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Original Article

Relationship between the β1-adrenergic receptor and the β2-adrenergic receptor in hypertension

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Abstract: Objective: To determine whether β1 adrenergic receptor (β1-AR) changes are related to β2-AR expression under conditions of hypertension. Methods: The mRNA and protein levels of β1- and β2-AR in the left ventricles (LVs) and peripheral blood mononuclear cells (PBMCs) obtained from Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were measured. The β1- and β2-AR mRNA levels in PBMCs from 22 healthy subjects and 17 hypertensive patients were measured. H9c2 cardiac myocytes were infected with AdAdrb1 or AdshAdrb1 to overexpress or knockdown β1-AR expression, and the β1-AR and β2-AR mRNA levels were analyzed. Results: β1-AR and β2-AR mRNA expression was significantly increased in the LV samples and PBMCs from the SHR group compared with the WKY group. Consistent with the animal study, the β1-AR and β2-AR mRNA levels were also increased in hypertensive patients compared with healthy subjects. H9c2 cells over-expressing β1-AR exhibited significantly increased β2-AR expression compared with AdGFP-infected cells. H9c2 cells in which β1-AR was knocked down exhibited significantly decreased β2-AR expression. Conclusions: Parallel changes in β1-AR and β2-AR expression were observed under hypertension conditions, highlighting the need to focus not only on the β-AR mechanism also on β1- or β2-AR dynamic changes to better understand the pathological mechanism of hypertension.

Keywords: Hypertension, β1-adrenergic receptors, β2-adrenergic receptors, H9c2 cardiomyocytes

Introduction

β adrenergic receptors (β-ARs) are involved in the pathogenesis of hypertension, which is a major risk factor for cardiovascular diseases, including coronary artery disease, heart failure, chronic kidney disease, peripheral vascular disease, and stroke [1]. As a member of the G-protein-coupled receptor superfamily, the β-AR family includes β1-AR, β2-AR, and β3-AR subtypes. Among them, β1-AR and β2-AR are abundantly expressed in the myocardium, with β1-AR being the predominant subtype [2-4]. Both β1-AR and β2-AR regulate cardiac contractility via the Gs-adenylate cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway [5]. Once stimulated, myocardial β1-AR and β2-AR activate the trimer protein Gs, resulting in the dissociation of different G protein subunits. Activated G_{α} interacts with intracellular AC, resulting in the conversion of adenosine triphosphate (ATP) into cAMP, which subsequently leads to the activation of PKA and phosphorylation of the L-type Ca(2+) channels (ICa, L) in ventricular myocytes. By enhancing Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} release, myocardial contractility is increased. In diastole, PKA induced the phosphorylation of membrane phospholipid proteins and troponin and enhanced the activity of Ca^{2+}-ATPase, resulting in myocardial relaxation [6]. However, β1-AR is exclusively coupled to the stimulatory subunit Gs and functions as described above. β2-AR is coupled to Gs in addition to inhibitory G protein Gi [7, 8]. The Gi-mediated action may oppose the effect mediated by Gs and in some cases may be the predominant β2-AR effect [9].

Pierroz et al. [10] reported that β1-AR gene expression was increased two-fold in the bones of Adrb2-/- mice, whereas β2-AR was reduced

Table 1. Adrb1-shRNA sequences

Target 1	GAAACAGGTGAAGAAGATC	GC%=42.1%	756-774 site
Target 2	GTGCTGCGATTTCGTCACCAA	GC%=52.4%	642-662 site
Target 3	GCTCAAGACACTGGGCATCAT	GC%=52.4%	933-953 site

Table 2. Primer sequences of β1-AR, β2-AR, and GAPDH

Species	Target	Sense primer (5'-3')	Anti-sense primer (5'-3')
Rat	Adrb1	CGTCGCCCTTTCGCTACCAG	CCGCCACCAGTGCAGTGCTGAGGAT
	Adrb2	TGCGTGATTGCAGTGGATCGCTAT	CTATCTTCTGCAGCTGCCTTTTGG
	GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
Human	Adrb1	ATTGCCCTGGACCGCTACCT	CGCCACCAGTGCATGAGGAT
	Adrb2	AGCAAAGGGACGAGGTGT	AAGTAATGGCAAAGTAGCG
	GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

in Adrb1-/- mice. In addition, Yin et al. [11] found that the expression of both β1-AR and β2-AR was decreased in a rat model of cardiac remodeling. Thus, a subtle balance exists between β1-AR and β2-AR in the myocardium. When cardiac pathology develops, β1-AR and β2-AR expression is altered, and the dynamic balance between these two receptors is disrupted.

In the present study, we hypothesized that β1-AR might regulate the expression of β2-AR in the pathological process of hypertension. We aimed to determine whether changes in β1-AR expression are related to β2-AR expression under conditions of hypertension.

Methods

Animal study

Experimental animals: Male Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were purchased from Vital River Experimental Animal Technology (Beijing, China). All rats (aged 14 weeks, weighing ~262 g) were allowed to adapt to their environmental conditions for one week before experiments were performed. The rats were fed at the Animal Experiment Center of Central South University (Changsha, China), exposed to a 12-h environmental light cycle, housed at 22±2°C and 55±5% humidity, and had free access to standard rat food and tap water in individual cages.

Treatment of animals: The systolic blood pressure (SBP) in conscious, resting rats was measured via a tail-cuff method using an electrosphygmomanometer coupled to a computerized recorder (Shanghai Alcott Biotech Co., Ltd., China) from 14:30 to 17:30 by the same investigator. SBP was measured 10 times by a single investigator, and the average value was recorded.

The rats were anesthetized with 10% chloral hydrate (300 mg/kg). Blood was collected from the abdominal aorta of the animals. After dissection, the organs were excised post-mortem, weighed, and immediately frozen and stored at -80°C. All procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and the animal regulations of Hunan Province.

Human study

Selection of study patients: Patients included in the study were selected from the Third Xiangya Hospital between December 2014 and April 2016. Patients with mild-to-moderate hypertension were recruited according to the following inclusion criteria: i) primary hypertension, age ≥25 and ≤55 years, male gender, and body mass index (BMI) ≥18 and ≤24; and ii) diastolic blood pressure (DBP) in the range of 90 to 114 mmHg (Korotkoff phase V, sitting position). The exclusion criteria were as follows: i) the presence of concurrent disease, such as heart failure, severe cardiovascular disease (CVD), secondary hypertension, malignant tumors, liver failure, mental disease, and hematologic diseases; and ii) the use of antihypertensive drugs. A control group included healthy individuals matched for age and sex. All patients provided written consent prior to enrollment, and the protocol was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University.

Clinical procedures: According to the standard recommended procedures, blood pressure was measured using a mercury sphygmomanometer with the patient in a sitting position after resting for 5 min in a quiet environment. The average of two measurements was used for the

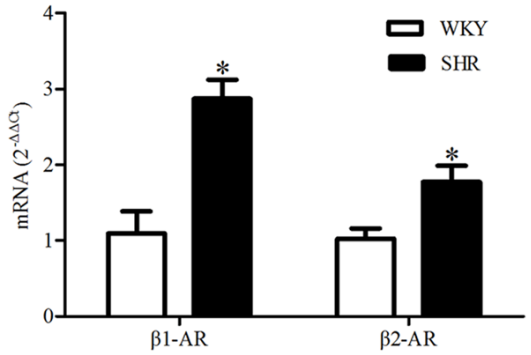

Figure 1. mRNA levels of β1-AR and β2-AR in the LV obtained from SHR and WKY rats. The data represent the means ± SEM of n=5 rats. *P<0.05 versus WKY. The statistical analysis was performed using Student's t test.

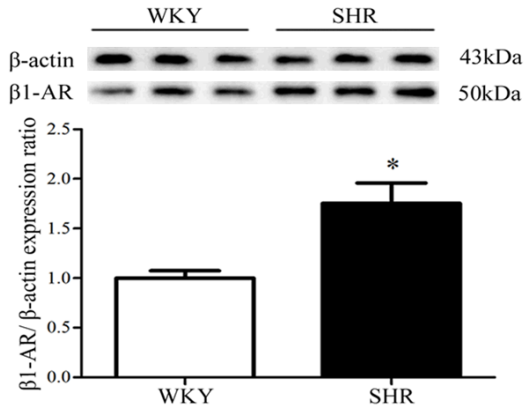

Figure 2. Protein levels of β1-AR in the LV obtained from SHR and WKY rats. The data represent the means ± SEM. *P<0.05 versus the control group. The statistical analysis was performed using Student's t test.

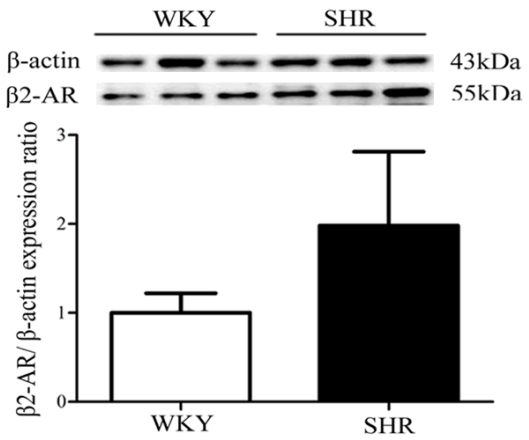

Figure 3. Protein levels of β2-AR in the LV obtained from SHR and WKY rats. The data represent the means ± SEM. The statistical analysis was performed using Student's t test.

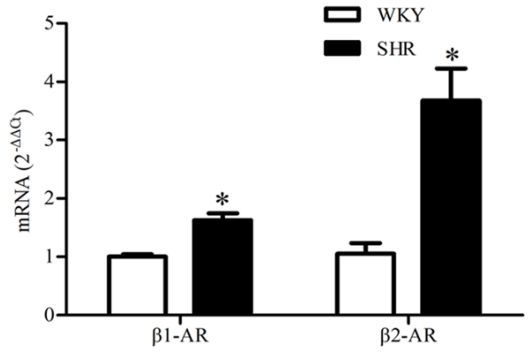

Figure 4. mRNA levels of β1-AR and β2-AR in the PBMCs obtained from SHR and WKY rats. The data represent the means ± SEM of n=5 rats. *P<0.05 versus WKY rats. The statistical analysis was performed using Student's t test.

data analysis. If the two measurements differed by greater than 5 mmHg, an additional measurement was obtained, and the average of the three measurements was used for the data analysis. After fasting overnight, blood samples were obtained in the morning for biochemical analysis. Lymphocytes were isolated, and total RNA was extracted for transcription to cDNA. Then, samples were stored at -80°C until further analysis.

Cell study

Cell culture: The rat H9c2 cardiomyocyte cell line was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in high-glucose DMEM supplemented with 10% FBS, 10,000 U/ml penicillin, and 10,000 μg/ml streptomycin (Genview, USA). The cells were incubated at 37°C with 5% CO_2 in a humidified incubator.

Construction of plasmids: The empty vectors pAAV-IRES-hrGFP and pAAV-ZsGreen-shRNA were provided by Yingrun Biotechnologies Inc. (Changsha, China). The full-length sequence of the Adrb1 gene was obtained through GenBank (NM_012701). We designed the three Adrb1-shRNA sequences (**Table 1**) using the online design program provided by Ambion. Then, pAAV-ZsGreen-Adrb1-shRNA was constructed and identified by restriction endonuclease digestion. Through careful selection and optimization, the best transfection conditions for pAAV-ZsGreen-Adrb1-shRNA were obtained.

Table 3. Demographic and biochemical characteristics of each study group

	Control	Hypertension
Age (years)	33±1	38±2
Body mass index (kg/m^2)	25.0±0.6	26.0±0.7
Office BP (mmHg)		
Systolic	125.3±2.1	162.8±3.6***
Diastolic	75.5±1.9	105.1±4.1***
Heart rate	72±1	79±3*
Total cholesterol (mg/dl)	4.6±0.1	4.6±0.2
HDL cholesterol (mg/dl)	1.20±0.02	1.13±0.03
LDL cholesterol (mg/dl)	2.6±0.1	2.5±0.2
Triglycerides (mg/dl)	1.2±0.1	1.5±0.1
Fasting blood sugar (mmol/l)	5.3±0.8	5.0±0.2
HB (g/l)	153.9±1.6	150.8±1.5
ALT (U/L)	26.1±2.2	23.9±3.0
TBIL (μmol/L)	17.4±0.9	18.0±1.4
BUN (mmol/L)	4.8±0.3	4.7±0.2
Scr (μmol/L)	76.7±2.4	81.2±5.0
UA (μmol/L)	322.1±16.0	382.6±26.1*
Proteinuria (%)	Negative (100%)	Negative (100%)

The values are expressed as the means ± SEM. BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HB, hemoglobin; ALT, alanine transaminase; TBIL, total bilirubin; BUN, blood urea nitrogen; Scr, serum creatinine; UA, uric acid. *P<0.05 vs. the control group. ***P<0.001 vs. the control group.

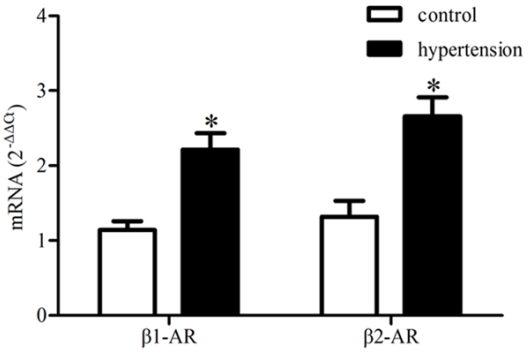

Figure 5. mRNA levels of β1-AR and β2-ARs in the PBMCs obtained from healthy subjects and hypertensive patients. The data represent the means ± SEM. *P<0.05 versus the control group. The statistical analysis was performed using Student's t test.

Cell transfection: H9c2 cells were seeded into 6-well plates the day before transfection to ensure 40 to 50% cell confluence at the time of transfection. According to the manufacturer's instructions for the Lipofectamine 2000 reagent, we infected cardiomyocytes with adenoviral short hairpin Adrb1 (AdshAdrb1), adenoviral Adrb1 (AdAdrb1), adenoviral short hairpin RNA (AdshRNA), or adenoviral green fluorescent protein (AdGFP) for 6 h. The culture medium was then replaced with freshly prepared low-glucose DMEM supplemented with 10% FBS. After 48 h of transfection, digital images were captured and infected H9c2 cells were harvested.

Quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA according to the manufacturer's instructions for the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. β1-AR and β2-AR mRNA expression was measured using SYBR Green Master Mix (Bio-Rad). The $2^{-\Delta\Delta Ct}$ method using GAPDH as the reference gene was used for the relative quantification of target genes. The specific primer-probe sets are listed in **Table 2**.

Western blot analysis

The left ventricle (LV) tissue was lysed with RIPA lysis buffer at 4°C for 30 min, and total protein was quantified using the BCA protein assay kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China). A total of 20 μg of protein was loaded, separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen), and transferred to polyvinylidene fluoride (PVDF) membranes that were subsequently blocked and incubated with the specific rabbit anti-rat Adrb1 (1:1,000, Abcam, USA) and Adrb2 (1:1,000, Abcam, USA) polyclonal antibodies. The membranes were washed thrice and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:8,000, Abcam, USA) for 2 h at room temperature. Finally, chemiluminescence was detected using an enhanced chemiluminescence (ECL) Western blotting substrate, and band intensity was assessed using a gel imaging analysis system (Bio-Rad, USA). The relative expression of the target gene was normalized to the expression of β-actin.

Statistical analyses

All values are expressed as means ± SEM. Between-group comparisons were performed

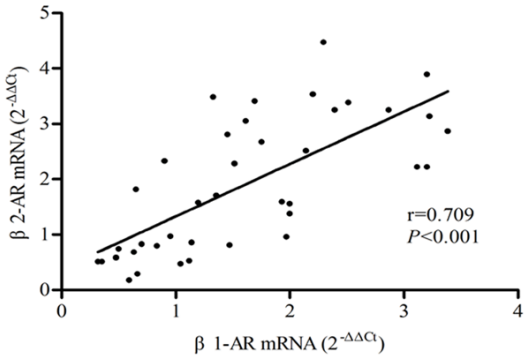

Figure 6. The linear regression observed between β1-AR and β2-AR mRNA levels in the PBMCs from healthy volunteers and hypertensive patients. Significant linear regression ($P<0.05$) is represented as a continuous line.

using a two-tailed Student's t-test. The association between β1-AR and β2-AR expression was analyzed using Pearson's correlation coefficient. *P<0.05* was considered significant.

Results

Changes in the expression of β1-AR and β2-AR in animal models of hypertension

The SBP of the SHR group was significantly increased compared with that of the WKY group (199.2±2.3 vs. 138.0±3.2, P<0.0001). β1-AR and β2-AR mRNA expression was significantly increased in the LV samples from the SHR group compared with those from the WKY group (2.87±0.25 vs. 1.09±0.29, *P*=0.0037; 1.77±0.22 vs. 1.03±0.13; *P*=0.0258; **Figure 1**). Western blot analyses (**Figures 2**, **3**) revealed that the WKY group displayed significantly decreased β1-AR protein levels compared with the SHR group (1.00±0.07 vs. 1.75±0.21, *P*= 0.0263). β2-AR protein levels were increased in the SHR group compared with the WKY group, although this difference was not significant (1.98±0.83 vs. 1.00±0.22, *P*=0.3183). Increased β1-AR and β2-AR expression was also observed in PBMCs from SHR compared with WKY rats (1.63±0.12 vs. 1.00±0.04, *P*=0.004; 3.68±0.55 vs. 1.05±0.18, *P*=0.004; **Figure 4**).

Changes in the expression of β1-AR and β2-AR in human PBMCs

In total, 22 healthy subjects and 17 hypertensive patients were included in the present study. The demographic characteristics of the study patients are presented in **Table 3**. As expected, significant differences in blood pressure values were observed between the hypertensive patients and healthy volunteers. The heart rate of the hypertensive group was significantly increased compared with that of the control group. However, no significant differences were observed between the groups in terms of age, body mass index, lipid profile,or other biochemical variables, confirming the absence of other abnormalities or alterations potentially acting as confounding factors. A slight but significant increase in uric acid levels was observed in the hypertensive group. As **Figure 5** illustrates, β1-AR and β2-AR expression was significantly increased in primary hypertensive patients compared with healthy subjects (2.21±0.22 vs. 1.14±0.12, *P*<0.0001; 2.66±0.25 vs. 1.32±0.21, *P*=0.0002). To confirm the relationship between changes in β1-AR and β2-AR expression, a linear regression analysis (**Figure 6**) was performed between the mRNA values of β1-AR and β2-AR in PBMCs obtained from each patient. In all the samples analyzed, a significant correlation was observed between β1-AR and β2-AR expression (r=0.709, *P*<0.001).

Changes in β1-AR affect β2-AR expression in H9c2 cardiac myocytes

To explore the influence of β1-AR on the expression of β2-AR, we infected H9c2 cardiac myocytes with AdAdrb1 or AdshAdrb1 to over-express or knockdown β1-AR expression. AdGFP and AdshRNA were used as control groups. The transfection efficiency was approximately 50% after 48 h of transfection (**Figure 7**). The mRNA of β1-AR and β2-AR was also analyzed (**Figure 8**). H9c2 cells over-expressing β1-AR exhibited significantly increased expression of β2-AR compared with AdGFP-infected cells. H9c2 cells in which β1-AR was knocked down exhibited significantly decreased β2-AR expression.

Discussion

As a member of the G-protein-coupled receptor superfamily, β-ARs play a crucial role in the onset and development of essential hypertension. Changes in β1- and β2-AR are critically reflected in the pathological process of hypertension. The present study described the expression of β1- and β2-AR inthe heart and PBMCs from animal models of SHR, PBMCs from patients with essential hypertension, and

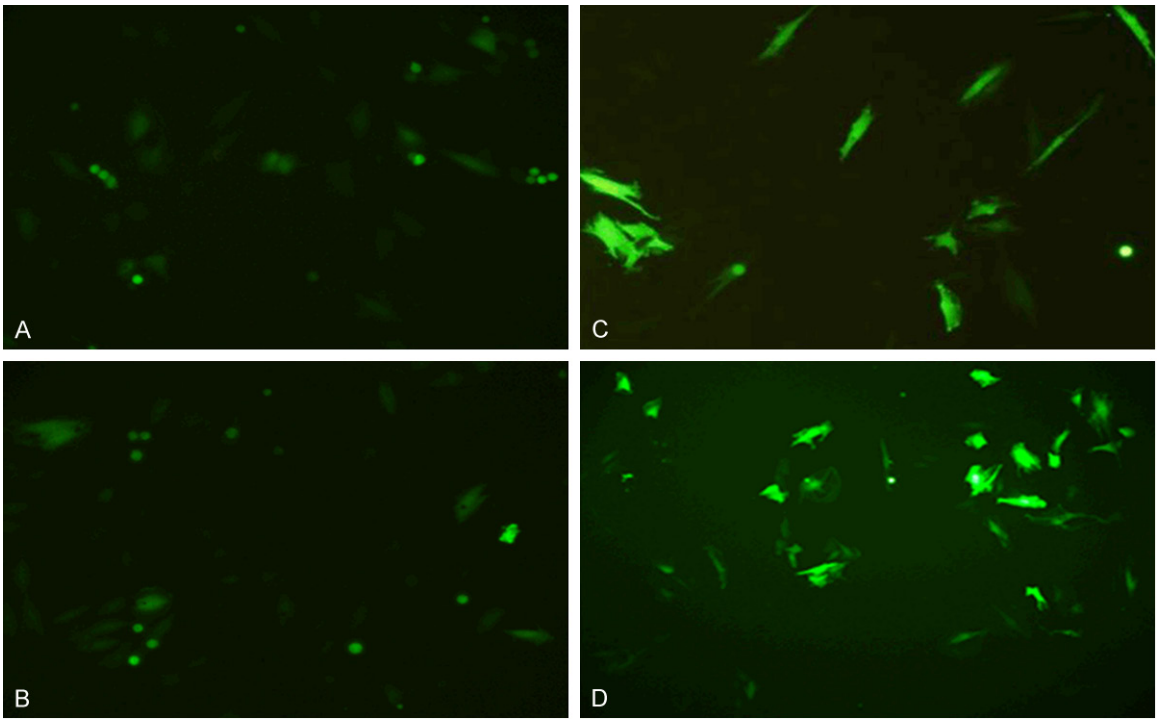

Figure 7. Fluorescence distribution of H9c2 cardiac myocytes after 48 h of transfection. A. H9c2 cardiac myocytes infected with AdGFP. B. H9c2 cardiac myocytes infected with AdAdrb1. C. H9c2 cardiac myocytes infected with AdshRNA. D. H9c2 cardiac myocytes infected with AdshAdrb1.

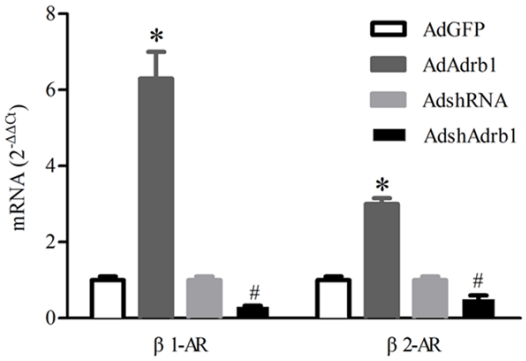

Figure 8. β1-AR and β2-AR mRNA levels in H9c2 cardiac myocytes infected with AdAdrb1 or AdshAdrb1. The data represent the means ± SEM (n=3). *P<0.05 versus the AdGFP group. #P<0.05 versus AdshRNA group. The statistical analysis was performed using Student's t test.

H9c2 cardiac myocytes with over-expression or knockdown of β1-AR. The main findings were that changes in β1-AR expression are related to β2-AR expression under conditions of hypertension.

In tissues in which β1-AR expression was increased (e.g., LV and PBMCs from SHR vs. WKY rats, PBMCs from hypertensive patients vs. healthy subjects), β2-AR expression was increased compared with that in the respective controls. Consistent with our study, Lang et al. [12] found that β2-AR expression exhibits a decreasing trend with decreased β1-AR expression in the human failing heart. Dominique D et al. found that β2-AR expression was decreased in bone tissues in Adrb1-/- mice compared with WT mice [10, 13]. However, Oliver et al. [14] showed that β1-AR expression levels were significantly increased in circulating lymphocytes from hypertensive patients compared with healthy subjects, but this difference was not observed for β2-AR expression. Given the small sample size in our study, further studies with larger sample sizes are required to clarify this issue. A significant correlation was observed between β1-AR and β2-AR expression. To further explore how changes in β1-AR affect β2-AR expression, we infected H9c2 cardiac myocytes with AdAdrb1 to over-express β1-AR or AdshAdrb1 to knockdown β1-AR expression. H9c2 cells over-expressing β1-AR exhibited significantly increased β2-AR expression compared with AdGFP-infected cells. H9c2 cells in which β1-AR was knocked down exhibited significant-

ly decreased expression of β2-AR. These findings suggest that parallel changes in β1-AR and β2-AR expression were observed under conditions of hypertension.

β1-AR, the predominant receptor subtype in the heart, increases myocardial contractility and regulates cardiac excitation-contraction coupling. Its stimulation results in the activation of the Gs-AC-cAMP-protein PKA signaling cascade [5, 15, 16]. In ventricular myocytes, the activation of PKA signaling triggers transient increases in calcium, contractility, and cardiac output, corresponding to a significant increase in blood pressure [17, 18]. Similarly, β2-AR also has a functional role in myocardial contraction [19]. However, in contrast to β1-AR, which couples only to G_s, β2-AR also couples to pertussis toxin (PTX)-sensitive G_i proteins. In contrast to the cardiovascular toxicity of persistent β1-AR activation, persistent β2-AR stimulation is cardioprotective via the Gβγ-phosphoinositol 3-kinase (PI3K)-Akt-endothelial nitric oxide synthase (eNOS) vasodilation pathway [20-23]. Thus,a subtle balance between β1-AR and β2-AR in the myocardium exists. However, β1-AR is the predominant subtype, and β-ARs mediate cardiac chronotropic and inotropic responses (primarily through β1-AR) may be less repaired by vascular vasodilator responses (primarily those mediated by β2-AR) [24]. As noted above, not withstanding the changes in β-AR responses, the net effect of β-ARs in hypertension is increases in cardiac contractility, peripheral vascular resistance and the pathological development of hypertension [25, 26].

In the present study, one important limitation is the small sample size. In future clinical work, we should increase the sample number to confirm our findings. Another issue is that we did not over-express or knockdown β2-AR to determine whether β2-AR can affect β1-AR expression in hypertension.

In conclusion, our data provide evidence of a close relationship between β1- and β2-AR mRNA levels based on animal, human, and cell studies. These findings highlight the need to focus not only on a single β-AR mechanism but also on β1- or β2-AR dynamic changes to completely understand the involvement of hypertension.

Acknowledgements

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Disclosure of conflict of interest

None.

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