The effects and possible mechanism of β_2AR gene expression in cardiocytes of canines with heart failure

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Abstract. The objective of the present study was to observe the changes of β_2-adrenergic receptor (β_2AR) protein expression in a canine model of heart failure (HF), and the function of cardiocytes after transfection with Adv-β_2AR. The canine model of chronic HF was induced by rapid right ventricular pacing and cardiocytes were isolated with collagenase II. Cardiocytes were transfected with Adv-β_2AR to observe contractile function with a motion edge-detection system of single cells. Expression of β_2AR protein in cardiocytes was measured by immunoblotting and the levels of intracellular cAMP were measured by ELISA. Compared with the control group (the sham group), the expression of β_2AR protein in HF cardiocytes did not change, but the basal (1 mM Ca^{2+}) contraction amplitude percentage (1.809±0.922 vs. 1.120±0.432%, P<0.05), the maximum contraction amplitude percentage (14.855±2.377 vs. 10.784±2.675%, P<0.01) and the basal levels of intracellular cAMP (9.39±2.54 vs. 5.26±0.95 pmol/ml, n=6, P<0.05) of HF cardiocytes were significantly decreased. However, when HF cardiocytes were transfected with Adv-β_2AR and cultured for 48 h, compared with the non-transfected group, the basal contraction amplitude percentage (0.851±0.324 vs. 1.629±0.522%, P<0.05), the maximum contraction amplitude percentage (9.260±2.208% vs. 12.205±1.437%, P<0.01) and the basal levels of intracellular cAMP (5.26±0.95 vs. 9.03±1.03 pmol/ml, n=6, P<0.05) of cardiocytes in the transfected group were significantly increased. In conclusion, the expression of β_2AR protein in HF cardiocytes did not change, but contraction function was impaired. The moderate overexpression of β_2AR gene in the HF cardiocytes increased the levels of intracellular cAMP and improved contraction function.

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Introduction

Chronic heart failure (CHF) is the result of the majority of cardiovascular diseases, and is a major cause of death. With the growth of the aging population, increased incidence of hypertension and coronary heart disease, and declining mortality from acute coronary heart disease, the prevalence of CHF is increasing. Previous studies have shown that the myocardial cell surface expresses 4 β-adrenergic receptors (βAR), the majority of which are β_1AR and β_2AR. In human ventricle, β_1AR constitutes 70-80% of total βAR, while β_2AR constitutes the remaining 20-30%. The effects of βAR activation are mainly mediated through β_1AR and β_2AR. In the heart, stimulation of βAR is the most potent means of increasing cardiac contractility and relaxation in response to stress or a 'fight-or-flight' situation. However, sustained βA stimulation promotes pathological cardiac remodeling such as myocyte hypertrophy, apoptosis and necrosis, thus contributing to the pathogenesis of CHF. Lack of cardiac contractility mediated by βAR was the most significant contradiction, and some suggested that increasing the expression of cardiac βAR in patients with HF by transgenic technology could reverse the lack of cardiac contractility. At present, research in transgenic mice (1,2), rat models of HF (3) and other small animals confirmed that increased expression of cardiac β_2AR significantly improved contractile function and demonstrated potential development of β_2AR gene therapy for HF. However, the effect of different expression levels of the β_2AR gene in cardiocytes of large mammals with HF for improving contractile function is less reported in the literature. In this study, an HF model in canines was established by rapid pacing. Changes in the expression of β_2AR protein during HF, and the function of cardiocytes after transfection with Adv-β_2AR were observed.

Materials and methods

Materials. Adult canines (male, 10-15 kg) were kindly supplied by the Medical College of Xuzhou (Xuzhou, China). SDS-polyacrylamide gel electrophoresis (PAGE) gel kit was from BiYunTian Biotechnology Research Institute (Nantong, China). Molecular weight Marker, anti-mouse IgG and anti-rabbit IgG were from Sigma (St. Louis, MO, USA). Anti-β-actin was from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-β_2AR (H-20):sc-569 was from Santa

Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). NBT/BCIP was from Promega Corp. (Madison, WI, USA). Protease inhibitor mixture was from Merck Millipore (Billerica, MA, USA). Single cell dynamic edge detection system was from IonOptix Corp. (Westwood, MA, USA). Full wavelength microplate reader was from Bio-Rad (Hercules, CA, USA). Gel electrophoresis system and semi-dry transfer system were from Bio-Rad (Berkeley, CA, USA). Decolorization shaker was obtained from Taicang Instrument (Taicang, China).

Establishment of HF model. Adult canines were weighed after intraperitoneal anesthesia by pentobarbital sodium (3%, 1 ml/kg). Next, canines underwent tracheal intubation and were connected to a ventilator (tidal volume was 200-300 ml, frequency was 12 times/min, exhale/inhale ratio was 1/1.5). A transverse incision at the 5th intercostal of the left chest was made to expose the heart, followed by a longitudinal pericardial incision. The anode of the pacing lead was placed on the surface of the right ventricle, the pericardium was sutured, and the cathode of the pacing lead was planted on the surface of the left pericardium. The chest was then closed. The anode and cathode of the pacing lead pierced the skin of the back and was connected with a pacemaker. We started the pacemaker after one week to maintain rapid pacing at 240 times/min, and measured cardiac function after four weeks of pacing by echocardiography. The animal experiments were approved by the Animal Ethics Committee of Xuzhou Medical University.

Determination of cardiac function in canines. Cardiac function was measured under anesthesia (method ibid.) by echocardiography in the control group (no pacing after surgery) at 1 and 5 weeks after surgery and in the HF group before pacing (1 week after surgery) and after pacing for four weeks (5 weeks after surgery). We took the section of the parasternal long axis, and used M-ultrasound to measure in the guided two-dimensional ultrasound. Measurement data averaged three consecutive cardiac cycles. Observational indices included: Internal diameter of left atrium (LA), internal diameter of left ventricular (LV) end-diastolic, internal diameter of LV end-systolic, interventricular septal thickness end-diastolic, LV ejection fraction (EF) and LV fractional shortening were obtained by the computer system of the ultrasonic detector.

Isolation and culture of cardiocytes. Each canine was anesthetized (method ibid.) and the chest was opened under mechanical ventilation. The heart was removed and quickly placed in frozen Tyrode's solution. The left anterior branch of the coronary artery of the heart was connected to a constant temperature perfusion device, and was successively perfused with Tyrode's solution, calcium-free solution and enzyme solution. Digested myocardium was cut-off and placed in a dish containing enzyme solution, cut into thin slices, oscillated, filtered, and centrifuged. The supernatant was removed and incubated twice. Cells were cultured in M199 medium.

Transfection of cardiocytes. Cultured cardiocytes were treated with E1 defective recombinant adenovirus as a carrier of the human β_2AR-enhanced green fluorescent protein (EGFP) or EGFP gene. The multiplicity of infection was 100.

Western blotting. Cardiocytes were collected after 48 h of adenovirus transfection, washed twice with M199 medium, centrifuged, and the supernatant was removed. Cell pellet was mixed with buffer containing protease inhibitor cocktail. Cells were lysed by sonication and cryopreserved at -80°C. Bovine serum albumin was used as standard to determine protein content.

The following operations were carried out at 4˚C. Samples were mixed with equal volumes of 4X Laemmli sample buffer and boiled in a water bath for 5 min for protein denaturation. Equal amounts of protein (100 μg) were separated by SDS-PAGE, and electrically transferred to nitrocellulose (NC) membranes by semi-dry method. After NC membranes were incubated at room temperature for 3 h in blocking solution, anti-β_2AR antibody (1:200) was added and incubated for 4 h at room temperature or overnight at 4˚C. Membranes were washed with TBST (5 min x 3), and incubated in alkaline phosphatase (AP) labeled secondary antibody working solution for 2 h at room temperature. Membranes were washed again in TBST (5 min×3) and washed in water. NBT/BCIP kit was used to color fresh AP reagent, and washed in running water to terminate the reaction. Color bands in the scanning film were analyzed with Image-Pro Plus software (version 10.0; Media Cybernetics, Silver Springs, MD, USA); SigmaStat (version 3.5; Jandel Scientific, San Rafael, CA) and SigmaPlot (version 11.0; Systat Software, San Jose, CA, USA). The optical density of the bands were marked by multiples of the control group on the same membrane.

Determination of intracellular cAMP levels. Cardiocytes were collected 48 h after transfection with adenovirus. Cells were lysed by freezing and thawing, supernatant was collected and intracellular cAMP was assayed by ELISA. Some cells were treated with isoproterenol (ISO) and allowed to react for 2 h. The final concentration of ISO was 10^{-6} mol/l.

Determination of contractile function of cardiocytes. The length-time curve of cardiocytes were recorded through a single cell dynamic edge detection system. Cells were perfused with Krebs-Henseleit solution containing 119 mM NaCl, 4.7 mM KCl, 0.94 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.5 mM Glucose·H_2O and 1 mM $CaCl_2$, pH 7.4, and were electrically stimulated at 0.5 Hz. Cells with stable contraction amplitude changes after intervention were selected to record the results. The percentage of cell contraction amplitude, time to peak contraction (TTP), time to 50% relaxation (R50) and time to R90 were obtained by IonWizard analysis software (IonOptix Corp.). The concentration of ISO that caused 50% maximum contraction was indicated as pD2.

Statistical analysis. Data are presented as mean ± standard deviation. Comparisons were made by Student's t-test or paired t-test when appropriate. Analysis of pD2 was through nonlinear regression analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical manifestations. Canines in the HF group showed loss of appetite and gradually decreased activity after two weeks

Table I. The result of the determination of the heart fuction of canines (n=10, mean ± standard deviation).

	Control group		Heart failure	
Characteristics	1 week after surgery	5 weeks after surgery	Before pacing	Pacing for 4 weeks
LA, mm	16.1±1.2	15.5±0.9	15.7±1.2	21.3±1.0[a]
IVS, mm	6.9±0.5	6.8±0.3	6.8±0.4	4.5±0.4[a]
LVEDD, mm	29.4±2.3	30.3±2.3	27.7±1.9	38.9±2.2[a]
LVESD, mm	18.8±2.1	20.3±2.1	17.8±1.5	32.9±2.1[a]
EF, %	67.5±4.4	63.0±4.2	67.7±6.5	32.7±6.9[a]
FS, %	36.2±3.4	33.1±3.1	36.2±4.9	15.2±3.7[a]

Compared with before pacing, [a]P<0.05. LA, left atrium; IVS, interventricular septum; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; EF, ejection fraction; FS, fraction shortening.

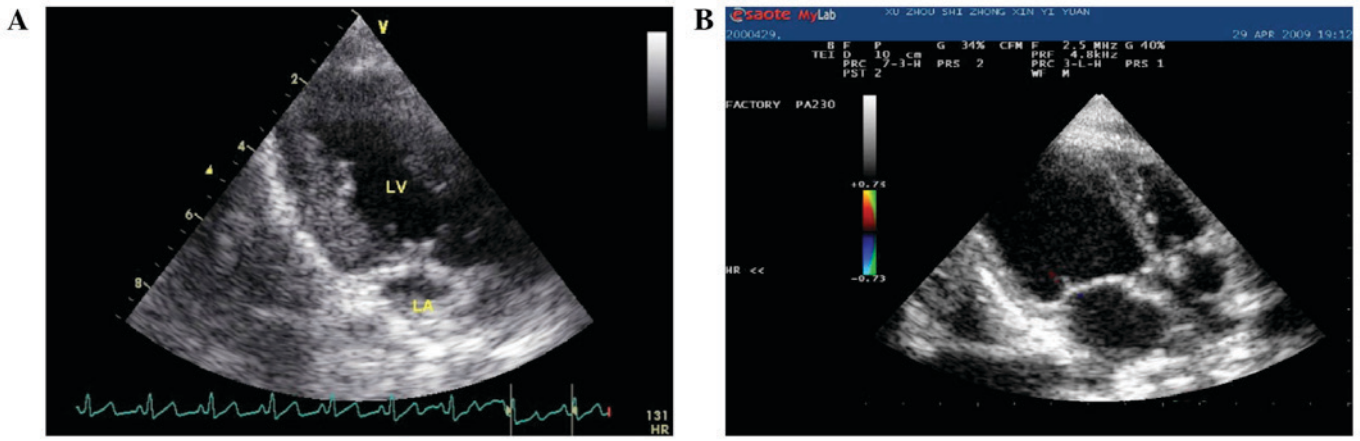

Figure 1. Four chambers of the heart before and after pacing. (A) Before pacing. (B) Pacing for 4 weeks.

of rapid pacing. After 4 weeks, they experienced shortness of breath and significantly decreased diet. Canines in the control group did not show these changes over the same period.

The change of echocardiography index before and after 4 weeks of rapid pacing. Canines in the HF group showed mitral regurgitation of different degrees and the related indexes showed significantly increased internal diameter of LA and left LV. In addition, myocardial thickness and EF decreased significantly (Table I and Fig. 1).

Identification of β_2AR protein expression in cardiocytes cultured for 48 h. Western blot analysis showed that, compared with untransfected cells, β_2AR protein expression of cardiocytes increased significantly in both the control group and HF group after transfection with β_2AR-EGFP for 48 h. Compared with the control group, β_2AR protein expression of cardiocytes in HF group did not change significantly. β_2AR protein expression of cardiocytes in the control group did not change significantly either after transfection with the EGFP gene for 48 h (Fig. 2).

The effect of β_2AR gene overexpression on intracellular cAMP concentration of cardiocytes. ELISA analysis showed that, compared with the control group, intracellular cAMP

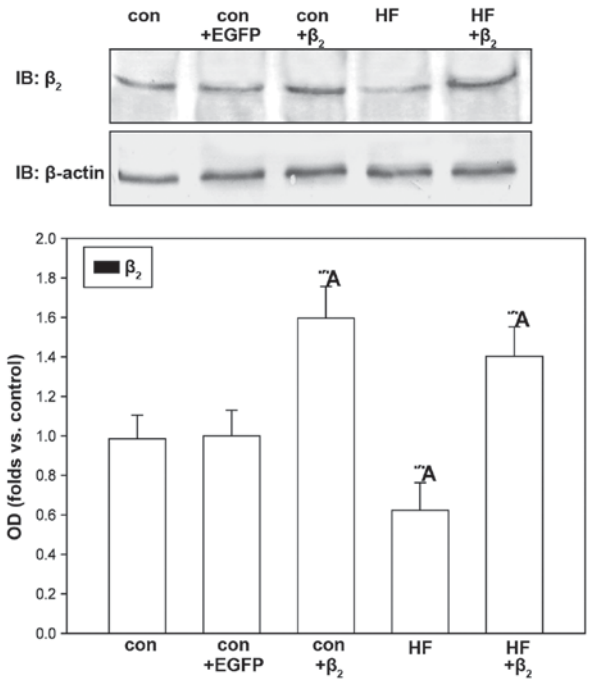

Figure 2. β_2AR protein expression of cardiocytes in control group and heart failure group after transfection with EGFP or β_2AR-EGFP for 48 h. Con vs. con + β_2, HF vs. HF + β_2, P<0.05, n=4. β_2AR, β_2-adrenergic receptor; EGFP, enhanced green fluorescent protein; con, control; HF, heart failure.

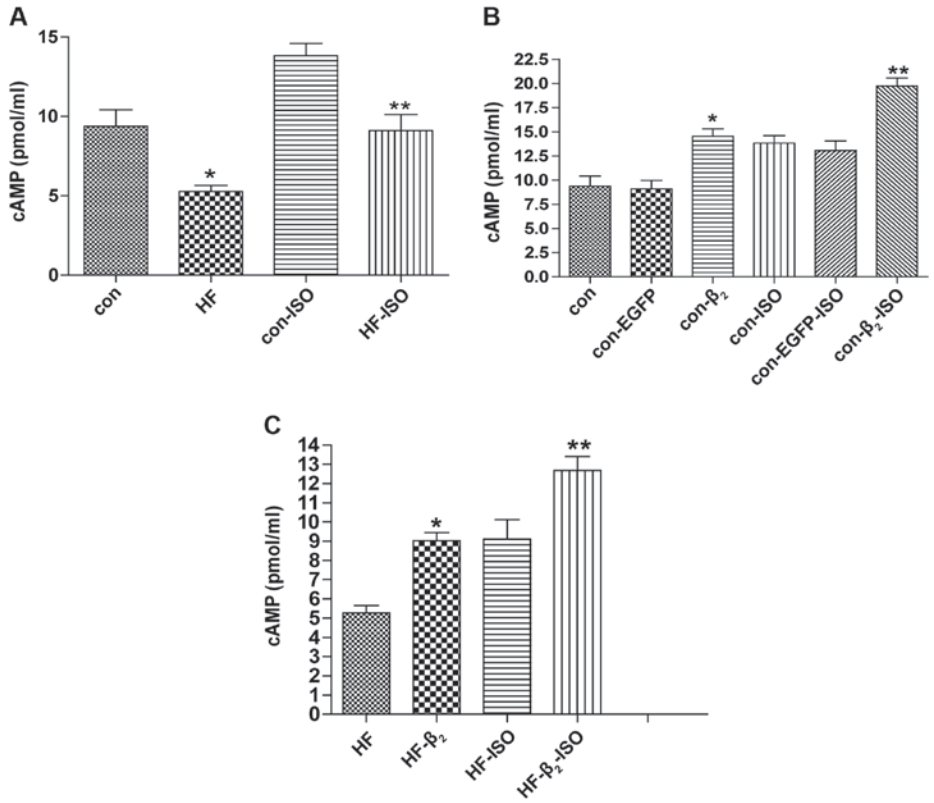

Figure 3. Changes of intracellular cAMP concentration after transfection with β_2AR-EGFP. (A and B) Compared with con, *P<0.05, n=6; compared with con-ISO, **P<0.01, n=6. (C) Compared with HF, *P<0.05, n=6; compared with HF-ISO, **P<0.01, n=6. β_2AR, β_2-adrenergic receptor; EGFP, enhanced green fluorescent protein; con, control; HF, heart failure; ISO, isoproterenol.

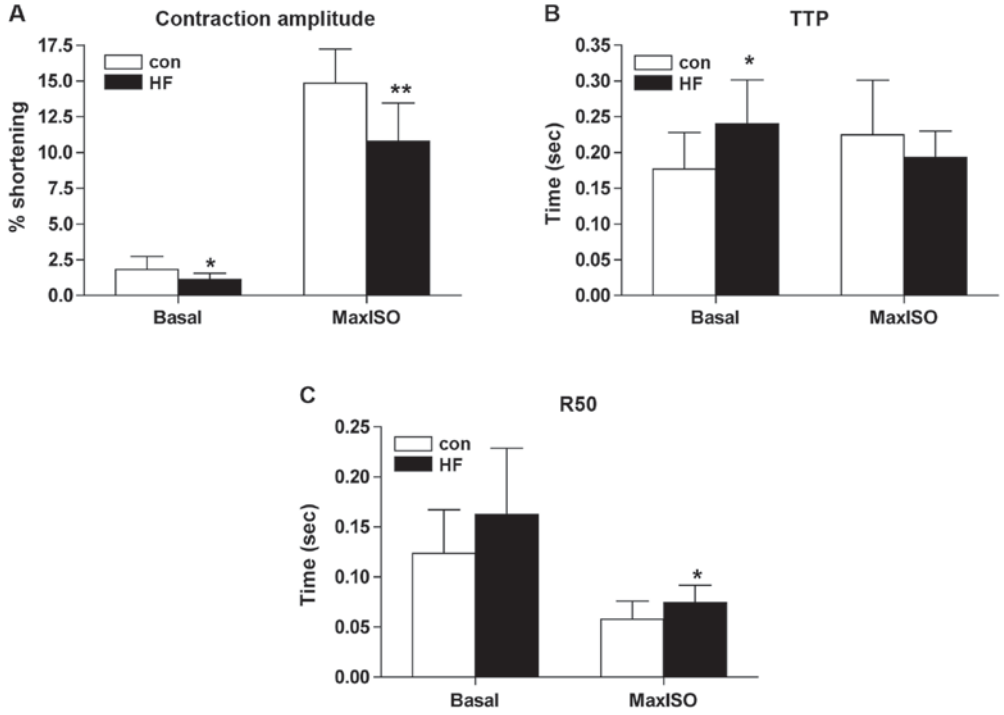

Figure 4. Contractile function of cardiocytes in canines separated immediately. (A) Compared with control group, *P<0.05 in basic state; **P<0.01 in the state of maximal contraction. (B) compared with control group, *P<0.05 in basic state. (C) Compared with control group, *P<0.05 in the state of maximal contraction. Con, control; HF, heart failure.

concentration in the HF group decreased significantly in the basic state (concentration of calcium was 1 mmol/l) and ISO stimulated state (concentration of ISO was 10^{-6} mol/l) (Fig. 3A). Cardiocytes of the control group were then transfected with

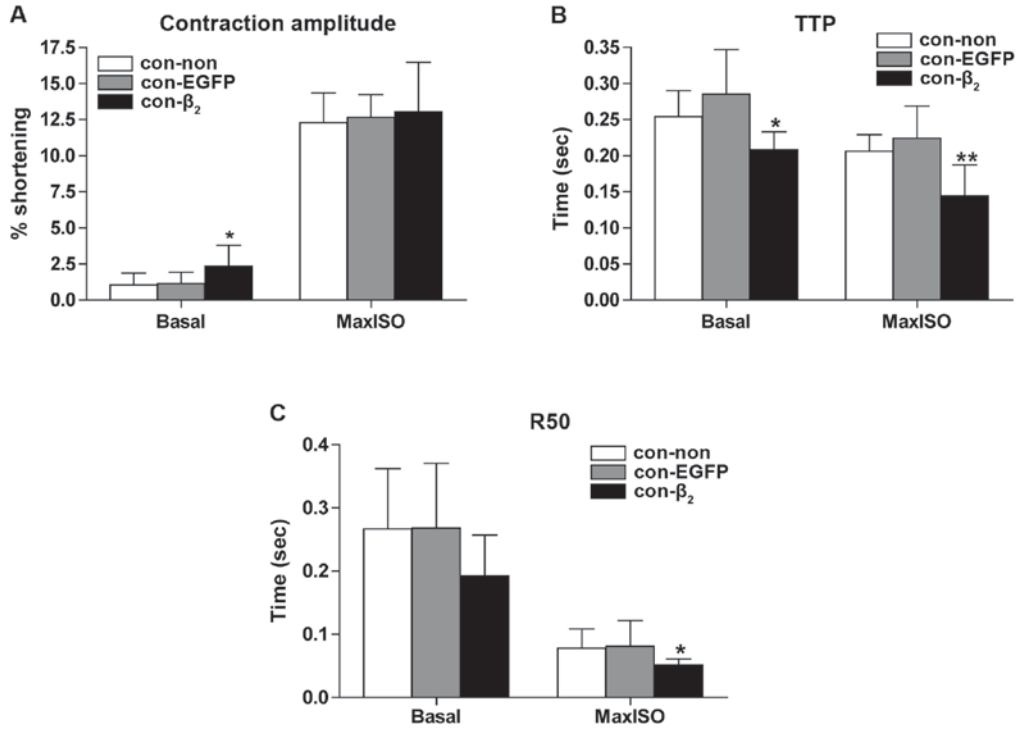

Figure 5. Contractile function of control cardiocytes after culture for 48 h. (A) Compared with control group, *P<0.05 in basic state. (B) Compared with control group, *P<0.01 in basic state; **P<0.01 in the state of maximal contraction. (C) Compared with control group, *P<0.05 in the state of maximal contraction. EGFP, enhanced green fluorescent protein; con, control.

β_2AR-EGFP or EGFP for 48 h. Compared with untransfected cells, intracellular cAMP concentration of the control group increased significantly in the basic and ISO stimulated states after transfection with β_2AR-EGFP, while intracellular cAMP concentration of the control group did not change significantly in either basic or ISO stimulated states after transfection with EGFP (Fig. 3B).

Cardiocytes of the HF group were also transfected with the β_2AR-EGFP gene for 48 h. Compared with untransfected cells, intracellular cAMP concentration of the HF group increased significantly in basic and ISO stimulated states after transfection with β_2AR-EGFP (Fig. 3C).

The effect of β2AR gene overexpression on contractile function of cardiocytes. Results of contractile function of cardiocytes separated immediately showed that compared with the control group (n=15), the percentage of cell contraction amplitude decreased significantly (Fig. 4A), TTP increased significantly (Fig. 4B) in the basic state (concentration of calcium was 1 mmol/l), the percentage of cell contraction amplitude decreased (Fig. 4A), and R50 increased significantly (Fig. 4C) in the state of maximal contraction stimulated by ISO of cardiocytes in the HF group.

Control cardiocytes were next divided into three groups: untransfected (n=11), transfected with β_2AR-EGFP (n=8), and transfected with EGFP (n=9). Contractile function was determined after culture for 48 h. Compared with untransfected, the percentage of cell contraction amplitude increased (Fig. 5A), TTP decreased (Fig. 5B) significantly in basic state, and TTP and R50 decreased (Fig. 5B and C) significantly in the state of maximal contraction of cardiocytes transfected with β_2AR-EGFP. Contractile function of cardiocytes in the control group did not change significantly in either basic or maximal contraction states after transfection with EGFP.

The values of the Ca^{2+} concentration vs percentage of contraction amplitude curve of cardiocytes transfected with β_2AR-EGFP gene was higher than in untransfected cells, which indicated that the percentage of contraction amplitude of cardiocytes transfected with β_2AR-EGFP was higher than in untransfected cells with the same Ca^{2+} concentration in the control group. The values of the semi-logarithmic cumulative concentration of ISO versus the percentage of contraction amplitude curve of cardiocytes transfected with β_2AR-EGFP were smaller in untransfected cells (Fig. 6). Compared with untransfected cells, pD2 of cardiocytes transfected with β_2AR-EGFP decreased significantly (3.000±0.063 vs. 0.126±0.039 nM, P<0.01).

Cardiocytes in the HF group were also divided into groups: Untransfected (n=11) and transfected with β_2AR-EGFP (n=10). Contractile function was determined after culture for 48 h. Compared with untransfected cells, the percentage of cell contraction amplitude increased (Fig. 7A) and TTP decreased (Fig. 7B) significantly in both basic and maximal contraction states of cardiocytes transfected with β_2AR-EGFP, while R50 of cardiocytes in the HF group did not change significantly in either the basic or maximal contraction state after transfection with β_2AR-EGFP (Fig. 7C).

The values of the curve of Ca^{2+} concentration versus the percentage of contraction amplitude of cardiocytes transfected with β_2AR-EGFP was higher than in untransfected cells, indicating that the percentage of contraction amplitude of cardiocytes transfected with β_2AR-EGFP was higher than in untransfected cells with the same Ca^{2+} concentration in the HF group. The values of the plot of semi-logarithmic cumulative

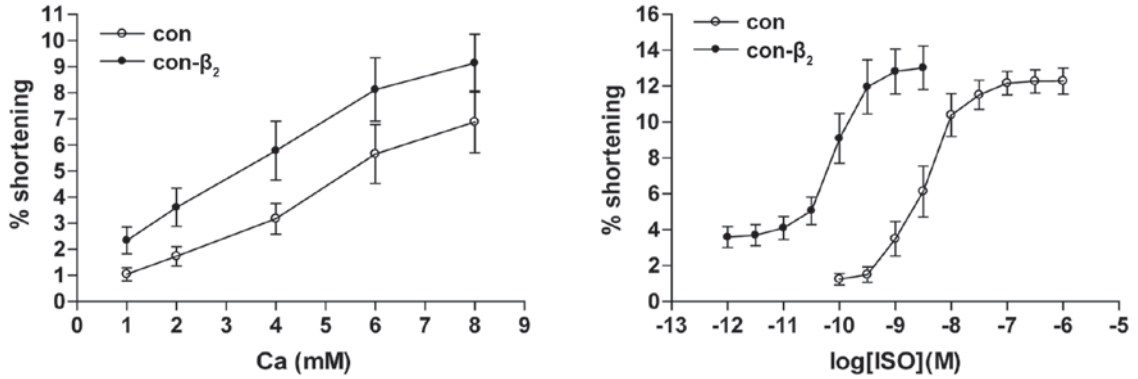

Figure 6. Ca^{2+} concentration and semi-logarithmic cumulative concentration of ISO-the percentage of contraction amplitude curve of cardiocytes in control group canines after cultured for 48 h.

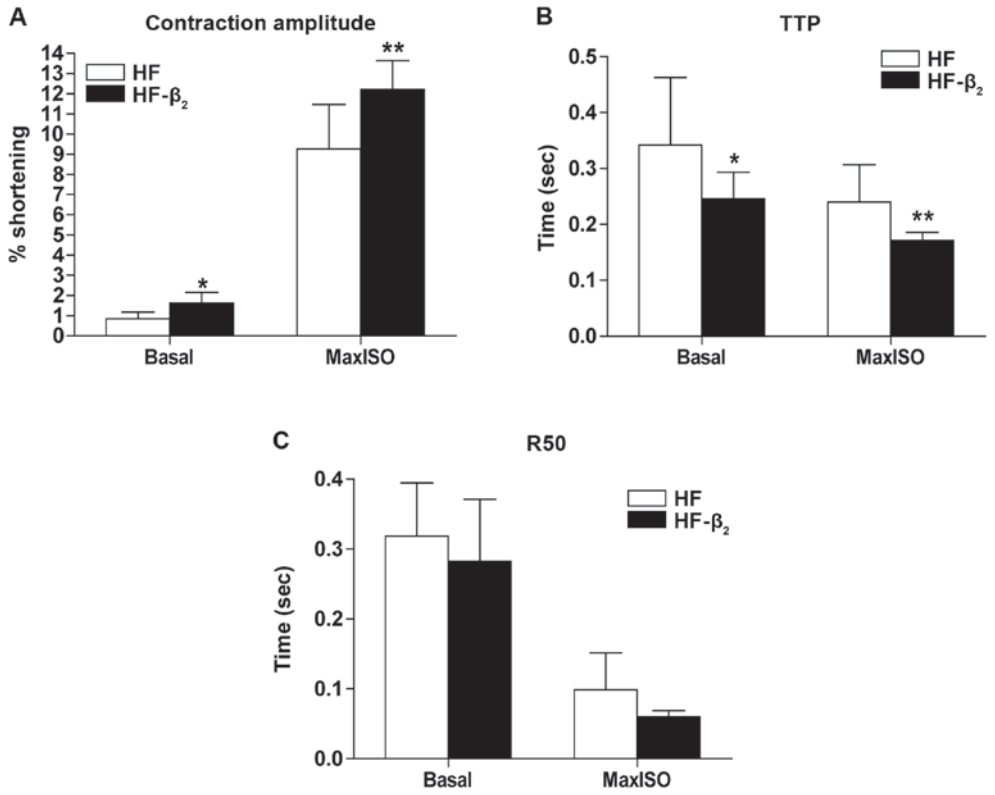

Figure 7. Contractile function of cardiocytes in heart failure (HF) group canines after cultured for 48 h. (A) Compared with control group, *P<0.05 in basic state; **P<0.01 in the state of maximal contraction. (B) Compared with control group, *P<0.05 in basic state; **P<0.01 in the state of maximal contraction. (C) R50 of cardiocytes in the HF group did not change significantly in either the basic or maximal contraction state after transfection with β_2AR-EGFP.

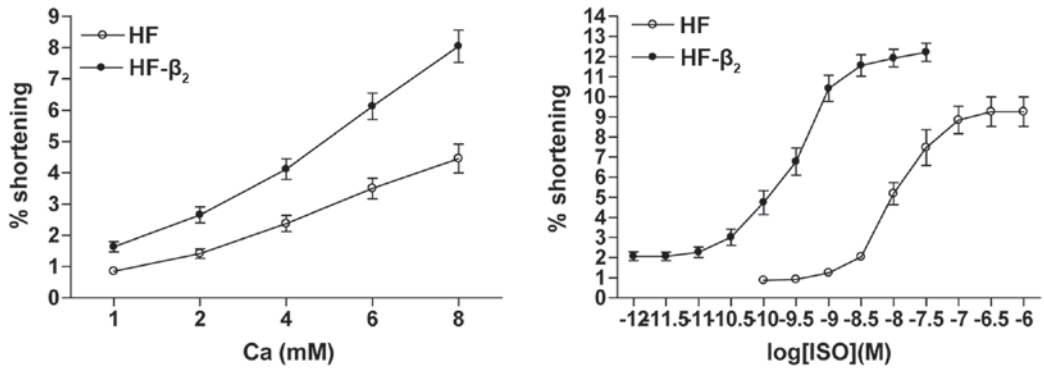

Figure 8. Ca^{2+} concentration and semi-logarithmic cumulative concentration of isoproterenol (ISO)-the percentage of contraction amplitude curve of cardiocytes in heart failure (HF) group canines cultured for 48 h.

concentration of ISO versus the percentage of contraction amplitude curve of cardiocytes transfected with β_2AR-EGFP were lower than the untransfected cells in the HF group, compared with untransfected (Fig. 8). The pD2 of cardiocytes transfected with β_2AR-EGFP decreased significantly (6.310±0.351 vs. 0.159±0.016 nM, P<0.01; Fig. 8).

Discussion

In mammals, including humans, the predominant βAR subtypes expressed in the heart are β_1AR and β_2AR. The traditional view of β_1AR signal transduction is that stimulation of the receptor activates the G_s-AC-cAMP cascade, leading to PKA-dependent phosphorylation of a set of regulatory proteins. Studies over the past decade have shown that stimulation of β_2AR activates both the G_s-AC-cAMP and $G_{i\alpha}$-$G_{i\beta\gamma}$-PI3K-Akt cascades (4). Therefore, βAR subtypes fulfill different, even opposite, physiological and pathophysiological roles via activating subtype-specific signaling pathways in the heart.

A series of studies showed that cardiac-specific overexpression of β_1AR and β_2AR had very different effects on the process of myocardial remodeling and the prognosis of HF. Studies in mice with modest (5-40-fold) cardiac-specific overexpression of human β_1AR revealed dilated cardiomyopathy, severe cardiac remodeling, and premature death (5), while cardiac-specific overexpression of human β_2AR (100-200-fold) in mice markedly enhanced cardiac contractility, even in the absence of β-agonist, without obvious pathological consequence at the age of 12 months (2). Studies in rats with HF also indicated that moderate over-expression of the β_2AR gene in cardiocytes could improve contractility (3).

The canine model of CHF was induced by rapid right ventricular pacing and is similar to human dilated cardiomyopathy in aspects of clinical manifestations, hemodynamics, activation of neurohumoral factors and ventricular remodeling. The results showed that clinical manifestations and echocardiographic performance after four weeks of rapid pacing in canines were consistent with CHF. The process of HF in the canine model was observed dynamically by echocardiography and had the advantages of being non-invasive, convenient, and reproducible.

The expression of β_2AR protein, intracellular cAMP and contractile function of cardiocytes in canines were measured in this study. The expression of β_2AR protein in the HF cardiocytes did not change, but contraction function and intracellular cAMP concentration decreased. This may be related to changes of βAR regulation in the heart during HF: i) CHF caused by diverse etiologies is characterized by elevated levels of circulating catecholamines and hyperadrenergic drive, as well as concurrent selective desensitization of β_1AR, leading to a markedly blunted βAR-mediated contractile response. ii) Sustained β_1AR stimulation caused by hyperadrenergic drive leading to changes of β_1AR signal transduction from the β_1AR-G_s-AC-cAMP-PKA cascade to β_1AR-G_s-Ca^{2+}-CaMK II (6), thus promoting cardiac hypertrophy and cardiocyte apoptosis (7). iii) Sustained β_2AR stimulation caused by hyperadrenergic drive leading to phosphorylation of β_2AR, mediated by PKA, inducing changes of β_2AR signal transduction from β_2AR-G_s-AC-cAMP-PKA cascade to β_2AR-$G_{i\alpha}$-$G_{i\beta\gamma}$-PI3K-Akt cascade (8). The β_2AR-G_i pathway could have anti-apoptotic effects (9,10), but cannot mediate positive inotropic effects, and the β_2AR-G_i pathway could compartmentalize the G_s-AC-cAMP-PKA cascade and then confine and negate the β_2AR-G_s-mediated positive inotropic effect, spatially and functionally (11-13).

β_2AR protein expression and intracellular cAMP concentration of cardiocytes in the control group increased significantly after transfection with β_2AR-EGFP, while the percentage of cell contraction amplitude in the maximal contraction state did not change significantly after transfection with β_2AR-EGFP. This may be related to the following factors: i) β1AR accounts for the majority of cardiac surface βAR in mammalian species, including humans. Contraction of cardiocytes induced by ISO is primarily mediated by the β_1AR-G_s pathway under normal physiological conditions. ii) β2AR coupled with the G_s, G_i and β_2AR-Gi pathways could compartmentalize the β2AR-G_s pathway. β_2AR-G_i and β_2AR-G_s coupling increased when β_2AR was overexpressed, so that the percentage of cell contraction amplitude of cardiocytes in the control group in the maximal contraction state did not change significantly after transfection with β_2AR-EGFP.

β_2AR protein expression, intracellular cAMP concentration and the percentage of cell contraction amplitude of cardiocytes in the HF group increased significantly after transfection with β_2AR-EGFP. There was a significant difference in the percentage of cell contraction amplitude of cardiocytes in the control group in the maximal contraction state, but it did not change significantly after transfection with β_2AR-EGFP. This may be because of concurrent selective desensitization of β_1AR in CHF. The advantages of β_2AR become more apparent under conditions of β_2AR overexpression. With increased β_2AR expression, increased β_2AR-G_s coupling can repair damaged contractile function. In addition, increased β_2AR-G_i coupling can inhibit cardiac hypertrophy, apoptosis and has other cell protective effects, inhibiting the harmful effects induced by excessive activation of the β_2AR-G_s pathway. β_2AR protein expression, intracellular cAMP concentration and contractile function of cardiocytes in the control group did not change significantly after transfection with EGFP. This demonstrated that EGFP co-transfected with β_2AR did not have non-specific effects.

At present, β_2AR overexpression in cardiocytes of HF has been considered as a potential means of gene therapy for CHF (14). This study demonstrated that transgenic β_2AR overexpression in cardiocytes effectively repaired their damaged contraction function, and may be related to changes of signal transduction of βAR during CHF. How safe and effective moderately overexpressed β_2AR *in vivo* is, and the long-term effects of β_2AR overexpression on survival require further exploration.

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