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Sustained β-AR stimulation induces synthesis and secretion of growth factors in cardiac myocytes that affect on cardiac fibroblast activation

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ABSTRACT

Paracrine factors, including growth factors and cytokines, released from cardiac myocytes following β-adrenergic receptor (β-AR) stimulation regulate cardiac fibroblasts. Activated cardiac fibroblasts have the ability to increase collagen synthesis, cell proliferation and myofibroblast differentiation, leading to cardiac fibrosis. However, it is unknown which β-AR subtypes and signaling pathways mediate the upregulation of paracrine factors in cardiac myocytes. In this study, we demonstrated that sustained stimulation of β-ARs significantly induced synthesis and secretion of growth factors, including connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF), via the cAMP-dependent and protein kinase A (PKA)-dependent pathways. In addition, isoproterenol (ISO)-mediated synthesis and secretion of CTGF and VEGF through the β_1-AR and β_2-AR subtypes. Paracrine factors released by cardiac myocytes following sustained β-AR stimulation are necessary for the induction of cell proliferation and synthesis of collagen I, collagen III and α-smooth muscle actin (α-SMA) in cardiac fibroblasts, confirming that β-AR overstimulation of cardiac myocytes induces cardiac fibrosis by releasing several paracrine factors. These effects can be antagonized by β-blockers, including atenolol, metoprolol, and propranolol. Thus, the use of β-blockers may have beneficial effects on the treatment of myocardial fibrosis in patients with heart failure.

1. Introduction

Under physiological condition, heart rate and contractility are predominantly controlled by the sympathetic and parasympathetic nervous systems mediated through adrenergic and cholinergic signals, respectively [1]. However, in pathophysiological conditions of the heart, there is a decrease in the blood pumped by the heart, leading to an increase in the sympathetic activity to improve cardiac output via β-adrenergic receptors (β-ARs) [1]. In the short term, these compensatory mechanisms preserve heart functions and produce benefits. However, prolonged overstimulation of β-ARs contributes to other pathological cardiac dysfunctions, including apoptosis [2], myocardial hypertrophy and heart failure (HF) [3], and cardiac insulin resistance [4]. Moreover, myocardial and circulating levels of the catecholamines epinephrine and norepinephrine (NE) in patients with HF are elevated and associated with poor outcomes [5].

A previous study demonstrated that populations of isolated human cardiac myocytes and human cardiac fibroblasts in primary cultures are able to synthesize and to secret interleukin-6 (IL-6) [6]. In addition, stimulation of β-ARs by isoproterenol (ISO) increased the IL-6 protein levels in serum and mouse myocardium [7]. Interestingly, the increased levels of other inflammatory cytokine, including IL-1β and IL-8, during cardiac injury have been reported [8,9]. In the case of growth factors, the synthesis and secretion of brain derived neurotropic factor (BDNF) and vascular endothelial growth factor (VEGF) by various normal cells as well as cardiac myocytes has been described [10,11]. Treatment with norepinephrine (NE) significantly increased VEGF mRNA expression in rat cardiac myocytes via a paracrine mechanism [11]. In addition, forskolin (an adenylate cyclase activator) has been shown to significantly enhance VEGF mRNA expression in cardiac myocytes [12]. Moreover, treatment with metoprolol affects the mRNA expression of CTGF and transforming growth factor-β (TGF-β) in cardiac myocytes [13]. In summary, it is possible that stimulation of the β-ARs of cardiac myocyte could increase the synthesis and secretion of several growth

Abbreviations: α-SMA, α-smooth muscle actin; AC, adenylate cyclase; 6-Bnz-cAMP, N^6-benzoyladenosine-3′,5′-cyclic monophosphate; cAMP, cyclic adenosine monophosphate; COL1A1, collagen type I alpha1; COL1A2, collagen type I alpha2; CTGF, connective tissue growth factor; DDA, 2′,5′-dideoxyadenosine; ECM, extracellular matrix; Epac, exchange protein directly activated by cAMP; ISO, isoproterenol; PKA, protein kinase A; PKI, myristoylated protein kinase A inhibitor amide 14–22; VEGF, vascular endothelial growth factor

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factors and cytokines. However, the relationship of β-AR stimulation to the synthesis and secretion of cytokines and growth factors in cardiac myocytes remains to be clarified.

The β-ARs, a type of G protein-coupled receptor (GPCR), have been classified into three subtypes, β_1-, β_2-, and β_3-ARs, which initially couple with the $G\alpha_s$ protein. In the normal heart, β_1-AR comprises 75–80% of the total cardiac β-ARs whereas β_2-AR is approximately 20% of the total cardiac β-ARs. The amount of β_3-AR in the heart is small [14]. Normally, catecholamine induces changes in cardiac functions through both β_1- and β_2-AR with different intracellular signaling. After ligand binding to the β-ARs, the $G\alpha_s$ protein dissociates from the $G_{\beta\gamma}$ subunit and then the $G\alpha_s$ protein activates adenylate cyclase (AC), leading to an increase in cAMP. The increase in cAMP then stimulates both protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) downstream signaling pathways [4,15] However, it is still unclear which β-AR subtypes and their signaling pathways mediate the upregulation of cytokines and growth factors in cardiac myocytes.

As mentioned above, growth factors and cytokines secreted from cardiac myocytes following β-AR stimulation might activate cardiac fibroblasts, leading to cardiac fibrosis. Cardiac fibrosis is characterized by the overproduction of the ECM proteins (e.g., collagen I and, collagen III) [16], the induction of cardiac fibroblast proliferation, the expression of pro-contractile proteins of the myofibroblasts (e.g., α-SMA) and the stiffening of the myocardium [16], and it is often a secondary condition that develops in response to heart failure [17]. However, it is unclear whether the overstimulation of the β-ARs of cardiac myocytes leads to the synthesis and secretion of several paracrine factors, which then induce cardiac fibroblast differentiation into myofibroblasts, the proliferation of cardiac fibroblasts and an abnormal production of ECM proteins.

The β-AR antagonists, or β-blockers, which block the overstimulation of catecholamine to β-ARs, are widely used for treating heart diseases, including heart failure, angina, and myocardial infarction. Considering that the β-ARs mediate the synthesis and secretion of several growth factors and cytokines, and these secreted paracrine factors activate cardiac fibroblasts, in this study, we hypothesized that the use of β -blockers may antagonize the β-AR-mediated synthesis and secretion of paracrine factors from cardiac myocytes. The mechanism of β-AR signaling and regulation of cardiac fibrosis significantly advances our understanding of this important β-AR signaling pathway in the heart.

2. Materials and methods

2.1. Materials

Isoproterenol (ISO), atenolol, metoprolol, propranolol, ESI-09, SR59230A, ICI118551, and CGP20712A were purchased from Sigma-Aldrich. Forskolin, 2′,5′-dideoxyadenosine (DDA), PKA inhibitor 14–22 amide (PKI), and KN-93 (CaMKII inhibitor) were purchased from Calbiochem. 8-pCPT-2′-O-Me-cAMP (8-CPT), ESI-09, and N^6-benzoyladenosine-3′-5′-cyclic monophosphate (6-Bnz-cAMP) were purchased from Tocris. Dulbecco's Modified Eagle Medium (DMEM), 0.25% trypsin-EDTA solution, penicillin/streptomycin solution, and fetal bovine serum (FBS) were purchased from Gibco. Collagenase type A was purchased from Roche Diagnostics.

2.2. Isolation of neonatal rat cardiac myocytes and fibroblasts

In this study, the animals were handled according to the approved protocol and the animal welfare regulations of the author's institutional Review Board of the Faculty of Pharmacy, Mahidol University (Protocol no. PYR002/2556 and PYR001/2560). Primary cell cultures of cardiac myocytes and fibroblasts were derived from 1- or 2-day old neonatal Sprague-Dawley rats which were purchased from the National Laboratory Animal Center, Mahidol University. Neonatal rat cardiac myocytes and fibroblasts were isolated as previously described [18]. Briefly, neonates were euthanized by decapitation and the hearts were removed, cut into small pieces, and digested with collagenase A solution. Cells were plated on 10-cm dishes and incubated for 3 h at 37 °C in a CO_2 incubator. After 3 h, cardiac fibroblasts were attached to the bottoms of 10-cm dishes, whereas non-adherent cardiac myocytes were removed by changing the culture medium. We plated cardiac myocytes on gelatin-coated 6-well plates (1×10^6 cells/well) and cultured them in DMEM containing 10% FBS, 5 mM taurine, 1 μg/ml insulin, 1 μg/ml transferrin, 10 ng/ml selenium and 1% (v/v) penicillin/streptomycin (P/S). Cardiac fibroblasts were maintained in DMEM containing 10% FBS and 1% P/S solution. All the fibroblast cells used in these experiments were taken from passage 1 or 2. The purity of cells was confirmed by both cellular morphology and immunostaining. The medium was changed to serum-free DMEM before stimulation.

2.3. mRNA expression analysis

Cardiac myocytes were seeded in gelatin coated 6-well plates at a density of 1×10^6 cells/well, whereas cardiac fibroblasts were cultured in 6-well plates at a density of 2×10^5 cells/well. The total RNA was extracted from cardiac myocytes and cardiac fibroblasts by using the GeneJET RNA Purification Kit (Thermo Scientific). The mRNA expression levels of growth factor genes, cytokine genes and fibrogenic genes were determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) using KAPA SYBR FAST One step RT-qPCR kits (KAPA Biosystems). Gene specific primers for growth factors, cytokines, collagen type I alpha1 (COL1A1), collagen type I alpha2 (COL1A2), collagen type III (COL3), and α-SMA were designed as shown in Supplementary Table 1. The expression of targeted genes was normalized to GAPDH. Comparisons of mRNA expression levels were performed based on the differences in ΔC_T of individual samples ($\Delta\Delta C_T$).

2.4. Enzyme-linked immunosorbent assays (ELISA) for VEGF and CTGF secretion

Cardiac myocytes were plated in gelatin coated 6-well plates (1×10^6 cells/well) and then treated with pharmacological agents in serum-free medium. After 24 h, the culture medium were collected and stored at − 80 °C. The CTGF and VEGF levels were assayed according to the manufacturer's instructions (VEGF Rat ELISA Kit ab100786 from Abcam, UK and CTGF ELISA Kit SEA010Ra from Cloud-Clone, USA). A 100 μl sample of each standard and each assay (culture medium) was added to an appropriate well. After the stop solution was added, the absorbance of each reaction was measured at 450 nm with an Infinite M200 microplate reader (TECAN, Switzerland).

2.5. Cell proliferation assay

Cell proliferation was investigated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays which depend on the ability of living cells to transform the yellow tetrazolium salt of MTT into an insoluble formazan salt. Cardiac fibroblasts were plated in 96-well plates at a density of 5×10^3 cells/well in DMEM with 1% FBS and 1% P/S solution and incubated overnight. The cells in each well were treated with 200 μl of culture medium from treated cardiac myocytes for 24 h. The experiments were performed in 3 replicate wells. After stimulation, the MTT solution (1 mg/ml) was added to each well and incubated for 3 h at 37 °C. The remaining MTT was removed, and the formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm using a microplate reader (TECAN). The percentage of viable cells was calculated according to the following equation.