stimulation alone (Fig. 4D). However, there was even greater β-agonist-mediated inhibition of TGFβ-stimulated collagen synthesis with GRK2 knockdown (748 ± 30 versus 1247 ± 164 cpm/mg protein, p < 0.04) (Fig. 4D). These overexpression and knockdown studies in normal adult human CF support the hypothesis that GRK2 plays an important role in the regulation of collagen synthesis in response to β-agonist and TGFβ stimulation, both of which are increased in the setting of HF.

Restoration of β-Adrenergic Signaling in Failing Cardiac Fibroblasts via Inhibition of GRK2 Inhibits Collagen Synthesis Stimulated by TGFβ—We then evaluated the potential therapeutic strategy of inhibiting GRK2 activity or expression in adult human CF isolated from failing left ventricles at the time of heart transplantation. The etiology of heart failure was ischemic cardiomyopathy in all patients. The previously described GRK2 inhibitor, GRK2ct, corresponding to the carboxyl-termi-
nal 194 amino acids of GRK2 (14) was expressed in failing CF using an adenoviral-mediated approach driven by the CMV promoter. Expression of GRK2ct was confirmed by protein immunoblotting (Fig. 5A, upper panel), and confocal imaging demonstrates less GRK2 membrane localization consistent with decreased activity (Fig. 5A, lower panel). Inhibition of GRK2 activity by GRK2ct led to decreased α-SMA expression following stimulation by isoproterenol (Fig. 5B). Inhibition of GRK2 activity by GRK2ct also restored normal β-AR signaling in these failing CF, as measured by increased cAMP production following stimulation with isoproterenol as compared with the uncoupled β-AR signaling present in failing CF infected with an empty (null) adenoviral vector (28.3 ± 1.4 versus 6.4 ± 0.3 pmol cAMP/ml, p < 0.01) (Fig. 5C). Base-line collagen types I, III, and VI expression in failing CF was not altered by expression of GRK2ct, but there was a significant decline following isoproterenol stimulation as measured by protein immunoblotting (10.1 ± 1.2 versus 20.2 ± 1.7, 9 ± 0.8 versus 18.7 ± 1.2, and 6.11 ± 0.8 versus 13.91 ± 1.7 arbitrary units, respectively, for collagens type I, III, and VI; p < 0.03 for each analysis) (Fig. 5D) and seen by confocal imaging (Fig. 5E). GRK2ct expression also significantly inhibited collagen synthesis in response to β-agonist stimulation in a dose-dependent fashion similar to that seen in normal control CF (Fig. 5F). TGFβ-stimulated collagen synthesis in failing CF was not altered by expression of GRK2ct, but there was restoration of the ability of isoproterenol to inhibit the stimulation of collagen synthesis by TGFβ as shown in normal CF (Fig. 5G). We also utilized a siRNA approach to inhibit GRK2 activity in failing human CF. GRK2 protein expression was decreased by ~75% using this strategy (Fig. 6A). Similar to our results with GRK2ct, siRNA-mediated knockdown of GRK2 resulted in a significant increase in isoproterenol-stimulated cAMP production in the failing CF (9.8 ± 1.3 versus 5.5 ± 1.1 pmol cAMP/ml, p < 0.04) (Fig. 6B). Knockdown of GRK2 expression led to β-agonist-mediated inhibition of collagen synthesis as present in the normal phenotype.
The ability of \(-\)agonist stimulation to inhibit TGF\(-\)mediated collagen synthesis in the failing CF was also restored by knockdown of GRK2 (1951 ± 154 versus 3992 ± 71 cpm/mg protein, \(p < 0.02\)) (Fig. 6C).

**DISCUSSION**

HF is a frequent complication of myocardial infarction (MI) that is associated with adverse ventricular remodeling (15). Well healed infarcts contain large amounts of ECM proteins, which can occupy up to 80% of the infarct area (16). However, collagen deposition also occurs in the non-infarcted remote myocardial region, predominantly in the interstitium, where it contributes to ventricular dysfunction. CF make up 60–70% of the total cell number in the heart and play a critical role in regulating normal myocardial function and in adverse remodeling. Cardiac fibrosis is characterized by overproduction of ECM, predominantly collagen types I and III, into the interstitial and perivascular space (17). 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 to activated myofibroblasts (18). Mechanisms of CF to myofibroblast transformation are just beginning to be understood; however, no targeted therapies currently exist to prevent myofibroblast formation and excessive collagen synthesis in the remote myocardium.

Our data show for the first time that GRK2 is robustly expressed in adult human CF and that expression and activity of this kinase are up-regulated in CF in the setting of chronic HF. This appears to be the primary mechanism of \(\beta\)-AR desensitization in CF, which results in impaired cAMP production, increased myofibroblast formation, and enhanced collagen synthesis, which are important mechanisms of adverse ventricular remodeling. Local myocardial and circulating levels of the catecholamines norepinephrine and epinephrine are elevated in advanced HF to stimulate myocyte contractility and increase cardiac output. This \(\beta\)-agonist stimulation would be expected to inhibit CF transformation and collagen synthesis through increased intracellular cAMP production; however, this effect is completely abolished as a result of GRK2-mediated \(\beta\)-AR desensitization. Overexpression of GRK2 in normal CF recapitulates this HF phenotype with loss of \(\beta\)-agonist-mediated inhibition of TGF\(\beta\)-stimulated collagen synthesis. However, knockdown of GRK2 expression by 50% in normal CF leads to decreased TGF\(\beta\)-mediated collagen synthesis following \(\beta\)-agonist stimulation. To investigate a potential novel therapeutic strategy to inhibit collagen synthesis in adult human CF isolated from patients with end-stage ischemic cardiomyopathy, we inhibited GRK2 activity through expression of the well...
FIGURE 5. Ad-GRK2ct inhibition of GRK2 activity in failing cardiac fibroblasts restores β-AR signaling and cAMP-mediated inhibition of collagen synthesis. A, representative immunoblot showing GRK2ct protein expression in failing CF infected with Ad-Null or Ad-GRK2ct. GAPDH was used as a loading control. n = 4 in each group. B, representative immunoblot (upper panel), densitometric analysis (center panel), and confocal images (lower panel) of GRK2ct-mediated decrease in α-SMA expression following isoproterenol (ISO) stimulation. *, p = 0.002 versus Ad-GRK2ct (untreated); *, p = 0.001 versus Ad-Null (ISO). n = 7 in each group. C, basal and ISO-stimulated (10 μM) intracellular cAMP levels in failing CF after adenoviral-mediated inhibition of GRK2 (Ad-GRK2ct) compared with Ad-Null. n = 6 in each group. *, p = 0.002 versus Ad-Null (ISO). D, representative immunoblot and densitometric analysis showing decreased collagens types I, III, and VI expression following ISO stimulation in Ad-GRK2ct compared with Ad-Null-infected failing CF. n = 4 in each group. *, p < 0.05 versus Ad-Null (ISO); **, p < 0.001 versus Ad-GRK2ct (untreated). E, confocal images (40×) of collagens types I, III, and VI (green) immunostaining in CF infected with either Ad-Null or Ad-GRK2ct with and without with ISO (10 μM) treatment. Nuclei were stained blue with DAPI. F, dose-response curves of ISO-stimulated (10^{-7} to 10^{-5} M) collagen synthesis measured by [3H]proline incorporation in failing CF after Ad-Null or Ad-GRK2ct infection, respectively. *, p < 0.01 versus Ad-Null. G, collagen synthesis in failing cardiac fibroblasts infected with either Ad-Null or Ad-GRK2ct under basal conditions, TGFβ stimulation, or pretreated with ISO (10 μM) for 20 min prior to 48 h of stimulation with TGFβ (10 ng/ml) in serum free-medium. n = 4 in each group. *, p < 0.05 versus Ad-Null (ISO + TGFβ).
described GRK2 inhibition using a siRNA approach in the normal phenotype was associated with enhanced collagen synthesis in a dose-dependent manner. Knockdown of GRK2 in the failing CF and CF transfected with siRNA-GRK2 diminished collagen synthesis in a dose-dependent manner. Inhibition of GRK2 in the failing CF also significantly diminished the profibrotic response to TGFβ (10 ng/ml) in serum-free medium. n = 4 in each group; *, p < 0.05 versus siRNA-scr (ISO) and siRNA-GRK2 versus siRNA-scr. B, siRNA-mediated inhibition of GRK2 in failing CF leads to enhanced ISO-stimulated collagen synthesis assessed by [3H]proline incorporation under basal conditions and in response to ISO (10 ng/ml) for 20 min prior to 48 h of stimulation with TGFβ (10 ng/ml) cAMP production. cAMP production represents the majority of connective tissue found in ischemic cardiomyopathy and contributes to adverse structural remodeling in the failing human heart (21). Our data suggest that GRK2 plays a prominent role in the regulation of CF biology in the normal and failing heart and that CF-specific inhibition of GRK2 may provide a mechanism to prevent the maladaptive ventricular remodeling and remote fibrosis that commonly occurs following myocardial infarction and eventually progresses to HF despite revascularization. Previous studies confirm that CF biology in response to various stimuli can differ significantly on the basis of species and level of development (1). This study involves only adult human CF isolated from the normal and failing left ventricles, providing greater clinical relevance. A recent study demonstrated that long-term myocardial expression of GRK2ct in the rat heart post-MI prevented adverse remodeling with decreased collagen I and TGFβ mRNA expression and resulted in improved left ventricular function (22). The adeno-associated viral vector was directly injected into the myocardium, and there was likely significant expression of GRK2ct in the fibroblasts in addition to cardiac myocytes. Thus, it appears that inhibition of GRK2 has a beneficial effect on post-infarction remodeling in both of these predominant cell types in the heart. This strategy may also be beneficial in preventing fibrosis of other organs, such as the lung, liver, and kidney, which also leads to significant morbidity and mortality.
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September 22, 2015
Molecular Bases of Disease:
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J. Biol. Chem. 2011, 286:15507-15516.
doi: 10.1074/jbc.M111.218263 originally published online February 25, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.218263

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September 22, 2015