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

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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 β₂AR TG mice. In conclusion, the present study demonstrated that cardiac contractility/relaxation and heart rate is increased in β₁AR TG 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 β₂ARs, 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/adenylyl 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 β₂ARs were demonstrated

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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 β_1- and β_2AR-mediated signaling mechanisms are involved in VEGF-A regulation in hemodynamically challenged hearts. The molecular mechanisms underlying the differences between β_1- and β_2AR signaling remain largely unknown. Therefore, the present study aimed to compare the expression of Gs, VEGF-A and their associated proteins, in hearts from β_1AR transgenic (TG), β_2AR TG and wild-type (WT) mice.

Materials and methods

TG mice. The generation of β_1AR and β_2AR TG mice that overexpress human cardiac-specific β_1- or β_2ARs was performed as previously described (6,7). In brief, wild-type human β_1- and β_2-AR cDNA was ligated into the *Sal*I 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 β_1AR, 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 β_1AR coding sequence. For β_2AR, forward primer 5'-GGAGCAGAGTGGATATCACG-3', located in the open reading frame, and reverse primer 5'-GTC ACACCACAGAAGTAAGG-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 100 mg/kg Nembutal and 1.5 units of heparin to prevent microthrombosis, the heart and aorta were attached to a 20-gauge cannula and subjected to temporary retrograde perfusion with oxygenated Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 0.5 mM Na-EDTA, 25 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 11 mM glucose; followed by saturation with 95% O$_2$+5% CO$_2$). A polyethelene-50 catheter was inserted into the apex of the left ventricle to measure intraventricular pressure. The pulmonary vein was connected to a second cannula and the antegrade perfusion was initiated with a basal workload of 300 mmHg ml/min (venous return, 6 ml; mean aortic pressure, 50 mmHg). Hearts were allowed to equilibrate for 20 min. Atrial pressure was monitored through a sidearm in the left atrial cannula (National Instruments Corporation, Austin, Texas, US). The left ventricular pressure signals were analyzed off-line using NI Biobench software version 1.0 (National Instruments Corporation). The first positive and negative derivatives of the left intraventricular pressure curve [that is, the maximal rate pressure development (+dP/dt) and the maximal rate pressure decline (-dP/dt)], the duration of contraction and relaxation [that is, time to peak pressure (TPP)], and the time to half relaxation (TR½) were calculated. TPP was normalized with respect to peak systolic pressure, calculated as: Systolic pressure-end diastolic pressure. TR½ was normalized with respect to ½ relaxation pressure, calculated as: (Systolic pressure-diastolic pressure)/2.

Hematoxylin and eosin (H&E) staining. Mice (n=5/group) 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 deooxycholate, 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

no. ab5245) obtained from Abcam (Cambridge, MA, USA). Membranes were then incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat no. SC-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. Protein bands were visualized using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Chalfont, UK) and the signals were acquired using a LAS-3000 charged coupled device camera (Fujifilm Holdings Corporation, Tokyo, Japan). Blots were semi-quantified using densitometric analysis and protein expression was normalized to GAPDH. Semi-quantification was performed using the Scion Image software version 4.0.3.2 (Scion Corporation, Walkersville, MD, USA). Experiments were performed in triplicate.

Statistical analysis. All data are presented as the mean ± standard error of the mean. Differences of the means amongst the groups were estimated by one-way analysis of variance followed by a post hoc Bonferroni test for multiple comparisons. All analyses were performed using SPSS version 20.0 (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Physiological parameters. The cardiac function of β_1AR TG and β_2AR TG mice was enhanced, and the cardiac contractility/relaxation and heart rate of β_1- and β_2AR TG mice were significantly increased compared with the WT mice (Table I). No significant differences between the β_1AR and β_2AR TG mouse hearts were identified; however, the clinical parameters, including left ventricular systolic pressure (SP), left ventricular diastolic pressure (DP), left ventricular end-diastolic pressure (EDP) and TPP in $\beta2$AR TG mice appeared to be slightly increased compared with in $\beta1$AR TG mice.

Myocardial hypertrophy. Heart/body weight ratio was significantly greater in β_1AR and β_2AR TG mice compared with in WT mice (3.91 ± 0.07 and 3.78 ± 0.06 mg/g vs. 3.61 ± 0.03 mg/g, respectively; $P<0.05$); however, no statistical significance was identified between the two transgenic groups. The diameter of the cardiomyocytes demonstrated a similar trend to the heart/body weight ratios; cell diameters were greater in βAR TG mice compared with in the WT mice, however, no significant differences in cardiomyocyte size were detected between the two transgenic groups (Fig. 1).

Expression levels of G proteins and GRK2. Protein expression levels of GRK2, Gs, Gi2 and Gi3 were examined by western blot analysis (Fig. 2). Gs protein expression level was significantly decreased, whereas Gi2, Gi3 and GRK2 expressions were significantly increased, in β_1AR and β_2AR TG mice compared with WT mice (Fig. 2). The upregulation of Gi2 was greatest in the β_2AR TG mice compared with the other two groups.

Expression levels of VEGF-A and GATA4. Upregulation of VEGF-A expression was detected in both β_1AR and β_2AR TG mice; however, the β_2AR TG mice exhibited a smaller increase compared with β_1AR TG mice. Furthermore, GATA4 serine

105 phosphorylation (p-GATA4) was similarly increased in both β_1 and β_2AR 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 β_1AR and β_2AR TG mice (Fig. 3).

Discussion

The present study examined physiological parameters in an isolated work-performing heart experiment of β_1AR TG and β_2AR TG mice. The results indicated that cardiac contractility/relaxation and heart rate were stronger in the β_2AR TG mice, compared with the β_1AR TG mice; however, this difference was not significant. The reduced hemodynamic functional response in the β_1AR 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 β_1- and β_2AR 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 β_1AR and β_2AR TG mice compared with WT. Furthermore, Gi2 expression was significantly higher in the β_2AR TG mice, compared with the β_1AR TG mice. Foerster *et al* (21) reported that, unlike β_1AR, the effects of β_2AR overexpression varied at the level of Gi2 and Gi3 proteins; β_2AR was associated with more prominent Gi2 upregulation and suggested that Gi2 may contribute to a longer survival and delayed cardiac pathology in β_2AR TG mice. Furthermore, Gi2 upregulation may reduce the deleterious effects of catecholamine signaling, contribute to several protective aspects ascribed to β_2AR/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 β_2AR and Gi may provide negative feedback to the β_2AR/Gs-mediated cAMP signal, thus resulting in reduced cardiac inotropy.

Previous research has indicated that the declined hemodynamic response in the β_2AR 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 β_1 and β_2AR TG mice, VEGF-A protein expression levels were significantly upregulated in myocardial tissue isolated from β_1AR TG mice compared with in tissue from β_2AR TG

Table I. Physiological parameters in the isolated work-performing hearts of the WT, β_1AR TG and β_2AR TG mice.

Physiological parameter	WT	β_1AR TG	β_2AR 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]

[a]$P<0.05$ 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.
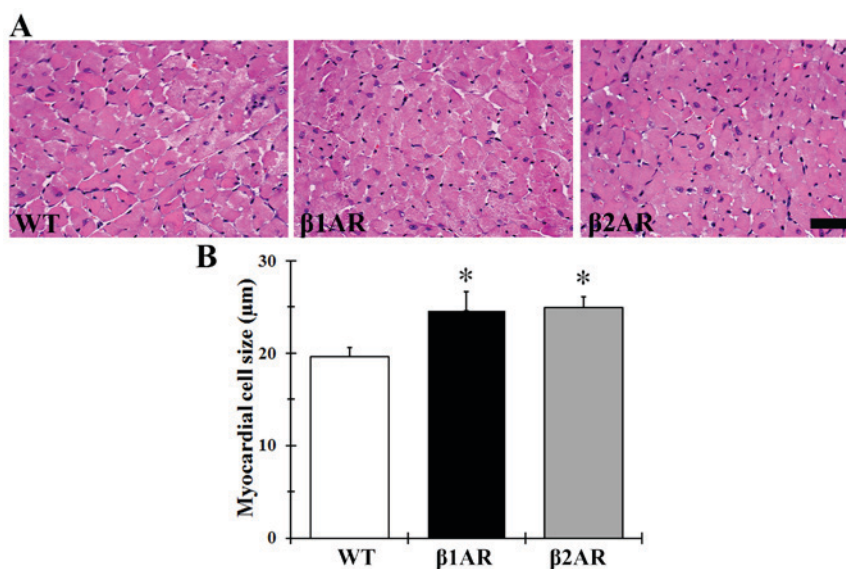

Figure 1. H&E staining and measurement of cardiomyocyte size in WT, β_1AR TG and β_2AR TG mice. (A) H&E staining revealed ventricular cardiomyocyte hypertrophy in both βAR TG mice. Scale bar, 50 μm. (B) Quantification of cardiomyocyte size in the transverse section in WT, β_1- and β_2AR TG mice. Data 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.
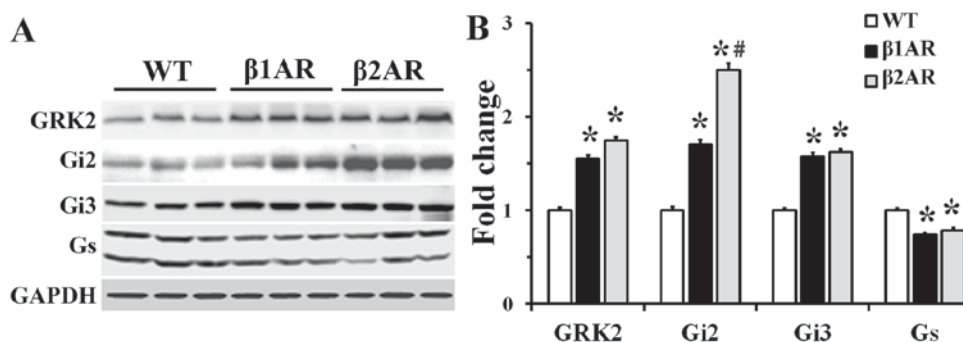

Figure 2. GRK2, Gi2, Gi3 and Gs protein expression in cardiac homogenates from WT, β_1AR TG and β_2AR TG mouse hearts. (A) Western blot analysis of GRK2, Gi2, Gi3 and Gs. (B) Quantification of western blot analysis; protein expressions are normalized to GAPDH. Values are the mean ± standard error of the mean. N=3 mice/group. *$P<0.05$, vs. WT mice. #$P<0.05$ vs. β_1AR TG 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.
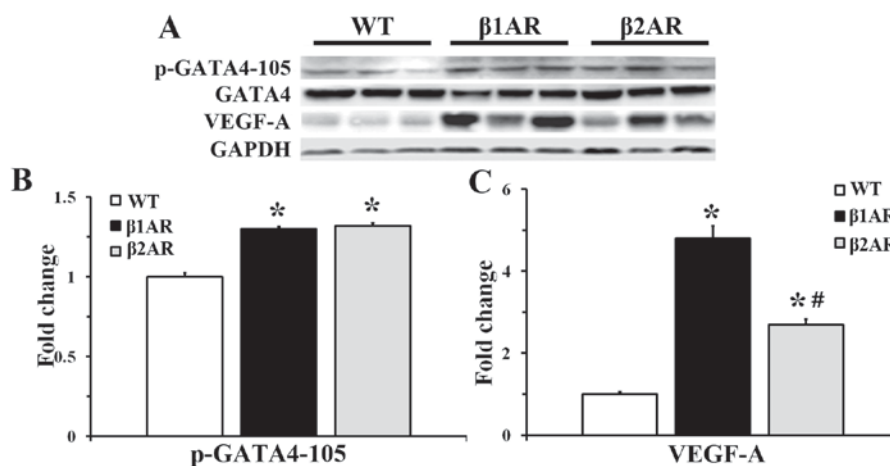
Figure 3. GATA4 and VEGF-A protein expression in WT, β_1AR TG and β_2AR TG mice. (A) Western blot analysis of heart protein extracts for p-GATA4-105, total GATA4 and VEGF-A. (B and C) Densitometric quantification of western blot analysis. Values are the mean ± standard error of the mean. N=3 mice/group; *P<0.05 vs. WT; #P<0.05 vs. β_1AR TG mice. AR, adrenergic receptor; GATA4, GATA binding protein 4; p-GATA4-105, GATA4 protein phosphorylated on serine 105; TG, transgenic; VEGF-A, vascular endothelial growth factor A; WT, wild type.

mice. In addition, p-GATA4 protein expression was similarly increased in myocardial samples from β_1 and β_2AR 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 β_2AR 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 β_1AR and β_2AR TG mice, and that these increases may be due to β_2AR-mediated upregulation of Gi protein expression and reduced upregulation of VEGF-A, in comparison with β_1AR TG mice.

Acknowledgements

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