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β -Arrestins Regulate Human Cardiac Fibroblast Transformation and Collagen Synthesis in Adverse Ventricular Remodeling

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Abstract

Aims—Cardiac fibroblasts (CF) produce and degrade the myocardial extracellular matrix and are critical in maladaptive ventricular remodeling that can result in heart failure (HF). β -arrestins are important signaling molecules involved in β -adrenergic receptor (β -AR) desensitization and can also mediate signaling in a G protein-independent fashion. We hypothesize that β -arrestins play an important role in the regulation of adult human CF biology with regard to myofibroblast transformation, increased collagen synthesis, and myocardial fibrosis which are important in the development of HF.

Methods and Results— β -arrestin 1&2 expression is significantly upregulated in adult human CF isolated from failing left ventricles and β -AR signaling is uncoupled with loss of β -agonist-mediated inhibition of collagen synthesis versus normal control CF. Knockdown of either β -arrestin 1 or 2 restored β -AR signaling and β -agonist mediated inhibition of collagen synthesis. Overexpression of β -arrestins in normal CF led to a failing phenotype with increased baseline collagen synthesis, impaired β -AR signaling, and loss of β -agonist-mediated inhibition of collagen synthesis. β -arrestin knockdown in failing CF diminished TGF- β stimulated collagen synthesis and also inhibited ERK phosphorylation. Overexpression of β -arrestins in normal CF increased basal ERK1/2 and Smad2/3 phosphorylation and enhanced TGF- β -stimulated collagen synthesis. This was prevented by pre-treatment with a MEK1/2 inhibitor.

Conclusions—Enhanced β -arrestin signaling appears to be deleterious in CF by promoting a pro-fibrotic phenotype via uncoupling of β -AR signaling as well as potentiating ERK and Smad

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Conflict of Interest

None of the authors has any conflicts of interest to disclose relevant to this work.

signaling. Targeted inhibition of β -arrestins in CF may represent a therapeutic strategy to prevent maladaptive myocardial fibrosis.

Keywords

heart failure; receptors; signal transduction

1. Introduction

Risk factors for the development of heart failure (HF) include coronary artery disease, particularly myocardial infarction, hypertension, diabetes, and idiopathic cardiomyopathy. Ventricular remodeling is a key process that contributes to the decline in both systolic and diastolic function in HF. During the development of HF, the left ventricle (LV) undergoes remodeling including increased mass, dilatation, and eccentric or concentric hypertrophy. At the cellular level, these changes are associated with myocyte hypertrophy, fibroblast proliferation, increased fibrillar collagen deposition, and fibrosis [1, 2, 3].

Cardiac fibroblasts (CF) are the most prevalent cell type in the heart accounting for over 60% of total cell number. CF are responsible for regulating normal myocardial function and play a critical role in the maladaptive ventricular remodeling that contributes to the development of HF [4]. Many of the functional effects of CF are mediated through differentiation, or transformation, to myofibroblasts which express contractile proteins including α -smooth muscle actin (α -SMA) and exhibit increased migratory, proliferative, and secretory properties [5]. CF are a key source of components of the extracellular matrix (ECM) which regulates the structure of the heart. Under normal conditions, cardiac collagen deposition is low, but this is markedly upregulated in many cardiac disease states including hypertrophy, post-MI remodeling, and HF.

Previous work has demonstrated that transforming growth factor- β (TGF- β) and angiotensin II are the major agonists that stimulate CF transformation and fibrosis in the heart and other organs [6, 7]. In addition, intracellular cyclic AMP (cAMP) has been shown to inhibit fibroblast transformation and collagen synthesis [8, 9]. We have recently reported that basal and β -agonist-stimulated cAMP production is markedly decreased in CF isolated from failing adult human LV contributing to unregulated collagen synthesis and fibrosis [10]. As previously described in cardiac myocytes, CF β -adrenergic receptor (β -AR) signaling is also uncoupled as a result of increased G protein-coupled receptor kinase (GRK) activity which is a hallmark of chronic HF [11]. Activation of GRKs and translocation to the membrane to phosphorylate agonist-occupied G protein-coupled receptors (GPCRs) leads to recruitment of arrestins to the receptor with subsequent uncoupling or desensitization. These phosphorylated receptors are additionally targeted by arrestins for internalization followed by degradation or recycling to the membrane [12]. Recent work has provided evidence that β -arrestin signaling in the heart may be beneficial, particularly in the setting of G protein-independent β -arrestin signaling, also referred to as arrestin-biased signaling [13]. These studies have primarily focused on cardiac myocytes, however, β -arrestin deficiency was found to be protective against pulmonary fibrosis in mice and prevented fibroblast invasion of ECM [14]. The role of β -arrestins specifically in the regulation of CF biology has not

been previously reported. In addition to their role in GPCR desensitization and downregulation, an important mechanism in regulating intracellular cAMP production via β -AR signaling and adenylyl cyclase, β -arrestin 1 and 2 are also known to activate downstream kinases including MAPK, PI3K, and Akt [15].

In this study we investigate the role of β -arrestins in regulating adult human CF transformation to myofibroblasts and collagen synthesis as relevant to the development of myocardial fibrosis and HF.

2. Materials and Methods

2.1 Cell culture and Reagents

All cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA, USA) except Fetal Bovine Serum (FBS) from Atlanta Biologicals (Lawrenceville, GA, USA). Unless stated otherwise, all additional chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 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 and the University of Wisconsin School of Medicine and Public Health. These studies conform to the principles of the Declaration of Helsinki and informed consent was obtained as part of the standard surgical consent process. Left ventricular (LV) tissue was taken from patients with end-stage HF undergoing LV assist device (LVAD) implantation. All patients had Stage D HF and were NYHA Class IV. Failing cardiac fibroblasts (CF) were isolated by a modified method of D'Souza et al [10]. Non-failing adult human LV CF (control) were purchased from Cell Applications Inc (San Diego, CA, USA). Each n value represents a unique patient from which the control CF were isolated and each set of control CF was obtained at a different time to ensure that they were from different patients. To prevent spontaneous differentiation, all studies were carried out in low serum (2.5% FBS) medium using early passage cells (3) plated at a density of approximately 200 cells per mm². HF fibroblasts were used within two weeks of culturing to ensure preservation of the failing phenotype.

2.3 siRNA transfection of cardiac fibroblasts

Target-specific siRNA duplexes were designed using the sequence from the open reading frame of human β -arrestin mRNA to knock-down mRNA and protein expression. Human β -arrestin 1 (sc-29741, 5'-AAAGCCUUCUGCGCGGAGAAU-3') and β -arrestin 2 (sc-29208, 5'-AAGGACCGCAAAGUGUUUGUG-3') were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Scrambled oligo-ribonucleotide complex was also obtained from Santa Cruz Biotechnology (sc-37007), which was not homologous to any mammalian genes and utilized as control. Cells were transfected with Lipofectamine 2000 (Invitrogen; CA, USA), according to manufacturers' instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

2.4 Adenoviral Infection of Cell Cultures

Normal control CF were infected at a multiplicity of infection (MOI) of 10 with either the adenovirus encoding β -arrestin 1, 2, or empty (Ad-GFP) adenovirus. The CF were incubated with the virus for 24 hours before stimulation with serum (DMEM/2.5% FBS) or agonist (10 μ M ISO or 10 ng/mL TGF- β) for the [3 H]proline incorporation assay. Protein expression was studied 48 hours after infection using appropriate antibodies.

2.5 Drug Treatment Protocol

CF were grown to desired confluence in 5ml of supplemented DMEM and treated with either transforming growth factor- β (TGF- β) to reach final concentrations of 0.5 to 10ng/mL, β -agonist isoproterenol/ISO (10 μ M) or no drug in DMEM with 2.5% FBS. Cells were collected following 60 minutes, 24 hours or 72 hours of treatment. For ERK-inhibitor studies, cells were pre-treated with 100 μ M of MEK1/2 inhibitor PD98059 or U0126 (Calbiochem, Billerica, MA) prior to stimulation with TGF- β or no drug.

2.6 Collagen Synthesis [3 H]Proline Incorporation

Cardiac fibroblasts (both control and HF) were treated with the β -agonist isoproterenol/ISO (10 μ M), TGF- β (0.5 to 10 ng/mL), MEK1/2 inhibitor PD98059 (100 μ M, Calbiochem, Billerica, MA) followed by TGF- β (10ng/mL), or MEK1/2 inhibitor U0126 (100 μ M, Calbiochem, Billerica, MA) followed by TGF- β (10ng/mL) as indicated in the figure legends. Cells were stimulated with ISO or TGF- β for 48 hours. [3 H]proline incorporation was measured according to the method of D'Souza et al [10]. Cells were grown to 80% confluence on 12-well plates, serum starved for 24 hours, and incubated with [3 H]proline (1 μ Ci/well, Perkin Elmer Life Sciences, Shelton, CT, USA) for 48 hr. Cellular protein was precipitated overnight with 20% trichloroacetic acid (TCA) and washed 3 times with 1ml of 5% TCA plus 0.01% proline, then dissolved in 0.2 M NaOH. The activity of [3 H]proline was determined by liquid scintillation counting.

2.7 Intracellular cAMP Quantitation

80% confluent CF cultured on 12-well plates were equilibrated for 24 hrs in low serum (2.5% FBS) DMEM and assayed for intracellular cAMP accumulation by 15 min incubation with 0.2 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor, followed by addition of isoproterenol/ISO (10 μ M) for an additional 15 min. Reactions were terminated by aspiration of culture medium and addition of 150 μ l of 0.1M hydrochloric acid (HCl) to each well. HCl extracts were assayed for cAMP content by Direct ELISA kit (Assay Designs, MI, USA).

2.8 Protein Immunoblotting

Cells were lysed in buffer containing (in mM): 25 HEPES, 1 EDTA, 125 NaCl, 0.5 NaF and 0.25% NP-40, 5% glycerol, protease inhibitor cocktail (Calbiochem, CA, USA) and phosphatase inhibitor cocktail (Sigma, MO, USA). Equal protein was run on 12% Tris-glycine polyacrylamide gels (Invitrogen) and transferred to PVDF membranes for immunoblotting. The following primary antibodies were used for immunoblotting: p-ERK1/2 (Cell Signaling 4377, 1:1,000), anti-t-ERK 1/2 (Cell Signaling 4376, 1:1000), anti-

β -arrestin 1 (Abcam ab32099, 1:1000), anti- β -arrestin 2 (Abcam ab31294, 1:500), anti-Collagen I (Abcam ab34710, 1:1000), anti-Collagen III (Abcam ab6310, 1:1,000), anti-p-smad (Santa Cruz sc-11769-R, 1:500), anti-t-smad (Santa Cruz sc-8332, 1:1000), anti-fibronectin (Santa Cruz sc-9068, 1:1000), vimentin (sc-5565), antibodies respectively. α -tubulin (Santa Cruz sc-32293, 1:1000) and GAPDH (Santa Cruz sc-25778, 1:1000) were used as a loading controls. Chemiluminescence detection was performed using the ECL reagent (Pierce). Band intensity was quantitated using Bio-Rad Quantity One software (<http://rsbweb.nih.gov/ij/>).

2.9 Confocal Microscopy

CF were grown to 80% confluence on 12 mm coverslips, washed with PBS, and fixed with 3.7% formaldehyde for 15 min. After the sections were pre-incubated for 30 min at room temperature in 0.3% Triton X-100 (PBS-Triton) and 10% normal donkey serum, the cells were treated with primary antibody (α -SMA and Vimentin 1:400 dilution; Collagen I, III, Fibronectin, p-ERK1/2, p-Smad2/3 1:200 dilution) overnight at 4°C and thereafter with either AlexaFluor 594 goat anti-mouse or rabbit IgG (1:1000; Invitrogen) or AlexaFluor 488 goat anti-rabbit or mouse IgG (1:1000, Invitrogen) secondary antibody for 1 hr. After extensive washing in PBS-T, cells were mounted in Fluoroshield™ with DAPI histology mounting medium from Sigma-Aldrich (MO, USA). Cells were visualized using a Leica SP2 Laser scanning microscope at the University of Chicago microscopy core facility.

2.10 Cell Proliferation [³H]Thymidine incorporation

Cell proliferation was assessed via [³H]Thymidine incorporation using techniques previously described by Olson et al.¹⁶ Briefly, equal numbers of CFs were plated on 12-well tissue culture plates in DMEM supplemented with 10% FBS. Cells were transfected with either siRNA or adenovirus as indicated in the figure legends. Cells were serum starved for 24 hours, and incubated with [³H]thymidine (1 μ Ci/well, Perkin Elmer Life Sciences, Shelton, CT, USA) for 48 hr. At the end of the treatments, the medium containing the label was removed, and the cells were washed two times with PBS, one time with 5% TCA, and two times with 95% ethanol. The cells were solubilized in 1 ml of 0.5 M sodium hydroxide for 30 min at room temperature. The activity of [³H]thymidine was determined by liquid scintillation counting.

2.11 Statistical Analysis

All data are expressed as mean $\pm$ SEM. cAMP quantitation was done using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). As appropriate student's t-test, one-way or two-way ANOVA followed by Tukey's HSD post hoc test were used. Values of $p < 0.05$ were considered significant.

3. Results

3.1 Fibroblasts isolated from failing human left ventricles display a myofibroblast phenotype, uncoupled β -AR signaling, and increased β -arrestin expression

CF isolated from failing human left ventricles had a 2-fold increase in α -smooth muscle actin (α -SMA) expression indicative of transformation to a myofibroblast phenotype

compared to the normal control CF. In addition, they displayed a robust (1.8-fold) increase in the ECM glycoprotein fibronectin, typical of the failing phenotype (Figure 1A). Confocal imaging again demonstrated the upregulation of α -SMA expression in the failing CF as well as increased expression of fibronectin (Figure 1B). Vimentin is a fibroblast marker protein which was studied to verify the purity of the CF cultures (Figures 1A and 1B). We then examined the effects of β -agonist (ISO) stimulation on collagen synthesis in normal and failing CF. Isoproterenol significantly inhibited collagen synthesis as measured by [3 H]proline incorporation in control adult human CF. In contrast, failing CF had a significantly higher baseline level of collagen synthesis which remained unchanged following β -agonist stimulation (Figure 1C). Increased collagen I and III expression in failing CF is illustrated in the confocal imaging studies relative to normal control fibroblasts (Figure 1D). ISO-stimulated cAMP production was severely blunted in the HF group versus normal control [5.0 ± 0.2 vs. 30.5 ± 3.2 pmol cAMP/mL; $p < 0.0002$] (Figure 1E). β_2 -ARs are the predominant adrenergic receptors expressed in CF that couple to adenylyl cyclase. Following chronic stimulation, β -ARs are phosphorylated by GRKs and then targeted by β -arrestins leading to uncoupling and endocytosis of these agonist-occupied receptors. To determine whether β -arrestin induced β -AR desensitization may be the mechanism of impaired cAMP production in the failing CF, we studied expression of β -arrestin 1 and 2. There was an approximately 2-fold increase in both β -arrestin 1 and β -arrestin 2 in the failing CF versus normal controls (Figure 1F).

3.2 Inhibition of β -arrestins decreases myofibroblast transformation and restores β -adrenergic signaling in failing cardiac fibroblasts

To investigate the potential role of β -arrestins in CF transformation and collagen synthesis, β -arrestin 1 or 2 expression was knocked down using an siRNA approach in failing CF. Expression of the β -arrestin 1 or 2 siRNA led to an approximately 31% decrease in β -arrestin 1 expression and a 71% decline for β -arrestin 2 compared to the scrambled siRNA control (Figures 2A and 2B). Knockdown of β -arrestin 1 decreased α -SMA expression by 21% and knockdown of β -arrestin 2 decreased α -SMA expression by 32% in the failing CF as demonstrated by immunoblotting and confocal imaging (Figures 2C&D). These data suggest that both β -arrestin 1 and 2 may play an important role in the transformation of CF into myofibroblasts in the setting of HF. We then studied the effects of inhibiting β -arrestin signaling on β -agonist stimulated collagen synthesis in failing CF. Knockdown of β -arrestins had no effect on baseline collagen synthesis [3058 ± 376 (β -Arr1 siRNA) and 2717 ± 117 (β -Arr2 siRNA) vs. 3087 ± 356 (scr-siRNA) cpm/ μ g protein], however, there was significantly greater isoproterenol-stimulated inhibition of collagen synthesis following both β -arrestin 1 or 2 knockdown versus scramble control [2128 ± 200 (β -Arr1 siRNA) and 1794 ± 105 (β -Arr2 siRNA) vs. 2841 ± 55 (scr-siRNA) cpm/ μ g protein, $p < 0.05$] (Figure 3A). Consistent with these findings, confocal microscopy showed ISO-induced inhibition of collagen I expression in the failing CF following knockdown of β -arrestins (Figure 3B). Furthermore, siRNA-mediated inhibition of β -arrestin expression significantly increased β -agonist-stimulated cAMP production compared to CF treated with the scrambled siRNA [18.6 ± 2.4 (β -Arr1 siRNA) and 18.0 ± 1.8 (β -Arr2 siRNA) vs. 10.5 ± 0.7 (scr-siRNA) pmol cAMP/mL; $p < 0.04$] (Figure 3C), which is consistent with restoration of β -AR signaling in the failing CF. CF proliferation was measured by [3 H]Thymidine incorporation to determine if changes in

proliferation were contributing to the observed decrease in collagen synthesis. Knockdown of either β -arrestin 1 or 2 had no effect on CF proliferation (Supplemental Figure 1).

3.3 Overexpression of β -arrestins uncouples β -AR signaling and increases collagen synthesis in normal human cardiac fibroblasts

To further investigate the role of β -arrestins in regulating collagen synthesis in normal adult human CF, adenoviral-mediated overexpression of β -arrestins was performed. The level of increased β -arrestin expression was similar to the failing CF, approximately 2-fold for β -arrestin 1 and 1.5-fold for β -arrestin 2 (Figures 4A and 4B). Overexpression of either β -arrestin 1 or 2 resulted in loss of ISO-stimulated inhibition of collagen synthesis versus adenoviral control [7669 ± 1438 (Ad- β -Arr1) and 6297 ± 788 (Ad- β -Arr2) vs. 3543 ± 50 (Ad-GFP) cpm/ μ g protein; $p < 0.05$] (Figure 4C). Both protein immunoblotting and confocal microscopy showed increased collagen I expression in normal CF with β -arrestin 1 or 2 overexpression. Importantly there was a loss of ISO stimulated decrease in collagen I expression with overexpression of either β -arrestin 1 or 2 indicating disruption of normal β -AR signaling (Figures 4D and E). Furthermore, we found that basal cAMP production was significantly lower in Ad- β -Arr1 infected normal CF compared to control [6.4 ± 1.2 (Ad- β -Arr1) vs. 16.8 ± 1.7 (Ad-GFP) pmol cAMP/mL; $p < 0.04$] and ISO-stimulated cAMP production was severely blunted in both β -arrestin 1 and 2 adenovirus infected CF versus control [9.5 ± 1.3 (Ad- β -Arr1), 13.2 ± 2.7 (Ad- β -Arr2) vs. 24.3 ± 2.2 (Ad-GFP) pmol cAMP/mL; $p < 0.009$] (Figure 4F). CF proliferation was again measured and no effect was seen following overexpression of β -arrestin 1 or 2 compared to Ad-GFP control (Supplemental Figure 2). These overexpression studies in normal adult human CF support the hypothesis that β -arrestins play an important role in the regulation of baseline collagen synthesis as well as in response to β -agonist stimulation in the setting of HF where β -arrestin expression is upregulated.

3.4 β -arrestin knockdown decreases TGF- β -stimulated collagen synthesis in failing CF via inhibition of ERK1/2 phosphorylation

The critical role of TGF- β has been well described in the development of fibrosis by promoting myofibroblast differentiation leading to sustained extracellular matrix overproduction. We investigated whether β -arrestins may be involved in the regulation of TGF- β -mediated pro-fibrotic signaling. Inhibition of β -arrestin expression by siRNA significantly decreased collagen synthesis in response to TGF- β stimulation in a dose-dependent fashion (Figure 5A). It is well described that TGF- β -induced phosphorylation of downstream ERK1/2 plays an important role in TGF- β -induced gene expression [6]. To demonstrate this in the adult human CF, ERK1/2 phosphorylation was measured by western blot and immunostaining studies in a temporal manner. In the presence of a non-targeting control siRNA (ScrsiRNA), TGF- β induces ERK1/2 phosphorylation (Figure 5B and 5C). The rapid (5 minute) and sustained stimulation of ERK1/2 phosphorylation by TGF- β is consistent with previous studies in different cell types including epithelial cells and chondrocytes. Furthermore, we found that either β -arrestin 1 or 2 knockdown reduces the 10 minute signal by approximately 70% and this is maintained up to 60 min (Figures 5B and 5C). Confocal microscopy also showed that knockdown of β -arrestins resulted in a

significant decrease in TGF- β -stimulated ERK1/2 phosphorylation in the failing CF (Figure 5D).

We went on to investigate the role of ERK signaling in β -arrestin-mediated regulation of TGF- β signaling and collagen synthesis in human CF. Control adult human CF demonstrated a dose-dependent increase in TGF- β stimulated collagen synthesis (Figure 6A). Adenoviral-mediated upregulation of β -arrestin expression in normal CF significantly enhanced TGF- β stimulated collagen synthesis for both β -arrestin 1 and 2 (Figure 6A). To determine a potential mechanism for enhanced TGF- β signaling we examined ERK1/2 phosphorylation. Basal p-ERK1/2 expression was significantly increased after overexpression of either β -arrestin 1 or 2 (Figure 6B). To determine whether increased ERK signaling contributes to the enhanced pro-fibrotic phenotype observed in the setting of β -arrestin overexpression we utilized the MEK1/2 inhibitors PD98059 or U0126. Both basal and TGF- β stimulated collagen synthesis were enhanced by overexpression of either β -arrestin 1 or 2 (Figure 6C). Importantly both these effects were blocked by the MEK1/2 inhibitors PD98059 or U0126 (Figure 6C). These overexpression studies in normal adult human CF support the hypothesis that β -arrestins play an important role in the regulation of collagen synthesis under baseline conditions and in response to TGF- β stimulation, which is increased in the setting of HF.

3.5 Regulation of TGF- β stimulated collagen synthesis by β -arrestins is mediated by Smad signaling

We investigated a potential mechanism for β -arrestin-mediated regulation of TGF- β signaling and collagen synthesis in human CF downstream of ERK activation. Smad2/3 phosphorylation was assessed in normal control CF treated with TGF- β for 1 hour with overexpression of β -arrestin 1 or 2 versus Ad-GFP control. It has been previously demonstrated that Smad2/3 can translocate to the nuclear compartment in response to activation of the TGF- β type I receptor and is mediated by ERK1/2 phosphorylation. Overexpression of either β -arrestin 1 or 2 increased basal and TGF- β stimulated Smad2/3 phosphorylation compared to Ad-GFP (Figure 7A). However, in the setting of pretreatment with the MEK1/2 inhibitor, U0126, basal or TGF- β -induced phosphorylation of Smad2/3 levels at 1 hour were similar in control (Ad-GFP) and in Ad- β -Arr1 or Ad- β -Arr2 (Figure 7A). To determine whether inhibition of the p42/44 MAPK pathway can alter the subcellular localization of Smad2/3, we pretreated CF with the MEK inhibitor U0126 for 20 minutes and then stimulated them with 10 ng/ml of TGF- β for 1 hour. As expected, Smad2/3 in unstimulated cells demonstrated a diffuse, mainly cytoplasmic staining (Figure 7B). When CF were treated with TGF- β , they exhibited a predominantly nuclear Smad2/3-specific staining compared with control, which was inhibited by U0126. Furthermore, TGF- β -induced nuclear translocation of phospho-Smad2/3 was significantly enhanced by β -arrestin 1 or 2 overexpression, however, this was nearly completely inhibited by the MEK inhibitor U0126 (Figure 7B). To determine, more directly, if the β -arrestin-induced increase in TGF- β -stimulated collagen synthesis is mediated via Smad signaling, we overexpressed β -arrestin 1 or 2 and simultaneously inhibited Smad2/3 expression using a siRNA approach. Inhibition of Smad expression was confirmed by immunoblotting (Figure 7C). Knockdown of Smad2/3 restored basal collagen synthesis in the setting of β -arrestin 1 or 2

overexpression (Ad- β -Arr1 or Ad- β -Arr2) to near control levels. Additionally, this experiment demonstrated that TGF- β -stimulated collagen synthesis under control conditions (Ad-GFP) or in the setting of β -arrestin overexpression was significantly blunted by Smad2/3 knockdown (Figure 7D). Thus, it appears that β -arrestins can regulate Smad signaling in an ERK-dependent manner and increased arrestin signaling in CF appears to promote a pro-fibrotic phenotype.

4. Discussion

The primary role of the myocardial ECM is to support tissue architecture to maintain chamber geometry and ventricular function. The cardiac ECM is composed primarily of fibrillar collagens, proteoglycans, and a basement membrane. Collagens I and III are the most abundant collagen subtypes in the heart [17]. Under normal conditions, there is a balance between synthesis of these collagen isoforms and degradation which is primarily mediated by matrix metalloproteinases and tissue inhibitors of metalloproteinases [18]. In response to injury, myocardial fibrosis results from abnormal regulation of both ECM synthesis and degradation, leading to an accumulation of collagen types I and III [19]. Excessive collagen deposition increases ventricular stiffness leading to diastolic dysfunction, arrhythmias, and eventually systolic dysfunction and HF.

Pathological fibrosis can be categorized into two distinct types: reactive fibrosis and reparative fibrosis. Reparative, or replacement fibrosis, occurs in the interstitium in response to myocyte apoptosis and necrosis which is most commonly present following myocardial ischemia and infarction as well as with aging. Reparative fibrosis in the infarcted myocardium is important to maintain structural integrity of the myocardium, however, it is also accompanied by reactive fibrosis both in the infarcted region and at sites remote from the MI [20]. Reactive fibrosis occurs in response to inflammatory processes and is characterized by excess collagen deposition in interstitial or perivascular regions [21]. Importantly, the degree of remote territory fibrosis is a critical component in the development of HF following infarction and represents the majority of fibrosis present in ischemic cardiomyopathy [22].

Several cytokine and growth factor signaling pathways have been shown to regulate CF transformation to myofibroblasts and subsequent collagen synthesis and fibrosis. TGF- β has both profibrotic and hypertrophic effects and has been linked to fibrosis in heart and kidney disease [23]. The primary source of TGF- β in the myocardium is the CF and expression is increased by a variety of stimuli including altered pH, hyperglycemia, generation of reactive oxygen species, mechanical stretch and vasoactive hormones such as angiotensin II [24]. Activation of TGF- β signaling leads to phosphorylation of Smad proteins, specifically Smad2/3, which form a functional transcription complex with Smad4 to initiate transcription responses [25]. In addition to Smad-mediated transcription, TGF- β can activate other signaling pathways, including extracellular signal regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), TGF- β -activated kinase 1 (TAK 1), and p38 mitogen activated protein kinase (MAPK) [26].

Recently, cAMP has been shown to have an antifibrotic action in CF via multiple intracellular signaling pathways. One is the inhibition of Smad-mediated transcription via competition between cAMP response element binding protein (CREB) and Smad for key transcriptional co-activators [8]. Another is cAMP-mediated inhibition of certain non-Smad signaling pathways activated by TGF- β , including ERK1/2 and JNK MAP kinases. These and likely other antifibrotic actions of cAMP appear to occur through activation of both PKA and exchange protein activated by cAMP-1 (Epac-1) [9]. Our lab has previously demonstrated that uncoupled β -adrenergic receptor signaling in CF isolated from failing adult human left ventricles may play a significant role in promoting myofibroblast transformation and unregulated collagen synthesis [10]. This is mediated primarily by increased G protein-coupled receptor kinase-2 (GRK2) activity which phosphorylates agonist-occupied β_2 -ARs. Impaired myocardial β -AR signaling and upregulation of GRK2 are a hallmark of chronic HF. Phosphorylation of these receptors promotes recruitment of β -arrestins which bind to and inactivate signaling of β_2 -ARs in a process known as desensitization.

Our data demonstrate, for the first time, significant upregulation of β -arrestin 1 and 2 in adult CF isolated from failing human left ventricles versus non-failing controls. In addition to significantly increased baseline collagen synthesis in the failing CF, there is loss of β -agonist-mediated inhibition of collagen synthesis due to β -AR uncoupling as demonstrated by impaired cAMP production in the failing CF following isoproterenol stimulation. Knockdown of either β -arrestin 1 or 2 expression by siRNA reversed the failing phenotype in HF CF as demonstrated by decreased transformation to activated myofibroblasts as well as decreased basal and β -agonist stimulated collagen synthesis. β -AR signaling was restored with increased cAMP production following β -agonist stimulation and this led to inhibition of collagen production. Adenoviral-mediated overexpression of either β -arrestin 1 or 2 in the normal control CF recapitulated the HF phenotype with increased basal collagen synthesis and collagen I expression. There was also loss of inhibition of collagen synthesis following β -agonist stimulation as a result of uncoupled β -AR signaling. These studies demonstrate that β -arrestins may play an important role in regulating CF transformation and increased collagen synthesis leading to myocardial fibrosis in the failing heart in a cAMP-dependent manner.

Recent studies have shown that β -arrestins can mediate signaling independent of classical second messenger-mediated signaling. β -arrestin mediated-signaling typically requires the formation of multiprotein signaling complexes in which β -arrestin acts as a scaffold, adaptor, and signal transducer. β -arrestins have been shown to facilitate mitogenic ERK1/2 signaling by scaffolding Raf, MEK1, and the non-receptor tyrosine kinase c-Src after activation of a number of GPCRs [27]. TGF- β stimulation also activates ERK MAP kinases, although to a much lower level than receptor tyrosine kinases [28]. Activation of the receptor complex activates T β RI which phosphorylates and activates Smad2 and Smad3. These proteins then complex with Smad4, translocate to the nucleus, and associate with DNA-binding complexes to regulate gene transcription. This is a critical signaling pathway for activation of fibroblasts and collagen synthesis. Despite its low level, ERK activation is important to TGF- β signaling.

ERK activation and Smad signaling are both necessary for TGF- β -induced epithelial-mesenchymal transformation [29]. In addition, ERK MAP kinases phosphorylate receptor-activated Smads to regulate their nuclear translocation and ERK substrates interact with Smads to regulate gene expression.

We therefore investigated the potential role of β -arrestin-mediated ERK activation in the regulation of TGF- β -stimulated collagen synthesis in the failing adult human CF. Interestingly, knockdown of either β -arrestin 1 or 2 significantly inhibited CF collagen synthesis following increasing doses of TGF- β . Both baseline and TGF- β -stimulated collagen I expression was also diminished after inhibition of β -arrestin expression. Under conditions of either β -arrestin 1 or 2 knockdown, ERK1/2 phosphorylation was inhibited following stimulation with TGF- β when measured at 5–60 minutes. To further establish this crosstalk between TGF- β signaling and β -arrestins at the level of ERK, either β -arrestin 1 or 2 was overexpressed in normal adult human CF and this led to enhanced collagen synthesis following TGF- β stimulation. β -arrestin overexpression also led to increased ERK1/2 phosphorylation as well as increased Smad phosphorylation which is critical for collagen synthesis in CF. Pre-treatment of these CF with the MEK inhibitor UO126 completely blocked TGF- β stimulated collagen synthesis in control CF. Additionally, the MEK inhibitor blunted both basal and TGF- β stimulated collagen synthesis following overexpression of β -arrestin 1 or 2. Inhibition of ERK signaling by UO126 also significantly diminished phosphorylation of Smad2/3 in the setting of β -arrestin 1 or 2 overexpression. These data support the hypothesis that β -arrestins can also regulate collagen synthesis in human CF in a cAMP-independent and ERK-Smad-dependent fashion.

We believe this is the first report on the potential role of β -arrestins in regulating myocardial fibrosis by CF. Recent work has shown that β -arrestin deficiency is protective against pulmonary fibrosis in both β -arrestin 1 or 2 knockout mice using a bleomycin-induced lung fibrosis model [14]. Loss of either β -arrestin 1 or 2 resulted in protection from mortality, inhibition of matrix deposition, and protected lung function. Interestingly, in this mouse model of pulmonary fibrosis, there was no difference in TGF- β -stimulated Smad3 phosphorylation or collagen I expression between fibroblasts isolated from wild type or either β -arrestin 1 or 2 knockout mice. This is in direct contrast to our findings in adult human cardiac fibroblasts where knockdown of β -arrestin had a profound impact on TGF- β -stimulated collagen synthesis and collagen expression. These findings may be explained by species- or organ-specific differences in the adult fibroblasts or in the model used to create pulmonary fibrosis compared to end-stage human heart failure. *In vivo* studies targeting β -arrestins in a CF-specific manner will greatly help to further elucidate the role of these signaling molecules in the initiation and progression of adverse ventricular remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. We investigated the role of beta-arrestins in regulating cardiac fibroblast biology.
2. Beta-arrestin is upregulated in failing fibroblasts.
3. Beta-arrestin signaling promotes a pro-fibrotic phenotype.
4. Inhibiting beta-arrestins may represent a novel strategy to prevent fibrosis.

Figure 1. Heart failure leads to uncoupling of β -adrenergic receptor signaling and up-regulation of β -arrestins

(A) Representative immunoblot (upper panel) showing significant increase in α -SMA and fibronectin in failing (HF) human CF compared with non-failing (control) CF. Vimentin and GAPDH were used as loading controls. Densitometric analysis of α -SMA and fibronectin expression normalized to vimentin and GAPDH is shown below. $n=3-4$ in each group; $*p<0.05$ vs. control. (B) Confocal images (40x) of α -SMA and fibronectin immunostaining with red AlexaFluor 594 dye, vimentin is stained with green AlexaFluor 488 dye shown in control and HF cells. Nuclei are stained blue with DAPI. Scale bar=10 μ m. (C) Collagen synthesis in control CF and heart failure CF under basal conditions and following ISO (10 μ M) treatment. $n=3-5$ in each group; $*p<0.03$ vs. control (untreated), $^{\#}p<0.001$ vs. control (untreated), $^{\#\#}p<0.001$ vs. control (Iso) and $p>0.05$ vs. HF (untreated). (D) Confocal images

(40x) of collagens types I and III in CF. Nuclei are stained blue with DAPI. Vimentin was used as a fibroblast marker protein visualized with green AlexaFluor 488. Scale bar=10 μ m. (E) Basal and ISO-stimulated intracellular cAMP levels in control and HF cardiac fibroblasts. n=4 in each group; * p <0.002 vs. control (untreated) and # p <0.002 vs. control (ISO). (F) Representative immunoblot (*upper panel*) and densitometric analysis (*lower panel*) demonstrate a 2.2-fold increase in β -arrestin 1 and a 2-fold increase in β -arrestin 2 in HF compared to control CF. Values are normalized to α -tubulin. n=3–5 in each group; * p <0.002 vs. control, # p <0.05 vs. control.

Figure 2. Inhibition of β-arrestins in failing CF inhibits transformation to myofibroblasts

(A) Failing CF were transfected with siRNA of β-arrestins for 48 hours. Representative immunoblot showing knockdown effects of β-arrestin 1 in HF. The lower panel shows densitometric analysis of β-arrestin 1 expression normalized to GAPDH. $n=4$ in each group; $*p=0.03$ vs. scr-siRNA control. (B) Failing CF were transfected with siRNA of β-arrestin 2 for 48 hours. Representative immunoblot showing knockdown effects of β-arrestin 2 in HF. The lower panel shows densitometric analysis of β-arrestin 2 expression normalized to α-tubulin. $n=2$ in each group; $*p<0.05$ vs. scr-siRNA control. (C) Representative immunoblot (upper panel) showing significant decrease in α-SMA after β-arrestin knockdown in failing CF when compared with scr-siRNA. GAPDH was used as a loading control. Densitometric analysis of α-SMA and vimentin expression normalized to GAPDH is shown below. $n=3$ in each group; $*p<0.006$ vs. scr-siRNA, $#p<0.001$ vs. scr-siRNA. (D) Confocal images (40x) of scr-siRNA vs. β-Arr1/2 siRNA in failing CF displaying inhibition of α-SMA and fibronectin expression after β-arrestin knockdown. Purity of the cultures was determined by staining for the fibroblast marker vimentin (red). Nuclei were stained blue with DAPI. Scale bar=10 μm.

Figure 3. Knockdown of β-arrestins in HF CF restores isoproterenol (ISO)-stimulated cAMP production and inhibition of collagen synthesis

(A) ISO (10 μM)-stimulated collagen synthesis in HF CF transfected with either β-arrestin 1 siRNA or β-arrestin 2 siRNA. n=4 in each group; * $P<0.03$ vs. scr-siRNA (ISO) & vs. β-arr1 siRNA (Untreated), ** $P<0.006$ vs. scr-siRNA (ISO) & vs. β-arr2 siRNA (Untreated). (B) Confocal images (40x) of β-Arr1 or 2 siRNA vs. scr-siRNA in HF fibroblasts demonstrating inhibition of collagen I expression with β-Arr1 or 2 knockdown. Nuclei were stained blue with DAPI. Scale bar=10 μm. (C) ISO (10 μM)-stimulated cAMP production in HF CF following β-arrestin 1 or 2 knockdown. n=3 in each group; * $p<0.003$ vs. scr-siRNA (ISO) & vs. β-arrestin 1 siRNA (Untreated), # $p<0.005$ vs. scr-siRNA (ISO) & vs. β-arrestin 2 siRNA (Untreated).

Figure 4. Overexpression of β -arrestins in normal cardiac fibroblasts uncouples β -AR signaling and prevents β -agonist-mediated inhibition of collagen synthesis
 (A) Normal CF were infected with adenoviruses overexpressing β -arrestin 1 (Ad- β -arr1), β -arrestin 2 (Ad- β -arr2), or control (Ad-GFP) for 48 hours. Representative immunoblot (upper panel) and densitometric analysis (lower panel) shows overexpression of β -arrestin 1 in normal CF. α -tubulin was used as a loading control. $n=3$ in each group; $*P<0.005$ vs. Ad-GFP & vs. Ad- β -arr2. (B) Normal CF were infected with adenoviruses overexpressing β -arrestin 1 (Ad- β -arr1) or β -arrestin 2 (Ad- β -arr2) for 48 hours. Representative immunoblot (upper panel) and densitometric analysis (lower panel) shows overexpression of β -arrestin 2 in normal CF. α -tubulin was used as a loading control. $n=2-4$ in each group; $*P<0.02$ vs. Ad-GFP. (C) Collagen synthesis assessed by [3 H]proline incorporation under basal conditions and in response to ISO (10 μ M) in the presence of 2.5% FBS. $n=3-4$ in each group; $*p<0.01$ vs. Ad-GFP (ISO), $\#p<0.05$ vs. Ad-GFP (ISO). (D) Representative immunoblot (upper panel) and densitometric analysis (lower panel) of Collagen I expression

in normal CF with β -arrestin overexpression or Ad-GFP control. α -tubulin was used as a loading control. n=3 in each group; * p <0.005 vs. Ad-GFP (untreated), # p <0.005 vs. Ad-GFP (ISO). (E) Confocal images (40x) of collagen I in normal CF with either Ad- β -arrestin 1/2 or Ad-GFP with and without ISO (10 μ M) treatment. Nuclei were stained blue with DAPI. Scale bar=10 μ m. (F) Adenoviral-mediated overexpression of β -arrestins in normal CF leads to impaired basal and ISO (10 μ M)-stimulated cAMP production n=3–4; * p <0.03 vs. Ad-GFP (untreated), # p <0.01 vs. Ad-GFP (ISO).

Figure 5. Inhibition of β-arrestin expression abolishes TGF-β-induced collagen synthesis and ERK1/2 phosphorylation in failing CF

(A) HF CF were transfected with β-arrestin siRNA and treated with 0.5, 1 and 10 ng/mL of TGF-β for 24 hours. Dose-response curves of TGF-β-stimulated collagen synthesis in HF CF transfected with β-arrestin siRNA. $n=4$ in each group; $*p<0.001$ (scr siRNA) vs. β-Arr1 siRNA & vs. β-Arr2 siRNA. (B) HF CF transfected with β-arrestin 1 or 2 siRNA were treated with 10ng/mL of TGF-β for 0, 5, 10, 20, 30, or 60 minutes. Representative immunoblot showing time course of TGF-β-stimulated p-ERK1/2 expression. (C) Densitometric analysis showing decreased p-ERK1/2 expression in β-arrestin knockdown HF CF vs. scr-siRNA control. p-ERK1/2 was normalized to t-ERK1/2. $n=4$ in each group; $*p<0.0001$ vs. scr-siRNA + Untreated (0min). (D) Confocal images (40x) of p-ERK1/2 stained green with FITC in HF CF transfected with either β-arrestin siRNA or scr-siRNA with TGF-β (10 ng/mL) treatment at different time points. Nuclei were stained blue with DAPI. Scale bar=10 μm.

Figure 6. TGF- β stimulated collagen synthesis is enhanced by overexpression of β -arrestins in normal CF and is ERK-dependent

(A) Normal CF infected with adenoviruses overexpressing β -arrestin 1 (Ad- β -arr1), β -arrestin 2 (Ad- β -arr2), or control (Ad-GFP) for 48 hours. Dose-response curves of TGF- β -stimulated (0.5, 1 and 10 ng/mL) collagen synthesis in normal CF. $n=3-8$ in each group; $*p<0.03$ (Ad- β -Arr1) vs. Ad-GFP, $^{\#}p<0.05$ (Ad- β -Arr2) vs. Ad-GFP. (B) Representative immunoblot shows p-ERK1/2 expression is upregulated by overexpression of β -arrestins in normal CF. p-ERK1/2 was normalized to t-ERK1/2. $n=3$ in each group; $*p<0.003$ vs. Ad-GFP, $^{\#}p<0.01$ vs. Ad-GFP. (C) Collagen synthesis in response to TGF- β stimulation (10ng/mL) under 2.5% serum conditions, or pre-treated with PD98059 (10 μ M) or UO126 (10 μ M) for 20 minutes prior to 48 hours of stimulation with TGF- β (10ng/mL). $n=4$ in each group; $*p<0.001$ vs. Ctrl, $**p<0.001$ vs. TGF- β , $^{\#}p<0.001$ vs. Ad-GFP (Ctrl).

Figure 7. β-arrestin-induced Smad2/3 phosphorylation is ERK-dependent and β-arrestin-mediated collagen synthesis is Smad-dependent

(A) Representative immunoblot (upper panel) and densitometric analysis (lower panel) show TGF-β-stimulated Smad2/3 phosphorylation is regulated by the ERK signaling pathway. p-Smad2/3 was normalized to t-Smad2/3. n=3 in each group; **P*<0.01 vs. Ad-GFP. (B) Confocal images (40x) of p-Smad2/3 in normal control CF infected with either Ad-β-arr1 or 2 or Ad-GFP with or without TGF-β (10 ng/mL) treatment. MEK inhibitor U0126 pre-treatment inhibits TGF-β-stimulated p-Smad2/3 translocation into nuclei. Scale bar=10 μm. (C) Normal CF were transfected with siRNA for Smad2/3 for 48 hours. Representative immunoblot showing effective knockdown of Smad2/3 in Normal CF. Smad2/3 was normalized to GAPDH. n=3 in each group. (D) Collagen synthesis in response to TGF-β stimulation (10ng/mL) under 2.5% serum conditions. Normal CF were infected with Ad-β-arrestin 1 or 2, then transfected with Smad2/3 siRNA. n=3–6 in each group; **p*<0.02 vs. scr-siRNA (untreated), #*p*<0.01 vs. scr-siRNA (TGF-β).