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A-kinase anchoring protein-Lbc promotes pro-fibrotic signaling in cardiac fibroblasts

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ABSTRACT

In response to stress or injury the heart undergoes an adverse remodeling process associated with cardiomyocyte hypertrophy and fibrosis. Transformation of cardiac fibroblasts to myofibroblasts is a crucial event initiating the fibrotic process. Cardiac myofibroblasts invade the myocardium and secrete excess amounts of extracellular matrix proteins, which cause myocardial stiffening, cardiac dysfunctions and progression to heart failure. While several studies indicate that the small GTPase RhoA can promote profibrotic responses, the exchange factors that modulate its activity in cardiac fibroblasts are yet to be identified. In the present study, we show that AKAP-Lbc, an A-kinase anchoring protein (AKAP) with an intrinsic Rho-specific guanine nucleotide exchange factor (GEF) activity, is critical for activating RhoA and transducing profibrotic signals downstream of type I angiotensin II receptors (AT_1Rs) in cardiac fibroblasts. In particular, our results indicate that suppression of AKAP-Lbc expression by infecting adult rat ventricular fibroblasts with lentiviruses encoding AKAP-Lbc specific short hairpin (sh) RNAs strongly reduces the ability of angiotensin II to promote RhoA activation, differentiation of cardiac fibroblasts to myofibroblasts, collagen deposition as well as myofibroblast migration. Interestingly, AT_1Rs promote AKAP-Lbc activation *via* a pathway that requires the α subunit of the heterotrimeric G protein G12. These findings identify AKAP-Lbc as a key Rho-guanine nucleotide exchange factor modulating profibrotic responses in cardiac fibroblasts.

1. Introduction

Cardiac fibroblasts constitute the predominant cell population in the myocardium and play a key role in maintaining the structural integrity of the heart through controlled proliferation and extracellular matrix (ECM) turnover [1,2]. In response to a neurohumoral or biomechanical stress placed on the heart, quiescent cardiac fibroblasts undergo a phenotypic transition to profibrogenic myofibroblasts, which are characterized by the expression of α-smooth muscle actin (α-SMA) [1,2], a contractile protein normally associated with smooth muscle cells. Myofibroblasts display a more mobile phenotype [3] and possess a greater synthetic ability to produce collagen-rich ECM proteins and profibrotic cytokines including the transforming growth factor β (TGF-β) [4]. Over the time, excessive formation of fibrous connective tissue and ECM leads to cardiac fibrosis. This impairs electrical coupling between cardiomyocytes and increases myocardial stiffness, leading arrhythmias and severe diastolic dysfunctions. The key role of cardiac fibroblast in the cardiac remodeling process associated with cardiac hypertrophy and heart failure makes them an attractive target for the treatment of heart failure [1].

Among the factors produced in the remodeling heart that affect the phenotype and function of cardiac fibroblasts, angiotensin II (Ang II) has been shown to contribute importantly to the development of cardiac fibrosis [1,3]. In this respect, several studies indicate that the profibrotic actions of Ang II are mainly mediated by the type 1 angiotensin II receptor (AT1R), a G protein coupled receptor linked to the heterotrimeric G proteins G_q and $G_{12/13}$ [5]. By promoting myofibroblast transdifferentiation, activated AT1Rs favor ECM accumulation and TGFβ-1 synthesis, and, as a consequence, fibrosis [3,6].

Several lines of evidence now suggest that the small molecular weight GTPase RhoA plays a crucial role in mediating several profibrotic responses in cardiac fibroblasts [7–10]. In this respect, recent findings indicate that RhoA can promote α-SMA expression in cardiac fibroblasts through a signaling pathway that involves actin polymerization and the nuclear translocation of myocardin related transcription factors (MRTFs) [9,10]. Moreover, exposure of cardiac myofibroblasts to an inhibitor of Rho-kinase, a downstream effector of RhoA, inhibits cardiac fibroblast migration [11]. Finally, disruption of the gene encoding the Rho-kinase ROCK1 reduces collagen deposition and prevents reactive cardiac fibrosis induced by Ang II infusion or pressure overload [12,13].

Abbreviations: GTP, guanosine triphosphate; AKAP, A-kinase anchoring protein; GEF, guanine nucleotide exchange factor; AT_1Rs, type I angiotensin II receptors; sh, short hairpin; ECM, extracellular matrix; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor β; Ang II, angiotensin II; MRTF, myocardin related transcription factor; α1-ARs, α1-adrenergic receptors; NVM, neonatal ventricular myocyte; AVF, adult ventricular fibroblast

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Rho GTPases act as molecular switches, cycling between active GTP-bound and inactive GDP-bound states. *In vivo*, the cycling between the GDP- and GTP-bound forms is regulated by guanine nucleotide-exchange factors (GEFs) that stimulate the exchange of GDP with GTP. We have previously identified an exchange factor mainly expressed in the heart, termed AKAP-Lbc, which functions as GEF for RhoA (Rho-GEF) as well as an A-kinase anchoring protein (AKAP) [14]. In cardiomyocytes, the Rho-GEF activity of AKAP-Lbc Rho-GEF is enhanced in response to the activation of α1-adrenergic receptors (ARs) through a signaling pathway that requires the α subunit of the heterotrimeric G protein G12 [14,15]. Silencing AKAP-Lbc expression in rat neonatal ventricular myocytes (NVMs) strongly inhibits the ability of α1-ARs to induce RhoA activation and hypertrophic responses, suggesting a role for this anchoring protein in cardiac remodeling [15,16].

While the implication of RhoA signaling in cardiac fibrosis has been proposed by several studies, the identity of the Rho-GEFs that transduces fibrotic signals in cardiac fibroblasts has remained elusive. In the present study, we used a lentivirus-based strategy to deliver AKAP-Lbc-specific short hairpin (sh) RNAs into primary cultures of rat adult ventricular fibroblasts (AVFs) or myofibroblasts (AVMyoFs). Using this approach, we could demonstrate that AKAP-Lbc plays a key role in mediating Ang II-induced fibrotic responses. In particular, we found that AKAP-Lbc participates in a signaling cascade activated by AT_1Rs that includes $G\alpha_{12}$, AKAP-Lbc, and RhoA. This pathway promotes α-SMA expression and myofibroblast differentiation and associated increased TGF-β1 production, collagen deposition as well as myofibroblast motility. Collectively, these findings identify AKAP-Lbc as the first Rho-GEF regulating profibrotic signals in cardiac fibroblasts.

2. Material and methods

2.1. Expression constructs

Double-stranded hairpin oligonucleotides based upon rat AKAP-Lbc mRNA sequences (GI: 198386327, bases 6347–6365 and 6626–6644) were cloned into the HindIII and BglII sites in the pSUPER vector. The following oligonucleotide sequences were used: AKAP-Lbc shRNA1 (sense strand) 5′-GCAAGTCGATCATGAGAAT-3′; AKAP-Lbc shRNA2 (sense strand) 5′-GGATAAGCGTTTCCAAGCC-3′; mutated AKAP-Lbc shRNA (sense strand) 5′-GCATGTCGATCATGCGATT-3′. Underlined base pairs in the mutated shRNA differ from the wild-type AKAP-Lbc shRNA. To generate lentiviral transfer vectors encoding AKAP-Lbc shRNAs, cDNA fragments containing the H1 RNA polymerase III promoter as well as the sequences encoding shRNAs were excised using BamHI/SalI from the pSUPER vector and subcloned into the pAB286.1 transfer vector. To generate the $G\alpha_{12}$ G228A-pAB286.1 vector, a BamHI/XhoI fragment containing the CMV promoter and the entire open reading frame for the $G\alpha_{12}$ G228A mutant was PCR-amplified from the $G\alpha_{12}$ G228A-pCDNA3.1 vector and subcloned into the BamHI and SalI sites in the pAB286.1 vector. To generate the RhoA N19-pAB286.1 vector, a BamHI/SalI fragment containing the CMV promoter and the entire open reading frame for the RhoA N19 mutant was PCR-amplified from the RhoA N19-pFLAG-CMV-6c vector and subcloned into the BamHI and SalI sites in the pAB286.1 vector. The lentiviral packaging vectors pCMVDR8.91 and pMD2.VSVG encode the viral capsid and the vesicular stomatitis virus-G envelope protein, respectively. Plasmids encoding GFP fusions of AKAP-Lbc and AKAP-Lbc Y2153F were described previously [14].

2.2. Peptide synthesis

Arg_{11}-SuperAKAP-*IS* (Arg_{11}-QIEYVAKQIVDYAIHQA) and Arg_{11}-scrambled SuperAKAP-*IS* (Arg_{11}-QDVEIHVKAAYYQQIAI) were synthesized and purified to >90% purity (GenWay Biotech).

2.3. Preparation of adult rat ventricular fibroblasts

AVFs were isolated from 10-week-old male Sprague–Dawley rats. Briefly animals were euthanized in a prefilled CO_2 chamber with 100% concentration of CO_2, and excised hearts were washed in ice-cold 1× ADS buffer (116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM D-glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$). Ventricular tissues were minced into small pieces and incubated overnight in a solution of 0.5 mg/ml trypsin (USB) at 4 °C on an orbital shaker at 80 rpm. Trypsinized tissues subsequently underwent four cycles of enzymatic digestion for 7 min at 37 °C in a solution of 80 mg/ml of type II collagenase (Worthington). Cells were pelleted by centrifugation (800 rpm, 10 min) and resuspended in DMEM supplemented with 10% FBS and penicillin/streptomycin (Gibco). The resuspended cells were then passed through a 70 μm-cell strainer, seeded onto uncoated plastic dishes and incubated for 2 h at 37 °C with 10% CO_2. DMEM was subsequently replaced with EGM-2 to maintain cardiac fibroblasts in an undifferentiated state [17]. Differentiation of AVFs to AVMyoFs was induced by culturing cells in DMEM supplemented with 10% FBS and penicillin/streptomycin. Purity of AVFs and AVMyoFs preparations was determined by immunofluorescence staining using mouse monoclonal anti-α-actinin (Sigma, 1:500 dilution), rabbit polyclonal anti-desmin (Cell Signaling, 1:25 dilution) and mouse monoclonal cy3-conjugated anti-vimentin antibodies (Sigma, dilution = 1:250). The presence of contaminant endothelial cells was assessed by incubating primary cultures for 4 h in the presence of Dil-acyl LDL (Biomedical Technologies Inc., dilution = 1:20). Cultures contained almost exclusively primary ventricular fibroblasts, as >95% of the cells were positive for the fibroblast marker vimentin, were negative for the cardiomyocyte marker α-actinin and the vascular smooth-muscle cell marker desmin, and were not labeled with Dil-acyl LDL (see Fig. S1A–D, in Supplementary material). Neonatal rat cardiomyocytes were used as a positive control for α-actinin and desmin staining and as a negative control for vimentin staining. The endothelial EA.hy 926 cell line was used as a positive control for Dil-acyl LDL labeling. Western blot analysis of AVF lysates confirmed the presence of vimentin but not α-actinin and desmin (see Fig. S1E–G, in Supplementary material). AVMyoF transdifferentiation was assessed by staining cells using mouse monoclonal anti-α-SMA antibodies (Sigma, dilution = 1:500) (see Fig. S1H, in Supplementary material).

2.4. Cell culture and treatment

Rat AVFs or AVMyoFs were seeded on 6-cm uncoated plastic dishes at 6×10^5 cells per dish for Rhotekin pulldown experiments, Western blots and real time PCR experiments. For indirect immunofluorescence experiments, ventricular fibroblasts were seeded on 4.2 cm^2 TPX Lab-Tek chamber slides at 5×10^4 cells per chamber. For [^{3}H]-proline incorporation assays, AVFs were seeded on uncoated 25-mm wells at 3×10^5 cells per well. When indicated, cells were treated with losartan (1 μM; Sigma), SB203580 (10 μM; Calbiochem), PD123319 (1 μM; Sigma), BMS-345541 (5 μM; Sigma), RO318220 (10 μM; Sigma), Gö6976 (0.5 μM; Sigma), or Na_3V0_4 (1 mM; Sigma) 6 h prior stimulation with Ang II (100 nM; Sigma).

2.5. Production of lentiviruses

VSV-G pseudotyped lentiviruses were produced by cotransfecting 293-T cells with 20 μg of the pAB286.1 vectors encoding either the AKAP-Lbc shRNAs, the $G\alpha_{12}$ G228A dominant negative mutant, or the Flag-RhoA N19 construct, 15 μg of pCMVDR8.91, and 5 μg of pMD2.VSVG using the calcium phosphate method. Culture medium was replaced by serum-free DMEM at 12 h after transfection. Cell supernatants were collected 48 h later, filtered through a 0.22-μm filter unit and concentrated using Centricon-Plus-70 MW 100,000 columns (Millipore). Virus titers were determined by infecting HEK293 cells using serial dilutions of the viral stocks and by scoring puromycin-