G protein, phosphorylated-GATA4 and VEGF expression in the hearts of transgenic mice overexpressing β₁- and β₂-adrenergic receptors

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Received April 28, 2016; Accepted February 22, 2017

DOI: 10.3892/mmr.2017.6526

Abstract. β₁- and β₂-adrenergic receptors (ARs) regulate cardiac contractility, calcium handling and protein phosphorylation. The present study aimed to examine the expression levels of vascular endothelial growth factor A (VEGF-A) and several G proteins, and the phosphorylation of transcription factor GATA binding protein 4 (GATA4), by western blot analysis, using isolated hearts from 6 month-old transgenic (TG) mice that overexpress β₁AR or β₂AR. Cardiac contractility/relaxation and heart rate was increased in both β₁AR TG and β₂AR TG mouse hearts compared with wild type; however, no significant differences were observed between the β₁- and β₂AR TG mouse hearts. Protein expression levels of inhibitory guanine nucleotide-binding protein (Gi) 2, Gi3 and G-protein-coupled receptor kinase 2 were upregulated in both TG mice, although the upregulation of Gi2 was more prominent in the β₂AR TG mice. VEGF-A expression levels were also increased in both TG mice, and were highest in the β₁AR TG mice. In addition, the levels of phosphorylated-GATA4 expression were increased in β₁- and β₂-AR TG mice. In conclusion, the present study demonstrated that cardiac contractility/relaxation and heart rate is increased in β₁AR and β₂AR TG mice, and indicated that this increase may be related to the overexpression of G proteins and G-protein-associated proteins.

Introduction

Heart failure occurs when the heart is unable to maintain adequate blood circulation to meet the body’s requirements, and is involved in the development of cardiac hypertrophy (1-3). Heart failure presents with an elevation of catecholamine levels, which are responsible for the functional uncoupling and downregulation of the adrenergic system (4,5). The cardiomyocyte adrenergic receptor (AR) subtypes β₁ and β₂ participate in the catecholamine-mediated increase of cardiac inotropy or chronotropy (6-8). The β₁- and β₂-ARs, which are homologous in structure, exert different effects on cardiac function (9); these differences may be explained by the distinct G-protein couplings associated with the βAR subtype (10). In particular, β₂AR activates stimulatory guanine nucleotide-binding proteins (Gs) and pertussis toxin-sensitive inhibitory guanine nucleotide-binding protein (Gi) 2 and Gi3 signaling pathways, whereas β₁AR exclusively couples to the Gs/adenyllyl cyclase/cyclic AMP (cAMP)/protein kinase A (PKA) signaling cascade (11). However, the differing underlying mechanisms between β₁AR and β₂AR remain unknown.

Notably, the vascular endothelial growth factor (VEGF) family has been revealed to exhibit an ability to initiate angiogenic cascades in the absence of ischemia or inflammation (12,13). Furthermore, β₁- and β₂-ARs were demonstrated

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Key words: β₁ and β₂ adrenergic receptors, transgenic mice, cardiomyocytes, G proteins, GATA4, VEGF
to mediate norepinephrine-induced VEGF upregulation (12). Important roles for VEGF-A have been suggested in the cardiovascular system, including angiogenesis and vasodilation (13). The transcription factor GATA4 has been reported to directly regulate VEGF expression, via binding to the promoter of the VEGF-A gene; GATA4 has been suggested to function as a stress-response regulator, by coordinating angiogenetic processes following hemodynamic load, through the modulation of non-hypoxic and load-responsive mechanisms (14). However, it is currently unclear whether distinct β1- and β2-AR-mediated signaling mechanisms are involved in VEGF-A regulation in hemodynamically challenged hearts. The molecular mechanisms underlying the differences between β1- and β2-AR signaling remain largely unknown. Therefore, the present study aimed to compare the expression of Gs, VEGF-A and their associated proteins, in hearts from β1,AR transgenic (TG),β2-AR TG and wild-type (WT) mice.

Materials and methods

TG mice. The generation of β1,AR and β2,AR TG mice that overexpress human cardiac-specific β1- or β2,ARs was performed as previously described (6,7). In brief, wild-type human β1- and β2-AR cDNA was ligated into the SalI site (exon 3) of the full-length 5.5-kb α-myosin heavy chain (MHC) promoter, obtained from Dr. Arthur R. Struch. (Mayo Clinic, Rochester, MN, USA). The linearized constructs were injected into male pronuclei of fertilized FVB/N mouse oocytes and implanted into pseudopregnant female oviducts (Taconic Biosciences, Rensselaer, NY, USA). Genomic DNA from tail-cuts was screened for the presence of transgenes, using targeted PCR with the following primers: For β1,AR, forward primer 5'-AGG ACT TCA CAT AGA AGC CTA G-3', located in the α-MHC promoter, and reverse primer 5'-TGT CCA CTG CTG AGA CAG CG-3', located in the β2,AR coding sequence. For β2,AR, forward primer 5'-GGAGCAGAGTGTGATACAGC-3', located in the open reading frame, and reverse primer 5'-GTC ACAACCGAGATG-3', located in the SV40 polyadenylation region. A total of 39 male mice (n=13 mice/group) were housed in individual cages (temperature, 23˚C; humidity, 60%) under 12/12 h light/dark cycles, and provided with commercial chow and water ad libitum. Mice were examined at 6 months of age, to ensure the presented phenotypes were independent of the confounding effects of cardiac growth and associated alterations to βAR functional coupling. Animal procedures were approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee.

Physiological parameters. Physiological parameters were evaluated in an isolated work-performing experiment, according to a previously published procedure (7). Briefly, mice (n=5/group; age, 6 months; weight, ~35 g) were anesthetized via intraperitoneal injection of pentobarbital sodium (100 mg/kg). Hearts were isolated and fixed with 10% buffered formalin for 2 days at room temperature. The heart/body weight ratio was calculated according to the following formula: Heart/body weight ratio=heart weight (mg)/body weight (g). Hearts were embedded in paraffin and sectioned (6 µm). The sections were stained with H&E, and digital images were captured using a light microscope and analyzed using the digital imaging analysis software Leica Steel Expert version 2.0 (Leica Microsystems GmbH, Wetzlar, Germany). Cardiomyocyte diameter was determined as the shortest distance across the nucleus in transverse cell sections. Cardiomyocytes (n=100) from 5 randomly selected microscope fields (x200 magnification) from the posterior wall of the left ventricle were measured to represent the average cardiomyocyte diameter.

Western blotting. The expression of heart proteins was detected according to previously published procedures (15). Briefly, proteins were extracted from heart tissue (~50 mg tissue; n=3 mice/group) using radioimmunoprecipitation assay buffer (1:4 w:v; R2002, Biosesang, Inc., Soungnam, Korea) containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris HCl (pH 7.5), 0.1% SDS, 2 mM EDTA (pH 8.0) and phosphatase inhibitor cocktail for 10 min over ice. Lysates were centrifuged at 19,000 x g for 10 min at 4°C and supernatants were collected. Protein concentrations were determined using a bicinchoninic acid assay kit. Equal amounts of extracted protein samples (200 mg/ml) were subjected to Tris-Glycine-SDS PAGE, transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% non-fat skim milk in TBS containing 0.1% Tween 20 for 2 h at room temperature and probed with the following primary antibodies overnight at 4°C: Anti-Gs (1:500; cat no. sc26766), anti-Gi2 (1:500; cat no. sc391), anti-Gi3 (1:500; cat no. sc262), anti-G-protein-coupled receptor kinase 2 (GRK2; 1:500; cat no. sc562), anti-GATA binding protein 4 (GATA4; 1:1,000; cat no. sc1237) and anti-GAPDH (1:2,000; cat no. sc166574), obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-VEGF-A (1:1,000; cat no. ab1316) and anti-phosphorylated (p)-GATA4 (1:500; cat
compared with VEGF-A expression was detected in both β1AR and β2AR TG mice, compared with the WT group. Phosphorylation on serine 105 is an event known to augment GATA4 activity. No significant difference in pGATA4 expression was observed between the β1AR and β2AR TG mice (Fig. 3).

Discussion

The present study examined physiological parameters in an isolated work-performing heart experiment of β1AR TG and β2AR TG mice. The results indicated that cardiac contractility/relaxation and heart rate were stronger in the β2AR TG mice, compared with the β1AR TG mice; however, this difference was not significant. The reduced hemodynamic functional response in the β1AR TG mouse heart may reduce cardiac cellular stress and metabolic expenditure.

The diameter of the cardiomyocytes was greater in the βAR TG mice compared with the WT mice, indicating the presence of hypertrophy. Previous studies reported that βAR signaling induces cardiomyocyte apoptosis through a Gs-mediated PKA-dependent mechanism (16-18), and β1- and β2AR may exert different effects on cardiac apoptosis (19,20), which may be due to the distinct G-protein couplings of βAR subtypes (11). In the present study, GRK2, Gi2 and Gi3 protein expression levels were significantly increased, whereas Gs expression levels were significantly decreased in β1AR and β2AR TG mice compared with WT. Furthermore, Gi2 expression was significantly higher in the β2AR TG mice, compared with the β1AR TG mice. Forster et al (21) reported that, unlike β1AR, the effects of β2AR overexpression varied at the level of Gi2 and Gi3 proteins; β2AR was associated with more prominent Gi2 upregulation and suggested that Gi2 may contribute to a longer survival and delayed cardiac pathology in β2AR TG mice. Furthermore, Gi2 upregulation may reduce the deleterious effects of catecholamine signaling, contribute to several protective aspects ascribed to β3AR/Gi coupling and reduce cardiac responsiveness against a number of Gαq-protein-related pro-growth factors (22). Based on these data, it may be hypothesized that the coupling of β1AR and Gi may provide negative feedback to the β1AR/Gs-mediated cAMP signal, thus resulting in reduced cardiac inotropy.

Previous research has indicated that the declined hemodynamic response in the β2AR TG mouse heart would result in reduced capillary growth (23). Furthermore, it has been suggested that regulatory circuits might exist between catecholamine-induced inotropy and VEGF expression in the heart, which adjusts hemodynamic load to myocardial blood supply (12). Heineke et al demonstrated that GATA4 directly regulates VEGF expression by binding to the VEGF-A gene promoter and functions as a stress-responsive regulator that coordinates angiogenesis following alterations to hemodynamic load via non-hypoxic and load-responsive mechanisms (14). Based upon these findings, the present study examined the expression levels of GATA4 and VEGF-A in both βAR TG mouse hearts. Although cardiac contractility/relaxation and heart rate were similarly increased in β1 and β2AR TG mice, VEGF-A protein expression levels were significantly upregulated in myocardial tissue isolated from β1AR TG mice compared with in tissue from β2AR TG
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Table I. Physiological parameters in the isolated work-performing hearts of the WT, β₁AR TG and β₂AR TG mice.

| Physiological parameter | WT          | β₁AR TG     | β₂AR TG     |
|-------------------------|-------------|-------------|-------------|
| Heart/body weight ratio (mg/g) | 3.61±0.03  | 3.91±0.07*a | 3.78±0.06*a |
| SP (mmHg)               | 133.4±1.7   | 159.4±3.5*a | 168.1±4.8*a |
| DP (mmHg)               | -8.1±1.36   | -33.7±1.3*a | -37.7±3.4*a |
| EDP (mmHg)              | 7.7±0.9     | 2.1±0.5*a   | 3.9±0.8*a   |
| +dP/dt (mmHg/s)         | 3961±41     | 5927±187*a  | 5775±342*a  |
| -dP/dt (mmHg/s)         | 2763±130    | 5179±206*a  | 5270±156*a  |
| HR (beats/minute)       | 247±3.9     | 334±4.3*a   | 316±0.9*a   |
| TPP (msec/mmHg)         | 0.40±0.01   | 0.25±0.02*a | 0.31±0.03*a |
| TR1/2 (msec/mmHg)       | 0.63±0.02   | 0.40±0.03*a | 0.47±0.03*a |

*aP<0.05 vs. WT. Data are expressed as the mean ± standard error of the mean. N=5 mice/group. +dP/dt, maximal rate pressure development; -dP/dt, maximal rate pressure decline; AR, adrenergic receptor; DP, left ventricular diastolic pressure; EDP, left ventricular end-diastolic pressure; HR, heart rate; SP, left ventricular systolic pressure; TG, transgenic receptor; TPP, time to peak pressure (normalized to peak pressure); TR1/2, ½ relaxation pressure (normalized to ½ relaxation pressure); WT, wild type.

Figure 1. H&E staining and measurement of cardiomyocyte size in WT, β₁AR TG and β₂AR TG mice. (A) H&E staining revealed ventricular cardiomyocyte hypertrophy in both βAR TG mice. Scale bar, 50 µm. (B) Quantification of cardiomyocyte size in the transverse section in WT, β₁; and β₂AR TG mice. Data represent the shortest cardiomyocyte diameter through the nucleus. The results are presented as mean ± standard error of the mean. *P<0.05 vs. WT mice. AR, adrenergic receptor; H&E, hematoxylin and eosin; TG, transgenic; WT, wild type.

Figure 2. GRK2, Gi2, Gi3 and Gs protein expression in cardiac homogenates from WT, β₁AR TG and β₂AR TG mouse hearts. (A) Western blot analysis of GRK2, Gi2, Gi3 and Gs. (B) Quantification of western blot analysis; protein expressions are normalized to GAPDH. Values are the mean ± standard error of the mean. *P<0.05 vs. WT mice. AR, adrenergic receptor; Gi, inhibitory guanine nucleotide-binding protein; GRK2, G-protein coupled receptor kinase 2; Gs, stimulatory guanine nucleotide-binding protein; TG, transgenic; WT, wild type.
mice. In addition, p-GATA4 protein expression was similarly increased in myocardial samples from β1 and β1AR TG mice.

Therefore, the present study hypothesized that VEGF-A-induced variation in angiogenesis may be associated with one of the mechanisms that halts hypertrophic remodeling of the heart in the β1AR TG mice. Notably, Tirziu et al (13) reported that increased endothelial cell mass and endothelial cell-cardiomyocyte interactions stimulated hypertrophic growth, via releasing paracrine factors, such as VEGF, from the vascular endothelium.

In conclusion, the present study demonstrated that cardiac contractility/relaxation and heart rate was increased in β1AR and β1AR TG mice, and that these increases may be due to β1AR-mediated upregulation of Gi protein expression and reduced upregulation of VEGF-A, in comparison with β1AR TG mice.

Acknowledgements

The authors would like to thank Mr Seung Uk Lee (Department of Neurobiology, School of Medicine, Kangwon National University, Chuncheon, Republic of Korea) for his technical help in this study. The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), the Ministry of Education (grant no. NRF -2014R1A1A2057263), the Priority Research Foundation of Korea (NRF), the Ministry of Science, ICT & Future Planning and the National Institutes of Health (grant no. HL077101).

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