

β_2 -adrenergic receptor autoantibodies alleviated myocardial damage induced by β_1 -adrenergic receptor autoantibodies in heart failure

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Aims

β_1 -adrenergic receptor autoantibodies (β_1 -AAs) and β_2 -adrenergic receptor autoantibodies (β_2 -AAs) are present in patients with heart failure (HF); however, their interrelationship with cardiac structure and function remains unknown. This study explored the effects of the imbalance between β_1 -AAs and β_2 -AAs on cardiac structure and its underlying mechanisms in HF.

Methods and results

Patients with left systolic HF who suffered from coronary heart disease (65.9%) or dilated cardiomyopathy (34.1%) were divided into New York Heart Association Classes I–II ($n = 51$) and Classes III–IV ($n = 37$) and compared with healthy volunteers as controls ($n = 41$). Total immunoglobulin G from HF patient serum comprising β_1 -AAs and/or β_2 -AAs were determined and purified for *in vitro* studies from neonatal rat cardiomyocytes (NRCMs). In addition, HF was induced by doxorubicin in mice. We observed that the increased ratio of β_1 -AAs/ β_2 -AAs was associated with worsening HF in patients. Moreover, β_2 -AAs from patients with HF suppressed the hyper-shrinking and apoptosis of NRCMs induced by β_1 -AAs from some patients. Finally, β_2 -AAs alleviated both myocardial damage and β_1 -AAs production induced by doxorubicin in mice.

Conclusion

β_2 -AAs were capable of antagonizing the effects imposed by β_1 -AAs both *in vitro* and *in vivo*. The imbalance of β_1 -AAs and β_2 -AAs in patients with HF is a mechanism underlying HF progression, and the increasing ratio of β_1 -AAs/ β_2 -AAs should be considered a clinical assessment factor for the deterioration of cardiac function in patients with HF.

Keywords

β_2 -adrenergic receptor autoantibodies • β_1 -adrenergic receptor autoantibodies • Cardiac function • Heart failure

1. Introduction

Despite the annual increase in the prevalence and incidence of heart failure (HF),¹ the underlying pathophysiology of this disease remains inadequately understood. Immune dysfunction is one of the key mechanisms underlying the occurrence and development of HF.² Initially identified in the serum of patients with dilated cardiomyopathy (DCM) in 1987, β_1 -adrenergic receptor autoantibodies (β_1 -AAs) can specifically bind to the second extracellular loop of the β_1 -adrenergic receptor (β_1 -AR)

(β_1 -ECII) and has β_1 -AR agonist-like effects.^{3,4} The β_1 -AAs can also induce sustained activation of the β_1 -AR,⁵ leading to HF. However, β -blockers cannot completely block damage to the myocardium induced by β_1 -AA⁶ and improve the survival rate of patients with HF who are positive for β_1 -AAs-immunoglobulin G.⁷ Therefore, it is important to identify an effective mechanism to ameliorate myocardial injury induced by β_1 -AAs.

Under physiological conditions, heart rate and contractility are regulated by cardiac β_1 -AR, whereas cardiac β_2 -ARs only play a minor role.

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However, in situations of stress or HF, when large amounts of catecholamine are released, β_2 -AR activation may restrain the overactivation of β_1 -AR via the Gi pathway.⁸ In addition, β_2 -AR activation plays a protective role in the injury of cardiomyocytes by inhibiting the apoptosis induced by β_1 -AR.⁹ β_2 -AR autoantibodies (β_2 -AAs) have also been detected in patients with HF¹⁰ and specifically bind to the second extracellular loop of the β_2 -AR (β_2 -ECII) to activate the receptor.¹¹

Therefore, a fundamental question that needs to be addressed is how the relationship between β_1 -AAs and β_2 -AAs is affected in relation to cardiac structure and function during HF when both autoantibodies co-exist. Furthermore, it needs to be determined if β_2 -AAs can inhibit the damage induced by β_1 -AAs by activating the β_2 -AR. To address these questions, this study explored the impact of the imbalance between β_1 -AAs and β_2 -AAs on the cardiac structure both in patients with HF and in experimental settings, and its underlying mechanisms *in vitro* and *in vivo*.

2. Methods

This research study conformed to the Declaration of Helsinki. Patient confidentiality was preserved, and the anonymity of all patient data was safeguarded throughout the study. The study was approved by the local research ethics committee (Beijing Anzhen Hospital, Capital Medical University, Beijing, China). The animal studies complied with the recommendations in the Guide for the Care and Use of Laboratory Animals protocol and National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals). This research study was approved by the Institutional Animal Care and Use Committee of Capital Medical University. The investigators understand the ethical principles by which the journal operates, and the study complied with the journal's animal ethics checklist (No. AEEI 2016-053). Before detecting cardiac function or collecting blood from the tip of the tail, mice were anaesthetized via a nasal mask with halothane (0.5 l/min) for about 30 s. At corresponding time points or the end of the experiment, the mice were euthanized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital.

Patients with left systolic HF who suffered from coronary heart disease (CHD) (65.9%) or DCM (34.1%) were divided into New York Heart Association (NYHA) Classes I–II ($n = 51$) and Classes III–IV ($n = 37$) compared with healthy volunteers as controls ($n = 41$). The relationships were identified between cardiac function and β_1 -AAs, β_2 -AAs, and their positive-to-negative (P/N) ratio values. Neonatal rat cardiac myocytes (NRCMs) were used to detect beat frequency, cell death, necrosis, and apoptosis after stimulation with IgGs comprising β_1 -AAs and/or β_2 -AAs from the serum of patients with HF. HF models were induced by doxorubicin (DOX), isoprenaline (ISO), or β_1 -AAs/ β_2 -AAs passive immunity using 10-week male Balb/c mice. Then cardiac function, and the level and ratio of β_1 -AAs and β_2 -AAs were detected. The detailed experimental procedures are described in the [Supplementary material online](#).

3. Results

3.1 The ratio of β_1 -AAs and β_2 -AAs P/N values and its relationship to worsening HF

The subjects were divided into three groups: healthy volunteers, patients with HF of Classes I–II, and HF Classes III–IV of NYHA. All patients had left systolic HF either induced by CHD (65.9%) or DCM (34.1%). Baseline and clinical characteristics of the study populations are shown in [Table 1](#). The serum optical density (OD) value of β_1 -AAs and β_2 -AAs of

the different groups is shown in [Table 1](#); if the P/N serum value was >2.1 , the sample was considered positive. The results showed that the positive rate and OD values of β_1 -AAs or β_2 -AAs in HF patients were higher than those in healthy volunteers ([Figure 1A–C](#), positive patients are shown above the dotted line). However, the double positive rate of β_1 -AAs and β_2 -AAs was decreased with the increasing class of NYHA [11.8% (I–II) vs. 5.4% (III–IV); [Figure 1A](#)]. Correlation analysis was used to explore the relationship between cardiac function and β_1 -AAs or β_2 -AAs. The results showed that a single β_1 -AAs or β_2 -AAs OD value had no correlation with left ventricular ejection fraction (LVEF) or left ventricular end-diastolic dimension (LVEDD) (see [Supplementary material online, Figure S1A–D](#)). However, the β_1 -AAs/ β_2 -AAs P/N ratio ($\beta_1/2$ -AAs P/N ratio) was negatively correlated with LVEF, but was positively correlated with LVEDD ([Figure 1D and E](#)) (P/N value was used to standardize the OD value, all P/N values were > 0). Furthermore, the ratio was higher between healthy volunteers and HF patients ([Figure 1F](#)). However, the correlation between the ratio and cardiac function was weak (correlation coefficient was -0.2 between the ratio and LVEF, and 0.2 between the ratio and LVEDD), and was driven by several extreme cases.

3.2 $\beta_1/2$ -AAs P/N ratio was negatively correlated with cardiac function in DOX-induced and ISO-induced HF mice

Two HF mouse models were established to investigate the correlation between P/N ratio and cardiac function. In the first model, HF was induced by DOX injection ([Figure 2A](#)). When compared with the saline group, the LVEF or LVEDD worsened, and the myocardium was damaged in the DOX-induced HF group (see [Supplementary material online, Figure S2A–D](#)). Moreover, the OD value of β_1 -AAs increased, the β_2 -AAs remained at a high level, and their P/N ratio increased with HF progression in the DOX-induced HF group at 30 days ([Figure 2B–D](#)). The ratio of the β_1 -AAs and β_2 -AAs P/N value was negatively correlated with LVEF and positively correlated with LVEDD at all time points ([Figure 2E and F](#)). When compared with patients with HF, the correlation between the ratio and cardiac function was stronger in DOX-induced HF mice (correlation coefficient was -0.526 between the ratio and LVEF, and 0.684 between the ratio and LVEDD). In the second mouse model, HF was induced by ISO injection (see [Supplementary material online, Figure SA–D](#)). Levels of both β_1 -AAs and β_2 -AAs were high halfway through the month, and then reduced in the end stage in ISO-induced HF mice (see [Supplementary material online, Figure S3E and F](#)). The other changes were similar to those in the DOX-induced HF mouse model including cardiac function deterioration, $\beta_1/2$ -AAs ratio increase, and a negative correlation with cardiac function (see [Supplementary material online, Figure S3G–I](#)) (correlation coefficient was -0.381 between the P/N ratio and LVEF, and 0.432 between the P/N ratio and LVEDD). Importantly, we found that a single β_1 -AAs or β_2 -AAs was not correlated, but the $\beta_1/2$ -AAs P/N ratio was negatively correlated with cardiac function in both patients with HF and the two HF mouse models. The high $\beta_1/2$ -AAs P/N ratio may have been due to the high level of β_1 -AAs and/or low level of β_2 -AAs. Because β_1 -AAs are highly expressed in HF,¹⁰ these results suggest that β_2 -AAs may play a protective role in HF patients.

3.3 β_1 -AAs and β_2 -AAs co-localize with β_1 -AR and β_2 -AR

To identify the potential protective role of β_2 -AAs in β_1 -AAs-positive patients with HF, four patients with HF containing different levels of

Table 1 Baseline and clinical characteristics of study population

Parameters	Cardiac function ^a of HF (n = 88)		P value	Health (n = 41)	P value
	I-II (n = 51)	III-IV (n = 37)			
Pathogeny ^b					
CHD, n(%)	34 (66.7)	24 (64.9)	NS	–	–
DCM, n(%)	17 (33.3)	13 (35.1)	NS	–	–
Age, y	59 ± 10	56 ± 10	NS	48 ± 20	NS
Male, n(%)	27 (47)	20 (54)	NS	20 (51)	NS
Overweight/Obesity, n(%)	13 (25)	10 (27)	NS	11 (21)	NS
Smoking, n(%)	22 (43)	15 (41)	NS	3 (6)	*
Heart rate, beats/min	75 ± 8	77 ± 9	NS	74 ± 9	NS
Diabetes, n(%)	21 (41)	17 (46)	NS	–	–
Hypertension, n(%)	27 (53)	21 (57)	NS	–	–
Dyslipidemia, n(%)	44 (86)	31 (84)	NS	–	–
SBP (mm Hg)	112 ± 16	109 ± 18	NS	–	–
DBP (mm Hg)	79 ± 17	75 ± 20	NS	–	–
LDH, IU/l	275 ± 96	240 ± 82	NS	–	–
Medications,					
Statins, n(%)	32 (63)	24 (65)	NS	–	–
Beta blockers ^c , n(%)	51 (100)	37 (100)	NS	–	–
Hypotensor ^d , n(%)	27 (53)	21 (57)	NS	–	–

Value are mean ± SD, n (%), or n.

NS, no significance; SBP, systolic blood pressure; DBP, diastolic blood pressure; q test after analysis of variance (ANOVA).

^aNYHA functional class.

^bLeft systolic HF induced by CHD or DCM.

^cCarvedilol/metoprolol/others.

^dACRI/ARB inhibitor/Aldosterone receptor antagonist/others.

*P < 0.05.

β₁-AAs and β₂-AAs were selected for the extraction of total IgGs. All four patients had similar baseline and clinical characteristics. P1 (β₁-AAs-/β₂-AAs-), P2 (β₁-AAs+/β₂-AAs-), P3 (β₁-AAs-/β₂-AAs+), and P4 (β₁-AAs+/β₂-AAs+) conformed to the tendency that the higher the β₁/β₂-AAs P/N ratio, the worse the cardiac function (P3 > P4 > P2) (see Supplementary material online, Table S1). The total IgGs from the four patients were of high purity (see Supplementary material online, Figure S4A–B). Moreover, to neutralize the effects of β₁-AAs and β₂-AAs, a high-purity peptide of the β₁-ECII and β₂-ECII was synthesized (see Supplementary material online, Figure S4C). To determine if total IgGs in P1–P4 could co-localize with β₁-AR or β₂-AR, isolated or confluent HEK293T cells stably expressing pEGFP-β₁-AR or pEGFP-β₂-AR were used. The results showed that P2 (β₁+/β₂-) and P4 (β₁+/β₂+) co-localized with β₁-AR rather than with P1 (β₁-/β₂-) or P3 (β₁-/β₂+) (Figure 3, see Supplementary material online, Figure S5, left panel). The co-localization between β₁-AAs and β₁-AR was neutralized by β₁-ECII (Figure 3, left panel). Conversely, P3 and P4 could co-localize with β₂-AR rather than with P1 or P2 (Figure 3, see Supplementary material online, Figure S5, right panel). The co-localization between β₂-AAs and β₂-AR was neutralized by β₂-ECII (Figure 3, right panel).

3.4 β₂-AAs from patients with HF inhibit the beat frequency of neonatal rat cardiomyocytes induced by β₁-AAs in vitro

Next, to evaluate the effects of β₁-AAs and/or β₂-AAs on cardiomyocyte function, neonatal rat cardiomyocytes (NRCMs) with spontaneous

beating were used (see Supplementary material online, Video). β_{1/2}-ECII was 100% homologous between rats and humans, as determined using the NCBI blast database (see Supplementary material online, Figure S4D); thus, IgGs from human serum were used to stimulate the rat cardiomyocytes. However, the peptides and receptor blockers had no effect on beat frequency, death, necrosis, and apoptosis of NRCMs (see Supplementary material online, Figure S6). Furthermore, the results showed that the P2 IgGs (β₁+/β₂-) could increase the beat frequency of the cardiomyocytes and maintain it for >12 h (Figure 4A); the effects could be neutralized by β₁-ECII and inhibited by the β₁-AR blocker metoprolol at 12 h (Figure 4B). When compared with P2, cardiomyocyte beat frequency and duration were less stimulated by P4 IgGs (β₁+/β₂+) (Figure 4A), and this effect could be neutralized by β₂-ECII and inhibited by the β₂-AR blocker ICI118551 at 12 h (Figure 4B). ISO, as a non-selective agonist of β-AR, had a transient increase in beat frequency similar to the effect of P4. However, when β₂-AR was blocked, ISO induced a sustained increase in beat frequency, similar to the effect of β₁-AAs single-positive IgGs (P2)⁵ (see Supplementary material online, Figure S7A). After blocking activation of β₂-AR induced by ISO, the increased beat frequency was maintained for a longer duration than P2¹² (see Supplementary material online, Figure S7B). The increase in beat frequency induced by P4 (β₁+/β₂+), rather than by P3 (β₁-/β₂+), could also be almost completely inhibited by metoprolol at 3 h (see Supplementary material online, Figure S7B). These results revealed that β₁-AAs, rather than β₂-AAs, induced the increase in beat frequency of NRCMs. To determine whether Gi or Gs protein of β₂-AR activated by β₂-AAs, cyclic adenosine monophosphate (cAMP) in cardiomyocytes

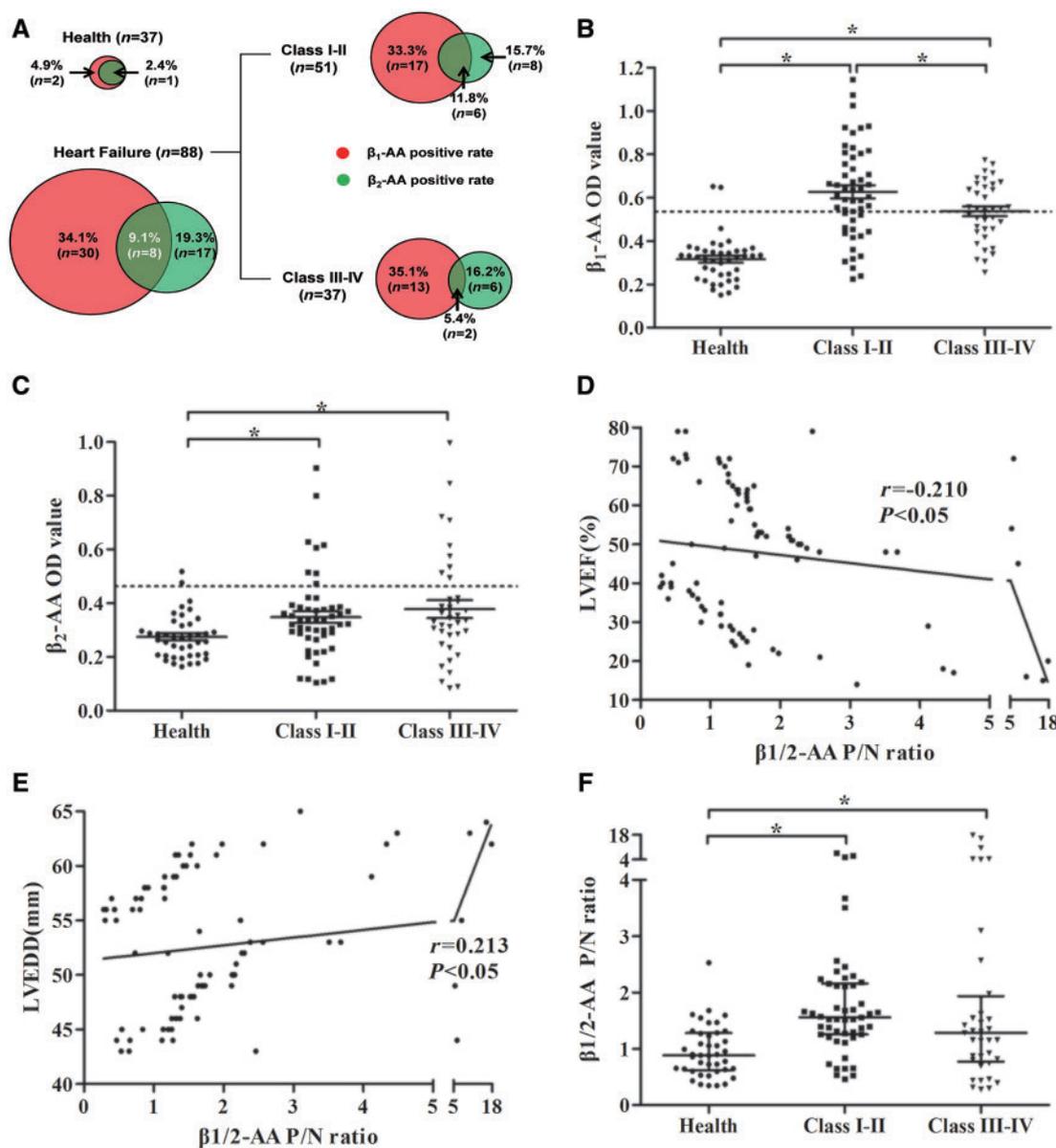


Figure 1 The β_1/β_2 -AAs P/N ratio is negatively correlated with cardiac function in patients with HF. (A) The double-positive rate of β_1 -AAs and β_2 -AAs was decreased with the class of NYHA. (Classes I–II vs. III–IV: 11.8 vs. 5.4%, chi-square test); (B and C). β_1 -AAs OD value distribution was reduced at higher NYHA class with HF patients [0.316 ± 0.016 (H) vs. 0.627 ± 0.030 (I–II) vs. 0.537 ± 0.022 (III–IV)] but β_2 -AAs OD value distribution changed little [0.275 ± 0.013 (H) vs. 0.348 ± 0.022 (I–II) vs. 0.378 ± 0.033 (III–IV)], (mean \pm S.E.M.; dotted line: positive or negative boundary, P/N value > 2.1 ; q test after one-way ANOVA). (D and E). β_1/β_2 -AAs P/N ratio was negatively correlated with LVEF ($r = -0.210$, $P < 0.05$) and positively correlated with LVEDD ($r = 0.213$, $P < 0.05$) at HF ($n = 88$, Pearson correlation analysis). (F) β_1/β_2 -AAs P/N ratio in health population was higher than patients with HF [0.881 (0.660) (H) vs. 1.558 (0.904) (I–II); 0.881 (0.660) (H) vs. 1.283 (1.164) (III–IV), median \pm interquartile range (IQR), $n = 129$, Kruskal-Wallis after one-way ANOVA]. *group vs. Health, $P < 0.05$; #group vs. Classes I–II, $P < 0.05$.

were detected after Gi or Gs were inhibited by PTX (pertussis toxin, Gi inhibitor) or DT (diphtheria toxin, Gs inhibitor). The results showed that the activation of β_2 -AR induced by β_2 -AAs of P3 or P4 IgGs resulted in decreased cAMP expression upon stimulation by P3 or P4 (Figure 4C). After PTX was used with P3 (β_1/β_2+) or P4, cAMP in cardiomyocytes significantly increased (Figure 4D). However, DT did not significantly change the

concentration of cAMP after stimulation by P3, and even decreased cAMP induced by P4 (β_1/β_2+) (see Supplementary material online, Figure S7C). These results revealed that the Gi rather than Gs of β_2 -AR played a role in the activation of β_2 -AR induced by β_2 -AAs. Thus, β_2 -AAs might play a protective role by activating the Gi of β_2 -AR to inhibit the high beat frequency and long duration of cardiomyocytes induced by β_1 -AAs.

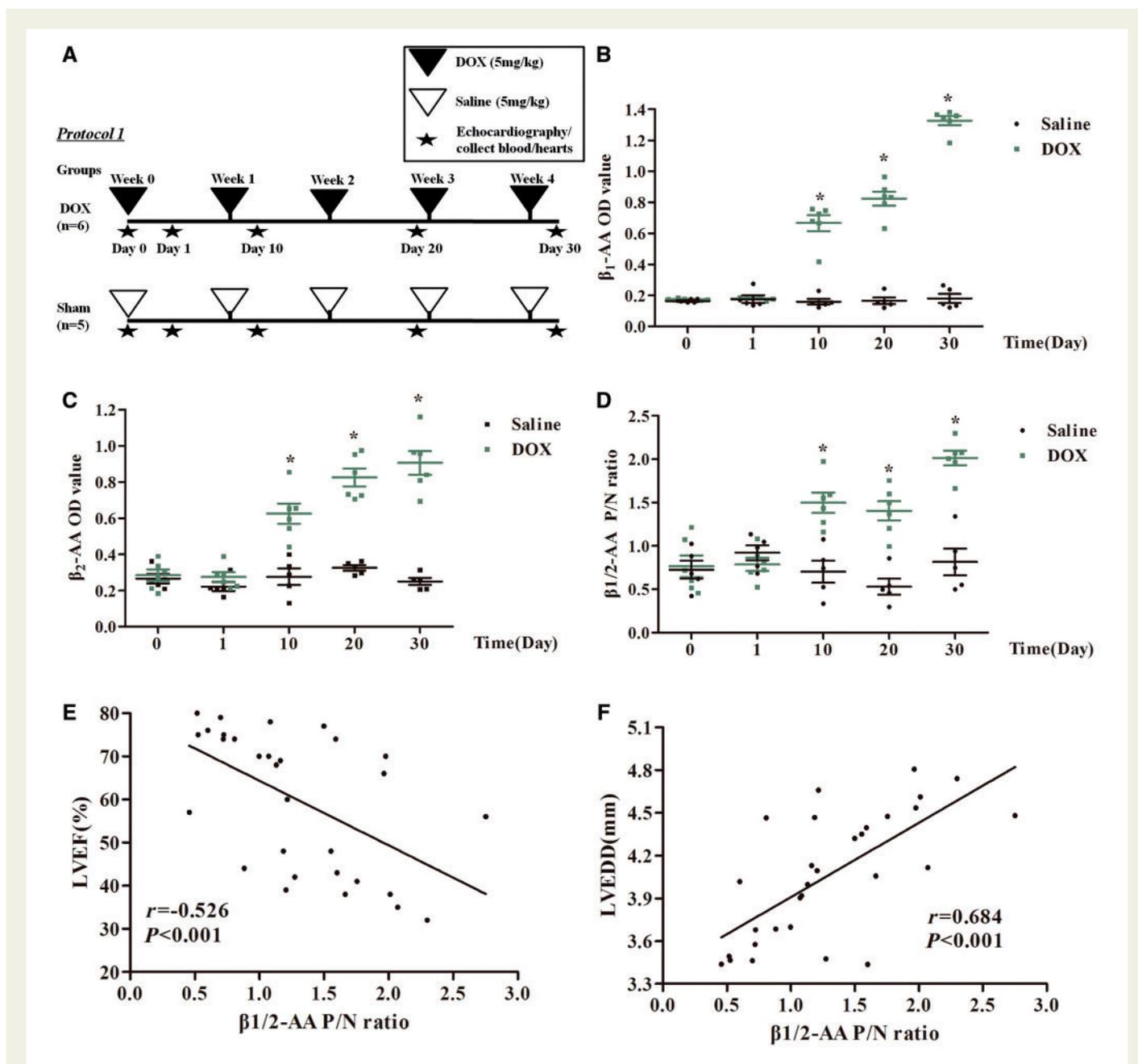


Figure 2 The $\beta_1/2$ -AAs P/N ratio is negatively correlated with cardiac function of HF mice induced by DOX. (A). The protocol of mice HF induced by DOX. (B–D). β_1 -AAs, β_2 -AAs, and $\beta_1/2$ -AAs P/N ratio gradually increased with HF progression induced by DOX from 0 to 30 days. [$n = 5$ (saline), $n = 6$ (DOX); mean \pm S.E.M.; *DOX vs. Saline, $P < 0.05$, q test after two-way ANOVA]. (E and F) The ratio of $\beta_1/2$ -AAs P/N value was negatively correlated with LVEF ($r = -0.526$, $P < 0.05$) and positively correlated with LVEDD ($r = 0.684$, $P < 0.05$) including all time-points at DOX group [$n = 30$ (6×5), Spearman correlation analysis].

3.5 β_2 -AAs from patients with HF inhibits the necrosis and apoptosis of NRCMs induced by β_1 -AAs *in vitro*

β_1 -AAs in P2 IgGs ($\beta_1 + / \beta_2 -$) significantly decreased the number of viable NRCMs, and the death rate was $\sim 40\%$ at 12 h, and this effect could be neutralized or blocked by β_1 -ECII or metoprolol. When compared with P2 IgGs, cardiomyocyte death was less stimulated by β_1 -AAs- and β_2 -AAs-double positive IgGs (P4), and the effect could be neutralized or

blocked by β_2 -ECII or ICI118551 (Figure 4E and F), indicating that β_2 -AAs-induced β_2 -AR activation attenuated the cardiomyocyte death rate imposed by β_1 -AAs. In addition, we investigated whether β_2 -AAs restrained cardiomyocyte death induced by β_1 -AAs through necrosis or apoptosis. The lactate dehydrogenase release test revealed that IgGs of P2 ($\beta_1 + / \beta_2 -$) stimulation could significantly increase the necrosis of NRCMs, and the effect could be neutralized or blocked by β_1 -ECII or metoprolol (see Supplementary material online, Figure S8A and B).

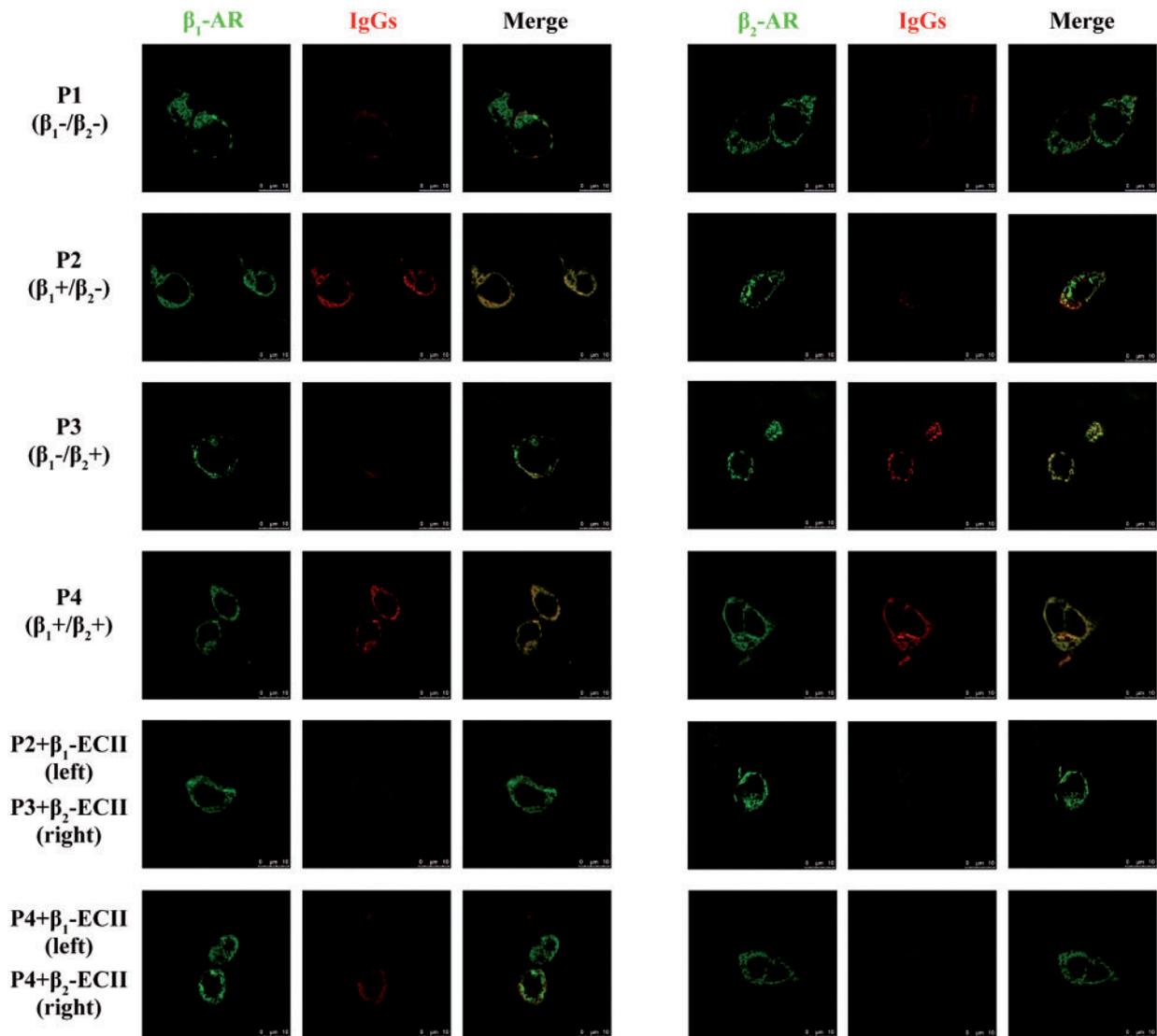


Figure 3 β_1 -AAs or β_2 -AAs in IgGs from HF patients co-localize with β_1 -AR or β_2 -AR at the cell surface in isolated HEK-293T cells. Left panel. IgGs from P2 (β_1 -AAs+/ β_2 -AAs-) and P4 (β_1 -AAs+/ β_2 -AAs+) could co-localize with EGFP- β_1 -AR in HEK-293T cells, and the phenomenon disappeared after β_1 -AAs was neutralized by β_1 -ECII. IgGs from P1 (β_1 -AAs-/ β_2 -AAs-) and P3 (β_1 -AAs-/ β_2 -AAs+) did not exhibit co-localization with β_1 -AR. Right panel. IgGs from P3 (β_1 -AAs-/ β_2 -AAs+) and P4 (β_1 -AAs+/ β_2 -AAs+) could co-localize with EGFP- β_2 -AR in HEK-293T cells, the phenomenon of co-localization disappeared after β_2 -AAs was neutralized by β_2 -ECII. IgGs from P1 (β_1 -AAs-/ β_2 -AAs-) and P2 (β_1 -AAs+/ β_2 -AAs-) did not show co-localization with β_1 -AR [$n = 6$, dilution ratio of IgGs: 1:500 (30 mg/l)]. $\beta_{1/2}$ -ECII: 30 mg/l. visualization at 63-fold magnification; size bar (10 μ m).

Moreover, the apoptosis rate of cardiomyocyte induced by P1–P4 was similar to the necrosis rate of NRCMs (Figure 5A–C). When compared with β_1 -AAs-single-positive IgGs (P2), cardiomyocyte necrosis or apoptosis was less stimulated by β_1 -AAs- and β_2 -AAs-double-positive IgGs (P4) and the effect could be neutralized or blocked by β_2 -ECII or ICI118551 (Figure 5, see Supplementary material online, Figure S8B). These results suggested that β_2 -AAs activated β_2 -AR to play a prohibitive effect against cardiomyocyte necrosis and apoptosis induced by β_1 -AAs via β_1 -AR. The protective effect of β_2 -AAs against cardiomyocyte injuries induced by β_1 -AAs was mainly exerted by activating β_2 -AR, thereby inhibiting the sustained beating, necrosis, and apoptosis of cardiomyocytes.

3.6 β_2 -AAs alleviates the cardiac dysfunction and structural injury induced by β_1 -AAs in vivo

To provide evidence that β_2 -AAs are capable of alleviating the cardiac dysfunction induced by β_1 -AAs, an *in vivo* mice model of HF induced by β_1 -AAs passive immunity was established (see Supplementary material online, Figure S9A). After 7 months, LVEF was decreased and LVEDD significantly increased in passive-immunized mice compared with control mice (see Supplementary material online, Figure S9B–D). Simultaneously, we observed increased β_1 -AAs levels and decreased β_2 -AAs levels (see Supplementary material online, Figure S9E and F). In addition, when maintaining the level of serum β_1 -AAs via β_1 -AAs passive immunity, β_2 -AAs

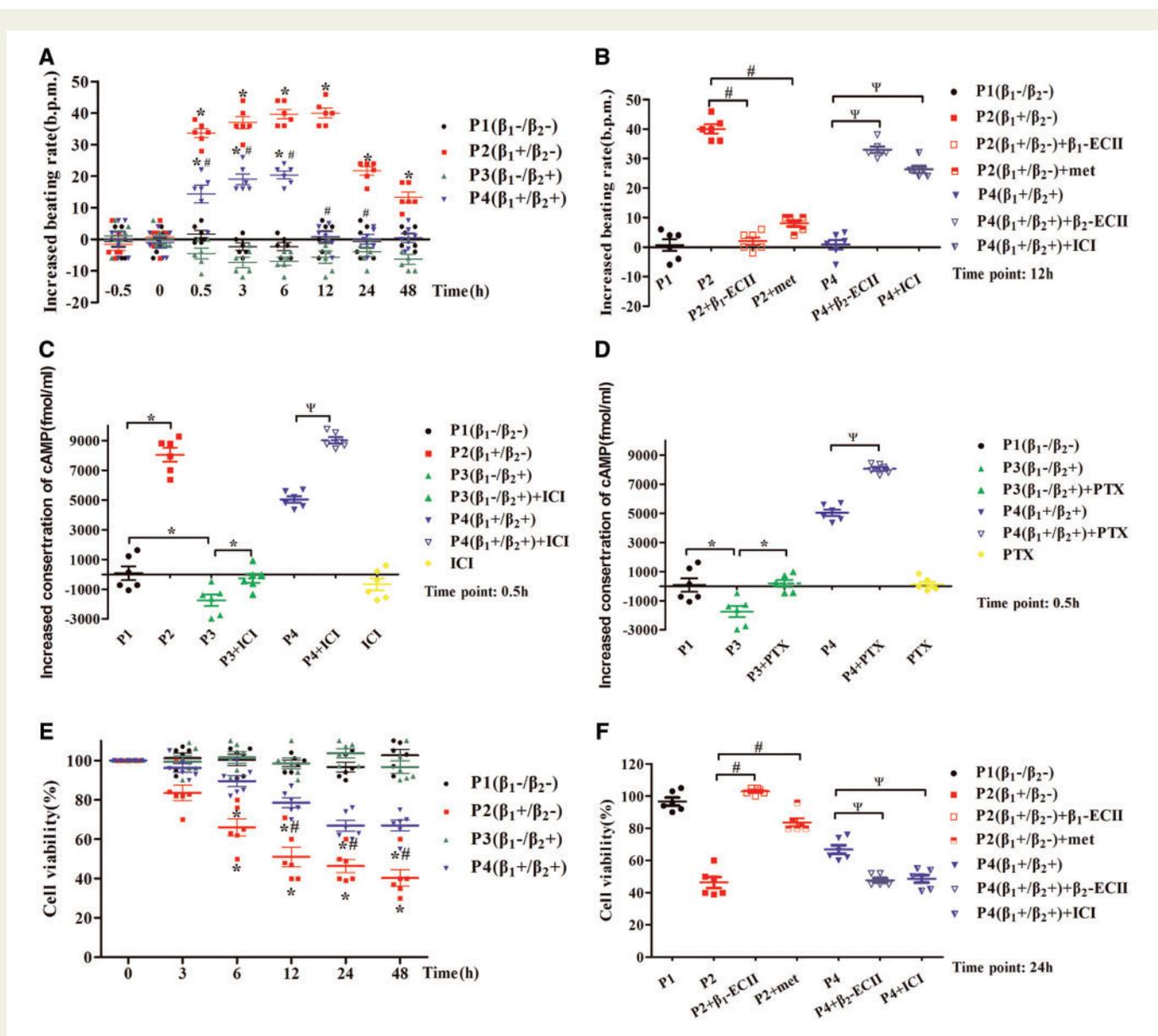


Figure 4 β_2 -AAs activates β_2 -AR Gi to inhibit beat frequency and cell death of NRCMs induced by β_1 -AAs. (A). NRCMs beating frequency and duration were less stimulated by β_1 -AAs- and β_2 -AAs-double positive IgGs (P4) compared with β_1 -AAs-single positive IgGs (P2) recorded by living cell station from 0 to 48h. (B) P4's IgGs played a similar effect to P2 that caused higher beating frequency and longer duration after β_2 -AAs was neutralized by β_2 -ECII or β_2 -AR was blocked by β_2 -AR inhibitor ICI118551 at 12 h. (C) β_2 -AAs in P3 or P4-induced lower concentration of cAMP by activating β_2 -AR in NRCMs at 0.5 h. (D) The decreasing in cAMP induced by β_2 -AAs in P3 or P4 was due to activation of Gi in NRCMs at 0.5h (PTX, Gi inhibitor). (E) The death of NRCMs were less stimulated by β_1 -AAs- and β_2 -AAs-double positive IgGs (P4) compared with β_1 -AAs-single positive IgGs (P2) by CCK8 from 0 to 48h. F. P4 played a similar effect to P2 that caused higher cell death of NRCMs after β_2 -AAs neutralized by β_2 -ECII or β_2 -AR inhibited by β_2 -AR blocker ICI118551 at 24 h. IgGs stimulus concentration: 0.1 μ M; Neutralization and block concentration: 1 μ M; n = 6; *group vs. P1; #group vs. P2; ψ group vs. P4, P < 0.05, mean \pm SEM, q test after two-way ANOVA.

OD value was positively correlated with LVEF and negatively correlated with LVEDD (see [Supplementary material online, Figure S9G and H](#)), suggesting that β_2 -AAs might alleviate the cardiac dysfunction induced by β_1 -AAs *in vivo*. The DOX-induced HF mouse model was used to identify the protective role of β_2 -AAs when β_1 -AAs levels were high. To this end, β_1 -ECII was injected into the tail vein (1 mg/kg, 10 days) to neutralize β_1 -AAs levels induced by DOX (see [Supplementary material online, Figure S10A–C](#)). In the β_1 -AAs-neutralized group, mice had better cardiac function than the untreated DOX-HF group (see [Supplementary material online, Figure S10D and E](#)). These results suggested that β_1 -AAs

played a role in DOX-induced deteriorating cardiac function.¹³ Passive immunity was also used to increase serum β_2 -AAs levels, and β_2 -ECII was injected into the tail vein to neutralize endogenous β_2 -AAs (Figure 6A). The passive immunity or neutralization of β_2 -AAs was successful (Figure 6B). The β_1 -AAs and $\beta_1/2$ -AAs P/N ratio decreased with increasing levels of β_2 -AAs (Figure 6C and D). When compared with the untreated group, β_2 -AAs could partly improve LVEF and LVEDD (Figure 6E–G) as well as myocardial structural injury and myocardial apoptosis induced by DOX (Figure 6H and I). Furthermore, in the process of HF, the increase in β_2 -AAs levels via passive immunity or decrease in

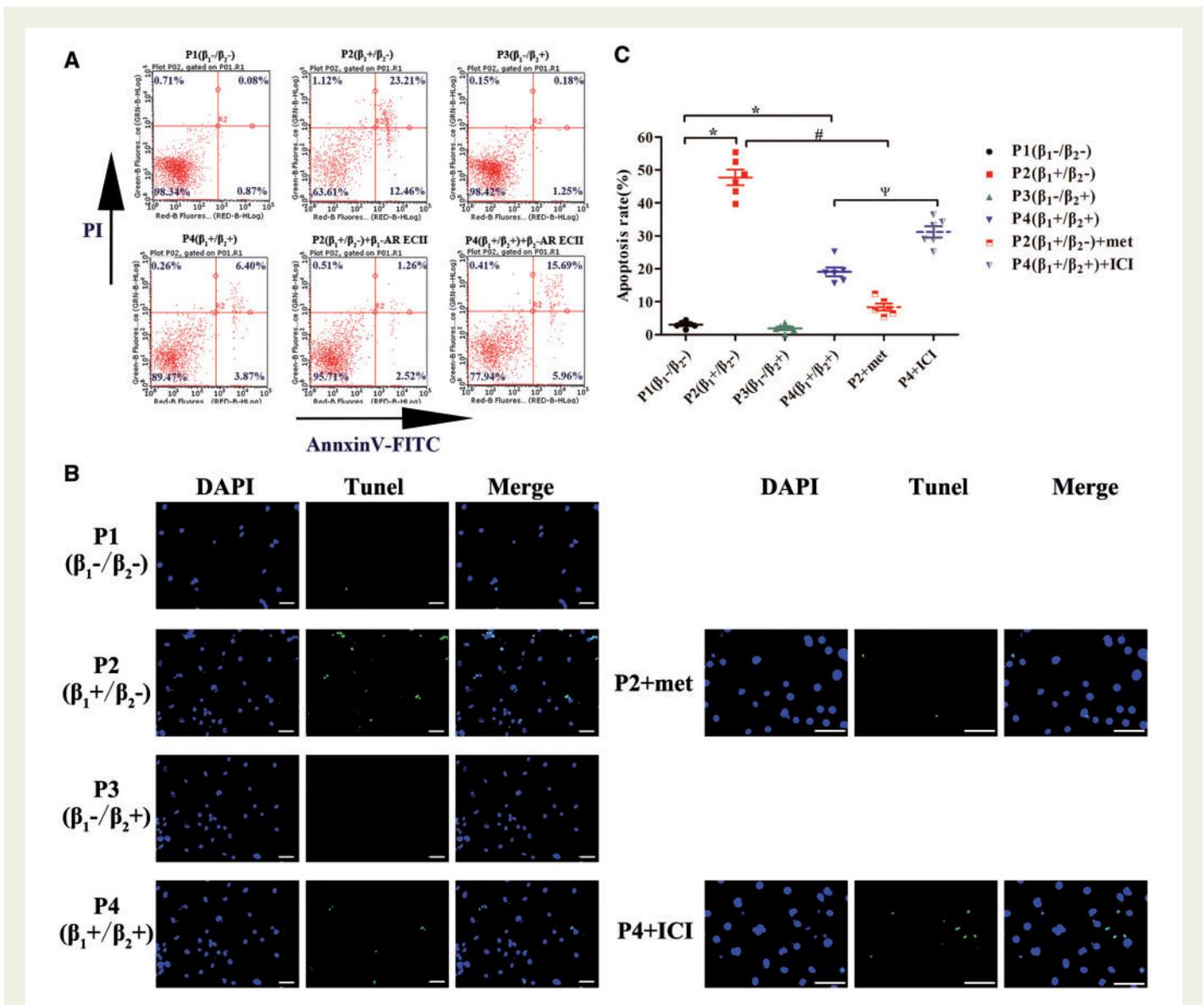


Figure 5 β_2 -AAs inhibits apoptosis of NRCMs induced by β_1 -AAs. (A) Flow cytometry analysis showed that the apoptosis rates of NRCMs were less stimulated by β_1 -AAs- and β_2 -AAs-double positive IgGs (P4) compared with β_1 -AAs-single positive IgGs (P2) via AnnxinV-FITC-PI kit. Effect of P4 could be neutralized by β_2 -ECII to play a similar effect stimulated by P2 to cause higher apoptosis rates. (B and C) TUNEL assay shows a similar result as flow cytometry analysis (mean \pm SEM, η test after one-way ANOVA), that P4 led to reduced apoptosis rates compared with P2, which could be blocked by β_2 -AR inhibitor ICI118551 (bar=30 μ m). IgGs stimulus concentration: 0.1 μ M; Time-point: 24h. Neutralization and block concentration: 1 μ M ($n = 6$, *group vs. P1; #group vs. P2; ψ group vs. P4, $P < 0.05$).

β_2 -AAs levels via neutralization peptide could increase or decrease the production of β_1 -AAs, both of which were detrimental for cardiac function (Figure 6C). Notably, single β_2 -AAs or $\beta_{1/2}$ -ECII without DOX did not affect cardiac function (see Supplementary material online, Figure S11). These data suggested that β_2 -AAs could alleviate cardiac dysfunction and structural injury induced by β_1 -AAs *in vivo*.

4. Discussion

Our findings demonstrated that an imbalance between β_1 -AAs and β_2 -AAs exists in patients with HF and is likely a part of HF pathogenesis. The higher β_1/β_2 -AAs P/N ratio was related to a worse cardiac function

in both patients and mice with HF. β_2 -AAs antagonized the damaging effects imposed by β_1 -AAs both *in vitro* and *in vivo*. A working model is illustrated in Figure 7.

4.1 The production and distribution of β_1 -AAs and β_2 -AAs in HF

β_1 -AAs and β_2 -AAs coexist in different cardiac diseases including CHD, DCM, arrhythmia.¹⁰ The relationship between HF and β -AAs is complicated, and in some cases, there is reciprocal causation. Different types of HF models can induce the production of β_1 -AAs or β_2 -AA,^{13,14} and conversely, β_1 -AAs can also result in HF.¹⁰ However, it is unclear how the generation of β_1 -AAs or β_2 -AAs in patients with HF can be achieved.

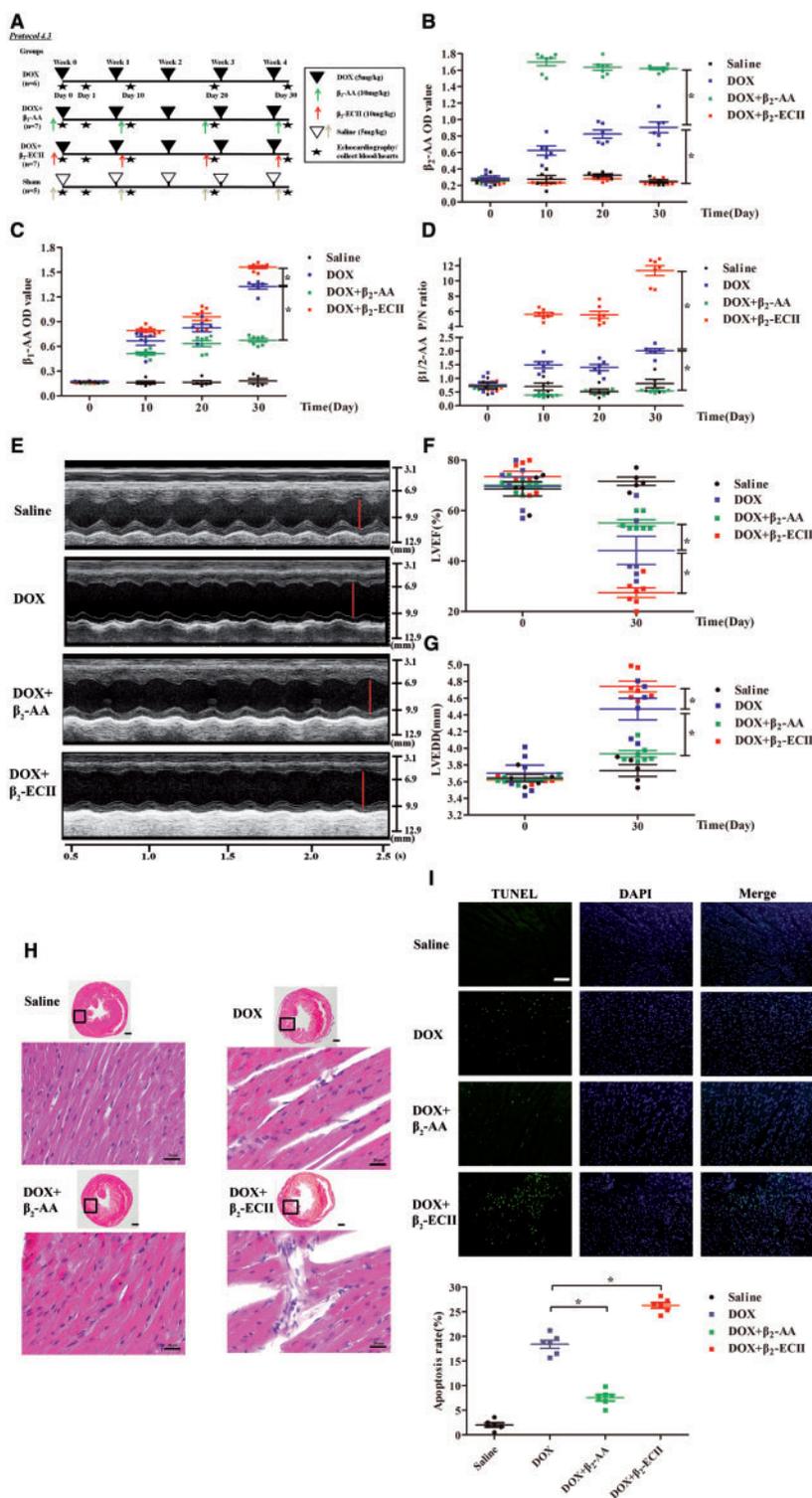


Figure 6 β_2 -AAs improves cardiac function and myocardial damage in mice HF induced by DOX. (A) The protocol to change β_2 -AAs endogenous level by β_2 -AAs passive immunity or β_2 -AAs neutralization peptide (β_2 -ECII) in a mouse HF model induced by DOX [$n = 5$ (saline), $n = 6$ (DOX), $n = 7$ (DOX+ β_2 -AAs/ β_2 -ECII)]. (B) The level of β_2 -AAs increased with β_2 -AAs passive immunity and decreased with β_2 -ECII significantly in DOX-HF mice. (C and D). The variation trend of β_1 -AAs level and $\beta_1/2$ -AAs P/N were opposite to β_2 -AAs level intervened by β_2 -AAs passive immunity or β_2 -ECII. (E–G) The LVEF and LVEDD of DOX-HF mice were partly improved with β_2 -AAs passive immunity and partly worsened when endogenous β_2 -AAs was neutralized. (H) Myocardial structural damage was partly improved with β_2 -AAs passive immunity, and increased in severity with β_2 -AAs neutralization in DOX-HF mice (time-point: 30 days; stereo picture: 1.6 \times ; bar = 500 μ M; micrograph: 40 \times ; bar = 200 μ M). (I) Upper: Apoptosis of cardiomyocytes induced by DOX was less with β_2 -AAs passive immunity and increased with β_2 -AAs neutralization in DOX-HF mice via TUNEL (time-point: 30 days, Amplification factor: 20 \times ; bar = 100 μ M). Below: The statistical results of apoptosis rate ($n = 6$ random sights/100 cells) ($n = 5$ –7, mean \pm S.E.M.; *group vs. DOX+ β_2 -AAs, $P < 0.05$, q test after two-way ANOVA).

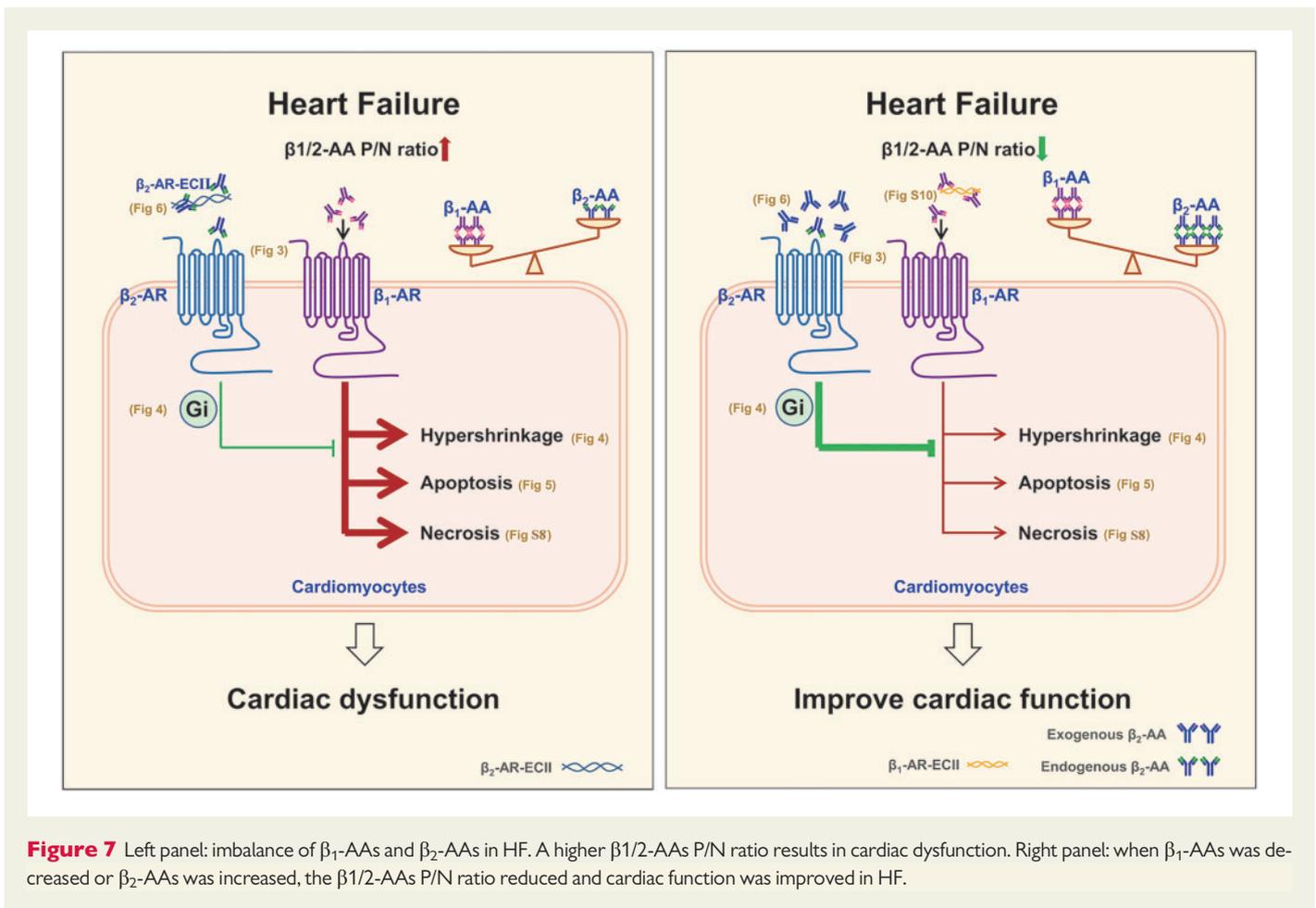


Figure 7 Left panel: imbalance of β_1 -AAs and β_2 -AAs in HF. A higher $\beta_1/2$ -AAs P/N ratio results in cardiac dysfunction. Right panel: when β_1 -AAs was decreased or β_2 -AAs was increased, the $\beta_1/2$ -AAs P/N ratio reduced and cardiac function was improved in HF.

Molecular homologies between cardiomyocyte surface proteins and microbial proteins are proposed as one possible mechanism underlying the cross-reaction of AAs directed against cardiac membrane receptors.¹⁴ Another, probable and more relevant mechanism leading to the formation of endogenous heart-directed AAs may be primary inflammation or cardiac injury followed by sudden (or chronic) release of a 'critical amount' of potential self-antigens from the myocyte surface or cytoplasm previously thought to be hidden from our immune system.¹⁴ Exposure of such antigens to the immune system may induce a host-directed autoimmune response, resulting in perpetuation of immune-mediated cardiac damage involving autoreactive T, B cells, or co-activation of both the innate and adaptive immune systems.¹⁴ In our research study, both β_1 -AAs and β_2 -AAs could be induced in three HF models (Figure 2, see Supplementary material online, Figures S2 and S9). However, the direct evidence is still needed to confirm the existence of mechanism to generate of AAs induced by inflammatory response in future research. The levels of β_1 -AAs in severe HF have been a debate up to date, as some studies have revealed that levels are high,¹⁵ whereas others have suggested they are low.¹⁶ These differences can be attributed to the different detection methods.¹⁷ Our findings showed a higher level of β_1 -AAs in NYHA I–II than in III–IV (Figure 1B), possibly due to downregulation of β_1 -AR in severe HF.¹⁸ Presumably, when β_1 -AR expression is decreased, exposure of abnormal antigen is reduced accordingly, thus leading to decreased production of AAs.¹⁹ At the same time, β_2 -AAs levels remain relatively constant because the expression of

β_2 -AR does not change in HF.¹⁸ In our study, levels of both β_1 -AAs and β_2 -AAs were high in the early stage of HF (Figure 2, see Supplementary material online, Figure S2), and were similar to the compensatory phase of patients with HF in NYHA I–II (Figure 1). The increased level can be attributed to the exposure of potential self-antigens for $\beta_{1/2}$ -AR in the myocardium. However, at the terminal stage of HF, different HF models had different levels of β_1 -AAs or β_2 -AAs compared with the early stage (Figure 2, see Supplementary material online, Figures S2 and S9).¹³ In mice HF induced by DOX, both of β_1 -AAs and β_2 -AAs levels were increased compared with the early stages (Figure 2). This may be attributed to the accumulation of β_1 -AAs and β_2 -AAs and increased of $\beta_{1/2}$ -AR in the course of HF induced by DOX.²⁰ However, the expression of $\beta_{1/2}$ -AR was decreased in the HF model induced by ISO²¹ because of the desensitization effect of $\beta_{1/2}$ -AR due to ISO stimulation. Therefore, the decrease in self-antigens resulted in decreased levels of β_1 -AAs and β_2 -AAs. In HF mice induced by β_1 -AAs, the levels of β_2 -AAs first increased and then gradually decreased (see Supplementary material online, Figure S9). However, future studies are needed to determine if the expression of β_2 -AR is changed in HF mice induced by β_1 -AAs passive immunity. β_2 -AAs were negatively correlated with cardiac function in the HF model induced by β_1 -AAs. This is direct evidence that β_2 -AAs play a protective role in HF induced by β_1 -AAs. Taken together with the study of HF patient serum samples and three HF mouse models, our findings revealed that β_1 -AAs and β_2 -AAs are closely related, and the level of the two AAs may affect the cardiac function of HF.

4.2 The β_1/β_2 -AAs P/N ratio is negatively correlated with cardiac function

To study the specific relationship between β_1 -AAs and β_2 -AAs in HF, correlation analysis of β_1 -AAs/ β_2 -AAs and cardiac function was used in patients with systolic HF. The results showed that the levels of β_1 -AAs or β_2 -AAs were not correlated with cardiac function (see [Supplementary material online, Figure S1](#)), similar to the results of previous studies.²² The effects of β_1 -AAs and β_2 -AAs on cardiac function might be due to the nature of the 'bidirectional' effect of receptor activation. Although β_1 -AAs can activate β_1 -AR to result in cardiomyocyte apoptosis or decrease autophagy to deteriorate the progress of HF,^{23,24} β_1 -AAs can also activate β_1 -AR to increase Ca^{2+} and cAMP leading to positive chronotropic and inotropic actions.^{25,26} β_1 -AAs can compensate for decreasing cardiac function partly in the early stage of HF. Thus, the 'bidirectional' effect of β_1 -AAs may be the reason why β_1 -AAs levels are not related to cardiac function in HF. The activation of β_2 -AR induced by β_2 -AAs has a similar mechanism as β_1 -AAs. In our research, β_2 -AAs can activate the β_2 -AR/Gi pathway ([Figure 4D](#)). Although the activation of β_2 -AR/Gi can inhibit cardiomyocyte apoptosis induced by β_1 -AR,²⁷ it can also inhibit the activity of adenylyl cyclase leading to a decrease of cAMP and calcium. This effect can inhibit the shrinkage of cardiomyocytes and be ultimately detrimental for cardiac function in HF. Thus, β_2 -AAs activate the β_2 -AR/Gi pathway and exhibit a 'bidirectional' effect of cardiac function in HF. Thus, it appears that the 'bidirectional' effect of β_1 -AAs and β_2 -AAs causes the lack of relationship to cardiac function in HF. Further studies showed that the β_1/β_2 -AAs P/N ratio was negatively related to cardiac function in patients with HF ([Figure 1D](#) and [E](#)). However, the correlation was weak and influenced by several extreme values, in accordance with previous studies.²⁵ β_1 -AAs levels in different patients with HF had different effects on cardiomyocyte function; some could significantly activate β_1 -AR, whereas others did not.²⁵ Individual differences of β_1 -AAs levels in patients with HF might be a reason that extreme values occurred and a weak correlation was observed due to different IgGs subtypes⁷ or affinity to β_1 -ECII.²⁸ Two HF models induced by ISO or DOX were established to examine determine if a correlation existed. The two HF models revealed that the ratio was negatively correlated to cardiac function, the correlation coefficient was larger, and the distribution of data was more concentrated ([Figure 2](#), see [Supplementary material online, Figure S2](#)). Thus, the integrated effects of β_1 -AAs and β_2 -AAs affect the cardiac function of patients with HF. We did not find that the ratio of β_1 -AAs and the three AAs (β_3 -AAs, α_1 -AAs, and AT1-AAs) were related to cardiac function in these patients with HF (see [Supplementary material online, Table S2](#)). This suggested that the β_1/β_2 -AAs P/N ratio might be negatively related to cardiac function in specific HF cases.

4.3 β_2 -AAs inhibits hyper-shrinking, apoptosis, and necrosis of cardiomyocytes stimulated by β_1 -AAs

The ratio of β_1 -AAs and β_2 -AAs P/N value was negatively related to cardiac function and the ratio in healthy volunteers was lower than in patients with HF ([Figure 1](#)). It is likely that the increased ratio might be caused by elevated β_1 -AAs levels and (or) lowered β_2 -AAs levels, suggesting that β_1 -AAs worsens or β_2 -AAs protects against cardiac function in HF. The fact that β_1 -AAs can cause HF is widely known. To study whether β_2 -AAs could reduce deterioration of cardiac function and the specific mechanism, NRCMs and mice HF induced by β_1 -AAs passive immunity or DOX were established ([Figures 2–7](#)). Our results indicated

that NRCMs beat frequency and duration were less upon stimulation by β_1 -AAs- and β_2 -AAs-double positive IgGs compared with β_1 -AAs-single positive IgGs ([Figure 4](#)). This phenomenon could be neutralized or blocked by β_2 -ECII peptides or β_2 -AR inhibitors. The β_2 -AR/Gi pathway was activated by β_2 -AAs to inhibit the overactivation of β_1 -AR induced by β_1 -AAs. β_1 -AAs could induce myocardial apoptosis and fibrosis via β_1 -AR.²⁹ By use of a mouse HF model, when the β_1 -AAs level was stabilized by passive immunity, worse cardiac function was observed at lower β_2 -AAs levels (see [Supplementary material online, Figure S9](#)), suggesting that β_2 -AAs play a protective role in cardiac function at HF caused by β_1 -AAs. In another mouse HF model induced by DOX, we decreased the level of β_1 -AAs by β_1 -ECII and consequently, the decreasing β_1 -AAs levels could increase cardiac function (see [Supplementary material online, Figure S10](#)). Upon injection of β_2 -AAs, the production of β_1 -AAs was decreased, the P/N ratio was significantly decreased, and the cardiac function and myocardial damage was improved. However, after injection of β_2 -ECII to neutralize the endogenous β_2 -AAs, the production of β_1 -AAs was increased, the P/N ratio significantly increased, and the cardiac function deteriorated and myocardial damage was severe ([Figure 6](#)). Thus, by increasing of β_2 -AAs to reduce β_1/β_2 -AAs P/N ratio, cardiac function can be improved and myocardial damage can be inhibited. Since it was related to cardiac function in HF, the P/N ratio might be more meaningful than individual β_1 -AAs or β_2 -AAs levels for assessing cardiac function in HF.

5. Conclusions

In summary, we observed that the increased ratio of β_1 -AAs/ β_2 -AAs was associated with worsening HF in patients. Moreover, β_2 -AAs from patients with HF was capable of suppressing the hyper-shrinking, necrosis, and apoptosis induced by β_1 -AAs from HF patients in NRCMs. Finally, β_2 -AAs was capable of alleviating myocardial damage and heart dysfunction induced by DOX in mice. The imbalance of β_1 -AAs and β_2 -AAs in patients with HF is a meaningful mechanism to progression of HF, and the increasing ratio of β_1/β_2 -AAs P/N might be a good clinical indicator of the deterioration state of cardiac function in patients with HF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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