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Regulation of Mitochondrial Oxidative Stress by β-arrestins in Cardiac Fibroblasts

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Abstract

Oxidative stress in cardiac fibroblasts (CF) promotes transformation to myofibroblasts and collagen synthesis leading to myocardial fibrosis, a precursor to heart failure (HF). NADPH oxidase 4 (Nox4) is a major source of cardiac reactive oxygen species (ROS), however, mechanisms of Nox4 regulation are unclear. β-arrestins are scaffold proteins which signal in G protein-dependent and independent manners including ERK activation. We hypothesize that β-arrestins regulate oxidative stress in a Nox4-dependent manner and increase fibrosis in HF. CF were isolated from normal and failing adult human left ventricles. Mitochondrial ROS/superoxide production was quantitated using MitoSox. β-arrestin and Nox4 expression were manipulated using adenoviral overexpression or siRNA-mediated knockdown. Mitochondrial oxidative stress and Nox4 expression in CF are significantly increased in HF. Nox4 knockdown resulted in significant inhibition of mitochondrial superoxide production and decreased basal and TGF-β stimulated collagen and α-SMA expression. CF β-arrestin expression is upregulated 4-fold in HF. β-arrestin knockdown in failing CF decreased ROS and Nox4 expression by 50%. β-arrestin overexpression in normal CF increased mitochondrial superoxide production 2-fold. These effects were inhibited by Nox or ERK inhibition. Upregulation of Nox4 appears to be a primary mechanism for increased ROS production in failing CF, which stimulates collagen deposition. β-arrestin expression is upregulated in HF and plays an important and novel role in regulating mitochondrial superoxide production via Nox4. The mechanism for this effect appears to be ERK-mediated. Targeted inhibition of β-arrestins in CF may decrease oxidative stress as well as pathological cardiac fibrosis.

TRANSLATIONAL IMPACT

Clinical issue

The incidence of heart failure (HF) continues to increase worldwide with no effective novel medical therapies. Adverse remodeling of the heart is associated with cardiac myocyte hypertrophy and dysfunction as well as myocardial fibrosis. This process of ventricular fibrosis is mediated by cardiac fibroblasts (CF) which make up nearly 70% of the total cell number in the human heart. Fibrosis initially leads to stiffening of the ventricle and diastolic dysfunction and this ultimately can progress to severe systolic HF. The mechanisms which regulate the transformation of quiescent CF to activated myofibroblasts that synthesize and deposit excess extracellular matrix are just beginning to be understood, but oxidative stress is one stimulus for this process. The focus of this study is to further elucidate how oxidative stress is regulated in adult human CF and to target this pathway as a potentially novel therapeutic strategy.

Results

Mitochondrial oxidative stress and NADPH oxidase (Nox4), a major source of ROS production in the heart, are significantly increased in CF isolated from failing human left ventricles. Nox4 knockdown resulted in significant inhibition of mitochondrial superoxide production and decreased basal and TGF-β stimulated α-SMA and collagen expression. β-arrestin expression in CF is upregulated 4-fold in failing CF. β-arrestin knockdown in failing CF decreased ROS and Nox4 expression by 50%. β-arrestin overexpression in normal CF increased mitochondrial superoxide production 2-fold. These effects were inhibited by Nox or ERK inhibition. Upregulation of Nox4 appears to be a primary mechanism for increased ROS production in failing CF, which stimulates ECM synthesis. β-arrestin expression is upregulated in HF and plays an important and novel role in regulating mitochondrial superoxide production via Nox4. The mechanism for this effect appears to be ERK-mediated.

Implications and future directions

Targeted inhibition of β-arrestins in CF may decrease oxidative stress as well as pathological fibrosis. This study provides the first evidence that β-arrestins can regulate oxidative stress in adult human CF via Nox4 in an ERK-dependent manner. Furthermore, we show that both TGF-β and β-arrestin signaling through ERK promotes an increase in Nox4 expression and mitochondrial ROS generation. Further investigation of specific mechanisms for the ERK-mediated increase in Nox4 expression is underway and *in vivo* fibroblast-specific inhibition of β-arrestins will be studied as a potential therapeutic strategy to prevent adverse ventricular remodeling. These findings may also have potential therapeutic implications for other organ systems that develop pathological fibrosis including lung, liver, and kidney.

Introduction

Cardiac fibroblasts (CF) make up 60-70% of the total cell number in the human heart and play a critical role in regulating normal myocardial function through maintenance of extracellular matrix (ECM) homeostasis (Weber KT, 2004). Additionally, CF play a key role in HF as they mediate the process of maladaptive remodeling. During this process, normally quiescent CF undergo phenotypic modulation to activated myofibroblasts (myoFb) (Petrov VV, et al., 2002). Myofibroblasts express α-smooth muscle actin (α-SMA), indicating acquisition of a secretory and contractile phenotype, a transition that correlates with increased synthesis and deposition of ECM proteins including collagen (Porter KE, Turner NA, 2009; Baudino TA et al., 2006). Transforming growth factor-β (TGF-β) is the most potent profibrotic stimulus for CF and myocardial TGF- β levels are elevated in HF. TGF-β stimulates both CF to myoFb transformation and marked increases in ECM deposition (Brown RD et al., 2005).

Oxidative stress is thought to play a critical role in the development and progression of cardiac remodeling associated with HF (Kinugawa S et al., 2000; Sia YT et al., 2002). Reactive oxygen species (ROS) modulate ECM remodeling by mediating CF function and stimulating collagen turnover (Murdoch CE et al., 2006). NADPH oxidases (Nox) are the major sources of ROS production in the heart (Octavia Y et al., 2012) and Nox2 and Nox4 are the primary isoforms expressed in the myocardium (Byrne JA et al., 2003). Nox4 is constitutively active, localized to the mitochondria, and a major source of superoxide generation in the heart and specifically in CF (Murdoch CE et al., 2006). It has recently been demonstrated that Nox4 mediates TGF-β stimulated myoFb transformation in normal human CF (Cucoranu I et al., 2005). Mechanisms by which Nox4-mediated ROS production is regulated remain unclear.

β-arrestins are scaffold proteins involved in G protein-coupled receptor (GPCR) desensitization and downregulation and also link GPCR activation to downstream signaling pathways including MAPK/ERK, PI3K, and AKT (Lefkowitz RJ et al., 2006). β-arrestins are critical in the impaired myocardial β-adrenergic receptor signaling that is a hallmark of chronic HF as β-arrestins target these receptors for internalization and degradation (Kelly E et al., 2008). The potential role of β-arrestins in regulating oxidative stress in fibroblasts has not been previously studied.

In this study, we investigate the role of Nox4-mediated mitochondrial oxidative stress in adult human CF biology in the setting of chronic HF. We hypothesize that β-arrestins may regulate Nox4-mediated mitochondrial ROS production and play a significant role in the development of the failing CF phenotype.

Results

Mitochondrial superoxide production and Nox4 are upregulated in failing cardiac fibroblasts

It is well established that oxidative stress is increased in the myocardium in the setting of HF. Markers of oxidative stress are increased in human HF that correlate with disease severity. We examined whether oxidative stress was specifically increased in human CF isolated from failing LV compared to normal controls. CF were stained with MitoSox to quantitate mitochondrial superoxide generation. There was a greater than 2-fold increase in mitochondrial superoxide levels in failing CF versus control (Figure 1A). We quantitated Nox4 protein expression in HF versus control CF as a potential mechanism for the increased mitochondrial superoxide production. There was more than a 3-fold increase in Nox4 expression in failing CF as demonstrated by immunostaining and immunoblotting (Figure 1B). Since Nox4 is constitutively active, this increase in expression appears to be an important mechanism of increased mitochondrial superoxide levels in failing CF. It is well established that TGF-β is an important pro-fibrotic stimulus for CF in both the healthy and failing myocardium (Weber KT, 2004; Petrov VV et al., 2002). We investigated whether TGF-β stimulation increases mitochondrial superoxide production. TGF-β stimulation increased MitoSox staining in normal CF (Figure 1A) to levels similar to those observed in failing CF. In failing CF, there was no additional increase in MitoSox fluorescence intensity following TFG-β stimulation (Figure 1A). Additionally, TGF-β significantly increased Nox4 expression in both control and failing CF (Figure 1C). These data are consistent with previous findings showing a link between TGF-β and Nox4 expression in normal human CF and further demonstrate that mitochondrial superoxide production and Nox4 expression increase with activation of TFG-β signaling in human CF.

Increased oxidative stress contributes to adverse remodeling in CF

The adverse remodeling phenotype observed in failing CF is characterized by increased myofibroblast transformation and ECM production. To determine whether increased superoxide production contributes to this maladaptive process, we utilized the non-specific Nox inhibitor apocynin. Pre-treatment with apocynin led to significant inhibition of TGF-β stimulated α-SMA expression, a marker of myofibroblast differentiation, in both control and failing CF as well as significant reduction in basal α-SMA expression in failing CF (Figure 1D). Consistent with inhibition of myofibroblast differentiation, apocynin pre-treatment decreased basal and TGF-β stimulated collagen expression as measured by western blot (Figure 1D) and collagen synthesis as determined by [^{3}H]proline incorporation (Figure 1E). These data provide evidence that mitochondrial oxidative stress in fibroblasts is increased in HF and that increased oxidative stress contributes to CF-mediated adverse remodeling.

Nox4 mediates TGF-β driven myofibroblast transformation and mitochondrial superoxide production in human CF

To more specifically investigate the role of Nox4 in TGF-β stimulated mitochondrial superoxide production and CF-mediated adverse remodeling, we used an siRNA approach to knockdown Nox4 expression. Inhibition of Nox4 protein expression was demonstrated by immunoblotting (Figure 2A). In control CF, siRNA knockdown of Nox4 (siNox4) significantly inhibited TGF-β stimulated mitochondrial superoxide production compared to scrambled control siRNA (Scr) (Figure 2B). Nox4 knockdown in HF CF returned superoxide production to control levels under basal conditions as well as following TGF-β stimulation (Figure 2A). To determine whether Nox4 contributed to CF-mediated myocardial fibrosis, α-

SMA expression and collagen production were examined after Nox4 knockdown. SiNox4 lead to significant inhibition of

TGF-β stimulated increases in α-SMA and collagen I protein expression in control CF (Figure 2C) providing evidence that TGF-β driven myofibroblast differentiation may be Nox4-dependent. In failing CF, both basal and TGF-β stimulated α-SMA and collagen I expression were inhibited by siNox4 versus scrambled control (Figure 2C), demonstrating that increased Nox4 expression may contribute to the maladaptive adverse remodeling phenotype in HF. Furthermore, Nox4 knockdown blunted TFG-β stimulated collagen synthesis in both control and failing CF (Figure 2D). Interestingly, knockdown of Nox4 decreased collagen synthesis in the failing CF to near control levels. These studies demonstrate that increased oxidative stress in the setting of HF and also following TGF-β stimulation appear to be mediated, to a large degree, by Nox4 and that knockdown of Nox4 reverses the activated pro-fibrotic phenotype present in failing CF.

Upregulation of β-arrestins in fibroblasts contributes to increased mitochondrial oxidative stress

β-arrestins are scaffold proteins linking G protein-coupled receptor activation to multiple downstream signaling pathways. To identify a potential mechanism by which oxidative stress is regulated in failing CF, we investigated the role of β-arrestins. Expression of both β-arrestin1 and 2 are increased over 4-fold in failing CF compared to controls as demonstrated by immunoblotting (Figure 3A) and immunostaining (Figure 3B). β-arrestin1&2 expression was inhibited using an siRNA approach (Figure 3C). Knockdown of either isoform resulted in significant inhibition of both basal and TGF-β stimulated mitochondrial superoxide production (Figure 3D). To further determine the mechanism by which β-arrestin inhibition decreased oxidative stress, we measured Nox4 mRNA expression

by qPCR. Knockdown of β-arrestin1 (Si-βarr1) led to a significant decrease in both basal and TGF-β stimulated Nox4 expression compared to scrambled controls (Scr) (Figure 3E). We have also measured Nox4 expression at the protein label. Knockdown of either β-arrestin1 (Si-βarr1) or β-arrestin2 (Si-βarr2) led to a significant decrease in both basal and TGF-β stimulated Nox4 expression compared to scrambled controls (Figure 3F). Importantly, TGF-β stimulation in normal control CF leads to upregulation of β-arrestin 1 and 2 expression (Figure 4A and B) and as TGF-β signaling is significantly increased in the setting of HF and pathological remodeling, this provides another mechanism for increased β-arrestin activity. We also quantitated Nox4 expression in separate cellular compartments. The ratio of mitochondrial to plasma membrane bound Nox4 is approximately 2:1 in both control and failing CF (Figure 4C). Interestingly, β-arrestin expression is greater in the mitochondrial fraction relative to the cytosolic fraction under control and failing conditions and is significantly upregulated in HF (Figure 4C).

β-arrestin overexpression increases mitochondrial superoxide production

We further investigated the role of β-arrestins in the regulation of CF oxidative stress utilizing adenoviral-mediated overexpression of β-arrestins in normal control CF. First we confirmed β-arrestin overexpression following adenoviral infection (Figure 5A). Overexpression of β-arrestin1 (Ad-βarr1) or β-arrestin2 (Ad-βarr2) in control CF led to a nearly 2-fold increase in mitochondrial superoxide levels as compared to a control null adenovirus (Ad-Null) (Figure 5B). The increase in mitochondrial ROS generation following β-arrestin overexpression was similar to that following TGF-β stimulation and no additional increase in MitoSox staining was demonstrated following TGF-β stimulation with overexpression of either isoform. Stimulation with TGF-β following infection with Ad-βarr2 resulted in a 2.7-fold increase in mitochondrial superoxide levels that was not statistically different than either Ad-Null + TGF-β or Ad-βarr2 alone. To demonstrate more specifically

that the increased oxidative stress in the setting of β-arrestin1 or 2 overexpression is due to increased Nox4 activity, we treated control CF with apocynin, a non-specific Nox inhibitor, following infection with Ad-βarr1, Ad-βarr2, or Ad-Null. Treatment with apocynin significantly decreased basal oxidative stress in both Ad-βarr1 and Ad-βarr2, reducing MitoSox staining to intensities similar to Ad-Null controls (Figure 5C). Additionally, pre-treatment with apocynin significantly inhibited TGF-β stimulated increases in mitochondrial superoxide production in all three groups. These data demonstrate that increased expression of β-arrestins appears to increase mitochondrial superoxide production in a Nox4-dependent manner.

β-arrestin stimulated oxidative stress is mediated by ERK

TGF-β is known to increase CF myofibroblast differentiation and ECM production through activation of the MAPK/ERK pathway (Zhang YE, 2009). ERK-phosphorylation is significantly increased in failing CF versus controls (Figure 6A). TGF-β stimulation increases ERK-phosphorylation in control but not in failing CF because the level of ERK phosphorylation is already maximal in the failing state (Figure 6A). We investigated whether β-arrestin stimulated increases in mitochondrial superoxide levels were ERK-mediated. Control CF were infected with Ad-βarr1, Ad-βarr2, or Ad-Null. Mitochondrial oxidative stress was quantified under basal conditions, after treatment with the ERK-inhibitor PD98059 (ERK-I), following TGF-β stimulation, or with ERK-I treatment prior to TGF-β stimulation (TGF-β + ERK-I). ERK inhibition significantly decreased basal mitochondrial superoxide production in the setting of β-arrestin1 or 2 overexpression (Figure 6B). Additionally, ERK inhibition blunted any TGF-β stimulated increases in mitochondrial superoxide levels (Figure 6B). To determine whether Nox4-mediated oxidative stress in failing CF may be due to ERK activation, we measured Nox4 expression following treatment with the ERK-I. ERK inhibition diminished basal and TGF-β stimulated Nox4 expression (Figure 6C). Figure 6D

confirms that ERK1/2 phosphorylation is blunted by ERK-I treatment. To determine whether ERK phosphorylation is critical for Nox4- mediated myofibroblast transformation, we measured α-SMA and collagen I expression under basal conditions, after treatment with the ERK-inhibitor PD98059 (ERK-I), following TGF-β stimulation, or with ERK-I treatment prior to TGF-β stimulation (TGF-β + ERK-I) in control fibroblasts. ERK inhibition significantly decreased basal α-SMA and collagen I expression and also decreased TGF-β-stimulated α-SMA and collagen I production (Figure 7A and B). These studies demonstrate that TGF-β and β-arrestin-stimulated increases in oxidative stress appear to be ERK-mediated and provide evidence that increased mitochondrial superoxide production in CF, in the setting of HF, may be regulated by increased ERK activity.

Discussion

Our data demonstrate significant upregulation of mitochondrial superoxide production and Nox4 expression in adult CF isolated from failing human LV compared to non-failing controls. Inhibition of Nox4, either by treatment with apocynin or via siRNA-mediated knockdown, decreased transformation to activated myofibroblasts, as measured by α-SMA expression, and decreased collagen synthesis in both failing and control CF. Our data also shows that upregulation of Nox4 and increased mitochondrial oxidative stress contribute to CF-mediated cardiac fibrosis and remodeling. TGF-β treatment led to significant increases in Nox4 expression and mitochondrial superoxide generation. TGF-β stimulated myoFb differentiation and collagen synthesis were also demonstrated to be Nox4-dependent. MitoSOX fluorescence is a well established and widely utilized method for quantification of mitochondrial superoxide generation. Our data provide clear evidence that changes in Nox4 expression significantly influence mitochondrial oxidant levels as measured by MitoSOX.

β-arrestin1 and 2 are upregulated in CF in the setting of HF. Knockdown of either β-arrestin isoform decreased both Nox4 expression and mitochondrial superoxide production. Furthermore, overexpression of β-arrestins led to a significant increase in mitochondrial superoxide levels through upregulation of Nox4 expression. The ERK inhibitor studies demonstrate that β-arrestin and TGF-β potentially drive increases in Nox4 expression and mitochondrial ROS through ERK activation. These data provide additional evidence for the role of β-arrestins in regulating CF biology and for the potential novel therapeutic role of inhibiting Nox4-mediated mitochondrial ROS production through β-arrestins in the prevention of maladaptive cardiac remodeling and the development of heart failure. This study provides the first evidence that β-arrestins can regulate oxidative stress in adult human CF via Nox4 in an ERK-dependent manner.

ROS production is elevated and contributes to the development of cardiac remodeling following myocardial infarction (Sun Y, 2009). There is significant evidence that ROS activate MAPKs and NF-kB accompanied by cardiac myocyte apoptosis, inflammation, and interstitial fibrosis (Al Ghouleh I et al, 2011). NADPH oxidases are the major sources of ROS in the myocardium in conditions such as MI, HF, and aging and are therefore important contributors to the oxidative stress, cytokine release, and cardiac dysfunction that underlie adverse myocardial remodeling (Kuroda J et al., 2010; Tetsuro A et al., 2010). Nox4 is a major source of ROS in the setting of myocardial injury and plays a critical role in activation of CF and cardiac remodeling (Murdoch CE et al., 2006). Nox4 has been shown to be localized to the mitochondria and was necessary for the development of pressure overload left ventricular hypertrophy in Nox4 transgenic and knockout mouse studies (Tetsuro A et al., 2010). Recent studies have also shown that inhibition of Nox4 by the plant phenolic alkaloid, leonurine, or an siRNA approach in neonatal rat CF attenuated angiotensin II-induced activation of ERK1/2, production of intracellular ROS, and expression of α-SMA and collagen I (Liu XH et al., 2012). Our data confirm the key role of ERK signaling in the upregulation of Nox4 and mitochondrial superoxide production.

Despite previous work on the role of Nox4 in maladaptive cardiac remodeling, mechanisms for the regulation of Nox4 expression and activity have been mostly unknown. β-arrestins are key signaling molecules involved in G protein-coupled receptor (GPCR) signaling. β-arrestin1&2 are the primary isoforms expressed in the heart and target chronically activated GPCRs for internalization and degradation following phosphorylation by GPCR kinases (GRKs) (Kelly E et al., 2008). Chronic HF is characterized by severely impaired β-adrenergic receptor (β-AR) signaling with a 50% decline in total β-AR density and uncoupled signaling in remaining receptors due to increased expression and activity of GRK2 (Bristow MR et al., 1982; Ungerer M et al., 1993). Inhibition of GRK2 in cardiac

myocytes *in vitro* and animal models of HF *in vivo* has been shown to restore β-AR signaling and cardiac function (Dorn GW, 2nd, 2009; Rengo G et al., 2009). We recently demonstrated that inhibition of GRK2 in adult human failing CF decreased myofibroblast transformation and collagen synthesis (D'Souza KM et al., 2011). It appears that β-arrestins regulate CF biology through Nox4/ROS-dependent mechanisms which appear to be ERK-mediated. It is well described that β-arrestin signaling also leads to activation of ERK (Lefkowitz RJ et al., 2006). TGF-β is a very potent stimulus for ERK activation and ERK signaling has been demonstrated to stimulate collagen synthesis (Leask A, Abraham DJ, 2004; Blanchette F et al., 2001). Thus, it appears that the upregulation of β-arrestin expression in HF contributes to increased ERK activation and subsequent upregulation of Nox4 and ROS generation in CF.

In summary, this is the first report demonstrating upregulation of Nox4 and mitochondrial superoxide production in failing adult human CF. This represents an important mechanism for CF-mediated myocardial fibrosis and adverse remodeling, a precursor to heart failure. Upregulation of Nox4 appears to be mediated by β-arrestins via ERK-dependent signaling. We also demonstrated increases in CF oxidative stress in the setting of TGF-β stimulation that are consistent with previous studies in normal adult mouse and human CF. Furthermore, we provide evidence that both TGF-β and β-arrestins signaling through ERK promotes an increase in Nox4 expression and mitochondrial ROS generation. Further investigation of specific mechanisms for the ERK-mediated increase in Nox4 expression is required. In conclusion, inhibition of β-arrestin signaling in a CF-specific manner may represent a novel therapeutic approach to prevent pathological cardiac fibrosis.

Materials and Methods

All cell culture reagents were purchased from Invitrogen Technologies (Eugene, OR) except fetal bovine serum (FBS), which was obtained from Atlanta Biologicals (Lawrenceville, GA). Unless stated otherwise, all additional chemicals were obtained from Sigma-Aldrich (St. Louis, MO). The α-SMA and vimentin antibodies were obtained from Sigma-Aldrich, Collagen types I and III and β-arrestin1&2 from Abcam (Cambridge, MA), phospho-p44/42 MAPK and ERK 1/2 from Cell Signaling Technology, Inc. (Danvers, MA), and GAPDH and α–tubulin from Santa Cruz Biotechnologies (Santa Cruz, CA).

Isolation and Culture of Adult Human Cardiac Fibroblasts (CF). All procedures for tissue procurement in this study were performed in compliance with institutional guidelines for human research and with 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. Left ventricular (LV) tissue was taken from patients with severe LV dysfunction undergoing LV Assist Device (LVAD) implantation. The indication for LVAD in all patients (n=10) was end-stage heart failure (HF), defined as New York Heart Association functional class IV, with deterioration of cardiac function despite maximal medical therapy. Failing CF were isolated by a modified method of D'Souza et al. (D'Souza KM et al., 2011). Non-failing adult human LV CF (control) were purchased from Cell Applications Inc (San Diego, CA). Five different control fibroblast cultures were obtained. To prevent spontaneous differentiation, all studies were carried out in low serum (2.5% FBS) medium using early passage cells (≤4) plated at a density of ~200 cells per mm^2. HF CF were used within two weeks of culturing to ensure preservation of the failing phenotype.

Protein Immunoblotting. Protein immunoblotting was performed following the method described by D'Souza et al (D'Souza KM et al., 2011). Band intensity was quantitated using NIH ImageJ software. GAPDH or α-tubulin was used as a loading control.

siRNA transfection of cardiac fibroblasts. Target-specific siRNA duplexes were designed using the sequence from the open reading frame of human β-arrestins and Nox4 mRNA to knock-down mRNA and protein expression of β-arrestins or Nox4. Human β-arrestin1 (sc-29741, 5′-AAAGCCUUCUGCGCGGAGAAU-3′), β-arrestin2 (sc-29208, 5′-AAGGACCGCAAAGUGUUUGUG-3′), Nox4 (sc-41586) were obtained from Santa Cruz Biotechnology. Scrambled oligo-ribonucleotide complex was also obtained (sc-37007), which was not homologous to any mammalian genes and utilized as control. Cells were transfected with Lipofectamine 2000 (Invitrogen; Carlsbad, CA), according to manufacturers' instructions. Silencing was quantified by immunoblotting and immunofluorescence. Only experiments with verified silencing were used.

Real-time PCR analysis. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μg) was used for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) with Nox4 primers. GAPDH was used to normalize the value. The PCR primer sequences used are as follows: for human Nox4 5′- TGTCCTGCTTTTCTGGAAAACC-3 (forward), 5′-GTAGTCAGAATTGGCTTGGTCG-3 (reverse) and *GAPDH* 5′-AGACCACAGTCCATGCCATC-3′ (forward) and 5′-TTGCCCACAGCCTTGGCAG-3′ (reverse).

Adenoviral Infection of Cell Cultures. Control CF were infected at a multiplicity of infection (MOI) of 10 with either an adenovirus encoding β-arrestin1 (Ad-βarr1), β-arrestin2 (Ad-βarr2), or empty (Ad-Null) adenoviruses. The CF were incubated with the virus for 18-24 hours before stimulation with serum (DMEM /2.5% FBS) or TGF-β (10ng/mL).

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 a final concentration of 10ng/mL or no drug in DMEM with 2.5% FBS. Cells were collected following 60 minutes, 24 hours or 72 hours of treatment. For Nox and ERK-inhibitor studies, cells were treated with 100 μM Apocynin or PD 98059, respectively, (Calbiochem, Billerica, MA) prior to stimulation with TGF-β or no drug.

Collagen Synthesis [^{3}H]proline Incorporation. CF were treated with TGF-β, no drug, PD98059, Apocynin, or a combination as indicated in the figure legends. [^{3}H]proline incorporation was measured according to the method described by Swaney et al. (Swaney JS et al., 2005) and D'Souza et al. (D'Souza KM et al., 2011).

Immunostaining and Confocal Microscopy. CF cells were grown on 12 mm coverslips and immunostaining was carried out following methods described by D'Souza et al (D'Souza KM et al., 2011) using the following primary antibodies (and dilutions): Nox4 (1:50), Vimentin (1:400), β-arrestin1&2 (1:200). Cells were mounted in Fluoroshield™ with DAPI mounting medium (Sigma-Aldrich) and visualized using an Olympus DSU Spinning Disk Confocal.

MitoSOX Staining. Cells were grown on 0.3% gelatin treated glass bottom dishes. After siRNA or adenoviral transfection and drug treatment, cells were washed with HBSS. Cells

were loaded with 5 μM MitoSOX Red (Invitrogen) and 1:1000 dilution of Hoechst 33342 (Invitrogen) in HBSS for 10 min at 37°C protected from the light. The cells were washed gently three times with warm buffer and placed in warm buffer for imaging. The indicator was detected using a confocal microscope at an excitation/emission maxima of 510/580 nm. The total fluorescence intensity per field was quantitated and standardized by total cell number using NIH ImageJ software analysis. The results are represented as fold change in relative fluorescence units.

Statistical Analysis. All data are expressed as mean ± SEM. Student's t-test was used to analyze differences between experimental groups. Single sample t-test was used to analyze experimental groups for data represented as fold change from control. One-way or two-way ANOVA followed by Tukey's HSD post hoc test were used as appropriate for experiments with three or more groups. Values of $p<0.05$ were considered significant.

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Disclosures

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

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Figures

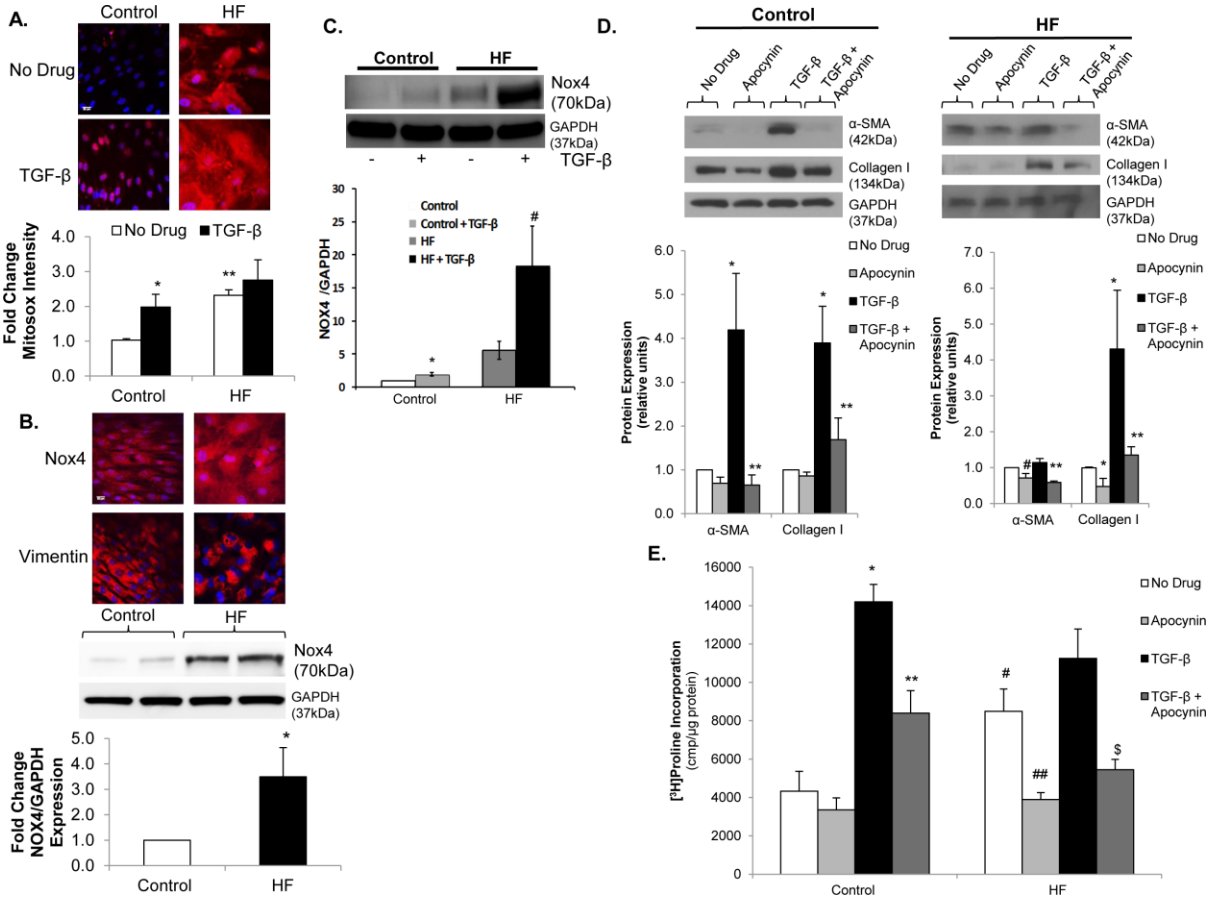

Figure 1. Mitochondrial superoxide production and Nox4 are upregulated in failing cardiac fibroblasts.

A, Confocal images of control and heart failure (HF) cardiac fibroblasts (CF) stained with MitoSOX (*red*) under basal conditions (No Drug) vs. TGF-β stimulation (*upper panel*). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below demonstrates over 2-fold increase in mitochondrial oxidative stress in Control CF in response to TGF-β. *p<0.03 vs. Control + No Drug; **p<0.005 vs. Control + No Drug; n=3-4 in all groups. B, Confocal images (*upper panel*) of Nox4 stained with *red* with Alexa Fluor 594 dye are show in in HF vs. Control CF. Nuclei are stained *blue* with DAPI. Representative immunoblot (*middle panel*) showing Nox4 expression in HF vs. Control CF. GAPDH was

used as a loading control. Densitometric analysis (*lower panel*) demonstrates over 4-fold increase in Nox4 expression in HF. *p<0.05 vs. Control; n=4 in all groups. This membrane was stripped, re-probed and quantitated in figure 3A. C, Representative immunoblot (*upper panel*) showing Nox4 expression under basal conditions and following TGF-β stimulation in Control and HF CF. GAPDH was used as a loading control. Densitometric analysis (*lower panel*) demonstrates increased Nox4 expression in response to TGF-β in both Control and HF. # p<0.05 vs. No Drug; n=5 in all groups. D, Representative immunoblots (*upper panels*) showing the effects of Apocynin on basal and TGF-β stimulated α-SMA and Collagen I expression in both Control (*left*) and HF (*right*) CF. GAPDH was used as a loading control. Densitometric analysis shown below. *p<0.02 vs. No Drug, **p<0.03 vs. TGF-β, #p<0.05 vs. No Drug; n=3-4 for all groups. E, Collagen synthesis in HF vs. Control CF under basal conditions, TGF-β stimulation, Apocynin treatment, and pre-treatment with Apo prior to stimulation with TGF-β. *p<0.001 vs. Control + No Drug; **p<0.002 vs. Control + TGF-β; #p<0.003 vs. Control + No Drug; ##p<0.01 vs. HF + No Drug; $p<0.002 vs. HF + TGF-β; n=3 in all groups.

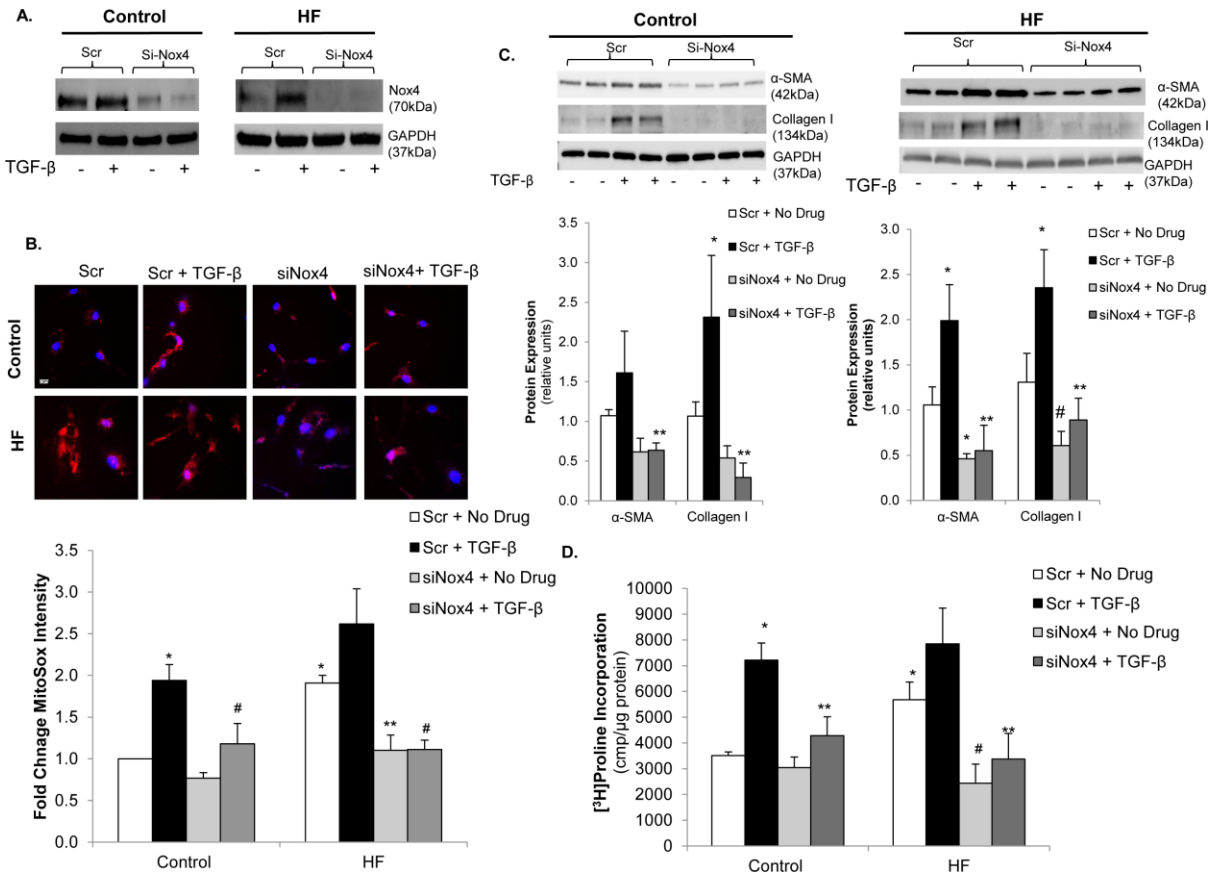

Figure 2. Nox4 mediates myofibroblast transformation and collagen synthesis via increased oxidative stress.

A, Representative immunoblots showing knockdown of Nox4 expression in Control (*left panel*) and heart failure (HF) cardiac fibroblasts (CF) *(right panel*) with Nox4 siRNA (siNox4) vs. scrambled control (Scr). GAPDH was used as a loading control. B, Confocal images of CF stained with MitoSOX (*red*) showing inhibition of mitochonodrial oxidative stress with siNox4 in Control and HF CF (*upper panel*). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. *p<0.003 vs. Control Scr +No Drug, **p<0.005 vs. Scr + No Drug, #p<0.02 vs. Scr + TGF-β; n=3-4 in all groups. C, Representative immunoblots (*upper panels*) showing the effects Nox4 knockdown on basal and TGF-β stimulated α-SMA and Collagen I expression in Control (*right panel*) and HF (*left*

panel) CF. GAPDH was used as a loading control. Densitometric analysis shown below. *p<0.05 vs. Scr + No Drug, **p<0.01 vs. Scr + TGF-β, #p=0.05 vs. Scr + No Drug; n=3-4 in all groups. D, Basal and TGF-β stimulated collagen synthesis in Control and HF CF following transfection with siNox4 or Scr control. *p<0.05 vs. Control Scr + No Drug, **p<0.005 vs. Scr +TGF-β; #p<0.01 vs. HF Scr + No Drug; n=3-4 in all groups.

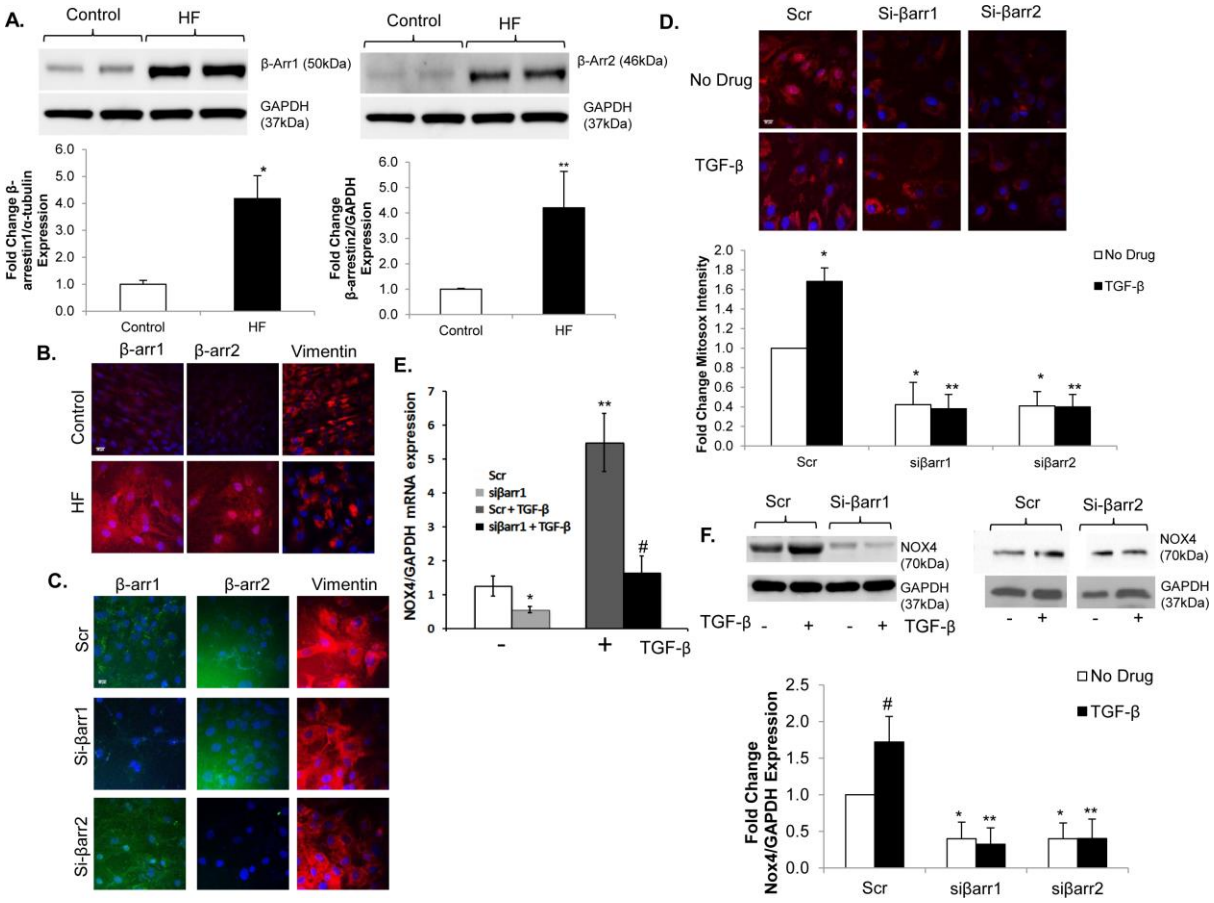

Figure 3. β-arrestins regulate mitochondrial superoxide production and Nox4 expression in cardiac fibroblasts.

A, The blot presented in Figure 1B was stripped and re-probed (*upper panels*) for β-arrestin1 (β-Arr1) (*left*) and β-arrestin2 (β-arr2) (*right*) showing expression in control vs. heart failure (HF) cardiac fibroblasts (CF) with the same GAPDH loading controls. Densitometric analysis (*lower panels*) demonstrates over 4-fold increase in β-arrestin1 and β-arrestin2 expression in HF. *p<0.01 vs. Control; **p<0.04 vs. Control; n=4 for β-arrestin1 expression groups, n=5 for β-arrestin2 expression groups. B, Confocal images of β-arr1, β-arr2, and vimentin stained with *red* with Alexa Fluor 594 dye are show in in HF vs. Control CF demonstrating increased β-arrestin expression in HF. Nuclei are stained *blue* with DAPI. C, Confocal images showing β-arr1 and β-arr2 stained *green* with FITC and vimentin stained

with *red* with Alexa Fluor 594 dye in HF CF following siRNA knockdown of β-arrestin1 (siβarr1), β-arrestin2 (siβarr2), or scrambled control (Scr) demonstrating successful siRNA knockdown of β-arrestins. Nuclei are stained *blue* with DAPI. D, Confocal images (*upper panel*) of failing CF stained with MitoSOX (*red*) following siRNA knockdown of β-arrestin1 (siβarr1), β-arrestin2 (siβarr2), or scrabmled control (Scr). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. *p<0.03 vs. Scr + No Drug; **p<0.001 vs. Scr + TGF-β; n=3 for all groups. E, Quantitative PCR showing basal and TGF-β stimulated Nox4 mRNA expression following siβarr1 vs. Scr control. Values normalized to GAPDH. *p<0.03 vs. siβarr1, **p<0.05 vs. Scr + TGF-β, #p<0.05 vs. siβarr1 + TGF-β; n=3 for all groups. F, Representative immunoblot (*upper panel*) showing basal and TGF-β stimulated Nox4 expression following siβarr1 or siβarr2 vs. Scr control. GAPDH was used as a loading control. Densitometric analysis shown below. *p<0.05 vs. Scr + No Drug, **p<0.001 vs. Scr + TGF-β; n=3 in each group.

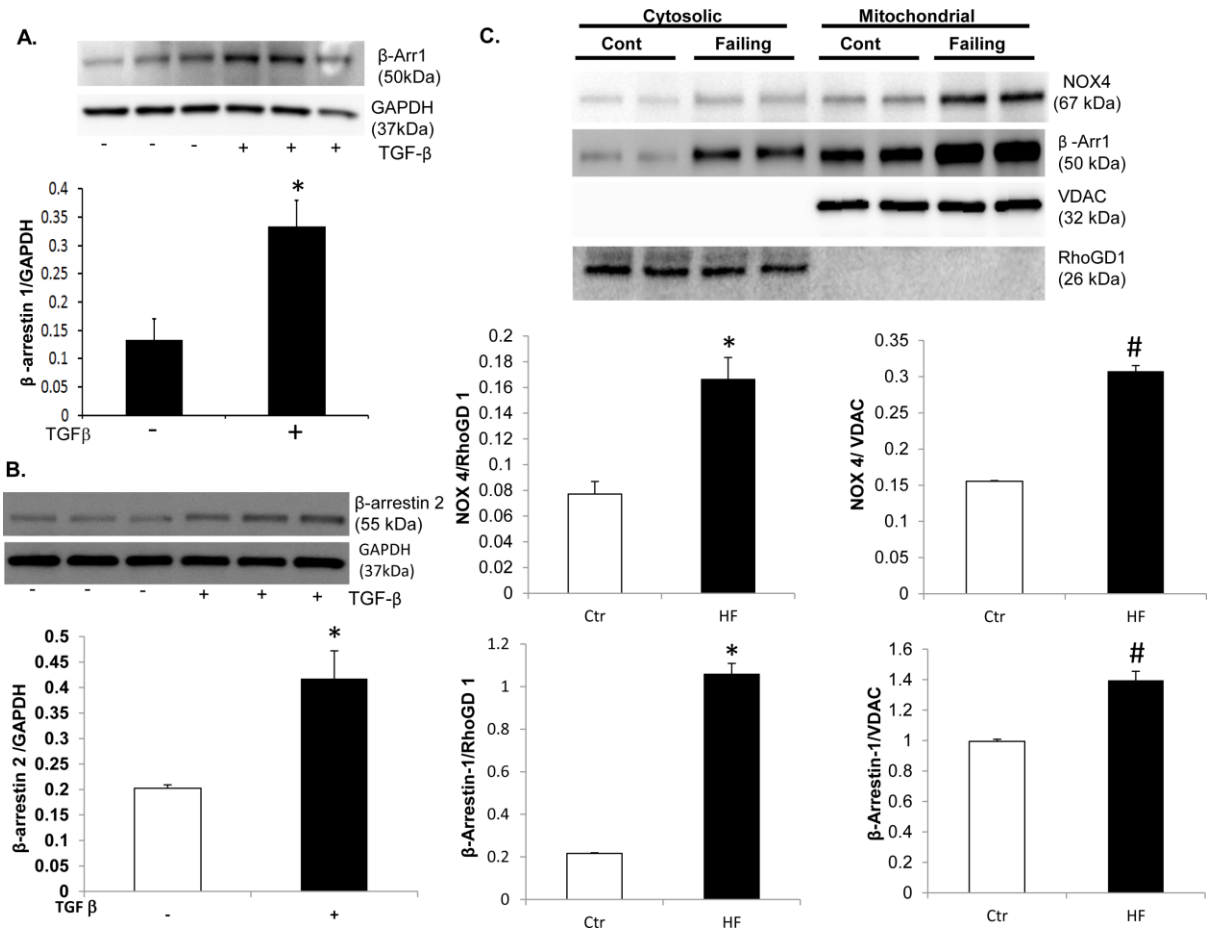

Figure 4. β-arrestin is robustly expressed in fibroblast mitochondria. A, Representative immunoblot (upper panel) showing β-arrestin1 expression under basal conditions and following TGF-β stimulation in control human cardiac fibroblasts. GAPDH was used as a loading control. Densitometric analysis (lower panel) demonstrates increased β-arrestin 1 expression in response to TGF-β in CF. * P<0.004 vs. No Drug. N=3 in each group. B, Representative immunoblot (upper panel) showing β-arrestin 2 expression under basal conditions and following TGF-β stimulation in control human cardiac fibroblasts. GAPDH was used as a loading control. Densitometric analysis (lower panel) demonstrates increased β-arrestin 2 expression in response to TGF-β in CF. * P<0.01 vs. No Drug. N=3 in each group. C, Cytosolic and mitochondrial fractionations were isolated from control and failing

human cardiac fibroblasts. Representative immunoblot (upper panel) showing Nox4 and β-arrestin1 expression in cytosolic and mitochondrial fractions of normal and failing human cardiac fibroblasts. This blot was stripped and re-probed for RhoGD1 and VDAC which were used as cytosolic and mitochondrial loading controls, respectively. Densitometric analysis (lower panel) demonstrates increased Nox4 expression in both cytosolic and mitochondrial fractions in the failing CF. *P=0.02 vs. control in the cytosolic fraction and $^{\#}$P=0.001 vs. control in the mitochondrial fraction. Densitometric analysis (lower panel) demonstrates increased β-arrestin1 expression in both cytosolic and mitochondrial fractions. *P=0.003 vs control in the cytosolic fraction and $^{\#}$P=0.01 vs. control in the mitochondrial fraction.

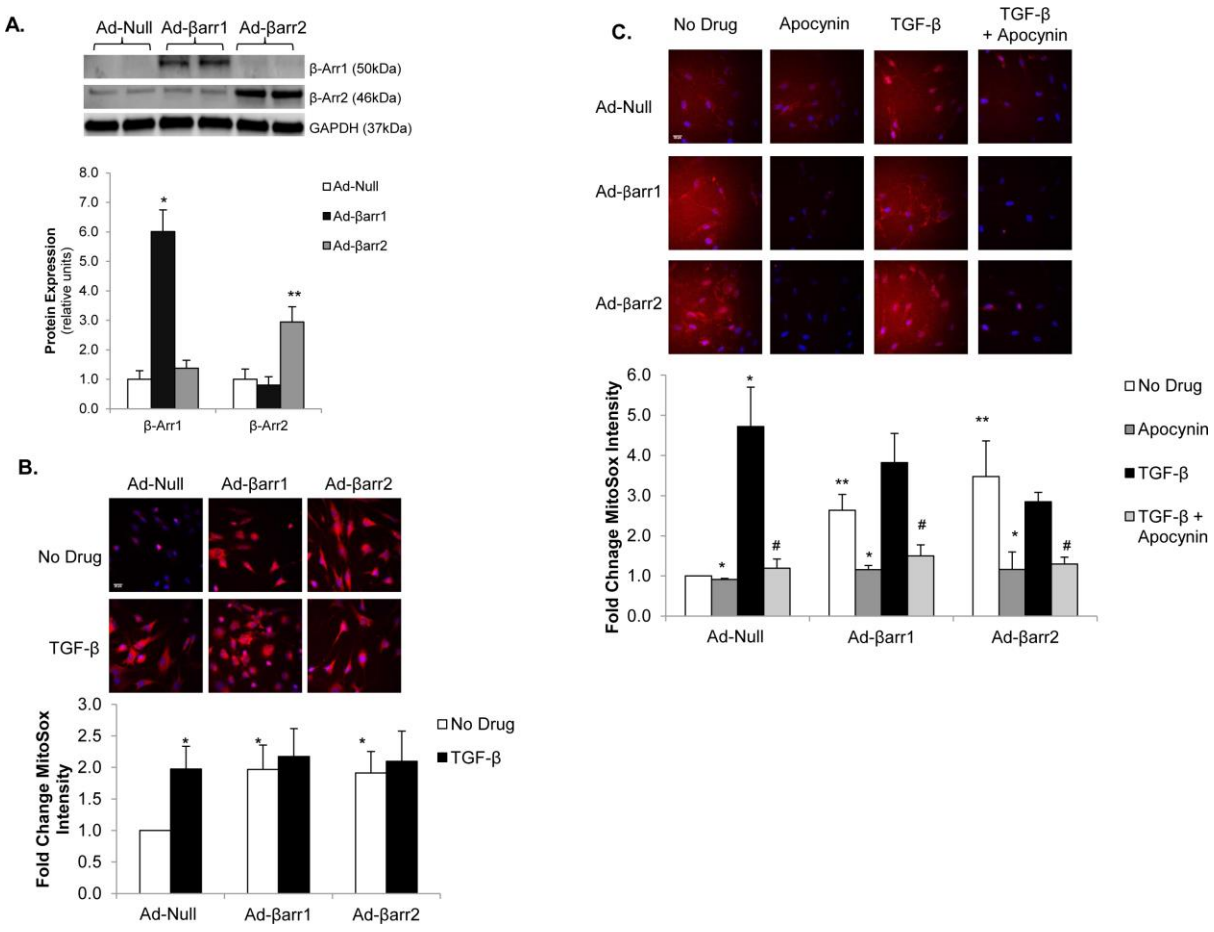

Figure 5. β-arrestin overexpression increases mitochondrial oxidative stress.

A, Representative immunoblot (*left panel*) showing β-arrestin1 (β-Arr1) and β-arrestin2 (β-Arr2) expression following transfection with Ad-βarr1, Ad-βarr2, or Ad-Null. GAPDH was used as a loading control. Densitometric analysis of β-arrestin1 and β-arrestin2 normalized to GAPDH expression shown to the right. *p<0.001 vs. Ad-Null & vs. Ad-βarr2, **p<0.03 vs. Ad-Null & vs. Ad-βarr1; n=3-4 in all groups. B, Confocal images (*upper panel*) of Control cardiac fibroblasts (CF) stained with MitoSOX (*red*) following transfection with adenoviruses overexpressing β-arrestin1 (Ad-βarr1), β-arrestin2 (Ad-βarr2), or control null virus (Ad-Null). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. *p<0.05 vs. Ad-Null + No Drug, n=3-5 in all groups. C, Confocal images of

transfected CF stained with MitoSOX (*red*) showing mitochondrial oxidative stress under basal conditions, TGF-β stimulation, Apocynin treatment, and pre-treatement with Apo prior to stimulation with TGF-β (TGF-β + Apocynin). Nuclei are stained *blue* with Hoechst 33342. Fluorescence quantitation shown below. *$p<0.02$ vs. No Drug; **$p<0.03$ vs. Ad-Null + No Drug; #$p<0.02$ vs. TGF-β, n=3 in each group.

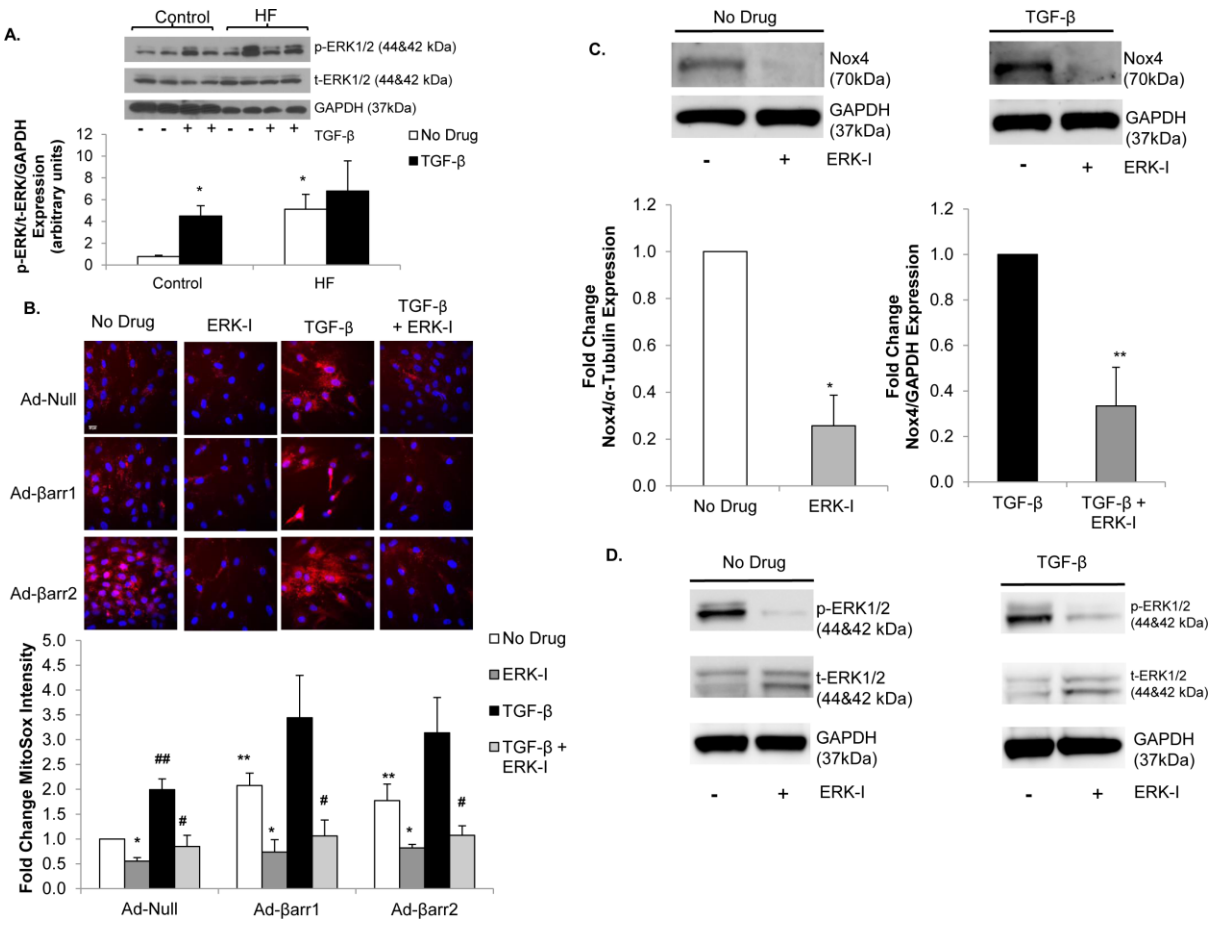

Figure 6. β-arrestin stimulated oxidative stress is mediated by ERK signaling.

A, Representative immunoblot (*upper panel*) showing phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 (t-ERK1/2) expression under basal conditions vs. TGF-β stimulation in Control and heart failure (HF) cardiac fibroblasts (CF). GAPDH was used as a loading control. Densitometric analysis shown below. *p<0.05 vs. Control + No Drug; n=3-6 in all groups. B, Confocal images (*upper panel*) of Control CF stained with MitoSOX (*red*) following transfection with adenoviruses overexpressing β-arrestin1 (Ad-βarr1), β-arrestin2 (Ad-βarr2), or control null virus (Ad-Null) under basal conditions, TGF-β stimulation, treatment with ERK inhibitor PD98059 (ERK-I), and pre-treatment with PD98059 prior to stimulation with TGF-β (TGF-β + ERK-I). Nuclei are stained *blue* with Hoechst 33342.

Fluorescence quantitation shown below. *p<0.05 vs. No Drug; **p<0.04 vs. Ad-Null + No Drug, #p<0.001 vs. TGF-β, ##p=0.05 vs. Ad-Null + No Drug, n=4 for all groups. C, Representative immunoblots (*upper panels*) showing effect of ERK inhibitor on basal (*right panel*) and TGF-β stimulated (*left panel*) Nox4 expression in failing CF. GAPDH were used as a loading controls. Densitometric analysis shown below. *p=0.01 vs. No Drug, **p<0.03 vs. TGF-β; n=3 in each group. D, Representative immunoblots showing decreased basal (No Drug) (*left panel*) and TGF-β stimulated (*right panel*) ERK1/2 phosphorylation following treatment with ERK inhibitor (ERK-I), PD98059. GAPDH was used as a loading control.

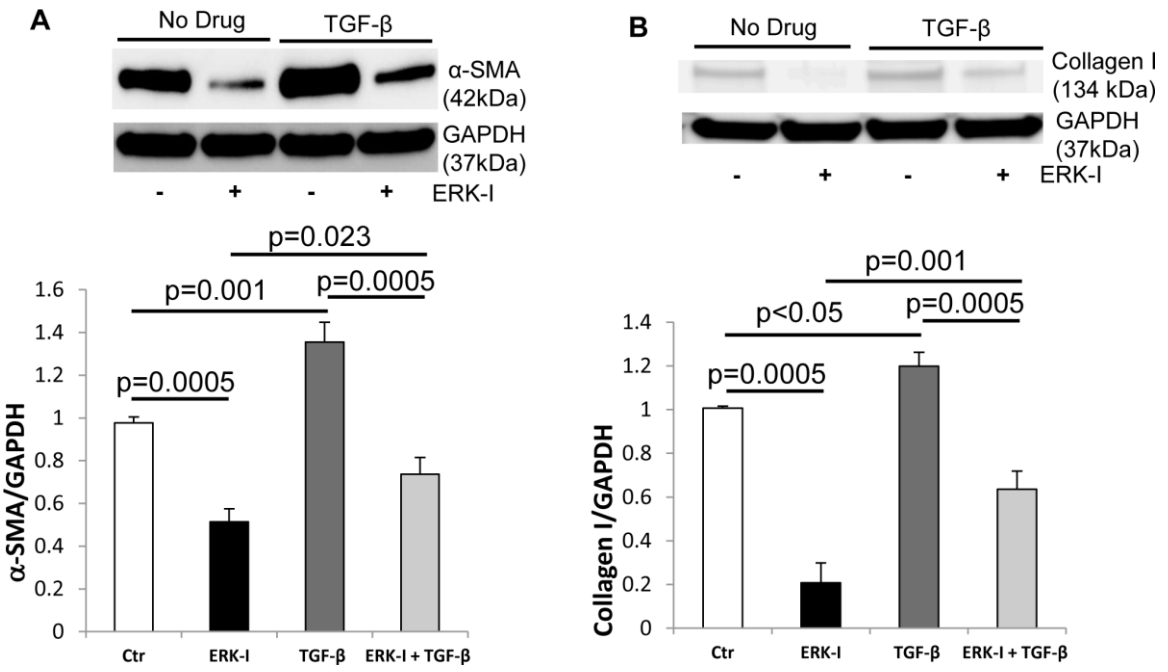

Figure 7. NOX4/oxidative stress-induced myofibroblast transformation is mediated by ERK signaling.

A, Representative immunoblot (*upper panel*) showing a-SMA expression in Control cardiac fibroblasts (CF), under basal conditions, TGF-β stimulation, treatment with ERK inhibitor PD98059 (ERK-I), and pre-treatment with PD98059 prior to stimulation with TGF-β (TGF-β + ERK-I). GAPDH was used as a loading control. Densitometric analysis shown below. p=0.0005 vs. Control + No Drug; p=0.001 vs. Control + TGF-β, p=0.023 vs. ERK-I vs. ERK-I +TGF-β, p=0.0005 vs. TGF-β vs. ERK-I +TGF-β, n=3 for all groups. B, Representative immunoblot (*upper panel*) showing collagen I expression in Control cardiac fibroblasts (CF), under basal conditions, TGF-β stimulation, treatment with ERK inhibitor PD98059 (ERK-I), and pre-treatment with PD98059 prior to stimulation with TGF-β (TGF-β + ERK-I). GAPDH was used as a loading control. Densitometric analysis shown below. p=0.0005 vs. Control + No Drug; p<0.05 vs. Control + TGF-β, p=0.001 vs. ERK-I vs. ERK-I +TGF-β, p=0.0005 vs. TGF-β vs. ERK-I +TGF-β, n=3 for all groups.