Chronic Activation of the G Protein-Coupled Receptor 30 with Agonist G-1 Attenuates Heart Failure

Shoulei Kang1, Ying Liu1, Di Sun1, Chunle Zhou1, Aiying Liu1, Chuanying Xu1, Yanling Hao1, Dongye Li2*, Changdong Yan1, Hong Sun1*

1 Department of Physiology, Xuzhou Medical College, Xuzhou, 221002, China, 2 Institute of Cardiovascular Disease Research, Xuzhou Medical College Affiliated Hospital, Xuzhou, 221002, China

Abstract

G protein-coupled receptor (GPR) 30 is a novel estrogen receptor. Recent studies suggest that activation of the GPR30 confers rapid cardioprotection in isolated rat heart. It is unknown whether chronic activation of GPR30 is beneficial or not for heart failure. In this study we investigated the cardiac effect of sustained activation or inhibition of GPR30. Female Sprague–Dawley rats were divided into 7 groups #2Q1: sham surgery (Sham), bilateral ovariectomy (OVX), OVX+estradiol (E2), OVX+isoproterenol (ISO), OVX+ISO+G-1, OVX+ISO+E2+G15, OVX+ISO+E2. ISO (85 mg/kg×17 day, sc) was given to make the heart failure models. G-1(120 μg/kg×14 day) was used to activate GPR30 and G15 (190 μg/kg×14 day) was used to inhibit GPR30. Concentration of brain natriuretic peptide in serum, masson staining in isolated heart, contractile function and the expression of β1 and β2 adrenergic receptor (AR) of ventricular myocytes were also determined. Our data showed that ISO treatment led to heart failure in OVX rats. G-1 or E2 treatment decreased concentration of brain natriuretic peptide, reduced cardiac fibrosis, and enhanced contraction of the heart. Combined treatment with β1 (CGP20712A) and β2-AR (IC118551) antagonist abolished the improvement of myocardial function induced by G-1. We also found that chronic treatment with G-1 normalized the expression of β1-AR and increased the expression of β2-AR. Our results indicate that chronic activation of the GPR30 with its agonist G-1 attenuates heart failure by normalizing the expression of β1-AR and increasing the expression of β2-AR.

Introduction

Heart failure (HF) is a complex clinical syndrome that can result from any structural or functional cardiac disorder, it impairs the ability of the ventricle to fill with or eject blood. Despite significant advances in understanding the mechanisms underlying this disease, current treatments for HF have not been satisfied. It is recognized that sympathetic nervous system is one of the most important mechanisms regulating cardiac function, mainly through activation of β-AR [1]. Catecholamine such as epinephrine and norepinephrine are agonists of adrenoceptor in vivo, and levels of circulating catecholamine increased in patients with heart failure [2]. The development of heart failure also associated with diminishment of β-AR responsiveness [3], which assumed that reduced the density of β1-AR, but β2-AR was unaffected [4,5]. Blockade of β1 and desensitization of β2-AR could reduce cardiac fibrosis which induced by ISO [6]. We and others have shown that overexpression of β2-AR protected the hearts against ischemia/reperfusion (I/R) or chronic hypoxia injury [7,8], and played a beneficial role in heart failure [9].

Pre-menopausal women have reduced risk for cardiovascular disease, and the incidence of cardiovascular disease increased after menopause. Studies on animal models have also suggested that estrogen played an important role in cardioprotection [10]. There are three different forms of the estrogen receptor, usually referred to as α (ERα), β (ERβ), and the third G protein-coupled estrogen receptor (GPER), here referred as GPR30. Previous study showed that GPR30 subcellular localized in the endoplasmic reticulum and plasma membrane [11,23,24], and expressed in a variety of tissues such as heart, vascular, liver and ovarian in human and rats [16,25]. Estrogen binds to the ERs, on the one hand translocates to the nucleus to produce genomic actions; on the other hand, confers rapid non-genomic actions [12]. Anne M. and his colleagues have reported that GPR30 specific agonist G-1 reduced post-infarction dysfunction and infarct size after I/R, they found that the protection was blocked by the addition of the PKC inhibitor [13]. Others have also found the similar results [14–16]. In addition to the rapid effects caused by activation of GPR30, its chronic effects have also been identified. It was reported that genetic deletion of GPR30 was associated with visceral adiposity in both male and female animals [17]. Jewell A. Jessup and his colleagues have shown that chronic GPR30 activation attenuated changes in left ventricular remodeling due to prolonged intake of a high salt diet [18]. We have reported oestrogen conferred cardioprotection by changing the expression of β1- and β2-AR [7], however oestrogen can bind to classical estrogen receptor and the novel estrogen receptor GPR30, whether separate activation of...
Results

General Features of Experimental Animals

Serum estrogen levels, uterine weight decreased and body weight increased significantly after the ovaries were removed. There were no significant differences between each group in body length. Compared with the Sham or OVX+E2 group, OVX treatment increased heart weight, but it was not significant. ISO plus OVX increased heart weight and heart weight/body length ratio compared with OVX group. G-1 or E2 but not E2 plus OVX increased heart weight, but it was not significant. ISO significant differences between OVX +OVX

G-1 Treatment Increased the Ratio of Phosphorylated AKT

In the experiment, OVX and OVX + ISO group have lower ratio of phosphorylated AKT than the Sham or OVX+E2 group, OVX + ISO +G-1 and OVX +ISO + E2 have higher ratio of phosphorylated AKT than OVX + ISO group, there was no significant differences between OVX +ISO + E2+G15 and OVX +ISO group (figure 1).

G-1 Treatment Decreased BNP Levels in Heart Failure Model

We assayed the concentration of brain natriuretic peptide (BNP) (in µg/L) in plasma before the animals were sacrificed. Ovariectomy induced a slight increase in the release of BNP compared with the Sham or OVX+E2 group. After ISO treatment, BNP release increased in all groups, but the OVX+ISO+G1/E2 group had a lower BNP release than OVX+ISO group (figure 2).

G-1 Treatment Improved Cardiac Function in Heart Failure Model

Hearts were equilibrated for 30 minutes with KHB (Krebs–Henseleit buffer), and then we recorded the cardiac function data of each group. We found that there were no significant differences before ISO treatment among all groups. ISO treatment decreased LVDP, ±dp/dt and RPP, and increased LVEDP significantly. Administration of G1 or E2 increased LVDP, ±dp/dt, RPP, and decreased LVEDP, however G15 treatment can not cause such changes (table 2).

G-1 Treatment Decreased the Fibrosis of the Failing Heart

Masson staining showed that fibrotic areas were stained green, and the normal cardiac myocytes were stained red. The green areas were lower in Sham, OVX, OVX+E2 groups, stained sections showed increased fibrosis in OVX+ISO and OVX+ISO+E2+G15 group. OVX+ISO+G1/E2 group has lower fibrotic areas than OVX+ISO group (figure 3).

G-1 Treatment Enhanced Contraction of Myocardial Cells

First we observed contractility of myocytes isolated from Sham, OVX, OVX+E2, OVX+ISO, OVX+ISO+G-1, OVX+ISO+E2+G15 and OVX+ISO+E2 which incubated with vehicle medium. We found that myocardial function decreased in OVX+ISO group compared with OVX group, which reflected in decreasing of contraction amplitude, and extending of systolic and diastolic. G-1 or E2 improved myocardial function, but E2+G15 had no effect on the improvement (figure 4A, 4B and 4C). This indicated that the protection was induced by activation of G protein-coupled receptor. To further study the relationship between the protective effect of G-1 and β-AR, we incubated myocytes isolated from OVX+ISO and OVX+ISO+G-1 with CGP, ICI or CGP+ICI. And then we found that, there were significant differences between OVX+ISO and OVX+ISO+G-1 in CGP or ICI medium, but in CGP+ICI medium, we didn’t find the difference (figure 4D, 4E, 4F). These indicated that the protection of G-1 may associate with both β1 and β2-AR.

β1- and β2-AR Protein Expressions in Ventricular Myocytes

Our research showed that the protective effect of G-1 in heart failure can be abolished by CGP+ICI, which indicated that the protective effect of G-1 may be relevant to β-AR, so we determined whether the expression of β1- or β2-AR was changed by G-1 treatment. We added two groups OVX+G-1 and OVX+E2 for ovariectomy could change the expression of β-AR [7]. We found that compared with Sham group, the expression of β2-AR increased in OVX group, G-1 or E2 treatment decreased the expression in OVX group. However OVX plus ISO treatment could also decreased the expression of β2-AR compared with OVX group, that may because sustained

| Table 1. General features. |
|----------------------------|
| Sham | OVX | OVX+ISO | OVX+ISO+G1 | OVX+ISO+E2+G15 | OVX+ISO+E2 |
| Number of rats | 10 | 10 | 10 | 10 | 10 |
| Body weight (g) | 243±7 | 350±10* | 345±10* | 360±9* | 353±7* | 252±8# |
| Body length (mm) | 203±7 | 210±5 | 195±10 | 198±7 | 205±4 | 212±8 |
| Heart weight (mg) | 850±23 | 910±19 | 1210±14*5 | 1007±25# | 1290±23 | 950±22# |
| Heart weight/body length ratio (mg/mm) | 4.25±0.13 | 4.31±0.11 | 6.50±0.16$ | 5.12±0.12# | 6.13±0.18 | 4.40±0.08# |
| Uterine weight (mg) | 650±90 | 120±30* | 160±51* | 119±20* | 623±31 | 662±28# |
| Serum estradiol (pg/ml) | 65.18±8.11 | 12.15±1.36* | 10.87±0.79* | 13.41±1.62* | 62.54±1.06* | 63.27±10.20# |

Each value represents the mean±S.E.M. n = 10,
*P<0.05 versus Sham
$P<0.05 versus OVX+ISO and Sp<0.05 versus OVX group.
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treatment with isoproterenol diminished $\beta$-AR responsiveness, the expression of $\beta_1$-AR decreased is one of the manifestations [4]. OVX+ISO+G-1/E2 treatment increased the expression of $\beta_1$-AR compared with OVX+ISO group. All these above indicated that G-1/E2 normalized the expression of $\beta_1$-AR: decreasing the expression in OVX group and increasing the expression in OVX+ISO group. We also found that the expression of $\beta_2$-AR decreased after ovariectomy no matter with ISO treatment or not, G-1 or E2 treatment increased the protein expression of $\beta_2$-AR (figure 5).

Discussion

In this study, we found that chronic treatment with GPR30 agonist G-1 attenuated heart failure in female SD rats. As a novel estrogen receptor GPR30 plays an important role in cardiac protection and gets more and more attention recently. In the paper, ISO treatment was used in ovariectomized rats to prepare for heart failure model [19]. BNP concentration in serum, hemodynamic, Masson’s trichrome staining in isolated heart, contractile function of ventricular myocytes were determined in the study, we also determined the effects of $\beta_1$- and $\beta_2$-AR antagonist on the function of myocytes, western blot method was used to determine the protein expression of p-AKT, $\beta_1$- and $\beta_2$-AR. Our data indicate that chronic G-1 treatment attenuated heart failure which induced by ISO, and the protective effect may be associated with regulating the expression of $\beta$-AR.

In our study, continuously and chronic effect of G-1 was observed. GPR30, as one of the estrogen receptor, can be activated by E2, in order to prevent the interference of E2, rats were ovariectomized. Here ISO treatment was used to get a heart failure model as previous described [19]. As our data shown, in OVX group, with out the protection of E2 (exogenous), ISO treatment caused significantly damage to the heart compared with E2 treatment, which indicated that estrogen played an important role in protection of the heart. To study the protective effect of GPR30, G1, E2+G15 and E2 were administrated. The amount of drugs was determined following the method mentioned in Lindsey SH.’s article [21]. First of all, determine drug affinity for GPR30 in the article [20,21], affinities as shown below: G-1 11 nM, G15 20 nM and E2 5.7 nM; second determine the concentration of G-1 and G15 according to the concentration of E2 which our laboratory used before [7] and then determine the ratio of their affinities to GPR30, the amount of drugs was determined: G-1 120 $\mu$g/kg·d, G15 190 $\mu$g/kg·d, E2 40 $\mu$g/kg·d.

We measured animals’ weight before they were killed, G-1 treatment didn’t change weight gain induced by ovariectomy, which was consistent with the results of Lindsey SH.’s research [21], and E2 or E2+G15 treatment decreased weight gain induced by ovariectomy which in line with our previous study [7,31,32], possibly because ER$\alpha$ and ER$\beta$ played a role in regulating body weight [21]. Other indications in our experiment showed that E2+...
abolish the improvement completely. This indicated that the contraction., but combination treatment with CGP and ICI could 
AR antagonit ICI118551, we found that treatment with CGP or 
raised and the secretion of BNP increased. The increase of the 
pressure, when heart failure occurred, ventricular filling pressure 
secretion is closely related to the changes of ventricular filling 
GPR30.

In the experiment, the concentration of BNP in OVX 
ISO and OVX 
2-AR. Interestingly, other 
2-AR in heart 
expression of 
example the antagonist drugs may reach to the liver, brain or 
other organs, which confer the systolic changes of the bodies.

The sympathetic nervous system is critically involved in the 
regulation of cardiac function through β-AR. Activation of β1-AR 
results in augmentation of cardiac activity (positive inotropic 
effect), including an increase in heart rate and atria-ventricle 
conduction velocity and enhancement of myocardial contraction 
[33]. Roth DM has pointed that overexpression of β1 receptors 
caused cardiac damage [25]. Our laboratory has found that the 
expression of β1-AR increased in ovariecetomized female rats 
compared with the Sham group [7], which indicated that estrogen 
may play an important role in regulate the expression of β1-AR 
thus conferred cardiac protection effect. In this paper, we found that 
the expression of β1-AR increased in ovariecetomized group. Taken together, G-1 
attenuated heart failure by increased the 
expression of 
just a risk factor for hearts. However ISO treatment decreased the 
expression of β1-AR and produced injury effect which may be 
attributed to continuous stimulation of catecholamine led to 
decline in receptor number and reduce of the function [4], G-1 or 
E2 treatment could reduce the injury and increased the expression of 
β1-AR compared with OVX+ISO group. Taken together, G-1 
or E2 treatment regulated protein β1-AR in the protective effects.

Unlike β1AR, activation of β2-AR plays a beneficial role in 
hearts. Sustained β2-AR stimulation promotes apoptotic death of 
cardiomycocytes, sustained stimulation of β2-AR protects myocytes 
against a wide range of apoptotic insults [27]. Similarly, some 
studies showed that overexpression of β2-AR conferred cardiac 
protective effect in the heart [8,28] which was consistent with our 
results.

In our opinion, treatment with the estrogen hormone agonist G- 
1 could increase the expression of β2-AR. Interestingly, other 
hormones or models could also regulate the expression of β2-AR 
in the body. For instance, Penna C has reported sub-chronic 
nandrolone pretreatment increased the expression of β2-AR 
[28], thyroid hormones increased the mRNA of β2-AR in heart 
[29], and in diabetic heart model, the expression of β2-AR 
decreased [30]. However whether the mechanism of protective 
effects of G-1 which changed the expression of β2-AR is direct or 
indirect effects such as regulating the secretion of other hormones 
is unknown, the mechanisms remain to be further studied.

Taken all together, in this study we found that chronic 
treatment with G-1 attenuated heart failure by increased the 
expression of β2-AR and normalized the expression of β2-AR in 
ovariectomized rats. This is the first time we have reported chronic 
treatment with G-1 is beneficial for the heart failure.

### Table 2. Cardiac function of each group.

| Group  | LVDP (mmHg) | LVEDP (mmHg) | -dp/dt (mmHg/s) | -dp/dt (mmHg/s) | HR (beats/min) | RPP (mmHg/min) |
|--------|-------------|--------------|----------------|----------------|---------------|---------------|
| Sham   | 89.7±8.6    | 5.9±0.4      | 1896.5±156.2   | 1672.3±123.2   | 283.5±16.8    | 26358.9±116.8 |
| OVX    | 82.6±7.5    | 5.8±0.7      | 18592.2±147.3  | 1536.7±115.6   | 281.2±17.1    | 23065.2±113.1 |
| OVX+ISO| 39.8±3.2*   | 16.8±2.9*    | 923.4±87.8*    | 565.2±64.6*    | 223.4±15.8*   | 8697.3±43.4*  |
| OVX+ISO+G-1| 47.8±3.6#&$ | 11.2±1.7*$   | 1394.9±97.1*   | 1022.4±78.1*   | 241.2±18.2*$  | 11327.5±67.9*  |
| OVX+ISO+E2+G15| 38.3±2.7*| 17.5±3.1*$  | 932.0±77.3*$  | 523.1±58.3*$  | 213.2±19.1*$  | 8093.8±47.6*  |
| OVX+ISO+E2 | 50.1±3.4*#| 10.8±2.2*   | 1411.3±106.3* | 1103.4±88.2*|$  | 234.2±18.3*$  | 11706.3±74.1*# |

Each value represents the mean±S.E.M. n = 10, 
*$P<0.05$ versus Sham. 
$P<0.05$ versus OVX, and Sp$<0.05$ versus OVX group. 
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G15 treatment didn’t play cardiac protection roles which indicated that the chronic activation of GPR30 is responsible, and not ERα 
and ERβ.

PI3K-AKT pathway is the downstream pathway of GPR30, 
and G-1 treatment increased phosphorylation of AKT. In our 
experiment, we determined the phosphorylation of AKT and found that G-1 or E2 treatment increased the phosphorylation of 
AKT, G15+E2 treatment didn’t increased the phosphorylation of 
AKT. This indicated that the special agonist G-1 activated 
GPR30.

BNP is mainly present in the left and right atria, the physiologic 
actions of it are similar to ANP (atrial natriuretic peptide) and 
include decrease in systemic vascular resistance and central venous 
pressure as well as an increase in natriuresis. The level of its 
secretion is closely related to the changes of ventricular filling 
pressure, when heart failure occurred, ventricular filling pressure 
raised and the secretion of BNP increased. The increase of the 
secretion was positively correlated to the degree of heart failure. So 
the concentration of BNP in serum could be an indicator to assess 
the severity of heart failure.

In the experiment, the concentration of BNP in OVX+ISO 
group increased significantly compared with OVX group, this is in 
accordance with the hemodynamics result. In OVX+ISO+G-1 
group, the concentration of BNP decreased compared with 
OVX+ISO group, this indicated that G-1 treatment conferred cardiac protective effect in ISO induced heart failure model.

We have detected hemodynamic in organ levels, found that ISO 
treatment diminished cardiac ejection and G-1 treatment 
enhanced the ability of the cardiac ejection, this indicated that 
G-1 conferred cardiac protective effect. As G-1 could reduce 
vascular tone and dilate rodent arterial blood vessels [17], and β-AR 
agonist also had the role of the vasodilator, in order to 
exclude the impact of these roles, we isolated cardiac myocytes 
with collagen digest method and detected systolic and diastolic 
function in single cells. In this way, we conclude that G-1, at least 
could act directly on myocardial cells in the protective effect of the 
failing heart.

We isolated cardiac myocytes of OVX+ISO and OVX+ISO+G-1 
group, cultured with β1-AR antagonist CGP20712A, β2-AR 
agonist IC118551, we found that treatment with CGP or 
ICI separately could not abolish the improvement of the cell 
contraction., but combination treatment with CGP and ICI could 
abolish the improvement completely. This indicated that the 
protective of G-1 may associate with both β1-AR and β2-AR.

#3Q3Although there is a group with antagonist group, the 
ligand specificity in vivo is still limitation in vivo study, for
Materials and Methods

Animals and Reagents
Female Sprague-Dawley (SD) rats weighing 180–220 g (200±20 g) were obtained from the Experimental Animal Centre of Xuzhou Medical College and all studies were approved by the Animal Ethics Committee of the Medical College of Xuzhou (permit number: xz11-12540) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health (NIH publication No. 85-23, revised 1996). The main reagents used in the experiments are as follows: isoproterenol hydrochloride (Sigma-Aldrich, St.Louis, MO, USA), G-1 (EMD Chemicals, San Diego, CA, USA), G15(EMD Chemicals, San Diego, CA, USA), 17β-estradiol (ABCR, Germany), ICI118551 (tocris, Bristol, UK), CGP20712A (Sigma-Aldrich, St.Louis, MO, USA), wortmannin (Merck, Darmstadt, Germany), in situ cell death detection kit, pod (Roche, Indianapolis, IN, USA), rabbit polyclonal anti β1-AR, β2-AR, p-AKT, t-AKT (Santa Cruz, CA, USA), brain natriuretic peptide (BNP) ELISA Kits (Fengxiang Bio-technology, Shanghai, China), Masson’s Trichrome Stain Kit (Yuanye Bio-technology, Shanghai, China).

The animals were anesthetized with sodium pentobarbitone (60 mg/kg, I.P.), corneal reflex disappeared, sensation of pain reduced and muscle tension decreased was the sign of the success of anesthesia. Incision was chosen at the side of the spine, separated the muscle layer and opened the peritoneum, then the ovaries were exteriorized, ligated and removed in OVX group. In Sham group, visualization of the ovaries through incisions into the abdominal cavity and then closure of the wounds. All surgeries were carried out under sterile conditions. ISO (85 mg/kg, sc) treatment was continued for 17 d to get the heart failure model [19]. Osmotic minipump (model 2006, Alza Corp., Palo Alto, CA, USA) implanted s.c. at the dorsum of the neck. In the surgery, pentobarbitone (60 mg/kg, I.P.) was used as an anaesthetic, corneal reflex disappeared, sensation of pain reduced and muscle tension decreased was the sign of the success of anesthesia. G-1, E2+G15 or E2 was pumped via the minipump at the dose of 120 mg/kg·d, 190 mg/kg·d, 40 μg/kg·d respectively, treatment lasted for 2 weeks. The dose of G-1, G15, E2 based on the affinity to GPR30 [20,21] and our previous studies [7]. In general, seven groups were divided: Sham, OVX, OVX+E2, OVX+ISO, OVX+ISO+G-1, OVX+ISO+E2+G15, OVX+ISO+E2.

Measurement of BNP Concentration in Plasma
We collected the blood from tail vein of the rat before it was killed, added an appropriate amount of heparin and then centrifuged at the rate of 2500 rpm for 15 min. The concentration of BNP was assayed as the instructions of ELISA kits. All samples were assayed in triplicate.

Isolation of the Heart and Heart Perfusion
Intraperitoneal injection with 5000 U/kg heparin was implemented, 15 minutes later, 150 mg/kg pentobarbital was injected for anesthesia, corneal reflex disappeared, sensation of pain reduced and muscle tension decreased was the sign of the success of anesthesia. A transverse incision was made, the abdominal cavity was exposed, hearts were quickly isolated and rinsed with ice-cold Ca2+-free buffer as we have reported before [7]. Oxygenated KHB was perfused through Mouse heart perfusion system (TME Technology, Chengdu, CHINA) in the experiment [22].

Figure 3. Masson staining of the heart. 3A: The fibrotic areas were stained green, and the normal cardiac myocytes were stained red and normal nuclei were stained blue. Representation of each group as following: a: Sham group; b: OVX group; c: OVX+ISO group; d: OVX+ISO+G-1 group; e: OVX+ISO+E2+G15 group; f: OVX+ISO+E2 group
3B: Each value represents the mean±S.E.M. n = 6, *P<0.05 versus Sham, #P<0.05 versus OVX+ISO, and $p<0.05 versus OVX group.
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Figure 4. Function determination of the single cardiac cell. Myocytes of the six groups incubated in veh medium, shortening amplitude, TTP and R90 are shown as figure (A), (B) and (C) respectively. Data shown are mean ± S.E.M. n = 10 hearts. *p < 0.05 versus Sham, #p < 0.05 versus OVX+ISO and $p < 0.05 versus OVX. OVX+ISO and OVX+ISO+G-1 groups were incubated in veh, CGP or ICI medium. Shortening amplitude, TTP and R90 are shown as figure (D), (E) and (F) respectively. Data shown are mean ± S.E.M. n = 10 hearts. #p < 0.05 versus OVX+ISO of each subgroup.

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Determination of Cardiac Function

Linked aortic side of the isolated heart to perfusion device, left ventricular pressure was recorded using a Biopac system (BIOPAC) via a pressure sensor Millar transducer instrument (1.4F, Millar). Hearts were equilibrated for 30 minutes with KHB. Heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular enddiastolic pressure (LVEDP), and the rate in rise and fall of ventricular pressure ($+\frac{dp}{dt}\text{max}$, $-\frac{dp}{dt}\text{max}$) were recorded as left ventricular functional parameters. The left ventricular developed pressure (LVDP) were calculated as $\text{LVDP} = \text{LVSP} - \text{LVEDP}$; rate pressure product (RPP) was calculated as $\text{RPP} = \text{HR}\times\text{LVDP}$.

Masson’s Trichrome Stain

Rat hearts were perfused with KHB for 20 minutes to remove the residual blood, and then fixed with 10% formalin before embedding in paraffin. All hearts were embedded in a cross section orientation, and all slices were cross sections of the heart. All slices were taken from the midpoint of the left ventricles. Four-micrometer slices were deparaffinized and rehydrated. Then the instruction of masson’s trichrome stain kit was followed. Microscope (IX 71, OLYMPUS, Japan) was used to get the pictures. The normal cardiac myocytes were stained red, and fibrotic areas were stained green.

Figure 5. The expression of beta-AR. The expression of $\beta$-actin was detected as an internal standard. The relative arbitrary unit for sham group was assigned as (A). Figure (B) and (C) showed the expression of $\beta_1$-AR and $\beta_2$-AR. Each value represents the mean±S.E.M. n = 10 hearts in each group, *$P<0.05$ versus Sham, $\#P<0.05$ versus OVX+ISO, $\$P<0.05$ versus OVX.

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Isolation and Culture of Myocardial Cells

Ventricular myocytes were isolated from the hearts as we described before [7,22]. Cardiac ventricular myocytes were isolated from the hearts then the cells were suspended in Dulbecco’s minimal essential medium (DMEM) at a density of 3×10^5 cells per well, finally took them to carbon dioxide incubator (Heraeus, Germany), parameters were set as below: 5% CO₂, 37°C, cultured for 1 hour to stabilize the cells. The rat cardiomyocytes were incubated with vehicle (veh), CGP20712A (CGP, 300 nM), or ICI118551 (ICI, 55 nM), for 2 h to study the impact of different β-AR antagonists on shortening amplitude in rat myocytes.

Myocyte Contractility

After myocardial cells were isolated, each group was divided into three subgroups: veh, CGP, ICI. A few drops of solution containing ventricular myocytes were added to an open chamber, they were superfused at 2 ml/min with KHB (containing ventricular myocytes were added to an open chamber, they were superfused at 2 ml/min with KHB (containing 2.0 mM Ca²⁺ and 100 nM ISO) adjusted to pH 7.4 by equilibration with 95% O₂–5% CO₂. The ventricular myocytes were paced with an electrical field stimulation (0.5 Hz). Myocytes used were rod shaped with clear sarcomeres, and 10 consecutive contractions were averaged. The whole process was recorded by a video recorder (Panasonic, Japan) connect to the microscope. Then the video was sent to a computer, cut into pictures by the software VirtualDub, the process of contraction and relaxation was recorded and transformed into digital data by the software OpticalMeasure (presented by China’s National Defense University of Science and Technology). Contractile function was assessed using the following indices: shortening amplitude, the amplitude myocytes shortened upon electrical stimulation, an indicative of peak ventricular; contractility time-to-peak contraction (TTCP), the duration of myocyte shortening, an indicative of systolic duration; and time-to-90% relaxation (R90), the duration to reach 90% relengthening, an indicative of diastolic duration.

Western Blot

Ventricular myocytes were frozen in liquid nitrogen for analysis of p-AKT, β₁- and β₂-AR protein by western blotting. Myocytes were homogenized in ice-cold homogenization. We followed our previous method in this part [7]. The membranes were scanned into the computer, and relative intensity of bands was analysed by the software photoshop (Adobe, San Jose, CA, USA).

Statistical Analysis

In each experimental series, data are presented as mean±S.E.M. Statistical analysis was performed with GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA). The contraction of the myocytes were analysed by two-way ANOVA, other data were analysed by one-way ANOVA followed by Bonferroni post hoc tests.

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Author Contributions

Conceived and designed the experiments: SK HS. Performed the experiments: SK YL DS CZ YH. Analyzed the data: SK YL DS CZ AL CX DL CY. Contributed reagents/materials/analysis tools: HS DL CY. Wrote the paper: SK HS YL DS CZ.

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