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β_1-Adrenergic Receptor Autoantibodies From Heart Failure Patients Enhanced TNF-α Secretion in RAW264.7 Macrophages in a Largely PKA-Dependent Fashion

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ABSTRACT

Autoantibodies against the second extracellular loop of β_1-adrenergic receptor (β_1-AA) not only contribute to increased susceptibility to heart failure, but also play a causative role in myocardial remodeling through their catecholamine-like effects via binding with the β_1-adrenergic receptor. The current study was designed to determine whether β_1-AA isolated from the sera of heart failure patients could cause TNF-α secretion from the murine macrophage-like cell line RAW264.7. Blood samples were collected from 40 patients who had suffered heart failure, as well as from 40 healthy subjects. The titer of β_1-AA and the level of TNF-α were detected using ELISA. The effect of β_1-AA on murine macrophage-like cell line RAW264.7 proliferation was detected by CCK-8 kits and CFSE assay. Western blot assay was used to analyze the expression of phospho-VASP. β_1-AA appeared more frequently in patients with heart failure than in healthy subjects. The β_1-AA isolated from heart failure patients promoted an increase of TNF-α levels, which could be completely blocked by the selective β_1-adrenergic receptor antagonist metoprolol and the second extracellular loop of β_1-adrenergic receptor (β_1-AR-EC_{II}), but only partially inhibited by PKA inhibitor H89. Furthermore, the β_1-AA could enhance the proliferation of RAW264.7 cells in vitro. Meanwhile, the expression of phospho-VASP was markedly increased in the presence of β_1-AA. These results demonstrate for the first time that the β_1-AA isolated from heart failure patients could bind with β_1-AR on the surface of RAW264.7 cells, causing the release of TNF-α largely in a PKA-dependent fashion. J. Cell. Biochem. 113: 3218–3228, 2012.

KEY WORDS: RECEPTOR, ADRENERGIC, BETA-1; AUTOANTIBODY; HEART FAILURE; MACROPHAGE; TNF-ALPHA

Abbreviations used: β_1-AA, autoantibodies against the second extracellular loop of β_1-adrenergic receptor; β_1-AR, β_1-adrenergic receptor; β_1-AR-EC_{II}, the second extracellular loop of β_1-adrenergic receptor; BSA, bovine serum albumin; CCK-8, colorimetric cell-counting-8; CFSE, carboxyfluorescein diacetatesuccinimidyl ester; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin fractions G; PBS, phosphate-buffered saline; PKA, protein kinase A; VASP, vasodilator-stimulated phosphoprotein.

The authors declare that they have no competing interests.

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Proinflammatory cytokines—including tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and IL-8—are important inflammatory mediators that are rapidly induced in the early stages of inflammatory diseases or during the injury process. They modulate a myriad of healing processes, but if overproduced, these cytokines can exacerbate the severity of many inflammatory diseases, such as atherosclerosis, rheumatoid arthritis, and acute ischemic stroke. TNF-α is a pleiotropic inflammatory cytokine mainly produced by activated macrophages. It can exert profound cytostatic and cytocidal effects on tumor cells and make changes to other cells, including cardiomyocyte growth and differentiation. Recent researches have shown that the elevation of plasma TNF-α levels is involved in many pathological processes [Morimoto et al., 2009; Stankovic-Popovic et al., 2011; Wang et al., 2011], but the mechanisms remain unclear.

Levine et al. [1990] first reported that the levels of TNF-α were significantly elevated in the plasma of patients with severe heart failure, cardiac cachexia, or chronic heart failure. Subsequently, Wiedermann et al. [1993] confirmed these results, and found that the levels of TNF-α in plasma correlated closely with the clinical characteristics of heart failure. These results suggested that certain elements may contribute to the release of a large number of TNF-α in the pathogenesis of chronic heart failure. However, the mechanisms involved in these processes have not been elucidated.

Other reports made in the 1990s showed that the autoimmune antibody against the second extracellular loop (197–223, the homology of β_1-AR-EC_{II} between human and mouse is 100% [Jahns et al., 1999]) of β_1-adrenergic receptor (β_1-AA) is present in the sera of patients with cardiovascular diseases [Wallukat and Wollenberger, 1987; Magnusson et al., 1990, 1994]. They proposed that β_1-AA mediates catecholamine-like actions leading to the beating rate in spontaneously beating neonatal rat cardiomyocytes [Jane-wit et al., 2007]. It has been reported that catecholamines can either activate [Guo et al., 2011; Nguyen et al., 2011] or suppress [Hasko et al., 1998; Zinyama et al., 2001] macrophage functions, such as the secretion of cytokines. Furthermore, published papers demonstrated that β_1-adrenergic receptor (β_1-AR) is expressed on the surface of RAW264.7 cells [Sun et al., 2005]. However, it remains unclear if the β_1-AA isolated from chronic heart failure patients, which is similar to catecholamine, could recognize the corresponding receptor, interfere with macrophages, and release a large number of TNF-α molecules.

To address these questions, this study explored the effect of β_1-AA, one of the catecholamine-like substances with β_1-adrenergic activity, on the production of TNF-α in murine macrophage-like cell line RAW264.7. In addition, we further investigated the possible pathways behind its effects.

MATERIALS AND METHODS

MATERIALS

Metoprolol (selective β_1-adrenergic receptor antagonist), isoproterenol (β_1/β_2-adrenergic receptor agonist), and H89 (selective PKA inhibitor) were purchased from Sigma–Aldrich Chemicals Company (St. Louis, MO). Carboxyfluorescein diacetatesuccinimidyl ester (CFSE) was purchased from Invitrogen Company (Merelbeke, Belgium). Polyclonal antibodies directed against phosphor-VASP Ser157 and total VASP were obtained from Cell Signaling Technologies (Beverly, MA). All chemicals utilized in this study were of analytical grade.

PATIENTS AND SAMPLES

This study adheres to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, revised November 13, 2001, effective December, 13, 2001. Forty chronic heart failure patients were recruited from People's Liberation Army Air Force General Hospital and General Hospital of Tonghua Mining Group Co., Ltd. Criteria for inclusion were symptoms of chronic heart failure (New York Heart Association functional class II–IV) for >1 year. All patients showed heart structure and functional changes as well as a left ventricular ejection fraction below 45% by echocardiography. Exclusion criteria were hypertrophic cardiomyopathy, hypertensive heart disease, valvular heart disease, diabetes, autoimmunopathy, and certain infectious diseases. The control group consisted of 40 healthy subjects randomly selected from the same community with normal clinical, ECG, and echocardiography examinations. On the basis of the resulting measurements of β_1-AA, the chronic heart failure patients were divided into a β_1-AA-positive group (n = 18) and a β_1-AA-negative group (n = 22). Clinical characteristics are summarized in Table I.

The research protocol was approved by the Institutional Committee for the Protection of Human Subjects of Capital Medical University. All patients were informed of the purpose and protocol

TABLE I. Clinical Data of Patients and Healthy Subjects (Mean ± SD)

	β_1-AA-positive group (n = 18)	β_1-AA-negative group (n = 22)	Healthy (n = 40)
Demographics			
Age (year)	59 ± 3	55 ± 6	49 ± 9
Gender (male/female)	15/3	17/5	30/10
NYHA	3.3 ± 0.4	2.8 ± 0.3	ND
Etiologies of HF			
CAD, n(%)	10	13	ND
Cardiomyopathy, n (%)	8	9	ND
Echocardiography			
EF (%)	30.1 ± 2.1*	38.5 ± 1.8*	69.4 ± 5.3

HF, heart failure; NYHA, New York Heart Association classification; CAD, coronary artery disease; EF, left ventricular ejection fraction. Values are expressed as mean ± SD.
*$P < 0.01$ versus healthy group.

of the investigational nature of the study. Both oral informed consent and written consent were obtained.

Venous blood samples were collected in vials without an anticoagulant agent. After centrifugation at 4°C, the serum was immediately separated and stored at −80°C until assay.

IMMUNOASSAYS WITH SYNTHETIC ANTIGENS

Peptide synthesis. The peptide corresponding to the sequence (amino acid residues 197–223) of the second extracellular loop of the human β_1-AR [Zuo et al., 2010], H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C, was synthesized by the solid-phase synthesis process, using an automated peptide-synthesizer. The peptide was judged by high-performance liquid chromatography analysis on an automated amino-acid analyzer, and 98% purity was achieved. This work was completed by Qiang Yao (Shanghai) Bio-Scientific Co., Ltd.

Enzyme-linked immunosorbent assay (ELISA). The titer of β_1-AA was measured by enzyme-linked immunosorbent assay (ELISA), as we have done previously, and the results were expressed as optical-density (OD) values [Liu et al., 1999]. Briefly, synthetic peptide 197–223 (H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C), which is the sequence of the second extracellular loop of β_1-AR (5 mg/ml) in a 100 mmol/L Na_2CO_3 solution (pH 11.0), was coated on microtiter plates overnight at 4°C. The wells were then saturated with 0.1% PMT buffer (0.1% [w/v] albumin bovine V, 0.1% [v/v] Tween-20 in phosphate-buffered saline [PBS], pH 7.4) for 1 h at 37°C. After washing three times with PBS-T, human-sera dilutions were added to the saturated microtiter-plates for 1 h at 37°C. After three washings, biotinylated goat antihuman IgG antibodies (Sigma; 1:1,000 dilutions in PMT) were added for 1 h at 37°C. Following three washings, streptavidin-peroxidase conjugate (Sigma) at 1:2,000 dilution in the same buffer was added to the wells and incubated under the same conditions. Finally, 2, 2-azino-di (3-ethylbenzothiazoline) sulfonic acid (ABTS)-H_2O_2 (Roche, Basel, Switzerland) substrate buffer was added and reacted for 30 min in the dark at room temperature. The OD values were measured at 405 nm using a microplate reader (Spectra Max Plus, Molecular Devices, Sunnyvale, CA). We also calculated the positive/negative (P/N) ratio [(specimen OD−blank OD)/(negative control OD−blank control OD)] of each sample. Those samples with a P/N value of at least 2.1 were considered to be β_1-AA positive.

PREPARATION OF IMMUNOGLOBULIN G

On the basis of a sera-positive response in an ELISA to peptides 197–223 of the β_1-AR, immunoglobulin fractions G (IgG) from the mixed sera of 18 β_1-AA-positive heart failure patients were prepared by MabTrap Kit (Amersham Bioscience, Uppsala, Sweden). The IgGs from the mixed sera of 22 β_1-AA-negative heart failure patients were acquired in an identical fashion and used as a control. The total purified IgG concentration (μg/ml) was determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, USA).

CELL CULTURE

RAW264.7 cells were obtained from the Institute of Basic Medical Sciences, Peking Union Medical College, cultured in DMEM medium (Sigma, St. Louis, MO), and supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere.

IMMUNOFLUORESCENCE STAINING

RAW264.7 cells were gently washed with PBS (pH 7.4) and immediately fixed with 4% paraformaldehyde (w/v) for 20 min. Cells were blocked in PBS containing 5% bovine serum albumin (BSA; w/v), and incubated overnight at 4°C with the IgG fractions (25 μg/ml) from β_1-AA-positive or β_1-AA-negative heart failure patients. Following 3 PBS washes, cells were incubated in donkey anti-human IgG tagged with fluorescein isothiocyanate (FITC) as the secondary antibody for 1 h in the dark at 37°C. After being rinsed with PBS, cover slips with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) stain nuclei. Negative controls were performed by omitting primary antibodies. Images were acquired using a Zeiss 510 Meta Confocal microscope (63 power oil 1.40 NA [Zeiss, Germany], pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software.

DETERMINATION OF CYTOKINE PRODUCTION

Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to quantify the TNF-α concentration in cell-free supernatants, according to the manufacturer's protocols. The absorbance at 450 nm was measured using a microplate reader with the wavelength correction set at 630 nm.

RAW264.7 CELLS PROLIFERATION ASSAY

The proliferation effect of β_1-AA on RAW264.7 cells was measured with a colorimetric cell-counting kit (CCK-8; Dojindo Laboratories, Japan) and carboxyfluorescein diacetatesuccinimidyl ester (CFSE) staining.

CCK-8 ASSAY

RAW264.7 cells (5×10^5 cells/ml) were cultured for 48 h in either the presence or absence of indicated concentrations of β_1-AA (25 μg/ml), H89 (1 μmol/L), metoprolol (1 μmol/L), or isoproterenol (0.1 μmol/L). Antagonists were added 1 h before the addition of agonist or β_1-AA. After the different treatments, 10 μl CCK-8 solution was added to each well, and the cells were incubated for 2 h at 37°C. The absorbance at 450 nm was measured using a microplate reader with the wavelength correction set at 630 nm.

CFSE-LABELING OF RAW264.7 CELLS

A CFSE stock (10 mmol/L in DMSO) was diluted in PBS. RAW264.7 cells were suspended in PBS supplemented with 1% BSA and 4 μmol/L CFSE (2×10^7 cells/ml) for 10 min at 37°C with 5% CO_2. Cells were washed and diluted in a 0.5 ml culture medium for 30 min at 37°C with 5% CO_2 to stabilize the CFSE-labeling. The efficiency of labeling was determined before the cells were used in the experiments and was >95%.

ANALYSIS OF cAMP PRODUCTION

RAW264.7 cells were washed twice in Tris buffer containing 120 mmol/L NaCl, 1 mmol/L $MgCl_2$, 5 mmol/L KCl, 0.6 mmol/L $CaCl_2$, 25 mmol/L Tris (hydroxymethyl-amino-ethane), 5 mmol/L glucose, and 0.1 mmol/L human albumin, adjusted to pH 7.4

with HCl. Cells were suspended in RPMI 1640/FBS medium to a final density of 2×10^6 cells/ml. The samples were incubated with 1-methyl-3-isobutylxanthine (0.5 mmol/L) for 10 min in either the absence or presence of the β_1-AA or agonist, without or with an antagonist. Reactions were terminated by adding 2 N HCl-0.1 mol/L EDTA followed by incubating the samples at 80°C for 10 min. After centrifugation of precipitated protein, the samples were neutralized with $CaCO_3$ and cAMP was measured using an enzyme immunoassay (Biotrak, Amersham, UK) as specified by the manufacturer. The cAMP concentrations are expressed as pg/ml.

ANALYSIS OF VASP-Ser157 PHOSPHORYLATION AND TOTAL VASP

Cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at 10,000 rpm for 20 min at 4°C. Protein concentration was determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, USA). An equal volume of 2× SDS sample buffer (0.1 M Tris–Cl, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) was added to an aliquot of the supernatant fraction from the lysates, and the mix was boiled for 5 min. Aliquots of 30 μg of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis for 1.5 h at 110 V. The separated proteins were transferred to PVDF membranes for 2 h at 20 mA with SD Semi-dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffed saline containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. The membranes were then incubated with rabbit polyclonal anti-mouse phospho-VASP (Ser 157) and anti-mouse VASP at 1:1,000 concentrations in 5% nonfat milk in TBS-T overnight at 4°C, and bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were washed and then developed using a Western Blotting Luminol Reagent system and autoradiography.

STATISTICAL ANALYSIS

Values are expressed as mean ± SD. Statistical analysis was performed with SPSS 13.0 programs. The *t*-test was used to compare two independent sample means, and one-way ANOVA was used to compare the means of more than two samples. A value of $P < 0.05$ was considered statistically significant.

RESULTS

SERA LEVELS OF β_1-AA AND TNF-α MARKEDLY INCREASED IN HEART FAILURE PATIENTS COMPARED WITH HEALTHY SUBJECTS

Using ELISA, we tested sera from 40 healthy subjects and 40 patients with chronic heart failure for TNF-α levels and the presence of autoantibodies directed against the second extracellular loop of β_1-AR. Clinical data are summarized in Table I. There was no difference in age or gender distribution at sampling. Compared with normal individuals, heart failure patients had markedly increased sera levels of TNF-α and β_1-AA (48.29 ± 13.29 pg/ml vs. 7.68 ± 4.86 pg/ml, $P < 0.01$; 0.582 ± 0.307 vs. 0.173 ± 0.115, $P < 0.01$; Fig. 1A,B). As illustrated in Figure 1C, only 3 of 40 normal subjects were β_1-AA positive (7.5%), whereas 18 of 40 heart failure-patients had a P/N value >2.1 (45%). These data demonstrated that sera levels of TNF-α and β_1-AA markedly increased in patients with heart failure when compared with control subjects.

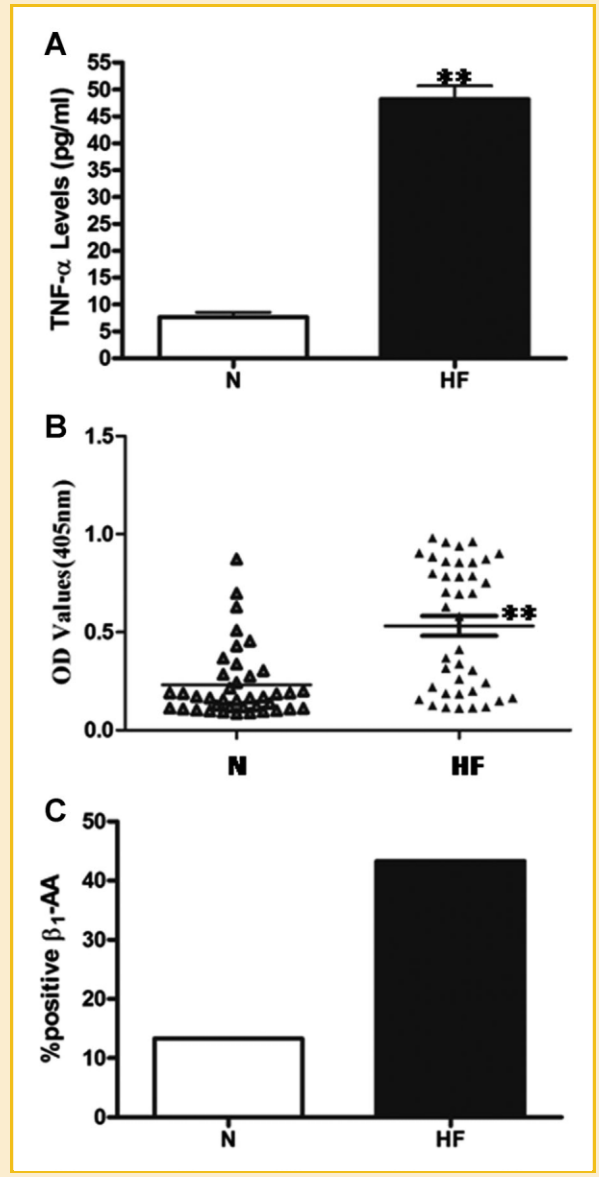

Fig. 1. Concentrations of TNF-α and β_1-AA in 40 healthy subjects and 40 patients with heart failure. A: Serum TNF-α level in 40 healthy subjects and 40 patients with heart failure. B: Concentration of β_1-AA from the sera of 40 healthy subjects (*open squares*) and 40 patients with heart failure (*filled squares*). Scatter plot represents concentration of β_1-AA by each subject in each group. Experiments were repeated twice per sample. C: Percentage of β_1-AA positive sera from two different groups. Experiments were repeated twice per sample. N, normal group; HF, heart failure. $^{**}P < 0.01$ versus N group.

β_1-AA BOUND TO β_1-ARs ON THE SURFACE OF RAW264.7 CELLS

We determined the specificity of IgG fraction isolated from β_1-AA positive sera of heart failure patients binding to β_1-ARs by immunoflurescense staining. We found that β_1-AA at a 1/1,000 dilution showed immunoflurescense staining of β_1-AR and DAPI (Fig. 2B). As shown, β_1-AR staining was predominantly in the membrane and cytoplasm with no merge with DAPI staining. In contrast, there was no detectable binding by IgG samples isolated from β_1-AA-negative sera of heart failure patients (Fig. 2C). From

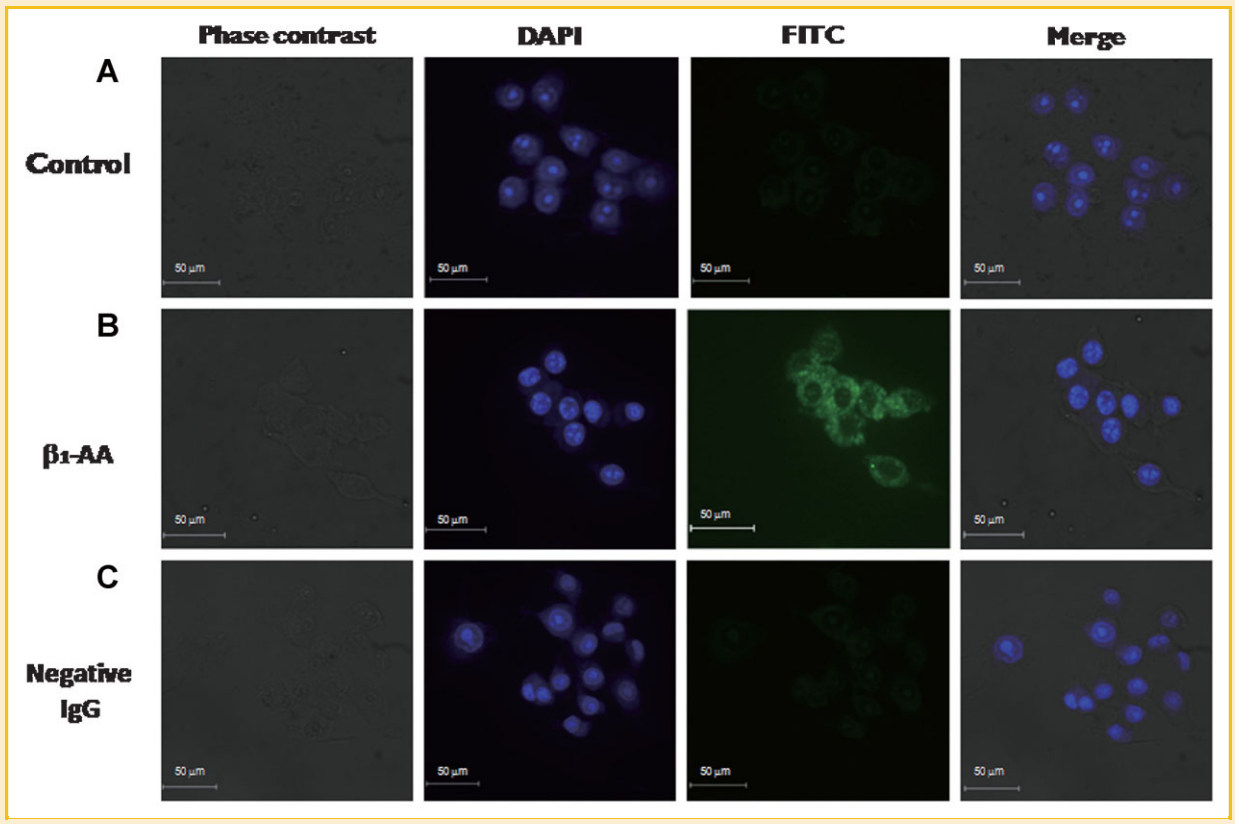

Fig. 2. β_1-AA isolated from HF patients bound to β_1-ARs on the surface of RAW264.7 cells. The binding of β_1-AA isolated from β_1-AA-positive HF patients (25 μg/ml) or negative IgGs obtained from β_1-AA-negative HF patients (25 μg/ml) with the β_1-ARs on the RAW264.7 cells was determined by confocal microscopy, respectively, and β_1-AR was identified using an anti-β_1-AR antibody (green). Nuclei were labeled with DAPI (blue). The negative control was performed by omitting primary antibodies during the incubation. Maginification ×400.

these results, we concluded that IgG fraction isolated from β_1-AA positive sera of heart failure patients showed a pattern of β_1-AR specific binding.

β_1-AA PROMOTED THE PRODUCTION OF TNF-α IN RAW264.7 CELLS

We examined the effect of different concentrations of β_1-AA (12.5, 25, 50 μg/ml) on TNF-α secretion, and found that β_1-AA enhanced TNF-α production in a concentration-dependent manner (Fig. 3A). Therefore, the middle concentration (25 μg/ml) was chosen for continued study, a dose comparable to the concentration in heart failure patients' sera. As illustrated in Figure 3B, the addition of IgG fraction (25 μg/ml) isolated from β_1-AA-positive sera of heart failure patients increased TNF-α production (212.64 ± 9.72 pg/ml vs. Vehicle group 37.69 ± 16.85 pg/ml, $P < 0.01$; 201.64 ± 9.72 pg/ml vs. negative IgG group 79.58 ± 17.94 pg/ml, $P < 0.01$). The enhanced TNF-α secretion observed following β_1-AA treatment was inhibited by preincubation with antigenic peptides corresponding to β_1-AR-EC_{II} (1 μmol/L; $P > 0.05$; Fig. 3C). Moreover, the effect was completely blocked by the addition of 1 μmol/L of the selective β_1-AR antagonist metoprolol ($P > 0.05$; Fig. 3D). However, the elevation of TNF-α levels treated by β_1-AA was partially inhibited by the PKA inhibitor H89 (1 μmol/L; 146.3 ± 23.34 pg/ml vs. β_1-AA group 201.64 ± 9.72, $P < 0.05$; 146.3 ± 23.34 vs. negative IgG group 79.58 ± 17.94, $P < 0.05$; Fig. 3E). Collectively, these results suggested that β_1-AA enhanced secretion of TNF-α.

β_1-AA PROMOTED THE PROLIFERATION OF RAW264.7 CELLS

RAW264.7 cells were treated in either the presence or absence of β_1-AA (25 μg/ml) for 48 h. As shown in Figure 4, the presence of β_1-AA increased RAW264.7 cell proliferation (viable cell number: $9,655 \pm 573$ vs. $31,495 \pm 4,687$, $P < 0.01$; Fig. 4A). However, administration of the same concentration of β_1-AA-negative IgG purified from the mixed sera of 22 heart failure patients revealed no proliferation effect on RAW264.7 cells ($P > 0.05$; Fig. 4A). The proliferation effect of β_1-AA was inhibited by the addition of antigenic peptides corresponding to β_1-AR-EC_{II} (1 μmol/L; $P > 0.05$; Fig. 4B). We also measured RAW264.7 cells proliferation by CFSE assay (Fig. 4C,D), finding the same proliferative effect as the CCK-8 assay.

β_1-AA ENHANCED RAW264.7 CELLS PROLIFERATION THROUGH THE β_1-AR/CAMP/PKA PATHWAY

The most common signaling mechanism initiated by β_1-AR stimulation is the β_1-AR/cAMP/PKA pathway [Chiale et al.,

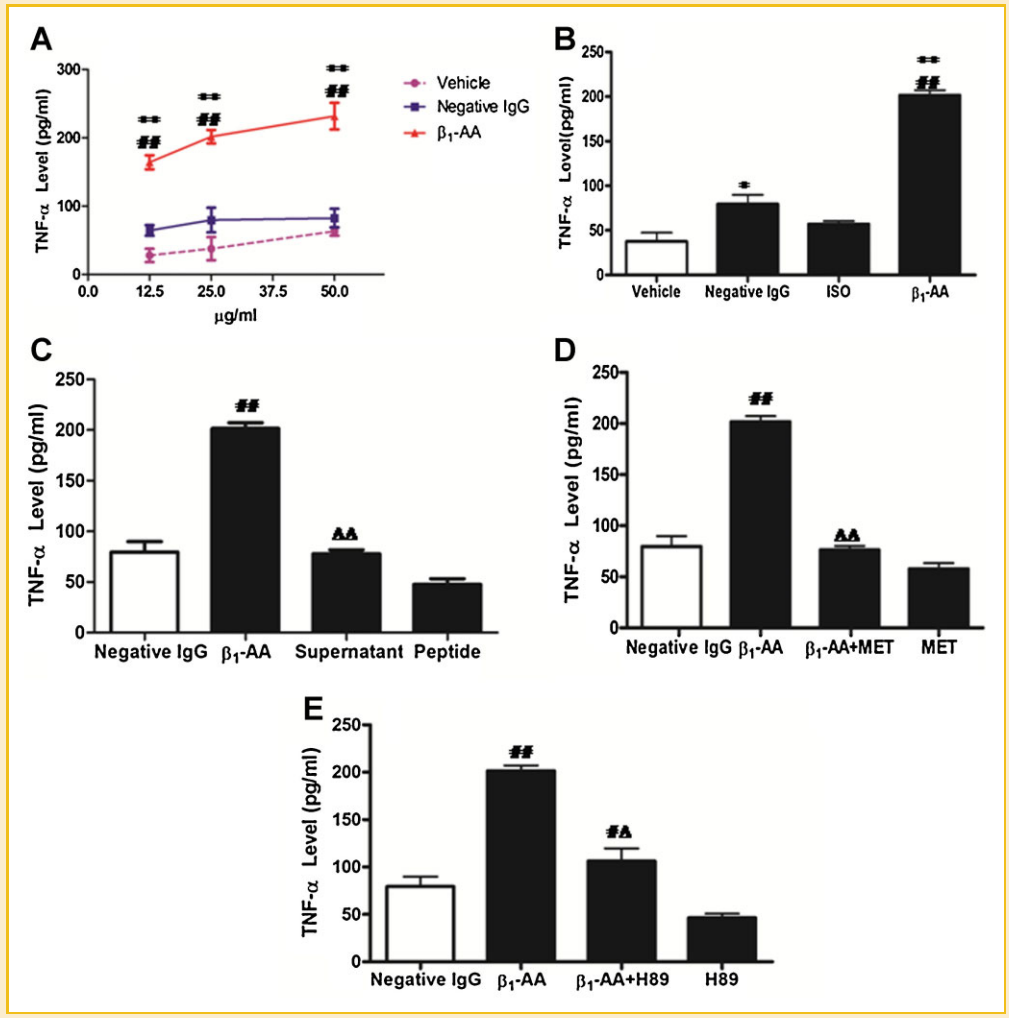

Fig. 3. β_1-AA isolated from HF patients promoted TNF-α production in RAW264.7 cells. A: Different concentrations of β_1-AA (12.5, 25, 50 μg/ml) promoted TNF-α production in RAW264.7 cells in a concentration-dependent manner. $^{**}P < 0.01$ versus vehicle group, $^{\#\#}P < 0.01$ versus negative IgG group. B: RAW264.7 cells (5×10^5 cells/ml) were stimulated for 48 h in the presence of β_1-AA (25 μg/ml) or isoproterenol (0.1 μmol/L), and then cell-free supernatants were collected to detect TNF-α levels by ELISA assay. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle group, $^{\#\#}P < 0.01$ versus negative IgG group. n = 9/group. C: β_1-AA (25 μg/ml) and the peptide corresponding to the sequence of the second extracellular loop of the human β_1-AR (β_1-AR-EC$_{II}$, 1 μmol/L) co-incubated for 1 h at 37°C, and then supernatants were collected to treat RAW264.7 cells. $^{\#\#}P < 0.01$ versus negative IgG group, $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. n = 9/group. D: RAW264.7 cells were treated with the selective β_1-AR antagonist metoprolol (1 μmol/L) for 1 h at 37°C and 5% CO_2 before β_1-AA. $^{\#\#}P < 0.01$ versus negative IgG group, $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. n = 9/group. E: RAW264.7 cells were pretreated with the selective PKA inhibitor H89 (1 μmol/L) for 1 h at 37°C and 5% CO_2 before being treated by β_1-AA. $^{\#\#}P < 0.01$ versus negative IgG group, $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. n = 9/group. Data were presented as means ± SD of six independent experiments. ISO: isoproterenol, MET: metoprolol; supernatant: β_1-AA + β_1-AR-EC$_{II}$; peptide: β_1-AR-EC$_{II}$.

1995]. To determine whether β_1-AA-stimulated RAW264.7 cells proliferation resulted from the triggering of this pathway, the selective β_1-AR antagonist metoprolol (1 μmol/L) and the PKA inhibitor H89 (1 μmol/L) were used to block the pathway prior to treatment with β_1-AA (25 μg/ml), and then the activity of PKA was determined. The results demonstrated that the proliferation of RAW264.7 cells mediated by β_1-AA was completely inhibited by metoprolol ($P > 0.05$; Fig. 5A) and H89 ($P > 0.05$; Fig. 5C). In addition, we determined the accumulation of intracellular cAMP in RAW264.7 cells treatment with β_1-AA (25 μg/ml). As summarized in Figure 5B, basal levels of cAMP (124 ± 4.33 pg/ml) were detected in RAW24.7 cells, and β_1-AA (25 μg/ml) significantly enhanced accumulation of intracellular cAMP levels (323 ± 7.1 pg/ml, $P < 0.01$), whereas metoprolol (1 μmol/L) antagonized the β_1-AA-induced accumulation of cAMP (Fig. 5B). In immunoblot analysis, following the addition of β_1-AA to RAW264.7 cells, phosphorylation of VASP at Ser157 rapidly and markedly increased. When cells were first treated with metoprolol and H89, subsequent β_1-AA-induced VASP phosphorylation was abrogated. However, β_1-AA treatment suppressed total VASP expression, which was also reversed by metoprolol and H89 (Fig. 5D,E). Collectively, these results suggest that the β_1-AR/cAMP/PKA pathway was involved in RAW264.7 cells proliferation treated by β_1-AA.

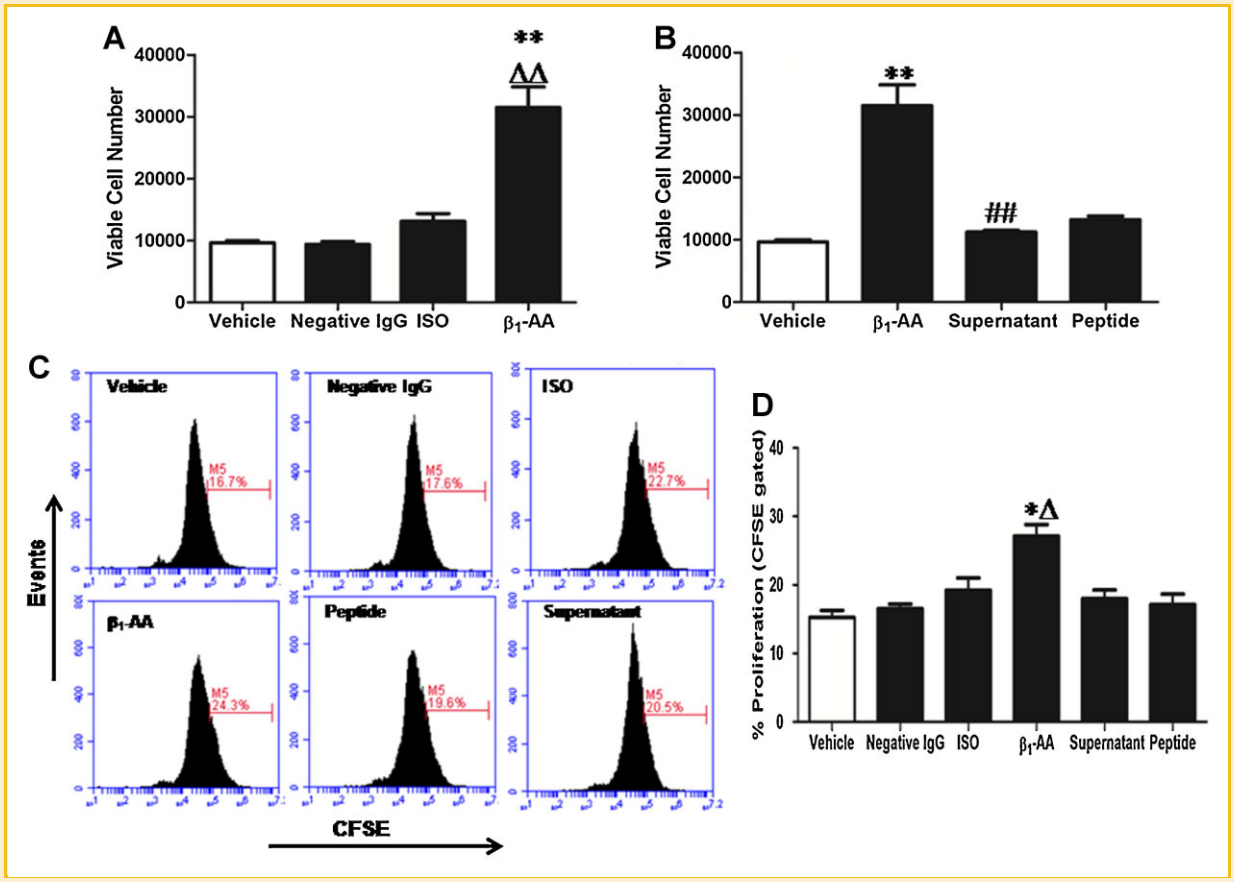

Fig. 4. β_1-AA isolated from heart failure patients significantly promoted the proliferation of RAW264.7 cells. A: RAW264.7 cells (5×10^5 cells/ml) were incubated for 48 h at 37°C and 5% CO_2 in the presence of β_1-AA (25 μg/ml) or Isoproterenol (0.1 μmol/L). Cell proliferation was measured by CCK-8 assay using OD450 nm. $^{**}P < 0.01$ versus vehicle group; $^{\#\#}P < 0.01$ versus negative IgG group. n = 9/group. B: β_1-AA (25 μg/ml) and β_1-AR-EC_{II} (1 μmol/L) co-incubated for 1 h at 37°C, and then supernatants were collected to treat RAW264.7 cells. $^{**}P < 0.01$ versus vehicle group; $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. n = 9/group. C: RAW264.7 cells were labeled with 4 μmol/L CFSE, and cell proliferation was measure by flow cytometry. Data shown here are from one of three different experiments with similar results. The bars and numbers stand for the percentage of proliferated living RAW264.7 cells in CFSE gate. D: Bar graph shows the percentage of proliferated ($CFSE^{lo}$) RAW264.7 cells among total RAW264.7 cells. N = 3, $^{*}P < 0.05$ versus vehicle group; $^{\Delta}P < 0.05$ versus negative IgG group, ISO: isoproterenol; supernatant: β_1-AA + β_1-AR-EC_{II}; peptide: β_1-AR-EC_{II}.

DISCUSSION

In previous studies, the concentrations of TNF-α [Byrkjeland et al., 2011; Tsarouhas et al., 2011] and of circulating autoantibodies directed against the second extracellular loop of β_1-AR were found to be increased in patients with heart failure [Liu et al., 1999; Arndt-Marić et al., 2010] when compared with healthy, control subjects. Our results confirmed these findings. In fact, we reported that autoantibodies directed against the second extracellular loop of β_1-AR existed in 45% of sera collected from 40 patients with heart failure, which was significantly higher than the levels in the 40 healthy, control subjects. Meanwhile, we found that the IgG fraction isolated from β_1-AA-positive sera of heart failure patients (as detected by binding to receptor fragments) recognized the native membrane-bound β_1-adrenergic receptors in RAW264.7 cells. All of these antibodies were directed against the second extracellular domain, which is known to affect ligand binding [Dohlman et al., 1990] and may induce immune responses, according to some suggestions [Magnusson et al., 1989]. Our results indicate that β_1-AA may be one of the abnormal immune phenomena that are characteristic of heart failure, suggesting its involvement in the pathophysiology of essential heart failure.

Recent clinical reports showed that the selective β_1-AR antagonist, metoprolol, decreased the frequency and the geometric mean titer of β_1-AA [Miao et al., 2006; Nagatomo et al., 2009]. Moreover, Wallukat et al. [1999] demonstrated that the β_1-adrenergic receptor antagonists were able to block the effect of the antibodies and displace the anti-β_1-adrenergic receptor antibodies from their binding sites on the receptor, leading to a decrease of β_1-AA titers in patients with heart failure. Therefore, the heart failure patients involved in the current study were required to stop treatment with β_1-AR antagonists for 1 week.

TNF-α is a proinflammatory cytokine with pleiotropic biological effects [Smith et al., 2012]. Elevated levels of TNF-α in the plasma occur in a variety of cardiovascular diseases, including acute myocarditis, cardiac allograft rejection, myocardial infarction, and congestive heart failure [Pérez et al., 2009; Lee et al., 2010; Hu et al., 2011; Tsarouhas et al., 2011]. TNF-α can cause a series of pathophysiological events involving left-ventricular systolic dysfunction, ventricular remodeling, oxygen free-radical release, and

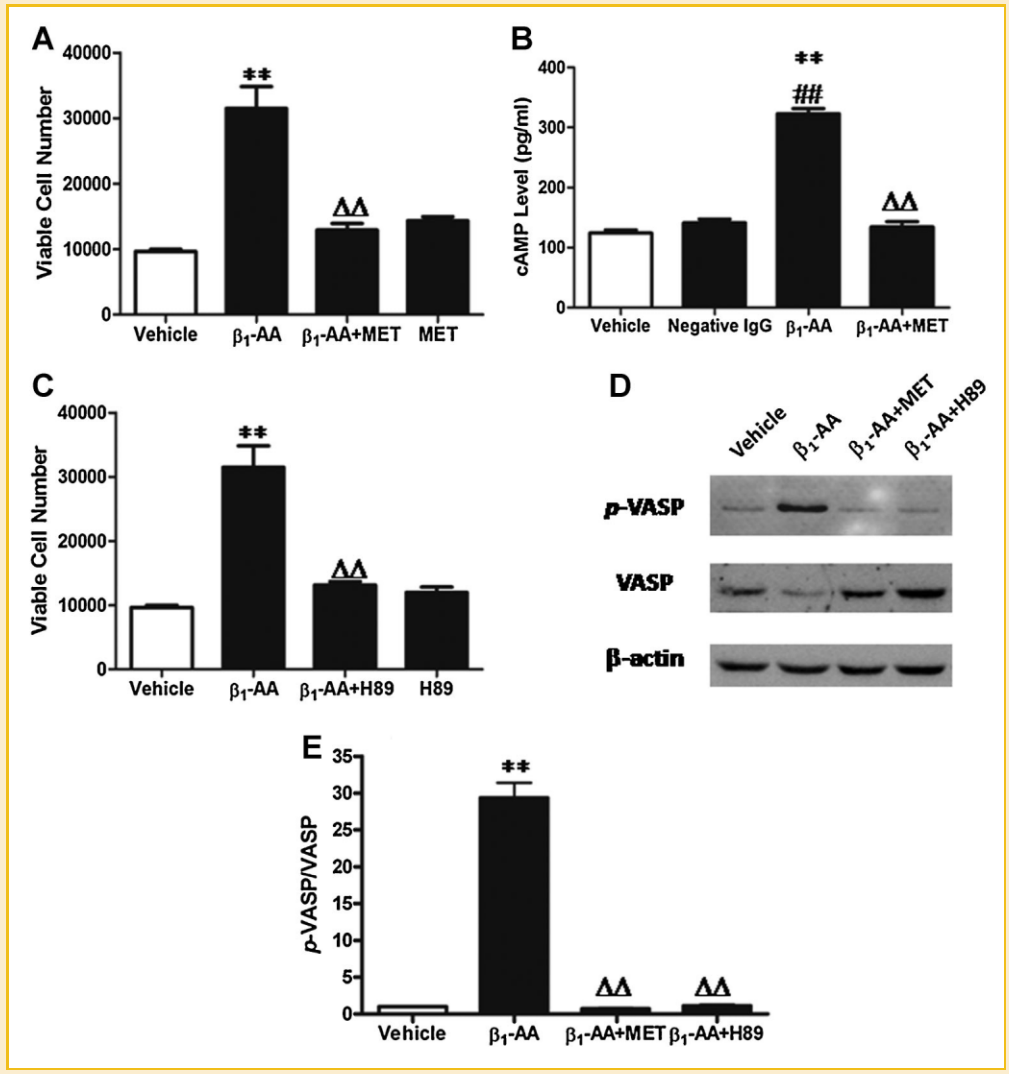

Fig. 5. β_1-AA isolated from heart failure patients proliferated RAW264.7 cells through the β_1-AR/cAMP/PKA pathway. A: RAW264.7 cells were treated with the selective β_1-AR antagonist metoprolol (1 μmol/L) for 1 h at 37°C and 5% CO_2 before β_1-AA (25 μg/ml). $^{**}P < 0.01$ versus vehicle group; $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. n = 9/group. B: The effect of β_1-AA or isoproterenol on the production of cAMP (expressed as pg/ml) in RAW264.7 cells was examined by ELISA, $^{**}P < 0.01$ versus vehicle group, $^{\#\#}P < 0.01$ versus negative IgG group, $^{\Delta\Delta}P < 0.01$ versus β_1-AA group. Data were presented as means ± SD of 6 independent experiments. C: RAW264.7 cells were pretreated with the selective PKA-inhibitor H89 (1 μmol/L) for 1 h at 37°C and 5% CO_2 before being stimulated by β_1-AA (25 μg/ml). $^{**}P < 0.01$ versus vehicle group; $^{\Delta\Delta}P < 0.05$ versus β_1-AA group. n = 9/group. D: Immunoblot detection of phosphorylated VASP (p-VASP) and total VASP from RAW264.7 cells treated with β_1-AA for 30 min. Images are representative of three independent experiments. E: Bar graph shows the ratio of p-VASP to total VASP. N = 3, $^{**}P < 0.01$ versus vehicle group, $^{\Delta\Delta}P < 0.05$ versus β_1-AA group. MET: metoprolol.

cardiomyocyte apoptosis [Chen et al., 2012]. Our results suggest that both β_1-AA-positive and -negative IgGs might directly activate RAW264.7 cells and promote the secretion of TNF-α, though the effect of β_1-AA-positive IgGs was more pronounced than that of β_1-AA-negative IgGs. These results indicate that heart failure itself may be a risk factor in activating RAW264.7 cells, and the effect may be magnified due to the presence of β_1-AA.

To explore the possible mechanisms behind β_1-AA-induced increases in TNF-α, the role of β_1-AA in RAW264.7 cells proliferation was investigated. We found that β_1-AA promoted the proliferation of RAW264.7 cells, which may in turn release a large number of TNF-α molecules. Moreover, we unexpectedly observed that isoproterenol, β_1-adrenergic receptor agonist, cannot enhance the proliferation of resting RAW264.7 cells, while promoting an increase of LPS-induced RAW264.7 cells (Supplementary Fig. 1), which suggested that compared to β_1-adrenergic receptor agonist, β_1-AA itself may activate macrophages. Additionally, according to published papers [Wallukat et al., 1991; Podlowski et al., 1998], the chronotropic activity of β_1-AA differs from the effect of isoproterenol in that the β_1-AR is not desensitized. Therefore, during the process of RAW264.7 cell proliferation, isoproterenol might induce the desensitization of the β_1-AR, while the β_1-AA may not desensitize the β_1-AR response.

It has been reported [Chiale et al., 1995] that the most common signaling mechanism initiated by β_1-AR stimulation is the β_1-AR/cAMP/PKA pathway. In order to explore whether β_1-AA activated

the RAW264.7 cells and promoted the secretion of TNF-α through this pathway, the β_1-AR selective antagonist metoprolol was added to RAW264.7 cells prior to treatment with β_1-AA. This completely blocked the effect of β_1-AA, suggesting that β_1-ARs on the surface of RAW264.7 macrophages could be activated by the β_1-AA from heart failure patients. Moreover, we observed the direct roles of β_1-AA in wide-type HEK293 cells lacking endogenous β_1-AR expression and HEK293 cells transfected with β_1-AR. We found that β_1-AA revealed no effect on wide-type HEK293 cells (Supplementary Fig. 2), but it enhanced the transfected HEK293 cells proliferation (Supplementary Fig. 3). However, when small interference RNA (siRNA) technique was used to down-regulate the endogenous expression of β_1-AR protein in RAW264.7 cells, we found that this treatment markedly reduced RAW264.7 cells proliferation and TNF-α secretion induced by β_1-AA (Supplementary Fig. 4), which further confirmed that β_1-AA promoted the proliferation and TNF-α production of RAW264.7 cells by binding to β_1-AR. Furthermore, we also found that each β_1-AA sample increased cAMP production in a receptor-mediated fashion.

Previous studies have asserted PKA-dependent effects in immune cells by either assessing agonist-stimulated PKA activity through in vitro assays or demonstrating the actions of pharmacologic PKA inhibitors and activators [Ganapathy et al., 2000; Aandahl et al., 2002]. For this report, we used a more direct approach, analyzing PKA activity by detecting the phosphorylation of VASP at Ser157, which is mediated directly and selectively by PKA [Smolenski et al., 1998; Sartoretto et al., 2009]. We found that β_1-AR activation by different concentrations of β_1-AA (12.5, 25, 50 μg/ml) rapidly led to VASP phosphorylation at Ser157, while isoproterenol and IgGs fractions isolated from β_1-AA-negative heart failure patients had no effect on phosphorylation levels of VASP at Ser157 (Supplementary Fig. 5). The selective β_1-AR antagonist metoprolol decreased the level of VASP phosphorylation stimulated by β_1-AA. In addition, inhibition of PKA by compound H89 abrogated β_1-AA-induced phosphorylation of VASP at Ser157. Taken together, all of these results strongly implicate the β_1-AR/cAMP/PKA pathway as the principal signaling system modulating the β_1-AA-induced phosphorylation of VASP at Ser157. Moreover, recent reports indicate that the activation of cAMP/PKA pathway enhances NF-κB activity [Gerlo et al., 2011], which induces the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 by macrophages [Cho et al., 2011]. Therefore, further researches need to be done to explore the effect of β_1-AA on cAMP/PKA/NF-κB pathway.

We noted that the immunoblots in this article revealed an apparent decrease in total VASP abundance following treatments with β_1-AA, an increase that enhanced VASP Ser157 phosphorylation. This result is not what we expected. We speculate that this decreased protein signal reflects a change in the apparent M_r of VASP as a consequence of its phosphorylation [Hamad et al., 2003]. The transient decrease in the immunoblotted VASP signal following β_1-AA treatment appears to be a consequence of VASP Ser157 phosphorylation, leading to slower migration on SDS–PAGE, which is also associated with decreased affinity of the antibody for the phosphorylated protein.

More interestingly, we found that the PKA inhibitor H89 could only partially block β_1-AA-induced TNF-α secretion. This suggested that the effects of β_1-AA on RAW264.7 cells may not be mediated solely by the β_1-AR/cAMP/PKA pathway. The study by Tutor et al. [2007] showed that the effect of β_1-AA on cardiomyocytes could be blocked by tyrosine kinase inhibitor PP2. Other studies have also reported that in the ischemic preconditioning heart, the role of β_1-AR could be mediated by PI3-kinase, PKC, or PKA [Robinet et al., 2005]. Meanwhile, other evidence suggests that p38MAPK molecules are involved in important signaling pathways that control the synthesis and release of pro-inflammatory mediators by activated macrophages during the inflammatory response [Guha and Mackman, 2001]. It has been reported that inhibition of p38MAPK suppressed TNF-α and IL-6 release in RAW264.7 cells [Avni et al., 2010]. Additionally, Tutor et al. [2007] found that β_1-AA from dilated cardiomyopathy (DCM) patients activated ERK1/2 pathway in cardiac cells, which is closely related to RAW264.7 cells proliferation [Cáceres et al., 2010]. Based on the studies mentioned, further researches are necessary to investigate the other possible pathways stimulated by β_1-AA.

In summary, we observed the direct effect of IgG fractions found in β_1-AA-positive sera from heart failure patients on murine macrophage-like cell line RAW264.7. We demonstrated, for the first time, that high sera levels of TNF-α in patients with chronic heart failure may have a close relationship with β_1-AA-induced macrophage activation. Nonetheless, our work leaves some unanswered questions for future researches. In our in vitro experiment, we used murine macrophage-like cell line RAW264.7, whose β_1-AR distribution may be different from that of heart failure mice. Therefore, the role of β_1-AA in pathological macrophages should be further investigated. Additionally, in our study, the β_1-AA used was not specific for the second extracellular loop of β_1-AR, and some nonspecific IgGs were involved. Thus, further studies using monoclonal antibodies specific for the second extracellular loop of β_1-AR should yield more conclusive results.

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