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β_3-adrenergic receptor activation induces TGFβ1 expression in cardiomyocytes via the PKG/JNK/c-Jun pathway

Zhongcheng Xu [a, b], Jimin Wu [b], Junzhou Xin [a, b], Yenan Feng [b], Guomin Hu [b], Jing Shen [b], Mingzhe Li [b], Youyi Zhang [b], Han Xiao [b, *], Li Wang [a, **]

[a] The 3rd Department of Cardiology, The First Affiliated Hospital of the Medical College, Shihezi University, Shihezi City, Xinjiang, 832008, China
[b] Department of Cardiology and Institute of Vascular Medicine, Peking University Third Hospital, Key Laboratory of Cardiovascular Molecular Biology and Regulatory Peptides, Ministry of Health, Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing Key Laboratory of Cardiovascular Receptors Research, Beijing, 100191, China

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ABSTRACT

In heart failure, the expression of cardiac β_3-adrenergic receptors (β_3-ARs) increases. However, the precise role of β_3-AR signaling within cardiomyocytes remains unclear. Transforming growth factor β1 (TGFβ1) is a crucial cytokine mediating the cardiac remodeling that plays a causal role in the progression of heart failure. Here, we set out to determine the effect of β_3-AR activation on TGFβ1 expression in rat cardiomyocytes and examine the underlying mechanism. The selective β_3-AR agonist BRL37344 induced an increase in TGFβ1 expression and the phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun in β_3-AR-overexpressing cardiomyocytes. Those effects of BRL37344 were suppressed by a β_3-AR antagonist. Moreover, the inhibition of JNK and c-Jun activity by a JNK inhibitor and c-Jun siRNA blocked the increase in TGFβ1 expression upon β_3-AR activation. A protein kinase G (PKG) inhibitor also attenuated β_3-AR-agonist-induced TGFβ1 expression and the phosphorylation of JNK and c-Jun. In conclusion, the β_3-AR activation in cardiomyocytes increases the expression of TGFβ1 via the PKG/JNK/c-Jun pathway. These results help us further understand the role of β_3-AR signaling in heart failure.

1. Introduction

The sympathetic–adrenergic system is over-activated in failing hearts, manifesting as a chronic increase in circulating catecholamine levels and sympathetic activation. As typical cardiac adrenergic receptors, β-adrenergic receptors (β-ARs) mediate positive inotropic effects following sympathetic activation in compensated cardiac remodeling and play an important role in progression to decompensated heart failure. In failing hearts, β_1-ARs are significantly desensitized and down-regulated upon sustained receptor activation, and β_2-ARs remain unchanged in their protein level but shift their signaling from a Gs-to Gi-dependent pathway [1]. In contrast, the expression of β_3-ARs gradually increases due to the lack of the phosphorylation sites of protein kinase A (PKA) and G protein-coupled receptor kinase (GRK) [2–4], which suggest the potential role of β_3-AR signaling in heart failure.

Although reported to mediate both Gs- and Gi-dependent signaling in other tissues, β_3-ARs primarily couple with Gi proteins in the myocardium and induce negative inotropic effects through the NO synthase/cyclic guanosine monophosphate/protein kinase G (NOS/cGMP/PKG) pathway [2,5,6]. This is opposite to the signaling effects of β_1/β_2-ARs, which mainly mediate positive inotropic effects via Gs protein-dependent signaling. Numerous recent animal studies have found that β_3-ARs inhibit cardiac remodeling and dysfunction in heart failure following

Abbreviations: AP-1, activator protein 1; β-AR, β-adrenergic receptor; cGMP, cyclic guanosine monophosphate; GRK, G protein-coupled receptor kinase; HFrEF, heart failure with reduced ejection fraction; HNF4α, hepatocyte nuclear factor 4 α; JNK, c-Jun N-terminal kinase; LVEF, left ventricle ejection fraction; NOS, nitric oxide synthase; NRCM, neonatal rat cardiomyocyte; PKA, protein kinase A; PKG, protein kinase G; siRNA, small interfering RNA; SREBP-1, sterol regulatory element-binding protein 1; TGFβ1, transforming growth factor β1.

* Corresponding author. Institute of Vascular Medicine, Peking University Third Hospital, Beijing, 100191, China.
** Corresponding author. The 3rd Department of Cardiology, The First Affiliated Hospital of the Medical College, Shihezi University, No. 107 North 2nd Road, ShiheziXinjiang, 832008, China.
E-mail addresses: sino100@outlook.com (Z. Xu), wujimin1989@126.com (J. Wu), 294344316@qq.com (J. Xin), fyn365@126.com (Y. Feng), guominhu@bjmu.edu.cn (G. Hu), sj152230@126.com (J. Shen), lmz@pku.edu.cn (M. Li), zhangyy@bjmu.edu.cn (Y. Zhang), xiaohan@bjmu.edu.cn (H. Xiao), 183304153@qq.com (L. Wang).

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hemodynamic overload and neurohormone stimulation [7–9]. However, inconsistent with these preclinical results, a clinical trial of a β_3-AR selective agonist, mirabegron, in patients suffering from heart failure with reduced ejection fraction (HFrEF) did not reach the primary end-point, that left ventricle ejection fraction (LVEF) was not altered by treatment with the β_3-AR agonist [10]. In fact, a few studies have reported that blocking β_3-AR signaling with antagonists is beneficial to cardiac function in some heart-failure animal models [11–14]. These results suggest that β_3-AR signaling may have a pathogenic role in addition to its therapeutic effect in heart failure. The precise role of β_3-AR signaling within cardiomyocytes in promoting cardiac remodeling is not yet clear, and understanding this role is very important for understanding the role of β_3-AR activation in the pathogenesis of heart failure and for improving β_3-AR-based drug discovery to treat heart failure.

Previous studies reported that transforming growth factor β1 (TGFβ1) is a crucial cytokine mediating the cardiac remodeling that plays a causal role in the progression of heart failure [15–17]. The humoral factors associated with hypertensive myocardial remodeling, especially angiotensin II, can stimulate myocardial cells to secrete TGFβ1 and cause cardiac remodeling. Angiotensin II induces the transcription of TGFβ1 by inducing the expression and activation of different transcription factors, such as activator protein-1 (AP-1) in cardiomyocytes [18], SREBP-1 in mesangial cells [19] and HNF4α in cardiac fibroblasts [20]. In this study, we investigated the effect of β_3-AR activation on the expression of TGFβ1 in cardiomyocytes and examined the underlying signaling pathway.

2. Materials and methods

2.1. Reagents

The β_3-AR agonist BRL37344 and antagonist SR59230A were purchased from Sigma-Aldrich (St. Louis, MO, USA), the c-Jun N-terminal kinase (JNK) inhibitor SP600125 was purchased from Selleck Chemicals (Houston, Texas, USA), and the PKG inhibitor KT5823 was purchased from Abcam (Cambridge, UK). Antibodies against JNK, phospho-JNK(Thr183/Tyr185), c-Jun, phospho-*c*-Jun (Ser73), and GAPDH were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Isolation and culture of primary neonatal rat cardiomyocytes (NRCMs)

All experimental procedures and animal protocols were approved by the Committee on the Ethics of Animal Experiments (LA2010-035) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 2011). Cardiomyocytes were isolated and cultured from 1-day-old Sprague-Dawley rats as described previously [21]. In brief, cardiomyocytes were dispersed by digesting hearts with 0.1% trypsin (Gibco, CA, USA) and 0.013% collagenase II (Gibco, CA, USA) at 37 °C. Dissociated cells were plated on 10-cm culture dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) containing 10% fetal bovine serum and antibiotics (Gibco, CA, USA) for 2 h. The non-attached cardiomyocyte-rich fraction was plated on culture dishes, and 10^{-4} mol/L bromodeoxyuridine was included to prevent fibroblast proliferation. The cells were incubated in serum-free medium for 12 h before drug or mock treatments.

2.3. β_3-AR overexpression in NRCMs

β_3-AR-overexpressing lentiviruses were constructed by Genechem Co. (Shanghai, China). The lentiviruses were thawed on ice and added into the corresponding wells at a MOI of 5 according to the experimental design. At 36 h after the transfection, the cells were starved with serum-free DMEM for 12 h and then treated and collected for further analysis.

2.4. Small interfering RNA (siRNA) transfection

NRCMs were transfected with c-Jun siRNA at 4 x 10^{-8} mol/L along with Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). After 24 h, cells were treated with drug or mock. c-Jun siRNA was designed and synthesized by Genepharma Co. (Shanghai, China). The sequences were as follows: sense, 5′−CCAAGAACUCGGACCUUCUTT-3’; antisense, 5′-AGAAGGUCCGAUUCUUGGTT-3′.

2.5. RNA extraction and real-time PCR

Total RNA was extracted from NRCMs using Trizol reagent (Invitrogen, CA, USA). One microgram of total RNA was reverse transcribed into cDNA. The expression of rat β_3-AR, TGFβ1 and c-Jun mRNA was determined by real-time PCR (Eppendorf Mastercycler ep realplex, Eppendorf, Hamburg, Germany). The reaction conditions included denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 20 s. The primer sequences used were as follows: β_3-AR, forward 5′-CGGCAGTAGTCCTGGTGT-3′, reverse 5′-CGTAGGGCATATTGGAGGC-3′; TGFβ1, forward 5′-GACCGCAACAACGCAATCT-3′, reverse 5′-GACAGCCACTCAGGCGTATC-3′; c-Jun, forward 5′-GCCAACTCATGCTAACGCAG-3′, reverse 5′-TCGCAACCCAGTCCATCTTG-3′; GAPDH, forward 5′-TCCCTCAAGATTGTCAGCAA-3′, reverse 5′-AGATCCACAACGGATACATT-3′.

2.6. ELISA for TGFβ1

The protein concentrations of TGFβ1 in NRCM culture media were measured with an ELISA kit (R&D Systems, Inc., MN, USA) according to the manufacturer's instructions. Absorbances were read at 450 nm (Microplate Reader Model 550, Bio-Rad, CA, USA). All absorbance values were in the linear range of the standard curve.

2.7. Western blot analysis

The levels of phospho-JNK, JNK, phospho-c-Jun, and c-Jun were examined by western blotting. All cell samples were lysed in a lysis buffer. Protein concentrations were assessed with a BCA protein assay kit (Life Technologies, IL, USA). Proteins (20 μg) were separated by electrophoresis on 10% SDS polyacrylamide gel and transferred to PVDF membranes. The membranes were incubated with primary antibodies overnight at 4 °C. After incubating the membranes with corresponding HRP-conjugated secondary antibodies, protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, MA, USA). Protein levels were quantified by calculating the gray value of each protein band using ImageJ software.

2.8. Statistical analysis

Data are expressed as the mean ± SEM from at least 6 independent experiments. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., CA, USA) and SPSS 24.0 (IBM Co., NY, USA). For parametric data with equal variances, analysis of variance (ANOVA) combined with Tukey's post hoc test was used to analyze the differences among groups. For data with unequal variances, Welch's ANOVA with the Games-Howell post hoc test was used. A *P*-value <0.05 was considered statistically significant.

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