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

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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+estrogen (E_2), OVX+isoproterenol (ISO), OVX+ISO+G-1, OVX+ISO+E_2+G15, OVX+ISO+E_2. ISO (85 mg/kg×17 day, sc) was given to make the heart failure models. G-1(120 μg/kg·d×14 day) was used to activate GPR30 and G15 (190 μg/kg·d×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 β_1 and β_2- 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 E_2 treatment decreased concentration of brain natriuretic peptide, reduced cardiac fibrosis, and enhanced contraction of the heart. Combined treatment with β_1 (CGP20712A) and β_2-AR (ICI118551) antagonist abolished the improvement of myocardial function induced by G-1. We also found that chronic treatment with G-1 normalized the expression of β_1-AR and increased the expression of β_2-AR. Our results indicate that chronic activation of the GPR30 with its agonist G-1 attenuates heart failure by normalizing the expression of β_1-AR and increasing the expression of β_2-AR.

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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 β_1-AR, but β_2-AR was unaffected [4,5]. Blockade of β_1 and desensitization of β_2-AR could reduce cardiac fibrosis which induced by ISO [6]. We and others have shown that overexpression of β_2-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-ischemic dysfunction and infarct size after I/R, they found that the protection was blocked by the addition of the PI3K 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 β_1- and β_2-AR [7], however oestrogen can bind to classical estrogen receptor and the novel estrogen receptor GPR30, whether separate activation of

GPR30 with G-1 is beneficial for ISO induced heart failure, or changes the expression of β-AR has not been reported.

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+E_2 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 E_2 but not E_2+G15 could eliminate the increasing of the heart weight caused by OVX plus ISO. E_2 and E_2+G15 but not G-1 could increase uterine weight (table 1).

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+E_2 group, OVX + ISO +G-1 and OVX +ISO + E_2 have higher ratio of phosphorylated AKT than OVX + ISO group, there was no significant differences between OVX +ISO + E_2+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+E_2 group. After ISO treatment, BNP release increased in all groups, but the OVX+ISO+G1/E_2 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 E_2 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+E_2 groups, stained sections showed increased fibrosis in OVX+ISO and OVX+ISO+E_2+G15 group. OVX+ISO+G-1/E_2 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+ E_2, OVX+ISO, OVX+ISO+G-1, OVX+ISO+E_2+G15 and OVX+ISO+E_2 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 E_2 improved myocardial function, but E_2+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 β_1 and β_2 -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 β_1- or β_2-AR was changed by G-1 treatment. We added two groups OVX+G-1 and OVX+E_2 for ovariectomy could change the expression of β-AR [7]. We found that compared with Sham group, the expression of β_1-AR increased in OVX group, G-1 or E_2 treatment decreased the expression in OVX group. However OVX plus ISO treatment could also decreased the expression of β_1-AR compared with OVX group, that may because sustained

Table 1. General features.

	Sham	OVX	OVX+ISO	OVX+ISO+G1	OVX+ISO+E_2+G15	OVX+ISO+E_2
Number of rats	10	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*$	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 $p<0.05 versus OVX group.
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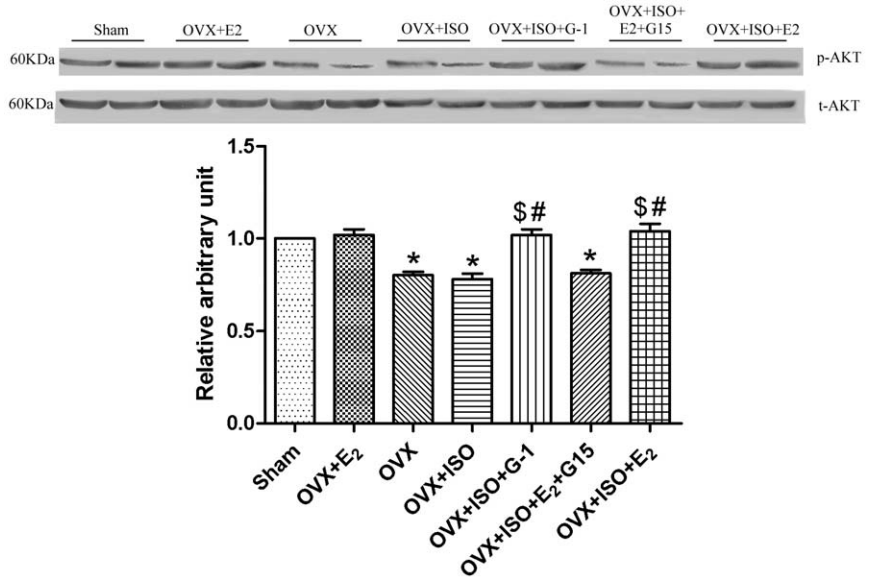

Figure 1. Expression of p-AKT. The relative arbitrary unit for sham group was assigned as figure (A). Figure (B) showed the expression of p-AKT. Each value represents the mean±S.E.M. n = 10 hearts in each group, *P<0.05 versus Sham, #P<0.05 versus OVX+ISO, $p<0.05 versus OVX.
doi:10.1371/journal.pone.0048185.g001

treatment with isoproterenol diminished β-AR responsiveness, the expression of β_1-AR decreased is one of the manifestations [4]. OVX+ISO+G-1/E_2 treatment increased the expression of β_1-AR compared with OVX+ISO group. All these above indicated that G-1/E_2 normalized the expression of β_1-AR: decreasing the expression in OVX group and increasing the expression in OVX+ISO group. We also found that the expression of β_2-AR decreased after ovariectomy no matter with ISO treatment or not, G-1 or E_2 treatment increased the protein expression of β_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 β_1- and β_2-AR antagonist on the function of myocytes, western blot method was used to determine the protein expression of p-AKT, β_1- and β_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 β-AR.

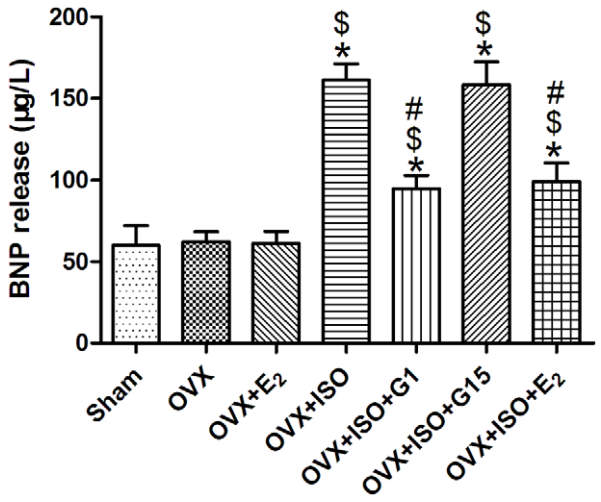

Figure 2. BNP activity of each group in serum. Each value represents the mean±S.E.M.; n = 10 hearts in each group. *P<0.05 versus Sham, #P<0.05 versus OVX+ISO, $p<0.05 versus OVX.
doi:10.1371/journal.pone.0048185.g002

In our study, continuously and chronic effect of G-1 was observed. GPR30, as one of the estrogen receptor, can be activated by E_2, in order to prevent the interference of E_2, 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 E_2 (exogenous), ISO treatment caused significantly damage to the heart compared with E_2 treatment, which indicated that estrogen played an important role in protection of the heart. To study the protective effect of GPR30, G1, E_2+G15 and E_2 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 E_2 5.7 nM; second determine the concentration of G-1 and G15 according to the concentration of E_2 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 μg/kg·d, G15 190 μg/kg·d, E_2 40 μg/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 E_2 or E_2+G15 treatment decreased weight gain induced by ovariectomy which in line with our previous study [7,31,32], possibly because ERα and ERβ played a role in regulating body weight [21]. Other indications in our experiment showed that E_2+

Table 2. Cardiac function of each 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	1859.2±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*$	11327.6±67.9*#$
OVX+ISO+E_2+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+E_2	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+ISO, and $p<0.05 versus OVX group.
doi:10.1371/journal.pone.0048185.t002

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+E_2 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 according with the hemodynamics resulst. 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 antagonist 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 β_1-AR antagonist CGP20712A, β_2-AR antagonit ICI118551, 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 β_1-AR and β_2-AR.

#3Q3Although there is a group with antagonist group, the ligand specificity in vivo is still limitation in vivo study, for 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 β_1-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 β_1 receptors caused cardiac damage [25]. Our laboratory has found that the expression of β_1-AR increased in ovariectomized female rats compared with the Sham group [7], which indicated that estrogen may play an important role in regulate the expression of β_1-AR thus conferred cardiac protection effect. In this paper, we found that the expression of β_1-AR increased in OVX group, G-1 or E_2 treatment decreased it, and we didn't observed cardiac damage indications in OVX group, here we speculated ovariectomized is just a risk factor for hearts. However ISO treatment decreased the expression of β_1-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 E_2 treatment could reduce the injury and increased the expression of β_1-AR compared with OVX+ISO group. Taken together, G-1 or E_2 treatment regulated protein β_1-AR in the protective effects.

Unlike β_1AR, activation of β_2-AR plays a beneficial role in hearts. Sustained β_1-AR stimulation promotes apoptotic death of cardiomyocytes, sustained stimulation of β_2-AR protects myocytes against a wide range of apoptotic insults [27]. Similarly, some studies showed that overexpression of β_2-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 β_2-AR. Interestingly, other hormones or models could also regulate the expression of β_2-AR in the body. For instance, Penna C has reported sub-chronic nandrolone pretreatment increased the expression of β_2-AR [28], thyroid hormones increased the mRNA of β_2-AR in heart [29], and in diabetic heart model, the expression of β_2-AR decreased [30]. However whether the mechanism of protective effects of G-1 which changed the expression of β_2-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 β_2-AR and normalized the expression of β_1-AR in ovariectomized rats. This is the first time we have reported chronic treatment with G-1 is beneficial for the heart failure.

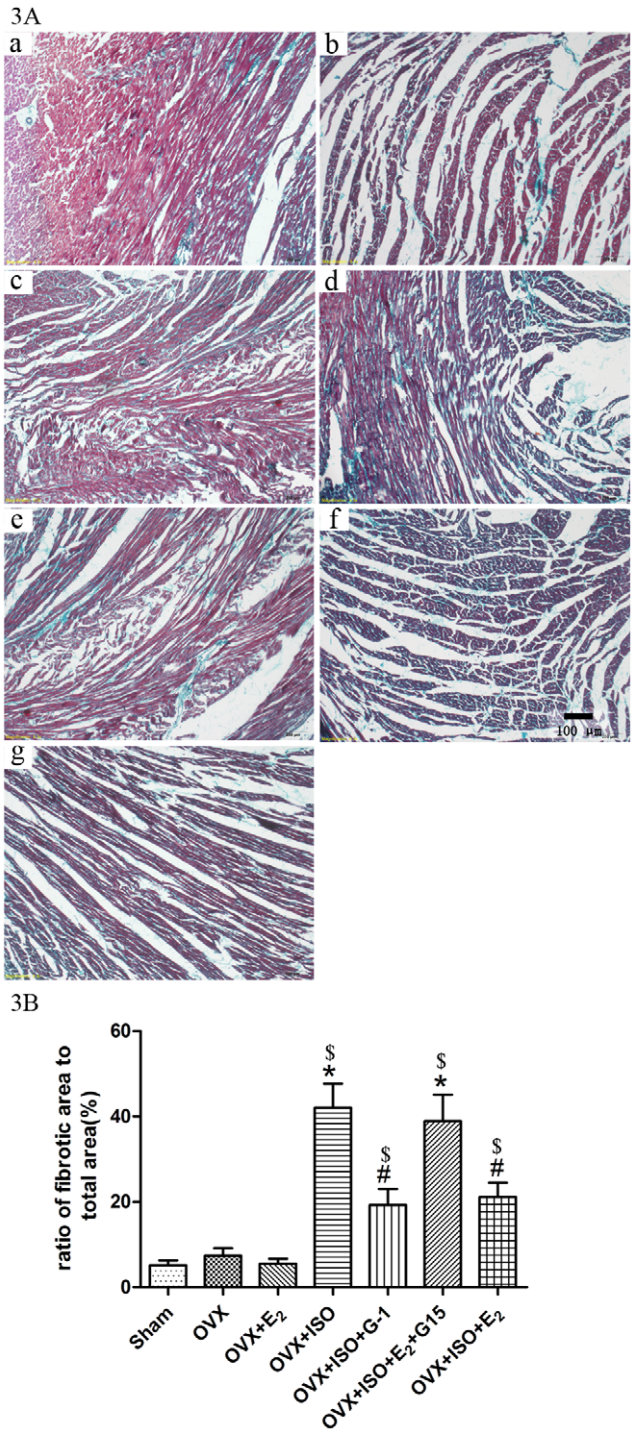

Figure 3. Masson staining of the heart. 3A: The fibrotic areas were stained green, and the normal cardiac myocytes were stained red and normal nuclei were stained blue. Representation of each group as following: a: Sham group; b: OVX group; c: OVX+ISO group; d: OVX+ISO+G-1 group; e: OVX+ISO+E_2+G15 group; f: OVX+ISO+E_2 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.
doi:10.1371/journal.pone.0048185.g003

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-estradlol (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 β_1-AR, β_2-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, E_2+G15 or E_2 was pumped via the minipump at the dose of 120 μg/kg·d, 190 μg/kg·d, 40 μg/kg·d respectively, treatment lasted for 2 weeks. The dose of G-1, G15, E_2 based on the affinity to GPR30 [20,21] and our previous studies [7]. In general, seven groups were divided: Sham, OVX, OVX+E_2, OVX+ISO, OVX+ISO+G-1, OVX+ISO+E_2+G15, OVX+ISO+E_2.

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 Ca^{2+} 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].

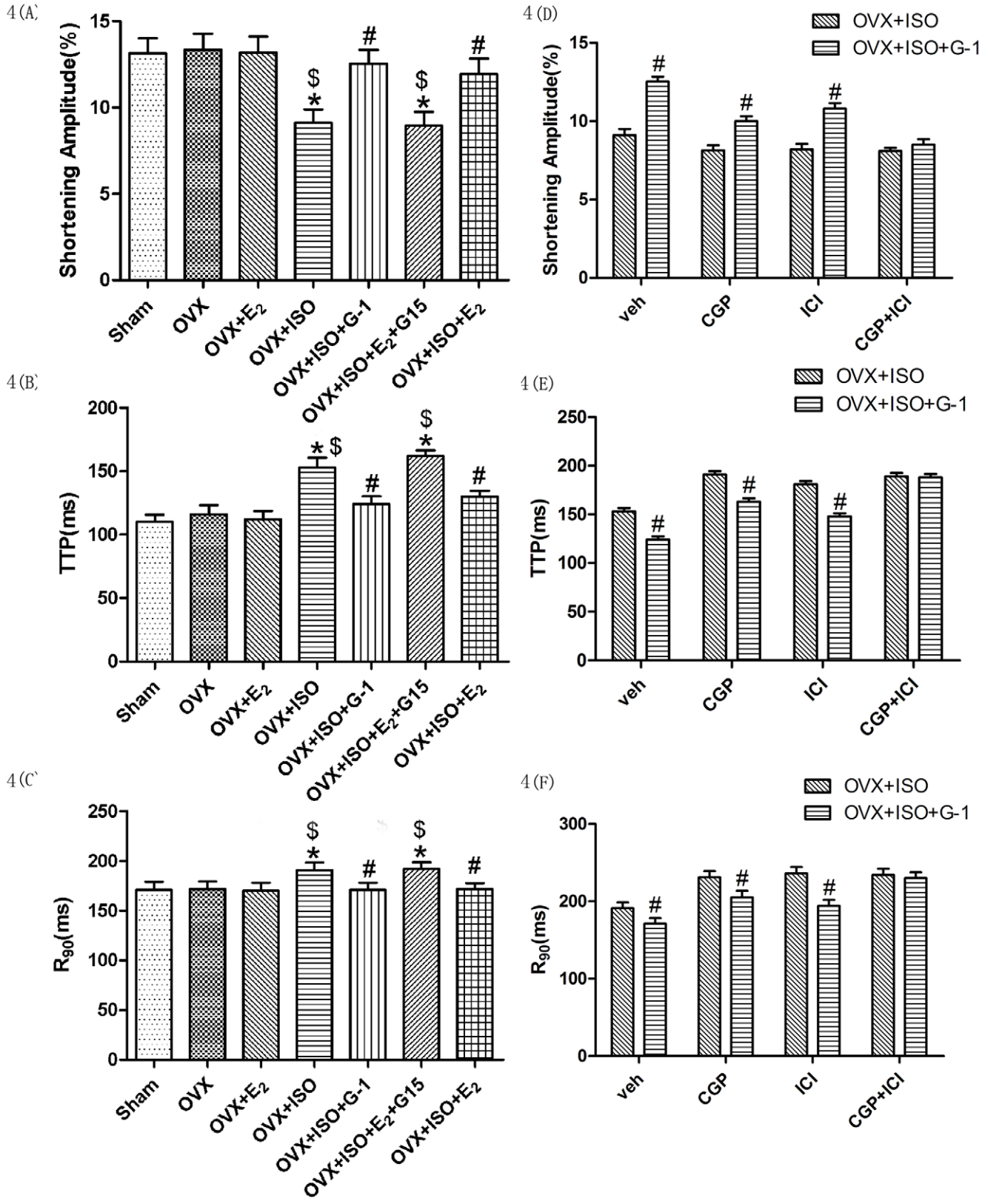

Figure 4. Function determination of the single cardiac cell. Myocytes of the six groups incubated in veh medium, shortening amplitude, TTP and R90 are shown as figure (A), (B) and (C) respectively. Data shown are mean± S.E.M. n = 10 hearts. *p<0.05 versus Sham, #p<0.05 versus OVX+ISO and $p<0.05 versus 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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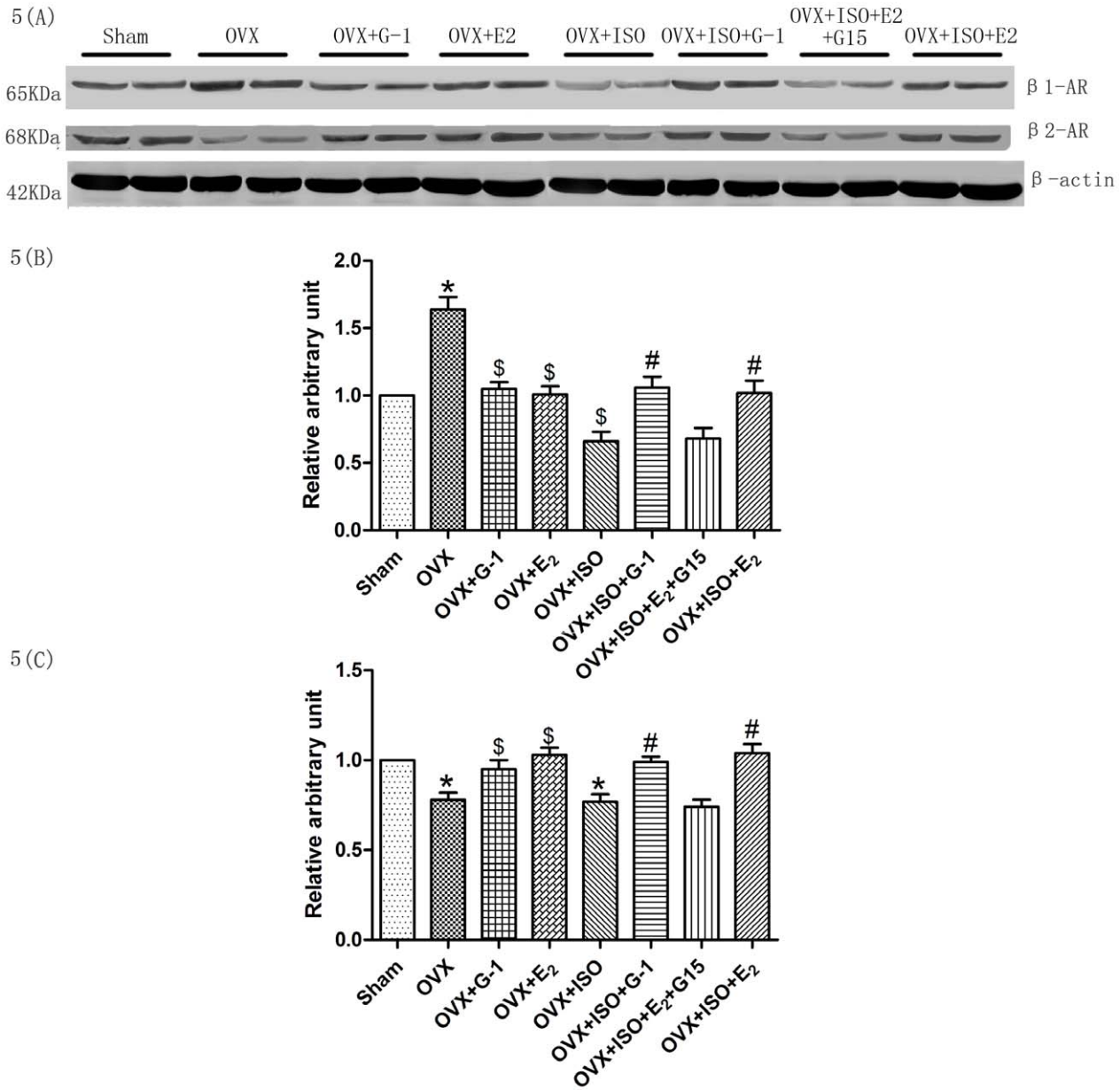

Figure 5. The expression of beta-AR. The expression of β-actin was detected as an internal standard. The relative arbitrary unit for sham group was assigned as (A). Figure (B) and (C) showed the expression of β_1-AR and β_2-AR. Each value represents the mean±S.E.M. n = 10 hearts in each group, *P<0.05 versus Sham, #P<0.05 versus OVX+ISO, $p<0.05 versus OVX.
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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 (+dp/dtmax, -dp/dtmax) were recorded as left ventricular functional parameters. The left ventricular developed pressure (LVDP) were calculated as LVDP = LVSP-LVEDP; rate pressure product (RPP) was calculated as RPP = HR ×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.

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% CO_2, 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 on the stage of an inverted microscope (Olympus, Japan). After 5 min, to allow cells to spontaneously attach to the floor of the 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 (TTP), the duration of myocyte shortening, an indicative of systolic duration; and time-to-90% relaxation (R_{90}), 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, β_1- and β_2-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 means±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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