

ARTICLE OPEN



Biased activation of β_2 -AR/Gi/GRK2 signal pathway attenuated β_1 -AR sustained activation induced by β_1 -adrenergic receptor autoantibody

Hao Chen^{1,6}, Ning Cao^{1,2,6}, Li Wang³, Ye Wu¹, Haojie Wei¹, Yuming Li⁴, Youyi Zhang², Suli Zhang^{1,5}✉ and Huirong Liu^{1,5}✉

© The Author(s) 2021

Heart failure is the terminal stage of many cardiac diseases, in which β_1 -adrenoceptor (β_1 -AR) autoantibody (β_1 -AA) has a causative role. By continuously activating β_1 -AR, β_1 -AA can induce cytotoxicity, leading to cardiomyocyte apoptosis and heart dysfunction. However, the mechanism underlying the persistent activation of β_1 -AR by β_1 -AA is not fully understood. Receptor endocytosis has a critical role in terminating signals over time. β_2 -adrenoceptor (β_2 -AR) is involved in the regulation of β_1 -AR signaling. This research aimed to clarify the mechanism of the β_1 -AA-induced sustained activation of β_1 -AR and explore the role of the β_2 -AR/Gi-signaling pathway in this process. The beating frequency of neonatal rat cardiomyocytes, cyclic adenosine monophosphate content, and intracellular Ca^{2+} levels were examined to detect the activation of β_1 -AA. Total internal reflection fluorescence microscopy was used to detect the endocytosis of β_1 -AR. IC118551 was used to assess β_2 -AR/Gi function in β_1 -AR sustained activation induced by β_1 -AA in vitro and in vivo. Monoclonal β_1 -AA derived from a mouse hybridoma could continuously activate β_1 -AR. β_1 -AA-restricted β_1 -AR endocytosis, which was reversed by overexpressing the endocytosis scaffold protein β -arrestin1/2, resulting in the cessation of β_1 -AR signaling. β_2 -AR could promote β_1 -AR endocytosis, as demonstrated by overexpressing/interfering with β_2 -AR in HL-1 cells, whereas β_1 -AA inhibited the binding of β_2 -AR to β_1 -AR, as determined by surface plasmon resonance. IC118551 biasedly activated the β_2 -AR/Gi/G protein-coupled receptor kinase 2 (GRK2) pathway, leading to the arrest of limited endocytosis and continuous activation of β_1 -AR by β_1 -AA in vitro. In vivo, IC118551 treatment attenuated myocardial fiber rupture and left ventricular dysfunction in β_1 -AA-positive mice. This study showed that β_1 -AA continuously activated β_1 -AR by inhibiting receptor endocytosis. Biased activation of the β_2 -AR/Gi/GRK2 signaling pathway could promote β_1 -AR endocytosis restricted by β_1 -AA, terminate signal transduction, and alleviate heart damage.

Cell Death Discovery (2021)7:340; <https://doi.org/10.1038/s41420-021-00735-2>

INTRODUCTION

Heart failure (HF) is the result of heart diseases and is a global threat to human health. Overactivation of the β_1 -adrenergic receptor (β_1 -AR) in cardiomyocytes is a core HF mechanism [1]. We and others have shown that in addition to the endogenous β_1 -AR ligand norepinephrine (NE), autoantibodies against β_1 -AR (β_1 -AA), harboring agonist-like effects, circulate in the sera of 30–40% of HF patients [2, 3]. Studies have demonstrated that β_1 -AA can directly lead to HF [4], suggesting the importance of β_1 -AA in HF development.

There are several potential approaches to protect against cardiac damage caused by β_1 -AA. β -blockers are widely used in treating chronic HF by blocking β_1 -AR activation [5]. However, the β_1 -AA-included pathological effects can only be partially abrogated by β -blockers, as suggested by animal [6] and clinical studies [7]. In vitro plasma exchange or immunoadsorption methods can increase the cardiac ejection fraction and restore cardiac function to a certain extent. However, these two schemes have poor specificity. Other

approaches, such as peptide homologs mimicking the epitope(s) or aptamers, are still underway. One of the reasons for the shortcomings of treatment plans is our insufficient understanding of the characteristics of β_1 -AA-mediated activation of β_1 -AR.

In contrast to transiently activating β_1 -AR by NE, β_1 -AA can persistently activate β_1 -AR, which leads to the overactivation of β_1 -AR downstream signaling and cardiac injury. Existing studies have suggested that this mode of action of β_1 -AA may be related to decreased β_1 -AR endocytosis [8]. However, there is a lack of convincing evidence on the role of limited endocytosis in the sustained activation of β_1 -AR caused by β_1 -AA and the related mechanisms. β_2 -adrenergic receptor (β_2 -AR) is another important adrenergic receptor in cardiomyocytes [9]. Our previous studies unexpectedly found that although β_1 -AA does not directly bind to β_2 -AR, the sustained increase in NRCM-beating frequency induced by β_1 -AA can be neutralized by the β_2 -AR autoantibody-mediated activation of β_2 -AR [2], indicating

¹Department of Physiology & Pathophysiology, School of Basic Medical Sciences, Capital Medical University, Beijing 100069, PR China. ²Institute of Vascular Medicine, Cardiology Department, Peking University Third Hospital, Beijing 100191, PR China. ³Department of Pathology, School of Basic Medical Sciences, Shanxi Medical University, Taiyuan 030001, PR China. ⁴Department of Physiology & Pathophysiology, Yanjing Medical College, Capital Medical University, Beijing 101300, PR China. ⁵Beijing Key Laboratory of Metabolic Disorders Related Cardiovascular Disease, Capital Medical University, Beijing 100069, PR China. ⁶These authors contributed equally: Hao Chen, Ning Cao. ✉email: sueney716@126.com; liuhr2000@ccmu.edu.cn

Received: 12 July 2021 Revised: 14 September 2021 Accepted: 19 October 2021

Published online: 08 November 2021

cross-talk between β_2 -AR and β_1 -AR signals under the action of β_1 -AA. Similar to β_1 -AR, β_2 -AR can couple to Gs protein and stimulate adenylyl cyclase (AC) [9]. The difference is that β_2 -AR can also couple to Gi protein, which mediates an opposite effect [9]. For example, upregulated β_2 -AR expression negatively regulates the overcontraction of cardiomyocytes caused by β_1 -AR through the Gi-signaling pathway in HF [10]. Utilizing a hypothesis-driven approach, we speculated that the insufficient regulatory function of the β_2 -AR/Gi pathway might be involved in β_1 -AA-induced limited β_1 -AR endocytosis and persistent signaling. Strengthening the β_2 -AR/Gi pathway may be a new rescue strategy for β_1 -AA-positive HF.

MATERIALS AND METHODS

Animals

The animals used in this study were 6- to 8-week-old Balb/c mice and 0- to 3-day-old neonatal rats. The animals were fed in the SPF animal room of Capital Medical University. At the end of the experiment, the mice were euthanized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital.

Patients

All patients gave informed consent. The detailed screening process is provided in the supplemental material. The information of patients was shown in Table S1.

Ethics statement

All animal experiments in this study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of Capital Medical University. The study complied with the journal's applicable checklists for animal ethics (AEEI-2015-097, AEEI 2016-053).

The research study in patients complied with the Declaration of Helsinki and passed the approval of the Beijing Anzhen Hospital's Ethical Review Committee.

NRCMs beating frequency and cyclic adenosine monophosphate measurements

Neonatal rats (0- to 3-day-old) were provided by the Capital Medical University animal laboratory. Neonatal rats were sacrificed by carbon dioxide inhalation. Hearts were immediately collected in iced-cold PBS. Ventricular cardiomyocytes were extracted by an enzymatic method and cultured in a cell incubator [11]. The spontaneous NRCMs contraction rate was monitored with a living cell station [2].

The cAMP content in NRCMs was measured by a cAMP [125 I] radioimmunoassay (RIA) kit (RK-509, Hungary) [12]. The detailed experimental procedure was provided in the supplemental material.

Total internal reflection fluorescence microscopy

Total internal reflection fluorescence microscopy (TIRF) was utilized to determine β_1 -AR endocytosis in HL-1 cells transfected with β_1 -AR-GFP. The myocardial cell line HL-1 was cultured in confocal dishes. A TIRF inverted microscope (Olympus, Japan) equipped with an EMCCD camera and an oil immersion objective lens ($\times 100$ magnification, NA = 1.49) was utilized. The depth of field, 120–130 nm, was selected to observe β_1 -AR fluorescence upon the plasma membrane but not the cytoplasm. Changes in cell surface fluorescence were recorded at 0, 5, 10, and 30 minutes (min) after cell administration. Recordings were made every 30 seconds (s) using MetaMorph software version 7.8.8.0. Image fluorescence intensity was analyzed using ImageJ 1.51J8 [13]. To detect the recruitment of β_1 -AR-GFP and β -arrestin-RFP, we recorded images at 5 min after drug treatment and utilized ImageJ 1.53c for colocalization analysis. Before analysis, all images were adjusted with ImageJ 1.53c to the same brightness and color. Pearson's *R* value (above threshold) was used as an indicator of the colocalization of β_1 -AR-GFP and β -arrestin-RFP [14].

Intracellular calcium ion detection

HL-1 cells were cultured at a density of $\sim 30\%$ in confocal dishes. The cells were incubated with the Ca^{2+} fluorescent probe Fluo-4 (10 μM , F14201,

Thermo Fisher, USA) for 90 min before the experiment. At the time of the experiment, the cell culture medium was changed to FluoroBrite DMEM (A1896701, Thermo Fisher, USA). Intracellular Ca^{2+} levels were measured using a turntable confocal microscope (UltraVIEW VoX, USA) [13].

Antibodies

Please refer to supplementary table S2 for detailed information.

Establishment of a mouse heart dysfunction model by injecting β_1 -AA

A mouse monoclonal β_1 -AA (synthesized from AbMax Biotechnology, China) [15] was mixed with saline before use (concentration of 0.5 mg/ml). Heart dysfunction models were established in male 6 to 8-week-old Balb/c mice randomly by intraperitoneal β_1 -AA injection (10 $\mu\text{g}/\text{kg}$, every two weeks, i.p.). In β_2 -AR inverse agonist treatment group, 32.7 $\mu\text{g}/\text{kg}$ ICI118551 was injected intraperitoneally every week for 2 months, accompanied by β_1 -AA injection every two weeks. Left ventricular function was determined in all mice by echocardiography, and morphological changes in the heart were observed by HE staining.

Statistical analysis

In this study, GraphPad Prism 8.0.2 software was used for statistical analysis and statistical graph production. The experimental results are expressed as the mean \pm SEM. The differences in dynamic changes in NRCM-beating frequency, cAMP concentration, and β_1 -AR endocytosis in the different groups were analyzed by 2-way ANOVA followed by a Bonferroni post hoc test. One-way ANOVA followed by Dunnett's test was used to compare multiple groups, and the t-test was used to compare the mean between two groups. $P < 0.05$ was considered statistically significant.

The detail of other material and methods was described in the supplementary material.

RESULTS

Similar to β_1 -AA (+)-IgG from patients, mouse-derived monoclonal β_1 -AA induces sustained activation and limited endocytosis of β_1 -AR

β_1 -AA and negative IgG used in the current study originated from mouse-derived hybridoma cells and sera from healthy Balb/c mice, respectively. We first compared the effect of mouse-derived β_1 -AA with that from HF patients. As shown in Fig. 1, patients' β_1 -AA (+)-IgG (β_1 -AA-positive total IgG, 10^{-7} M) elicited persistent activation of β_1 -AR, evidenced by the increasing NRCM-beating frequency for 1 hour, an effect partially inhibited by the β_1 -AR blocker metoprolol (Met, 10^{-5} M) and completely neutralized by the peptide corresponding to the extracellular second loop of β_1 -AR (β_1 -AR-ECLII, 10^{-5} M) (Fig. 1A, Table S3). After preincubating NRCMs with phenoxybenzamine (Phe, 10^{-5} M) for 30 min to block the α_1 -adrenergic receptor (α_1 -AR), NE (10^{-5} M) increased the NRCM-beating frequency for a much shorter time, ~ 5 min (Fig. 1A). The mouse monoclonal β_1 -AA (10^{-7} M) also continuously increased NRCM-beating frequency through β_1 -AR, whereas negative IgG had no effect (Fig. 1B, Table S4). Moreover, monoclonal β_1 -AA increased the Ca^{2+} level in HL-1 cells, which lasted until the end of the observation (300 s). This effect could be blocked by the β_1 -AR-ECLII peptide. In contrast, after NE stimulation, the intracellular Ca^{2+} increased rapidly and then decreased rapidly, maintaining for only 5 s (Fig. 1C, Table S5, Video S1, and S2). Met, Phe, and the β_1 -AR-ECLII peptide alone had no effects on either NRCM-beating or intracellular Ca^{2+} levels (Fig. S1A, B, Table S7 and S8). These results indicated that the mouse monoclonal β_1 -AA and the β_1 -AA from HF patients acted very similarly, and the former was used in subsequent experiments.

G protein-coupled receptor (GPCR) endocytosis can terminate the downstream signal over time. To investigate the potential role of endocytosis in long-term β_1 -AR activation, we transiently transfected the β_1 -AR-GFP plasmid into an HL-1 cell line (Fig. S2A, B, Fig. S14 A1, A2). TIRF was used to determine the degree of β_1 -AR endocytosis reflected by the reduced β_1 -AR-GFP

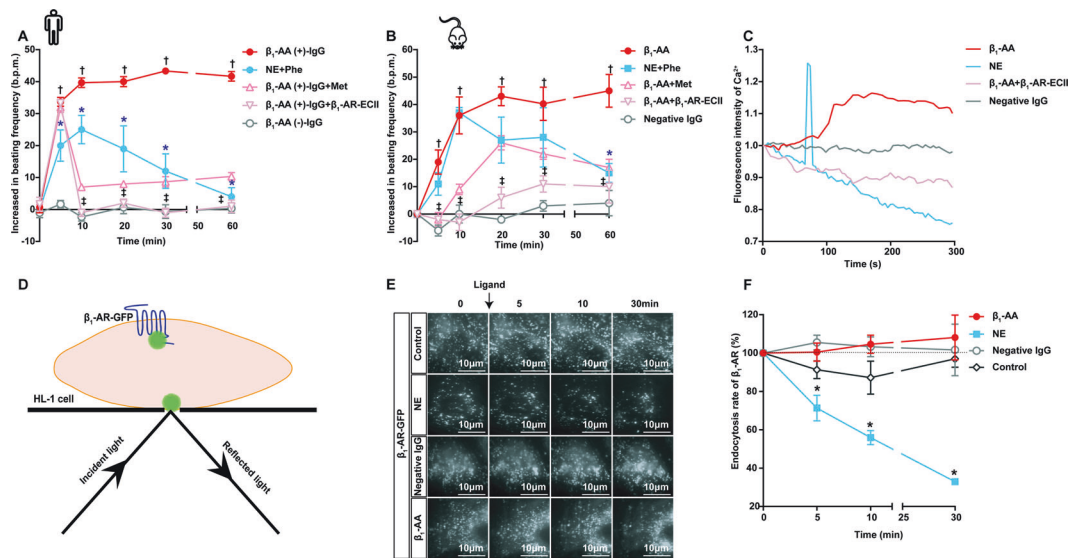


Fig. 1 Monoclonal β_1 -AA purified from mouse-derived hybridoma cells caused long-term activation of β_1 -AR but restricted β_1 -AR endocytosis. **A** and **B** NRCMs were utilized to evaluate the effects of β_1 -AA (-)-IgG (β_1 -AA-positive total IgG) isolated from HF patients and mouse-derived monoclonal β_1 -AA on β_1 -AR activation. β_1 -AA (-)-IgG (β_1 -AA-negative total IgG) from HF patients and negative IgG from healthy mice were treated as the antibody controls. NE acted as a physiological ligand control. Phe and Met were α -adrenoceptor (α -AR) and β_1 -AR blockers, respectively. The peptide-based on β_1 -AR-ECII could specifically neutralize β_1 -AA. Data were analyzed by two-way ANOVA, followed by a Bonferroni post hoc test. $n = 4-6$ /group, *P vs β_1 -AA, $^{\dagger}P$ vs β_1 -AA (-)-IgG (**A**) or negative IgG (**B**), $^{\ddagger}P$ vs β_1 -AR-ECII, $P < 0.05$. **C** HL-1 cells were incubated with Fluo-4 (10 μ M) for 90 min and then treated with different stimuli to dynamically measure the intracellular Ca^{2+} changes. $n = 3$ /group. **D-F** TIRF was used to determine the endocytosis of β_1 -AR on the membrane of HL-1 cells, reflected by the decreased percentage of fluorescence value of β_1 -AR-GFP. **D** is the schematic diagram of TIRF detection. **E** and **F** are typical pictures and statistical charts of the effects of different stimuli on β_1 -AR-GFP endocytosis, respectively. Scale bar = 10 μ m. Data were analyzed by two-way ANOVA, followed by a Bonferroni test. $n = 3$ /group. *P vs β_1 -AA, $P < 0.05$.

fluorescence intensity on the cell membrane. TIRF detection showed that the fluorescence value was reduced by 28% at 5 min after treatment with NE plus Phe and by 44% and 67% after 10 min and 30 min (Fig. 1D-F, Fig. S20), respectively, indicating marked β_1 -AR endocytosis. However, the fluorescence intensity showed no significant change in the β_1 -AA treatment group or in the plasmid transfection control and negative IgG groups (Fig. 1F, Table S6).

Weakened β_1 -AR endocytosis is an important factor in the continuous activation of β_1 -AR

To clarify the causal relationship between endocytosis and β_1 -AR activation duration, we assessed the changes in β_1 -AR-mediated signaling and the effect after decreasing or increasing β_1 -AR endocytosis. As shown in Fig. 2A, after blocking α_1 -AR with Phe (10^{-5} M), NE (10^{-5} M) induced a short-term increase in NRCMs beating and intracellular cyclic adenosine monophosphate (cAMP) levels. In contrast, after preincubation with the GPCR endocytosis inhibitor Dynasore (10^{-4} M) for 30 min, NE increased the beating frequency of NRCMs for 60 min and the cAMP level for 30 min, similar to that caused by β_1 -AA (Fig. 2A, B, Tables S9 and S10). The result of Dynasore treatment alone was negative for the NRCMs beating (Fig. 2A).

β -arrestin1/2 are key scaffold proteins that initiate β_1 -AR endocytosis. Bioluminescence resonance energy transfer detection showed that it was difficult for β_1 -AR to recruit β -arrestin1 or β -arrestin2 upon β_1 -AA stimulation (Fig. S3 A-G, Fig. S14 B1-D2, Tables S15 and S16). Therefore, we overexpressed β -arrestin1-RFP (β -arr1 OE) or β -arrestin2-RFP (β -arr2 OE) in HL-1 cells transiently transfected with β_1 -AR-GFP to improve β_1 -AR endocytosis (Fig. S4 A, B, Fig. S15 A1-C2). As determined by TIRF, either β -arrestin1-RFP or β -arrestin2-RFP overexpression markedly enhanced β_1 -AA-induced β_1 -AR endocytosis. At 30 min after β_1 -AA administration (10^{-7} M), β_1 -AR-GFP fluorescence decreased by 42% in the β -arr1 OE group (Fig. 2C, D, Fig. S21 A, Table S11) and by 25% in the

β -arr2 OE group (Fig. 2E, F, Fig. S21 B, Table S12). Moreover, both β -arrestin1-RFP and β -arrestin2-RFP overexpression significantly attenuated the sustained elevated levels of Ca^{2+} in HL-1 cells caused by β_1 -AA (Fig. 2G, H, Tables S13 and S14), suggesting that the prevention of enhanced β_1 -AR endocytosis led to the continuous activation of β_1 -AR. We concluded that β_1 -AA-initiated sustained activation of β_1 -AR could be at least partially attributed to insufficient β_1 -AR endocytosis.

Insufficient regulation of β_2 -AR is involved in the limited endocytosis and sustained activation of β_1 -AR caused by β_1 -AA

β_2 -AR regulates β_1 -AR signaling by interacting with it. To assess the possible role of β_2 -AR in β_1 -AA-restricted endocytosis, we first observed the effect of β_1 -AA on the binding of β_1 -AR to β_2 -AR. Purified human β_1 -AR and β_2 -AR proteins were isolated and used in the following experiment. The 300 nM purified β_2 -AR protein was coupled on the surface of a chip, and different concentrations (58.75 nM - 1880 nM) of purified β_1 -AR protein were added. Surface plasmon resonance (SPR) detection showed that purified β_1 -AR and β_2 -AR proteins could bind directly in a dose-dependent manner; the KD value was 2.147 μ M (Fig. 3A, Table S17). β_1 -AA reduced the binding of β_1 -AR to β_2 -AR (Fig. 3B, Table S18) with an IC₅₀ of 1.16 ± 0.7 μ M (Fig. 3C, Table S19). Moreover, β_1 -AA did not bind directly to β_2 -AR. These results suggested that β_1 -AA might attenuate the regulatory effect of β_2 -AR on β_1 -AR.

To investigate the influence of the insufficient regulatory function of β_2 -AR on β_1 -AR endocytosis, endogenous β_2 -AR expression in HL-1 cells was inhibited by transfection with siRNA β_2 -AR (Fig. S5A, B, Fig. S15 D1, D2). As observed with TIRF, after stimulation with NE (10^{-5} M) for 5 min, 10 min, and 30 min, the fluorescence intensities of β_1 -AR-GFP on the cell membrane were reduced by approximately 1%, 5%, and 11%, respectively, in the siRNA β_2 -AR group, which was markedly lower than that of the siRNA control group (reduced by 6%, 27%, and 29%). This

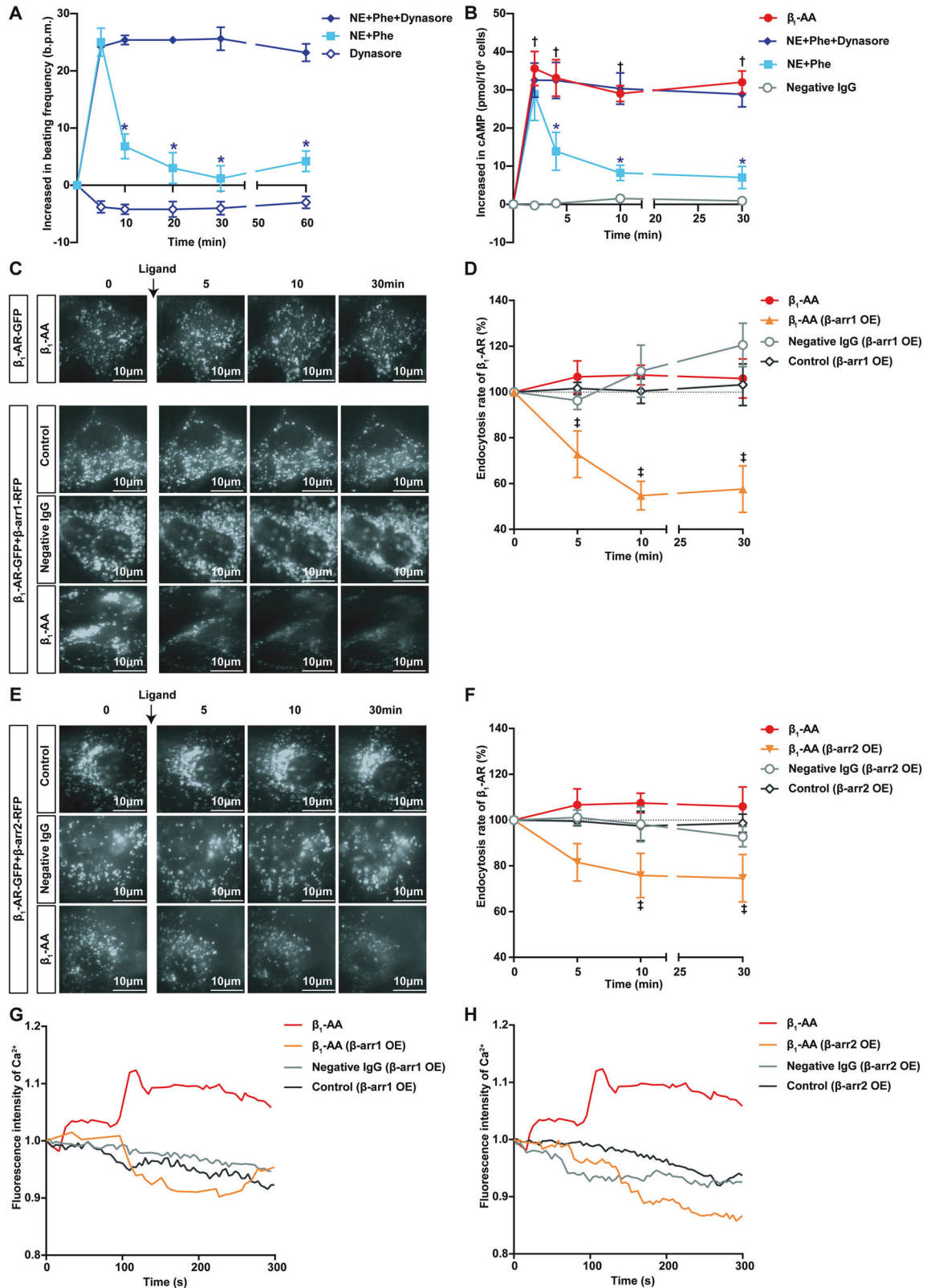


Fig. 2 Inhibiting/promoting β_1 -AR endocytosis enhanced/weakened the sustained activation of β_1 -AR. **A** The effect of the receptor endocytosis inhibitor Dynasore on the NE-induced increase in NRCMs beating frequency. $n = 5$ /group, $*P$ vs NE + Phe, $P < 0.05$. **B** An radioimmunoassay kit was used to detect the cAMP concentration in NRCMs after treatment with different stimuli. $n = 4$ /group. $*P$ vs NE + Phe, $^\dagger\beta_1$ -AA vs negative IgG, $P < 0.05$. **C–F** After overexpressing β -arrestin1 (β -arr1 OE) or β -arrestin2 (β -arr2 OE), the effect of β_1 -AA on β_1 -AR-GFP endocytosis was measured via TIRF. HL-1 cells were transiently transfected with β_1 -AR-GFP, combined with or without transfection with β -arrestin1-RFP (**C** and **D**) or β -arrestin2-RFP (**E** and **F**). **C** and **E** are typical pictures, and **D** and **F** are the corresponding statistical charts. Scale bar = 10 μ m. $n = 3$ –4/group. $^\dagger P$ vs β_1 -AA, $P < 0.05$. Data in **A–F** were analyzed by two-way ANOVA with a Bonferroni post hoc test. **G** and **H** The impacts of β_1 -AA on intracellular Ca²⁺ in HL-1 cells with or without β -arr1 OE or β -arr2 OE. Fluo-4 (10 μ M) was used as a Ca²⁺ indicator. $n = 3$ –4/group.

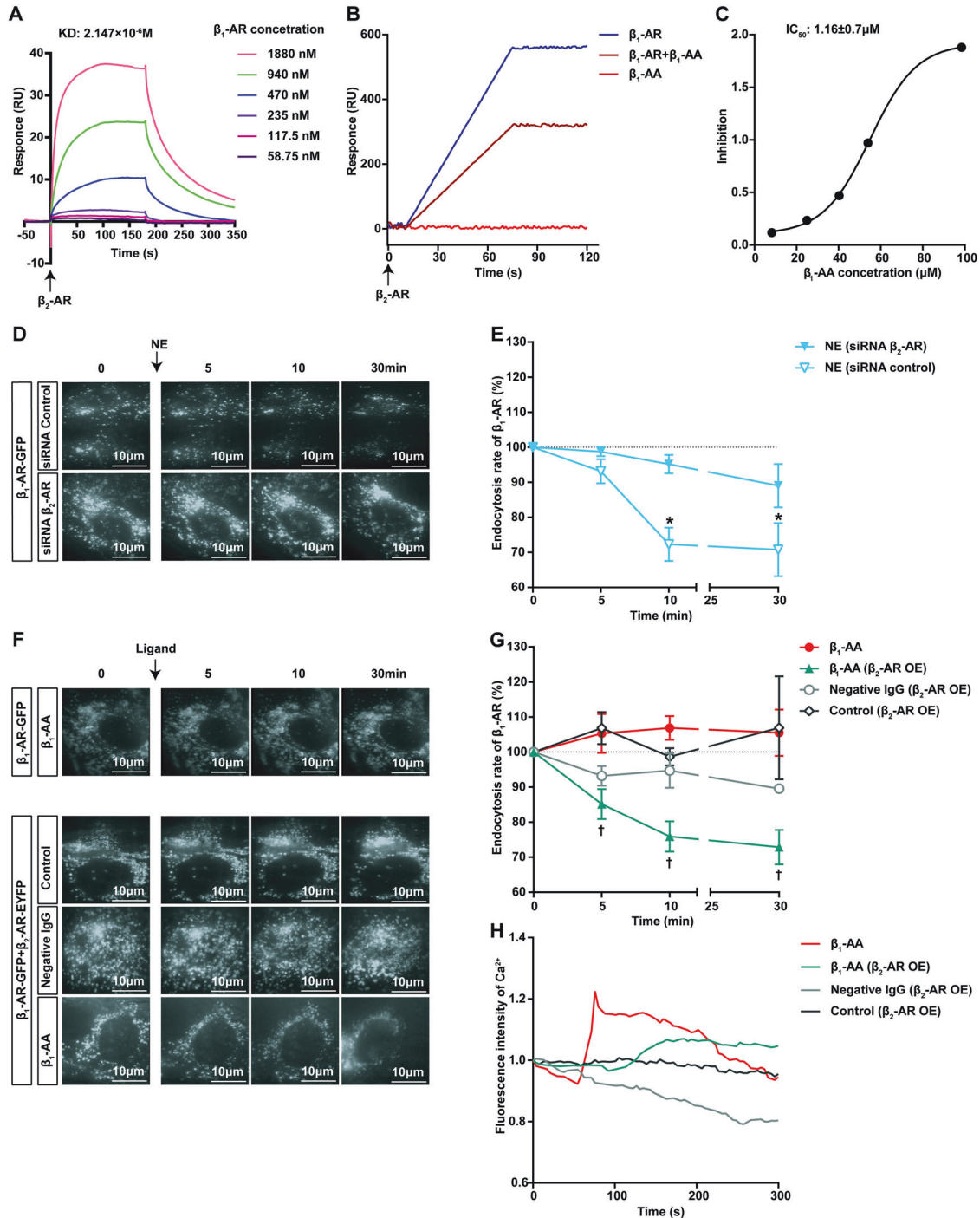


Fig. 3 The ability of β_2 -AR to promote β_1 -AR endocytosis was reduced upon β_1 -AA stimulation. **A** The binding ability of purified β_1 -AR to β_2 -AR proteins was detected by SPR. The β_2 -AR protein was first coupled to the chip, and then different concentrations of β_1 -AR proteins were added. **B** SPR was used to observe the binding of β_1 -AA and β_2 -AR protein and the influence of β_1 -AA on the binding of β_1 -AR and β_2 -AR. **C** The IC_{50} of β_1 -AA on binding β_1 -AR and β_2 -AR. **D**, **E** TIRF was used to assess NE-induced β_1 -AR endocytosis when HL-1 cells were transfected with siRNA β_2 -AR or an siRNA control. Representative images and quantification of changes in β_1 -AR-GFP fluorescence density are shown in **D** and **E**, respectively. Scale bar = 10 μ m. $n = 3$ /group. * P vs NE, $P < 0.05$. **F**, **G**: Changes in β_1 -AR endocytosis caused by β_1 -AA in HL-1 cells with and without the overexpression of β_2 -AR-EYFP (β_2 -AR OE). Scale bar = 10 μ m. $n = 3$ /group. † P vs β_1 -AA, $P < 0.05$. Data in **D–G** were analyzed by two-way ANOVA with the Bonferroni test. **H** The effects of β_1 -AA on intracellular Ca^{2+} in HL-1 cells with and without the overexpression of β_2 -AR-EYFP (β_2 -AR OE). Fluo-4 (10 μ M) acted as a Ca^{2+} indicator. $n = 3$ /group.

indicated that the lack of β_2 -AR restricted β_1 -AR endocytosis (Fig. 3D, E, Fig. S22 A, Table S20).

To further clarify the influence of β_2 -AR on β_1 -AA-induced limited β_1 -AR endocytosis, β_2 -AR-EYFP was overexpressed in HL-1 cells transiently transfected with β_1 -AR-GFP (Fig. S6A, B, Fig. S16

A1, A2). As detected by TIRF, β_1 -AA (10^{-7} M) administration decreased the fluorescence intensities of β_1 -AR-GFP by 14%, 24%, and 27% at 5 min, 10 min and 30 min after β_2 -AR overexpression, respectively. These results indicated the promoting effect of the increased β_2 -AR expression on β_1 -AA-induced β_1 -AR endocytosis

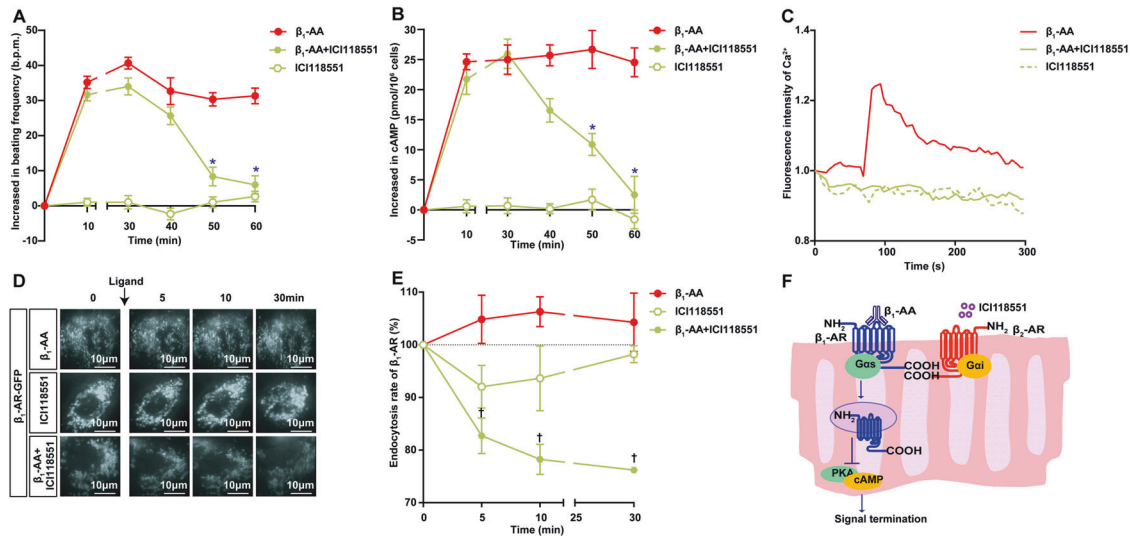


Fig. 4 By activating β_2 -AR/Gi, ICI118551 promoted β_1 -AA-induced β_1 -AR endocytosis and prevented continuous β_1 -AR activation. **A, B** The impact of ICI118551 on β_1 -AA-induced increased beating frequency and cAMP level of NRCMs, respectively. ICI118551 was added 10 min after administration of β_1 -AA. $n = 3$ –6/group. * P vs β_1 -AA + ICI118551, $P < 0.05$ (two-way ANOVA with Bonferroni test). **C** The reversal effect of ICI118551 on the persistent rise in intracellular Ca^{2+} caused by β_1 -AA in HL-1 cells. HL-1 cells were pretreated with Fluo-4 for 90 min, ICI118551 was added, and then β_1 -AA was added after 5 min. $n = 3$ /group. **D** and **E** The reversal effect of ICI118551 on the limited endocytosis of β_1 -AR caused by β_1 -AA. Scale bar = 10 μm . $n = 3$ –6/group. † P vs β_1 -AA, $P < 0.05$ (two-way ANOVA with Bonferroni test). **F** illustrates that β_2 -AR/Gi-biased activation by ICI118551 could reverse the limited endocytosis and sustained activation of β_1 -AR induced by β_1 -AA.

(Fig. 3F, G, Fig. S22 B, Table S21). We further observed the impact of β_2 -AR on the sustained activation of β_1 -AR signaling. The β_2 -AR-EYFP plasmid was transfected into HL-1 cells, and a Fluo-4 fluorescence probe was used to identify intracellular Ca^{2+} changes. As shown in Fig. 3H (Table S22), compared with the nonoverexpression group, β_2 -AR-EYFP overexpression significantly reduced the amplitude increase in Ca^{2+} induced by β_1 -AA and delayed the time of increase in Ca^{2+} . Transfection of β_2 -AR-EYFP alone or the addition of negative IgG had no effect on β_1 -AR endocytosis or Ca^{2+} levels in HL-1 cells. The above results suggested that a lack of β_2 -AR regulation might be responsible for weakened endocytosis and the long-term activation of β_1 -AR caused by β_1 -AA.

Biased activation of β_2 -AR/Gi promotes β_1 -AR endocytosis and then inhibits the persistent activation of β_1 -AR elicited by β_1 -AA

Accumulating reports have demonstrated that β_2 -AR negatively regulates β_1 -AR by coupling with Gi. We demonstrated that as a specific β_2 -AR antagonist, ICI118551 (10^{-5} M) also acted as a biased agonist of the β_2 -AR/Gi-signaling pathway (Fig. S7A and B, Fig. S16 B1–B3). This effect could be inhibited by the Gi inhibitor pertussis toxin (PTX, 1.5 $\mu\text{g}/\text{ml}$, pretreatment for 13 h) (Figure S7C and D, Figure S17 A1 and A2). The cultured NRCMs were treated with β_1 -AA (10^{-7} M) with or without ICI118551 (10^{-5} M). As shown in Fig. 4, the β_1 -AA-induced increased beating frequency and the intracellular cAMP content of NRCMs were significantly relieved by ICI118551 supplementation, but ICI118551 alone had no effects on these parameters (Fig. 4A, B, Table S23 and S24). Furthermore, the continuous increase in intracellular Ca^{2+} induced by β_1 -AA disappeared after preincubation with ICI118551 in HL-1 cells (Fig. 4C, Table S25). Then, HL-1 cells transfected with β_1 -AR-GFP were used to detect β_1 -AR endocytosis via TIRF. After HL-1 cells were preincubated with ICI118551 for 5 min and subsequently stimulated with β_1 -AA, β_1 -AR-GFP fluorescence was reduced by 17%, 21%, and 24% at 5 min, 10 min, and 30 min, respectively, indicating marked β_1 -AR endocytosis (Fig. 4D, E, Fig. S22 C, Table S26). However, the above effects related to ICI118551 supplementation disappeared after interference with the expression of endogenous β_2 -AR (Fig. 5A–C,

Table S27, Fig. S23A, Table S28) or with PTX (1.5 $\mu\text{g}/\text{ml}$) treatment (Fig. 5D–F, Table S29, Fig. S23B, Table S30) in HL-1 cells. We further treated HL-1 cells overexpressing β_2 -AR with PTX. The results showed that after PTX inhibited Gi, the effects of β_2 -AR overexpression on promoting β_1 -AR endocytosis and inhibiting the intracellular Ca^{2+} increase caused by β_1 -AA disappeared (Fig. 5G–I, Table S31, Fig. S23C, Table S32). To identify the role of β_2 -AR/Gi-signaling in NE-induced endocytosis of β_1 -AR, we tested the β_1 -AR endocytosis in HL-1 cells overexpressed Gi by TIRF. The results showed that overexpression of Gi did not enhance the endocytosis of β_1 -AR upon NE stimulation (Fig. S8A, B, Fig. S24 A, Table S33). In summary, activating the β_2 -AR/Gi-signaling pathway reversed β_1 -AA-induced weakened β_1 -AR endocytosis, leading to the termination of β_1 -AR signaling over time (Fig. 4F).

β_2 -AR/Gi promotes β_1 -AR endocytosis by activating GRK2

β -arrestin1/2 initiates GPCR endocytosis after binding to receptors phosphorylated by intracellular protein kinases. G protein-coupled receptor kinase 2 (GRK2) and protein kinase A (PKA) are two predominant kinases that promote β_1 -AR phosphorylation in cardiomyocytes. To explore the mechanism of promoting β_1 -AR endocytosis after the biased activation of β_2 -AR/Gi, we tested the activities of GRK2 and PKA in HL-1 cells. As shown in Fig. 6, both β_1 -AA (10^{-7} M) and ICI118551 (10^{-5} M) costimulation and ICI118551 single stimulation increased GRK2 phosphorylation (Fig. 6A, Fig. S17 B1, B2), whereas negative IgG (10^{-7} M) stimulation showed no significant effect (Fig. 6A). However, neither β_1 -AA or ICI118551 alone nor their combination affected PKA phosphorylation (Fig. 6B, Fig. S18 A1, A2). These results indicated that GRK2 activation might promote the recruitment of β -arrestin and β_1 -AR, subsequently triggering β_1 -AR endocytosis. Therefore, we detected the recruitment of β_1 -AR-GFP and β -arrestin1/2-RFP on the surface of the HL-1 cell membrane by TIRF. The results showed that owing to the activity of ICI118551, β_1 -AA stimulation increased the recruitment of β_1 -AR and β -arrestin. During β_1 -AA stimulation, the Pearson coefficients (Pearson's R values) that measured the colocalization of β_1 -AR with β -arrestin1 and β -arrestin2 were 0.160 ± 0.024 and 0.330 ± 0.083 as the mean \pm SEM, respectively, whereas in the ICI118551 plus β_1 -AA

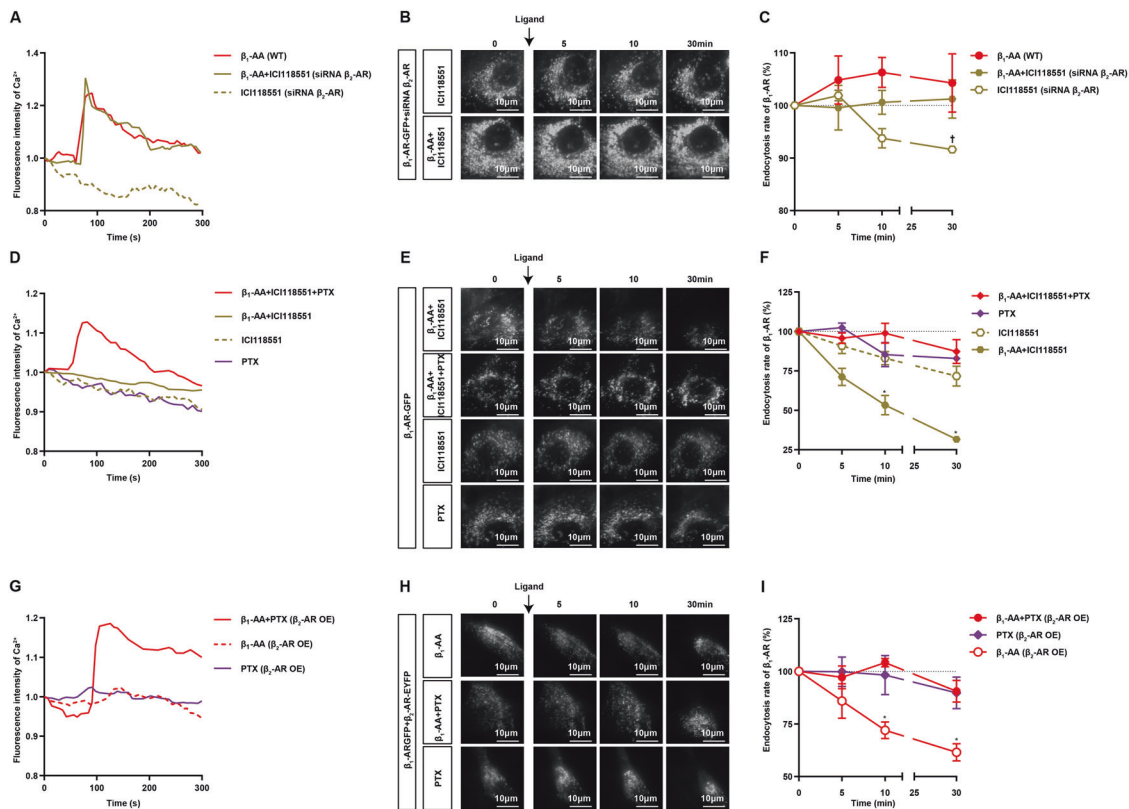


Fig. 5 siRNA β_2 -AR or PTX blocked the endocytosis-promoting effect of β_2 -AR/Gi-signaling pathway for β_1 -AR. **A** The effect of ICI118551 on β_1 -AA-induced Ca^{2+} change in HL-1 cells after interfering with β_2 -AR (siRNA β_2 -AR). $n = 3$ /group. Because the experiments in Fig. 4C and Fig. 5A were done at the same time, the same β_1 -AA (WT) group in Fig. 4C were re-used as control here. **B** and **C** The effect of ICI118551 on β_1 -AA restricting β_1 -AR endocytosis in HL-1 cells which were transfected with siRNA β_2 -AR. Scale bar = 10 μm . $n = 3$ –6/group. **C** The β_1 -AA (WT) group represented that β_1 -AA stimulated β_1 -AR overexpressed HL-1 cells. Because the experiments in Fig. 4E and Fig. 5C were done at the same time, the same β_1 -AA (WT) group in Fig. 4E were re-used as control here. **D** The effect of ICI118551 on β_1 -AA-induced Ca^{2+} change in HL-1 cells pretreated with PTX (1.5 $\mu\text{g}/\text{ml}$, 13 hours). $n = 3$ /group. **E**, **F** The effect of ICI118551 on β_1 -AA restricting β_1 -AR endocytosis in HL-1 cells, which were pretreated with PTX. Scale bar = 10 μm . $n = 3$ /group. **G** The effects of β_1 -AA on intracellular Ca^{2+} when the HL-1 cells overexpressed β_2 -AR-EYFP (β_2 -AR OE) and pretreated with PTX. $n = 3$ /group. **H**, **I**, The change of β_1 -AR endocytosis caused by β_1 -AA after the HL-1 cells overexpressing β_2 -AR-EYFP (β_2 -AR OE) and pretreated with PTX. Scale bar = 10 μm . $n = 3$ /group. * P vs β_1 -AA + PTX, $P < 0.05$. Data of **C**, **F**, and **I** were analyzed by two-way ANOVA with Bonferroni test. **B**, **E** and **H** were the representative images. **C**, **F** and **I** were the quantitative graphs, respectively.

stimulation group, the Pearson coefficient was increased to 0.687 ± 0.110 (β -arrestin1) and 0.753 ± 0.068 (β -arrestin2) as the mean \pm SEM (Fig. 6C, D). In addition, after interference with GRK2 expression in HL-1 cells, the reversal effect of ICI118551 on β_1 -AA-restricted β_1 -AR-GFP endocytosis disappeared (Fig. S9 A–D, Fig. S18 B1, B2, Fig. S24 B, Table S34). These results suggested that after the biased activation of the β_2 -AR/Gi-signaling pathway, ICI118551 could activate GRK2, thereby increasing the recruitment of β_1 -AR and β -arrestin, promoting receptor endocytosis and terminating the continuous activation of β_1 -AR under the action of β_1 -AA.

Biased β_2 -AR/Gi activation ameliorates cardiac structural damage and dysfunction in β_1 -AA-positive mice

To verify that activating the β_2 -AR/Gi-signaling pathway protected β_1 -AA-induced cardiac injury in vivo, wild-type Balb/c male mice (6–8 weeks old) without β_1 -AA were divided into four groups: saline (3.7 $\mu\text{g}/\text{kg}$, once a week, i.p.), β_1 -AA (10 $\mu\text{g}/\text{kg}$, every 2 weeks, i.p.), and therapy groups β_1 -AA combined with ICI118551 and ICI118551 alone (32.7 $\mu\text{g}/\text{kg}$, once a week, i.p.) (Fig. S10A). Eight weeks later, the β_1 -AA levels detected by enzyme-linked immunosorbent assay were significantly higher in the β_1 -AA and therapy groups than in the saline control group (Fig. S11, Table S35). M-mode echocardiography detection showed that the left ventricular ejection fraction (LVEF) decreased by 12.6% after 8 weeks of β_1 -AA treatment, and the left ventricular

end-diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD) increased by 15% and 42%, respectively. However, LVEF, LVESD, or LVEDD in the saline control group and the ICI118551 treatment group showed no significant change compared with preadministration measures (Fig. S10B, C, Table S36). Hematoxylin–eosin (HE) staining showed broken myocardial fibers in the β_1 -AA group, which were significantly alleviated after treatment with ICI118551 (Figure S10D). These results suggested that β_1 -AA caused cardiac dysfunction and structural damage in mice, and ICI118551, a biased agonist of β_2 -AR/Gi, had a certain therapeutic effect on cardiac injury caused by β_1 -AA.

DISCUSSION

Previous studies have shown that β_1 -AA promotes the occurrence and development of HF by continuously activating β_1 -AR. However, the specific mechanism is not fully understood. Receptor endocytosis is one of the important mechanisms for terminating signal transduction. The main study findings were that β_1 -AA continuously activated β_1 -AR by restricting β_1 -AR endocytosis and that biased activation of the β_2 -AR/Gi pathway could activate GRK2, promote β_1 -AR endocytosis restricted by β_1 -AA in vitro and alleviate the morphologic and functional damage in the mouse heart in vivo Fig. 7.

β_1 -AA is produced against β_1 -AR-ECII [16]. In 1987, Wallukat et al. [17] found this autoantibody in dilated cardiomyopathy for

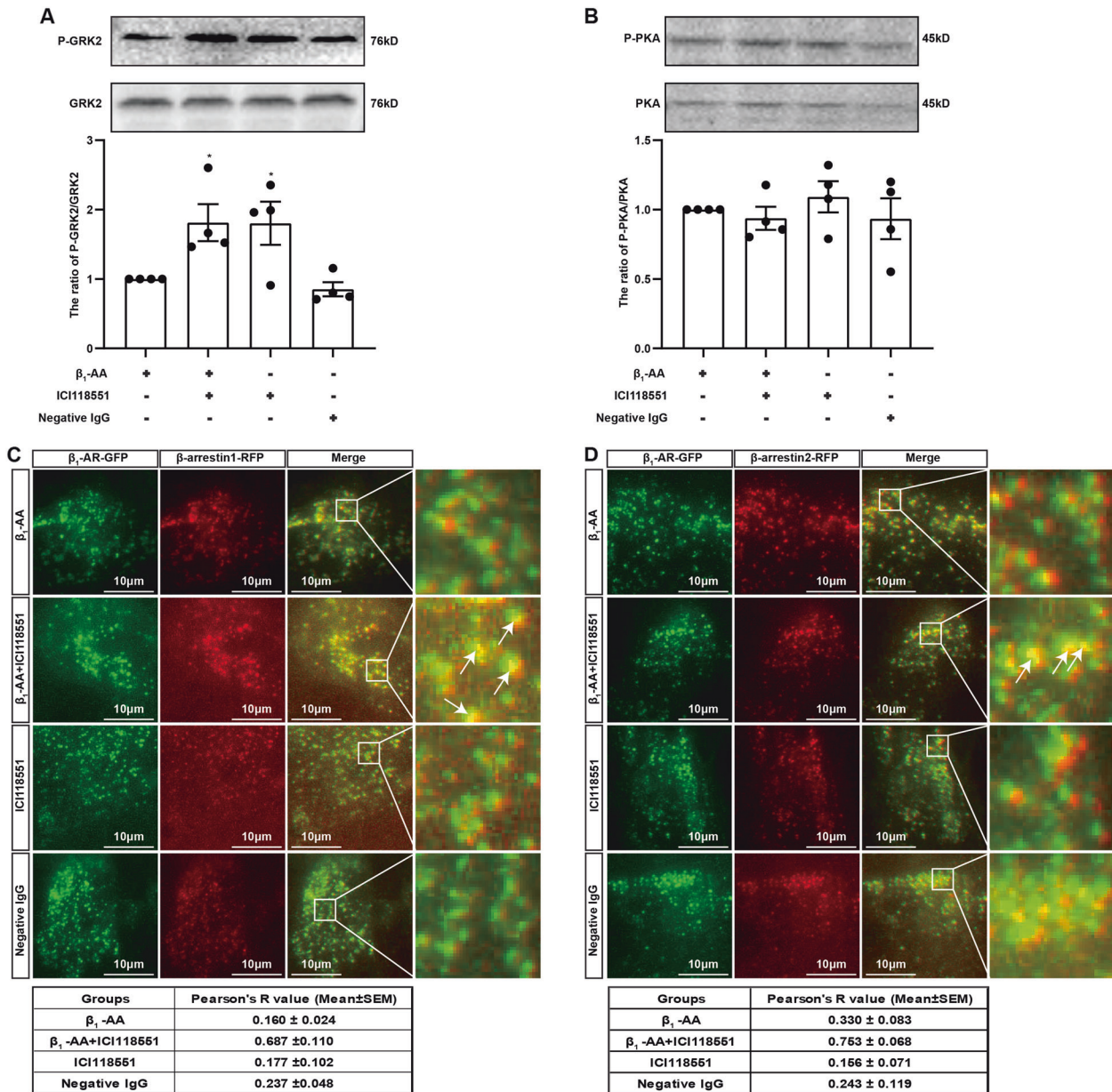


Fig. 6 Activation of GRK2 enhanced the recruitment of β_1 -AR and β -arrestin stimulated by β_1 -AA. **A** The phosphorylation of GRK2 was detected after HL-1 cells were stimulated with ICI118551 alone or in combination with β_1 -AA for 5 min. $n = 4$ /group. * P vs β_1 -AA, $P < 0.05$ (one-way ANOVA with Dunnett test). **B** The detection of PKA phosphorylation. $n = 4$ /group. **C, D** TIRF was used to detect the recruitment of β_1 -AR and β -arrestin1/2 (above pictures), and Pearson's coefficient (below tables) was calculated to reflect the colocalization between the two proteins. White arrows indicate the colocalization of β_1 -AR with β -arrestin1 (**C**) or β -arrestin2 (**D**). $n = 3$ /group.

the first time. Subsequently, high positivity rates of β_1 -AA have been detected in a variety of cardiovascular diseases, especially in HF [3, 18, 19]. Therefore, the relationship between β_1 -AA and HF has gradually attracted researchers' attention. β_1 -AA elicits various pathological effects, such as triggering cardiomyocyte apoptosis [20], reducing the effective refractory period of the atrium and inducing atrial fibrillation [18], and inducing calcium overload [21], to cause cardiac damage. It is worth noting that continuously activating β_1 -AR is a common mechanism underlying these effects. Elucidating the related mechanism is conducive to drug development and clinical treatment. As an agonistic ligand control, NE was selected. It is an endogenous α_1/β_1 -AR selective agonist belonging to the catecholamine category.

As a GPCR, the activation of β_1 -AR is a complicated process. The agonist ligand binds to β_1 -AR and induces its structural change,

which in turn exposes the Gs protein-binding sites. The Gs protein undergoes the GTP cycle to convert to GTP-Gs and binds to β_1 -AR. Then, GTP-Gs activates AC and mediates signal transduction through cAMP-PKA [9, 22, 23]. To avoid the cytotoxicity caused by sustained GPCR activation, activated β_1 -AR needs to be desensitized and endocytosed over time. GPCR desensitization is divided into transient desensitization and long-term desensitization. Transient desensitization proceeds through the interaction of β -arrestin and GPCRs over a short period of time (minutes). Long-term desensitization proceeds through GPCR degradation in the lysosome over a long period of time (hours or days), during which the mRNA level of GPCR is reduced [24]. In this study, all experiments were completed in a short time; thus, receptor desensitization involved transient desensitization. The process is roughly as follows: GRK phosphorylates activated β_1 -AR, and then,

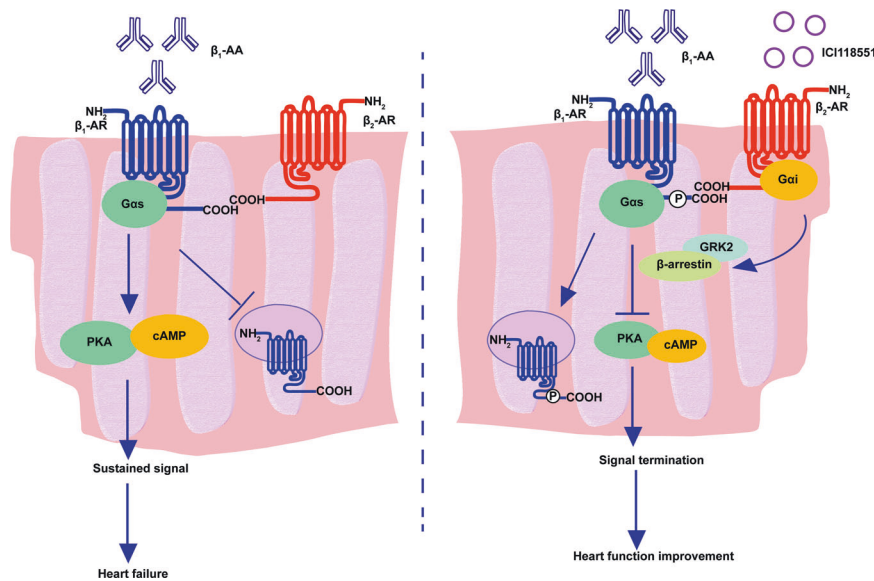


Fig. 7 Work model. Under the action of β_1 -AA, the sustained activation of β_1 -AR is due to the decrease of endocytosis. Biased activation of β_2 -AR/Gi/GRK2 signaling pathway can promote the endocytosis of β_1 -AR that restricted by β_1 -AA, leading to the termination of β_1 -AA-induced continuous activation of β_1 -AR and improvement of cardiac structure and function.

β -arrestin1/2 in the cell recognizes and binds to phosphorylated β_1 -AR, blocking the binding between Gs protein and β_1 -AR and promoting the endocytosis of β_1 -AR [24, 25]. β -arrestin is a key protein in receptor endocytosis. Upon receptor activation, β -arrestin1/2 promotes receptor desensitization and endocytosis by binding to phosphorylated receptors. Overexpression of β -arrestin has been proved to be an effective way to enhance their recruitment to the receptor and promote receptor endocytosis [26]. In the present study, overexpressing β -arrestin promoted β_1 -AA endocytosis restricted by β_1 -AA in HL-1 cells.

To detect the β_1 -AR activation and endocytosis, NRCMs and HL-1 cells were used. NRCMs are rhythmic and their contraction profile just meets the needs of beating frequency experiments [27]. However, the rhythmic contraction will greatly disturb the TIRF observation and intracellular Ca^{2+} determination. Thus, a mouse myocardial HL-1 cell line, with good adhesion, was chosen as well. It has been studied that HL-1 cells can be used as a tool cell for β_1 -AR and β_2 -AR signaling pathways [28–30]. Here we confirmed that the proteins of β_1 -AR, β_2 -AR, β -arrestin1, and β -arrestin2 were expressed in HL-1 cells (Fig. S12A, Fig. S18 C1–D4). Furthermore, when given NE stimulation, the NRCMs need to be preincubated with Phe to block α_1 -AR, whereas HL-1 cells do not, because NE has a higher affinity for α_1 -AR than β_1 -AR in cardiomyocytes, whereas HL-1 cells do not express α_1 -AR (Fig. S12 B, Fig. S18 E1, E2).

In HL-1 cells, the change in cytosol Ca^{2+} upon NE stimulation was transient. However, a long time of elevated cytosol Ca^{2+} was observed under the β_1 -AA condition. The sustained high concentration of Ca^{2+} in the cytosol of cardiomyocyte was a fascinating phenomenon, which we cannot fully explain yet. We speculated that it may be owing to the change of activity of Ca^{2+} channels or calcium ATPase in sarcoplasmic reticulum when stimulated by β_1 -AA.

β_2 -AR is also important in cardiomyocytes because it can bind to either Gs or Gi protein [9, 31]. By coupling to the Gi protein, β_2 -AR inhibits AC activation to block β_1 -AR signaling and exerts cardioprotective effects [31]. In addition, β_2 -AR can target the C-terminus of β_1 -AR in a phosphodiesterase 4-dependent manner to limit signal diffusion to avoid cytotoxicity after β_1 -AR activation [32]. However, some studies found that the formation of the β_1 - β_2 -AR heterodimer strengthens the Gs signaling pathway in normal adult mouse cardiomyocytes and restricts β_2 -AR endocytosis [33, 34].

These reports point out the complexity of the relationship between β_1 -AR and β_2 -AR in different situations. Here, we verified that β_2 -AR could promote β_1 -AR endocytosis upon stimulation with either β_1 -AA or NE.

ICI118551 is used as a specific antagonist of β_2 -AR [35]. However, our study and other studies have found that ICI118551 preferentially activates β_2 -AR/Gi [10, 36]. ICI118551 is reported to have a protective effect on the cardiovascular system after activating the β_2 -AR/Gi pathway by reducing the contraction of myocytes in failing hearts [10] and reducing pulmonary artery tension [36]. We demonstrated that by activating the β_2 -AR/Gi pathway, ICI118551 could increase GRK2 activity, enhance the recruitment of β -arrestin1/2 to β_1 -AR, and improve β_1 -AA-induced β_1 -AR endocytosis. GRK2 is the predominant GRK and the primary regulator of β_1 -AR and β_2 -AR desensitization in the heart. GRK2 can phosphorylate activated β -adrenergic receptors, which are recognized by β -arrestin and initiate receptor endocytosis [37]. Contrary to expectation, we found that β_1 -AA upregulated β_1 -AR phosphorylation, whereas ICI118551 supplementation reduced it (Fig. S13A, B, Fig. S19 A1, A2). As reported, differences in receptor conformation stabilized by various ligands may expose different phosphorylation sites, which may elicit distinctive β -arrestin activation states and receptor endocytosis [38, 39]. We speculate that although β_1 -AR phosphorylation was decreased in the ICI118551 treatment group, the exposed phosphorylation sites were prone to coupling with β -arrestin. Of course, more work is needed to test this hypothesis.

Although the close relationship between β_1 -AA and HF is known, how to effectively block β_1 -AA remains difficult. β_1 -AR blockers are among the most commonly used medications in treating HF and the most convenient method because of their oral administration. However, β_1 -AR blockers cannot sufficiently block the continuous activation of β_1 -AR induced by β_1 -AA [40]. This problem was evident in this study, which may be owing to the differences in the binding sites of β_1 -AA and β_1 -AR blockers. Metoprolol recognizes the β_1 -AR ligand-binding pocket [23, 41, 42], whereas β_1 -AA binds to the second extracellular loop of β_1 -AR [16]. Animal studies have demonstrated that the combination of the β_2 -AR agonist fenoterol and β_1 -AR blocker metoprolol can improve the heart function of rats with HF. In addition, preferentially activating β_2 -AR/Gi can antagonize the apoptosis-promoting effect of β_1 -AR on cardiomyocytes through the PI3K-Akt pathway [43]. Here, we mainly found that activating

β_2 -AR/Gi could exert a cardioprotective effect by promoting β_1 -AR endocytosis and terminating its signal in a timely manner. Therefore, biased activation of the β_2 -AR/Gi-signaling pathway may be a new strategy for the treatment of HF patients with β_1 -AA. However, the effectiveness of this program in clinical treatment needs to be further confirmed.

DATA AVAILABILITY

The original data sets are available from the corresponding author upon request.

REFERENCES

- Wang J, Gareri C, Rockman HA. G-protein-coupled receptors in heart disease. *Circ Res.* 2018;123:716–35.
- Cao N, Chen H, Bai Y, Yang X, Xu W, Hao W, et al. β_2 -adrenergic receptor autoantibodies alleviated myocardial damage induced by β_1 -adrenergic receptor autoantibodies in heart failure. *Cardiovasc Res.* 2018;114:1487–98.
- Nagatomo Y, Li D, Kirsop J, Borowski A, Thakur A, Tang WH. Autoantibodies specifically against β_1 adrenergic receptors and adverse clinical outcome in patients with chronic systolic heart failure in the beta-blocker era: the importance of immunoglobulin G3 subclass. *J Card Fail.* 2016;22:417–22.
- Jahns R, Boivin V, Hein L, Triebel S, Angermann CE, Ertl G, et al. Direct evidence for a β_1 -adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. *J Clin Invest.* 2004;113:1419–29.
- van der Meer P, Gaggini HK, Dec GW. ACC/AHA versus ESC guidelines on heart failure: JACC guideline comparison. *J Am Coll Cardiol.* 2019;73:2756–68.
- Matsui S, Persson M, Fu HM, Hayase M, Katsuda S, Teraoka K, et al. Protective effect of bisoprolol on β_1 adrenoceptor peptide-induced autoimmune myocardial damage in rabbits. *Herz.* 2000;25:267–70.
- Nagatomo Y, McNamara DM, Alexis JD, Cooper LT, Dec GW, Pauly DF, et al. Myocardial recovery in patients with systolic heart failure and autoantibodies against β_1 -adrenergic receptors. *J Am Coll Cardiol.* 2017;69:698–77.
- Bornholz B, Weidtkamp-Peters S, Schmitmeier S, Seidel CA, Herda LR, Felix SB, et al. Impact of human autoantibodies on β_1 -adrenergic receptor conformation, activity, and internalization. *Cardiovasc Res.* 2013;97:472–80.
- Ali DC, Naveed M, Gordon A, Majeed F, Saeed M, Ogbuke MI, et al. β_1 -Adrenergic receptor, an essential target in cardiovascular diseases. *Heart Fail Rev.* 2020;25:343–54.
- Gong H, Sun H, Koch WJ, Rau T, Eschenhagen T, Ravens U, et al. Specific β_2 AR blocker ICI 118,551 actively decreases contraction through a G(i)-coupled form of the β_2 AR in myocytes from failing human heart. *Circulation.* 2002;105:2497–503.
- Shi HH, Wang ZQ, Zhang S. MIR-208a participates with sevoflurane post-conditioning in protecting neonatal rat cardiomyocytes with simulated ischemia-reperfusion injury via PI3K/AKT signaling pathway. *Eur Rev Med Pharm Sci.* 2020;24:943–55.
- Zhou Y, Ji H, Lin BQ, Jiang Y, Li P. The effects of five alkaloids from *Bulbus Fritillariae* on the concentration of cAMP in HEK cells transfected with muscarinic M(2) receptor plasmid. *Am J Chin Med.* 2006;34:901–10.
- Bian J, Lei J, Yin X, Wang P, Wu Y, Yang X, et al. Limited AT1 receptor internalization is a novel mechanism underlying sustained vasoconstriction induced by AT1 receptor autoantibody from preeclampsia. *J Am Heart Assoc.* 2019;8:e11179.
- Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A, et al. Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc Natl Acad Sci USA.* 2013;110:743–8.
- Xu W, Wu Y, Wang L, Bai Y, Du Y, Li Y, et al. Autoantibody against β_1 -adrenoceptor promotes the differentiation of natural regulatory T cells from activated CD4(+) T cells by up-regulating AMPK-mediated fatty acid oxidation. *Cell Death Dis.* 2019;10:158.
- Soave M, Cseke G, Hutchings CJ, Brown A, Woolard J, Hill SJ. A monoclonal antibody raised against a thermo-stabilised β_1 -adrenoceptor interacts with extracellular loop 2 and acts as a negative allosteric modulator of a sub-set of β_1 -adrenoceptors expressed in stable cell lines. *Biochem Pharmacol.* 2018;147:38–54.
- Wallukat G, Wollenberger A. Effects of the serum gamma globulin fraction of patients with allergic asthma and dilated cardiomyopathy on chronotropic β_1 -adrenoceptor function in cultured neonatal rat heart myocytes. *Biomed Biochim Acta.* 1987;46:S634–9.
- Shang L, Zhang L, Shao M, Feng M, Shi J, Dong Z, et al. Elevated β_1 -adrenergic receptor autoantibody levels increase atrial fibrillation susceptibility by promoting atrial fibrosis. *Front Physiol.* 2020;11:76.
- Sun R, Mak S, Haschemi J, Horn P, Boege F, Luppa PB. Nanodiscs incorporating native β_1 adrenergic receptor as a novel approach for the detection of pathological autoantibodies in patients with dilated cardiomyopathy. *J Appl Lab Med.* 2019;4:391–403.
- Wang L, Li Y, Ning N, Wang J, Yan Z, Zhang S, et al. Decreased autophagy induced by β_1 -adrenoceptor autoantibodies contributes to cardiomyocyte apoptosis. *Cell Death Dis.* 2018;9:406.
- Jane-wit D, Altuntas CZ, Johnson JM, Yong S, Wickley PJ, Clark P, et al. β_1 -adrenergic receptor autoantibodies mediate dilated cardiomyopathy by agonistically inducing cardiomyocyte apoptosis. *Circulation.* 2007;116:399–410.
- Hilger D, Masureel M, Kobilka BK. Structure and dynamics of GPCR signaling complexes. *Nat Struct Mol Biol.* 2018;25:4–12.
- Xu X, Kaindl J, Clark MJ, Hubner H, Hirata K, Sunahara RK, et al. Binding pathway determines norepinephrine selectivity for the human β_1 AR over β_2 AR. *Cell Res.* 2020.
- Rajagopal S, Shenoy SK. GPCR desensitization: acute and prolonged phases. *Cell Signal.* 2018;41:9–16.
- Black JB, Premont RT, Daaka Y. Feedback regulation of G protein-coupled receptor signaling by GRKs and arrestins. *Semin Cell Dev Biol.* 2016;50:95–104.
- Gaborik Z, Szaszak M, Szidonya L, Balla B, Paku S, Catt KJ, et al. Beta-arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor. *Mol Pharmacol.* 2001;59:239–47.
- Morisco C, Marrone C, Galeotti J, Shao D, Vatner DE, Vatner SF, et al. Endocytosis machinery is required for β_1 -adrenergic receptor-induced hypertrophy in neonatal rat cardiac myocytes. *Cardiovasc Res.* 2008;78:36–44.
- Schinner C, Erber BM, Yeruva S, Waschke J. Regulation of cardiac myocyte cohesion and gap junctions via desmosomal adhesion. *Acta Physiol (Oxf).* 2019;226:e13242.
- Gizak A, Zarzycki M, Rakus D. Nuclear targeting of FBPase in HL-1 cells is controlled by β_1 -adrenergic receptor-activated Gs protein signaling cascade. *Biochim Biophys Acta.* 2009;1793:871–7.
- Erickson CE, Gul R, Blessing CP, Nguyen J, Liu T, Pulakat L, et al. The β_1 -blocker nebivolol is a GRK/ β -arrestin biased agonist. *PLOS One.* 2013;8:e71980.
- Woo AY, Song Y, Xiao RP, Zhu W. Biased β_2 -adrenoceptor signalling in heart failure: pathophysiology and drug discovery. *Br J Pharmacol.* 2015;172:5444–56.
- Yang HQ, Wang LP, Gong YY, Fan XX, Zhu SY, Wang XT, et al. β_2 -adrenergic stimulation compartmentalizes β_1 signaling into nanoscale local domains by targeting the c-terminus of β_1 -adrenoceptors. *Circ Res.* 2019;124:1350–9.
- Zhu WZ, Chakir K, Zhang S, Yang D, Lavoie C, Bouvier M, et al. Heterodimerization of β_1 - and β_2 -adrenergic receptor subtypes optimizes β -adrenergic modulation of cardiac contractility. *Circ Res.* 2005;97:244–51.
- Lavoie C, Mercier JF, Salahpour A, Umaphathy D, Breit A, Villeneuve LR, et al. β_1/β_2 -adrenergic receptor heterodimerization regulates β_2 -adrenergic receptor internalization and ERK signaling efficacy. *J Biol Chem.* 2002;277:35402–10.
- Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, et al. β_2 -adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science.* 2010;327:1653–7.
- Wenzel D, Knies R, Matthey M, Klein AM, Welschoff J, Stolle V, et al. β_2 -adrenoceptor antagonist ICI 118,551 decreases pulmonary vascular tone in mice via a G(i/o) protein/nitric oxide-coupled pathway. *Hypertension.* 2009;54:157–63.
- Kim K, Chung KY. Many faces of the GPCR-arrestin interaction. *Arch Pharm Res.* 2020;43:890–9.
- Latorraca NR, Masureel M, Hollingsworth SA, Heydenreich FM, Suomivuori CM, Brinton C, et al. How GPCR phosphorylation patterns orchestrate arrestin-mediated signaling. *Cell.* 2020;183:1813–25.
- Zhou XE, He Y, de Waal PW, Gao X, Kang Y, Van Eps N, et al. Identification of phosphorylation codes for arrestin recruitment by G protein-coupled receptors. *Cell.* 2017;170:457–69.
- Dungen HD, Dordevic A, Felix SB, Pieske B, Voors AA, McMurray J, et al. β_1 -adrenoceptor autoantibodies in heart failure: physiology and therapeutic implications. *Circ Heart Fail.* 2020;13:e6155.
- Warne T, Serrano-Vega MJ, Baker JG, Moukhametianov R, Edwards PC, Henderson R, et al. Structure of a β_1 -adrenergic G-protein-coupled receptor. *Nature.* 2008;454:486–91.
- Zhou Q, Yang D, Wu M, Guo Y, Guo W, Zhong L, et al. Common activation mechanism of class A GPCRs. *Elife.* 2019;8.
- Talan MI, Ahmet I, Xiao RP, Lakatta EG. β_2 AR agonists in treatment of chronic heart failure: long path to translation. *J Mol Cell Cardiol.* 2011;51:529–33.

ACKNOWLEDGEMENTS

The authors thank Mrs. Weiwei Hao for her contribution in heart tissue paraffin sectioning and HE staining, Mrs. Qing Xu for her expertise in Doppler ultrasound for cardiac function detection, Mrs. Lina Liu for her proficiency in TIRF, and Mrs. Ying

Yang for SPR in the Core Facility Center, Capital Medical University. The authors also thank professor Jinpeng Sun for kindly providing plasmids (β -arrestin1-Rluc and β -arrestin2-Rluc), professor Qian Fan for collecting HF patient samples. This work was supported by funding from the National Natural Science Foundation of China (81970334, 81770393, 31871177).

AUTHOR CONTRIBUTIONS

Conception and design of the study: H.C., N.C., S.L.Z. and H.R.L. Acquisition of data: H. C., N.C., H.J.W. Analysis and interpretation of data: Y.W. Writing of the manuscript: H. C., Y.M.L. Revision of the manuscript: L.W., Y.Y.Z., S.L.Z., H.R.L.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41420-021-00735-2>.

Correspondence and requests for materials should be addressed to Suli Zhang or Huirong Liu.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021