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Chronic therapy with an $ET_{A/B}$ receptor antagonist in conscious dogs during progression of congestive heart failure Intracellular Ca^{2+} regulation and nitric oxide mediated coronary relaxation

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Abstract

Background: Although it is known that endothelin (ET-1) is elevated in heart failure (HF), it remains unclear if chronic $ET_{A/B}$ receptor antagonism affects the progression of HF, particularly by affecting coronary vasoactivity and left ventricular (LV) diastolic function. **Methods:** We examined the effects of an $ET_{A/B}$ receptor antagonist, L-753,037 (oral bid for 6 weeks, $n=7$), and vehicle ($n=8$) in conscious dogs with previously implanted aortic, coronary sinus and left atrial catheters, LV pressure gauge, aortic flow probe, LV dimension crystals and pacers. **Results:** Baseline hemodynamics were similar in the two groups. During the development of rapid pacing-induced HF, treatment with the $ET_{A/B}$ antagonist significantly reduced total peripheral resistance and increased cardiac output compared to vehicle. After 2 weeks of pacing, LV diastolic function (tau) was improved ($P<0.05$) in the $ET_{A/B}$ antagonist group ($+6\pm2$ ms) compared to the vehicle group ($+12\pm2$ ms). In addition, $ET_{A/B}$ antagonist treatment attenuated the increase in mean left atrial pressure and LV end-diastolic pressure that occurred during heart failure in vehicle-treated animals. However, LV systolic function (LV dP/dt, fractional shortening and Vcfc) neither at rest nor in response to dobutamine was altered by $ET_{A/B}$ antagonist treatment. Also, $ET_{A/B}$ antagonist treatment did not affect the progressive increases in LV dimension. After 6 weeks of pacing, maximal Ca^{2+} transport in isolated cardiac sarcoplasmic reticulum (SR) was reduced ($P<0.02$) in the vehicle-treated compared to the $ET_{A/B}$ antagonist-treated dogs (1.34 ± 0.09 vs. 1.60 ± 0.06 μmol/mg/min, respectively). The improvement in SR function in the $ET_{A/B}$ antagonist-treated dogs was associated with a significant attenuation of the reduction in protein expression of SERCA2a and calsequestrin observed in the vehicle-treated dogs. Coronary arteries isolated from the dogs treated with the $ET_{A/B}$ antagonist exhibited enhanced ($P<0.01$) coronary endothelium-dependent relaxation compared to the vehicle group, while coronary responses to an NO donor were identical in the two groups. Plasma NO levels in the coronary sinus during the late stage of HF were higher ($P<0.05$) in the $ET_{A/B}$ antagonist group (40 ± 2 μM) compared to the vehicle group (18 ± 2 μM). **Conclusions:** We conclude that in conscious dogs during the development of HF induced by rapid pacing, chronic inhibition of $ET_{A/B}$ receptors does not affect resting myocardial contractile function nor reserve, but reduces vascular resistance and improves LV diastolic function. After 6 weeks of pacing, the reduction in intracellular Ca^{2+} regulation by the SR is also attenuated, and endothelium-dependent coronary relaxation is improved, which appears to be related to the preservation of coronary NO levels.

Keywords: Contractile function; Endothelins; Heart failure; SR (function); Vasoconstriction/dilation; Ventricular function

1. Introduction

Congestive heart failure is characterized by multiple alterations in hemodynamic function and neurohumoral activation. Experimental and clinical studies have suggested that the circulating plasma concentration of endothelin (ET-1), a potent vasoconstrictor peptide produced by vascular endothelium, is closely associated with the pathophysiological processes of congestive heart failure [1–6]. The functional effects of ET-1 are mediated by two

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specific receptor subtypes, i.e. ET_A and ET_B receptors, which have been cloned [7,8].

Although several experimental studies have reported on the efficacy of ET receptor antagonists in heart failure [9–18], in most of these studies the antagonists were administered acutely [11,12,14,18] or for a relatively short period, i.e. 2–3 weeks [13,17]. Most long-term chronic studies with ET receptor antagonists have been conducted using small animal models with minimal measurements [9,11,15,16]. Importantly, the results, particularly those regarding myocardial contractile performance, were inconsistent, even in similar animal models and using the same ET receptor subtype antagonists [13,17]. Since the majority of these prior studies were conducted using either anesthetized or tranquilized animals and without continuous, direct measurements of cardiac and systemic hemodynamics during the development of congestive heart failure, it is difficult to reconcile the different results. Therefore, it remains unclear whether chronic treatment with an $ET_{A/B}$ receptor antagonist affects cardiovascular dynamics during the progression of heart failure, or alters coronary vasoactivity during the advanced stages of heart failure.

Accordingly, the primary goal of the present investigation was to use a prolonged rapid ventricular pacing-induced heart failure model, i.e. 6 weeks of rapid pacing in chronically instrumented, conscious dogs, to determine whether the chronic oral administration of an $ET_{A/B}$ receptor antagonist, L-753,037 (J-104132) [19], affects the altered LV systolic and diastolic function, and myocardial contractile reserve during the development of heart failure. To determine whether inhibition of both ET_A and ET_B receptors would affect the level of cardiac nitric oxide synthesis, the release of nitric oxide from the coronary circulation was measured via a chronically implanted coronary sinus catheter during heart failure development. In addition, coronary vasoactivity and cardiac sarcoplasmic reticulum Ca^{2+} regulation and protein expression were assessed in vitro at the end stage of heart failure.

2. Methods

2.1. Implantation of instrumentation

Fifteen adult mongrel dogs, weighing 15–20 kg, were anesthetized with pentothal (12–15 mg/kg, i.v.). Following tracheal intubation and ventilation, anesthesia was maintained with isoflurane (1.5–2.0 vol% in oxygen). A left thoracotomy was performed at the fifth intercostal space. Tygon catheters (Norton Plastics, Akron, OH) were implanted in the descending aorta and left atrium for measurement of their respective pressures. A Silastic catheter, used to collect blood samples, was inserted directly into the coronary sinus. The left circumflex coronary artery was isolated, and a flow probe (Transonic Inc., Ithaca, NY) was implanted to measure coronary blood flow. A solid-state miniature pressure gauge (Konigsberg, Pasadena, CA) was implanted in the left ventricular (LV) cavity through the apex for measurements of LV pressure and rate of change of LV pressure (LV dP/dt). A flow probe (Transonic Inc., Ithaca, NY) was placed around the ascending aorta to measure aortic blood flow, i.e. cardiac output. One pair of piezoelectric ultrasonic dimension crystals was implanted on opposing anterior and posterior endocardial surfaces of the left ventricle to measure LV internal diameter. Another pair of dimension crystals were placed transmurally across the LV free wall regions to measure wall thickness. A pacing lead (Medtronic Inc., Minneapolis, MN) was attached to the right ventricular free wall, and stainless steel pacing leads were attached to the left atrial appendage. Catheters and electrical leads were externalized between the scapulae, and the chest was closed in layers. The animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Institute of Laboratory Animal Resources, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the Merck Research Laboratories Institutional Animal Care and Use Committee.

2.2. Experimental measurements

2.2.1. LV and systemic hemodynamic measurements

Hemodynamic recordings were made using a data tape recorder and a multiple-channel oscillograph (Gould, Cleveland, OH). Aortic and left atrial pressures were measured using strain gauge manometers (Argon, Athens, TX), which were previously calibrated using a mercury manometer connected to the fluid-filled catheters. The solid-state LV pressure gauge was cross-calibrated with aortic and left atrial pressure measurements. LV dP/dt was obtained by electronically differentiating the LV pressure signal. A triangular wave signal was substituted for the pressure signals to directly calibrate the differentiator (Triton Inc., San Diego, CA). Ascending aortic and coronary blood flows were measured using a volume flow meter (Transonic Inc., Ithaca, NY). Stroke volume was calculated as the quotient of cardiac output and heart rate. Cardiac output and stroke volume also were normalized by body weight to yield cardiac index and stroke volume index, respectively. LV dimension was measured with an ultrasonic transit-time dimension gauge (Triton Inc., San Diego, CA). Total peripheral resistance was calculated as the quotient of mean arterial pressure and cardiac output. LV end-diastolic dimension (EDD) was measured at the time that coincided with beginning of the upstroke of the LV dP/dt signal. LV end-systolic dimension (ESD) was measured at minimum LV dP/dt. The percent shortening of LV internal diameter was calculated as (EDD−ESD)/EDD*100. Mean velocity of LV circumferential fiber shortening corrected for heart rate (Vcfc) was formulated

as [(EDD−ESD)/EDD]/ET/R-R] ($s^{-1/2}$), where ET and R-R denote ejection time and R-R interval (in seconds), respectively. Ejection time was measured as the interval between maximum and minimum LV d*P*/d*t*. LV wall stress was calculated at LV end-systole and at LV end-diastole using a cylindrical model: Stress=1.36(LVP*ID)/(2*WT), where ID and WT are the LV internal diameter and wall thickness, respectively. LV isovolumetric relaxation time constant, tau, was calculated beat-by-beat and on-line, from the minimum value of LV d*P*/d*t* (LV d*P*/d*t* min) to 36% of LV d*P*/d*t* min (Modular Instruments, Malvern, PA). An LV pressure–diameter loop (P–D) was constructed for each animal before and after heart failure, i.e. after 4 weeks of pacing. A cardiotachometer triggered by the LV pressure pulse provided instantaneous and continuous records of heart rate.

2.2.2. Assessments of NO levels and isolated coronary arterial relaxation

Coronary and arterial blood samples were collected for NO determination at baseline (before heart failure) and after 2, 4 and 6 weeks of pacing. The samples were placed in Na EDTA tubes on ice and centrifuged at 4°C to separate the plasma, which was stored at −70°C. NO measurements were made using a NO chemiluminescence analyzer (270B, Sievers Instruments, Boulder, CO).

Coronary arteries were removed when the dogs were sacrificed, and placed in Krebs–Henseleit (K–H) solution. The arterial rings, i.e. 3–5 mm in length, were mounted onto stainless steel hooks, suspended in 7.5-ml tissue baths, and connected to transducers (World Precision Instruments, Sarasota, FL) to record changes in force. The rings were initially stretched to give an optimal preload of 2 *g* of force and equilibrated for 60 min. After equilibration, U-46619, a thromboxane A_2 mimetic, was added to a concentration of 75–100 nM to generate maximal vasoconstriction. After the response stabilized, the U-46619 was washed out of the bath and force was allowed to return to the baseline value. The rings were then contracted submaximally by adding U-46619, and cumulative relaxation curves to acetylcholine (Ach) (0.01–10.0 μM) and acidified $NaNO_2$ (0.01–10 μM) were obtained. Coronary artery rings isolated from three non-heart failure control dogs were also assessed.

2.2.3. Isolation and characterization of sarcoplasmic reticulum

Cardiac sarcoplasmic reticulum (SR) was isolated from the LV for the initial homogenization steps. Oxalate-supported, steady-state, Ca^{2+} transport was measured using $^{45}Ca^{2+}$ and rapid filtration over a free [Ca^{2+}] range of 0.01–17 μM in 0.5 mM EGTA, 10 mM K-oxalate, 5 mM NaN_3, 20 mM imidazole (pH 7.15), 3.75 mM MgATP and a free [Mg^{2+}] of 0.6 mM at a total ionic strength of 0.16 M with 0.1 mg/ml SR. The expression of SR protein was estimated using Western blot analysis. Isolated SR proteins were separated by SDS–PAGE using 10% bis-Tris gels and NuPAGE technique (Novex, San Diego, CA). Proteins were loaded at 1, 2, 3 or 4 μg/well using one protein amount/gel. Each gel contained three normal, five vehicle and five $ET_{A/B}$ antagonist cardiac SR preparations. The proteins were transferred either to 0.2-μm nitrocellulose membranes (SERCA2a and calsequestrin) or to 0.2-μm PVDF membranes for phospholamban. The blots were washed in TBS then blocked with 1% blocking solution. The blots were then incubated in primary antibody against either SERCA2a (MA3-919, Affinity Bioreagents, Albany, NY), calsequestrin or phospholamban (mAb 1D11). The blots were washed in TBST then incubated in ^{125}I secondary antibody (Amersham, Piscataway, NJ) (0.2 μCi/ml). The blots were washed in TBST then dried. The proteins of interest were located by using the MW markers and complementary blots stained with secondary antibody to visualize the bands, cut out and counted. The counts were plotted against protein load and normalized to the number of counts in the normal SR samples on the same blot.

2.3. Experimental protocol

The dose of the $ET_{A/B}$ receptor antagonist, L-753,037 (J-104,132) required to block the effect of ET-1 was determined using the pressor response to ET-1. In the conscious normal dogs (n=4) treated with a single oral dose (5 mg/kg) of $ET_{A/B}$ antagonist, ET-1 (0.2 μg/kg, left atrial injection) increased mean arterial pressure by 2±1, 8±3, 18±5 and 28±8 mmHg at 4, 8, 12 and 24 h after dosing, while the mean arterial pressure was increased by 31±4, 30±3, 31±7 and 25±4 mmHg during the same time period after vehicle was given (n=4). Based on these results, L-753,037 was given twice a day (5 mg/kg, ×2) for 6 weeks beginning on the day when rapid pacing was initiated. Either L-753,037 or vehicle was prepared in a gelatin capsule for oral dosing. A total of 15 dogs were divided into $ET_{A/B}$ antagonist (n=7) and vehicle (n=8) groups. In addition, five normal dogs were served as non-heart failure controls.

Heart failure was initiated 2 weeks after the dogs had been surgically instrumented, while they were conscious and lying quietly on their left side. Baseline hemodynamic recordings were made from 15 dogs. Coronary and arterial blood samples were taken to measure plasma levels of NO. Inotropic responses to β-adrenergic receptor stimulation were assessed by infusion of norepinephrine (NE, 0.1, 0.2 and 0.4 mg/kg/min, i.v.) and dobutamine (2.5, 5.0, 7.5 and 10.0 mg/kg/min, i.v.) for 5 min at each dose. After baseline experiments, right ventricular pacing at a rate of 230 beats/min using a programmable pacemaker was initiated and continued for 2 weeks, followed by continuous pacing at 240 beats/min for another 4 weeks.

Hemodynamic status and inotropic responses to NE and

dobutamine were reassessed weekly for 6 weeks after initiation of pacing. After the final hemodynamic measurements were made, i.e. following 6 weeks of pacing, the dogs were anesthetized with pentobarbital sodium and the heart rapidly excised and placed in ice-cold saline. The atria and right ventricle were removed. LV tissue containing large coronary arteries was excised for in vitro study and the remaining LV tissue used for the isolation of cardiac SR. Body weight was measured before and after removing the abdominal fluid, since during the late stages of congestive heart failure significant ascetics is often evident. Two dogs in the $ET_{A/B}$ antagonist-treated group died a few days before the end of the 6-week pacing period (death occurred at 37 and 39 days). The death which occurred at 37 days before the end of the pacing period was due to instrumentation-induced acute arterial rupture. The other death was possibly due to the severity of heart failure.

2.4. Data analysis

Data before and after development of heart failure and responses to inotropic challenge were compared using the Student's *t*-test for paired data with a Bonferroni correction. Data between the $ET_{A/B}$ antagonist-treated and vehicle-treated groups were compared using unpaired Student's *t*-test. Regression lines were compared by differences in both slope and elevations of the lines by use of the *F*-test. SR Ca^{2+} uptake rates and Western blot quantification were compared between groups using a one-way ANOVA analysis and a Student–Newman–Keul's post-hoc comparison. All values are expressed as the mean±S.E. Statistical significance was accepted at the $P<0.05$ level.

3. Results

3.1. Effects of $ET_{A/B}$ antagonist on basal hemodynamics before and after heart failure development

The basal systemic hemodynamics and LV function in the $ET_{A/B}$ antagonist-treated and vehicle-treated groups before and 2, 4 and 6 weeks after initiation of treatment and rapid pacing are shown in Tables 1 and 2. There were no differences in any of the indices at baseline, i.e. before initiation of rapid ventricular pacing, between the groups treated with either vehicle or $ET_{A/B}$ antagonist. Fig. 1 shows representative waveforms from vehicle- and $ET_{A/B}$ antagonist-treated conscious dogs before and after 2, 4 and 6 weeks of rapid pacing. During the development of heart failure, treatment with the $ET_{A/B}$ antagonist decreased LV systolic pressure and aortic pressure, and increased mean

Table 1
Hemodynamic changes in conscious dogs during the development of heart failure[a]

	Baseline	Weeks of pacing		
		2	4	6
Mean arterial pressure (mmHg)				
Vehicle ($n=8$)	89±4	−1±3	−4±5	−4±4
$ET_{A/B}$ antagonist ($n=7$)	89±3	−14±4*#	−14±3*#	−16±5*
Mean left atrial pressure (mmHg)				
Vehicle ($n=8$)	6±1	+8±1*	+16±2*	+20±2*
$ET_{A/B}$ antagonist ($n=7$)	7±1	+6±1*	+11±2*	+12±3*#
Stroke volume index (ml/kg)				
Vehicle ($n=8$)	1.93±0.14	−0.54±0.11*	−0.69±0.13*	−0.89±0.17*
$ET_{A/B}$ antagonist ($n=7$)	1.75±0.18	−0.06±0.08#	−0.20±0.12#	−0.37±0.22
Cardiac index (ml/min/kg)				
Vehicle ($n=8$)	168.5±14.8	−13.1±12.5	−11.2±18.2	−31.6±21.2
$ET_{A/B}$ antagonist ($n=7$)	161.0±15.6	+52.5±14.7#	+42.2±21.7	+24.6±20.2
Total peripheral resistance (mmHg/ml/min/kg)				
Vehicle ($n=8$)	0.55±0.04	+0.07±0.05	+0.07±0.10	+0.23±0.10*
$ET_{A/B}$ antagonist ($n=7$)	0.58±0.06	−0.21±0.05*#	−0.18±0.05*#	−0.18±0.07*#
Coronary blood flow (ml/min)				
Vehicle ($n=6$)	20.4±2.9	−1.1±2.2	+0.3±2.3	−4.1±3.7
$ET_{A/B}$ antagonist ($n=6$)	19.8±1.3	+2.8±2.8	+3.5±2.8	+3.3±4.5
Heart rate (beat/min)				
Vehicle ($n=8$)	87±4	+23±3*	+40±6*	+48±7*
$ET_{A/B}$ antagonist ($n=7$)	93±6	+36±7*	+41±4*	+36±5*

[a] Data are mean±S.E.; *n*: number of animals, $n=5$ at 6 weeks of pacing in $ET_{A/B}$ antagonist group; * $P<0.05$ vs. baseline; # $P<0.05$ vs. vehicle.

Table 2
Left ventricular function in conscious dogs during the development of heart failure

	Baseline	Weeks of pacing		
		2	4	6
LV systolic pressure (mmHg)				
Vehicle (n=8)	113±6	−7±6	−11±5	−15±5*
$ET_{A/B}$ antagonist (n=7)	110±4	−15±4	−21±4*	−21±7*
LV end-diastolic pressure (mmHg)				
Vehicle (n=8)	6±1	+8±1*	+11±1*	+11±1*
$ET_{A/B}$ antagonist (n=7)	6±1	+5±1*	+7±1*	+6±3*#
LV dP/dt (mmHg/s)				
Vehicle (n=8)	2961±140	−1096±121*	−1156±176*	−1111±165*
$ET_{A/B}$ antagonist (n=7)	2875±215	−1009±205*	−1295±184*	−1363±348*
LV dP/dt/end-diastolic diameter (mmHg/s/mm)				
Vehicle (n=8)	86±4.6	−35±3.0*	−39±4.6*	−45±4.2*
$ET_{A/B}$ antagonist (n=7)	83±4.5	−33±5.0*	−42±4.7*	−44±8.7*
LV relaxation time constant, tau (ms)				
Vehicle (n=8)	32.6±1.6	+11.6±1.7*	+13.2±1.0*	+11.1±2.8*
$ET_{A/B}$ antagonist (n=7)	32.6±1.7	+5.7±1.8#	+9.6±2.0*	+11.6±2.7*
LV end-diastolic diameter (mm)				
Vehicle (n=8)	34.4±1.1	+2.3±0.6*	+4.3±0.9*	+4.5±1.0*
$ET_{A/B}$ antagonist (n=7)	34.5±0.9	+2.9±0.7*	+5.1±1.0*	+5.2±1.2*
LV fractional shortening (%)				
Vehicle (n=8)	16.0±1.2	−7.3±1.1*	−8.5±1.4*	−8.6±1.2*
$ET_{A/B}$ antagonist (n=7)	16.2±0.7	−5.8±0.8*	−7.5±1.5*	−7.1±1.2*
Vcfc ($s^{-1/2}$)				
Vehicle (n=8)	0.70±0.05	−0.35±0.05*	−0.40±0.06*	−0.40±0.05*
$ET_{A/B}$ antagonist (n=7)	0.73±0.04	−0.32±0.04*	−0.39±0.07*	−0.39±0.07*
LV systolic wall thickening (mm)				
Vehicle (n=8)	3.1±0.4	−1.0±0.10*	−1.4±0.3*	−1.5±0.3*
$ET_{A/B}$ antagonist (n=7)	3.4±0.3	−0.8±0.80*	−0.8±0.2*#	−1.0±0.2*
LV end systolic stress (g/cm^2)				
Vehicle (n=8)	160±10	+35±9*	+49±14*	+46±10*
$ET_{A/B}$ antagonist (n=7)	158±13	+17±8	+26±8	+39±13*
LV end diastolic stress (g/cm^2)				
Vehicle (n=8)	13.6±2.3	+21.2±1.9*	+30.5±4.3*	+30.4±4.6*
$ET_{A/B}$ antagonist (n=7)	12.9±1.1	+13.6±3.6*#	+23.1±3.3*	+26.0±10.0*

[a] Data are mean±S.E.; n: number of animals, n=5 at 6 weeks of pacing in $ET_{A/B}$ antagonist group; * $P<0.05$ vs. baseline; # $P<0.05$ vs. vehicle.

aortic blood flow compared to the vehicle. The time course of hemodynamic changes during the 6-week pacing period in these two groups are presented in Figs 2 and 3.

During the development of heart failure, LV dP/dt, LV fractional shortening, Vcfc and systolic wall thickening were significantly ($P<0.05$) decreased, while mean left atrial pressure, LV end-diastolic pressure, LV isovolumetric relaxation time constant (tau), LV end-diastolic diameter, LV systolic and diastolic stress, and heart rate were significantly ($P<0.05$) increased in the vehicle-treated group. In addition, cardiac index was decreased and total peripheral resistance was increased, but these changes were not significantly different from the baseline. The changes in LV dP/dt, LV dP/dt/end-diastolic diameter, LV fractional shortening and Vcfc during the development of heart failure in the group treated with $ET_{A/B}$ antagonist were similar to those observed in the vehicle-treated group. However, treatment with $ET_{A/B}$ antagonist significantly ($P<0.05$) reduced mean arterial pressure and total peripheral resistance, and increased cardiac index compared to the vehicle-treated group. In addition, the reduction in stroke volume index after heart failure was significantly less ($P<0.05$) in the group treated with $ET_{A/B}$ antagonist than in the vehicle-treated group. The changes in mean left atrial pressure and LV end-diastolic pressure were less ($P<0.05$) in the group treated with the $ET_{A/B}$

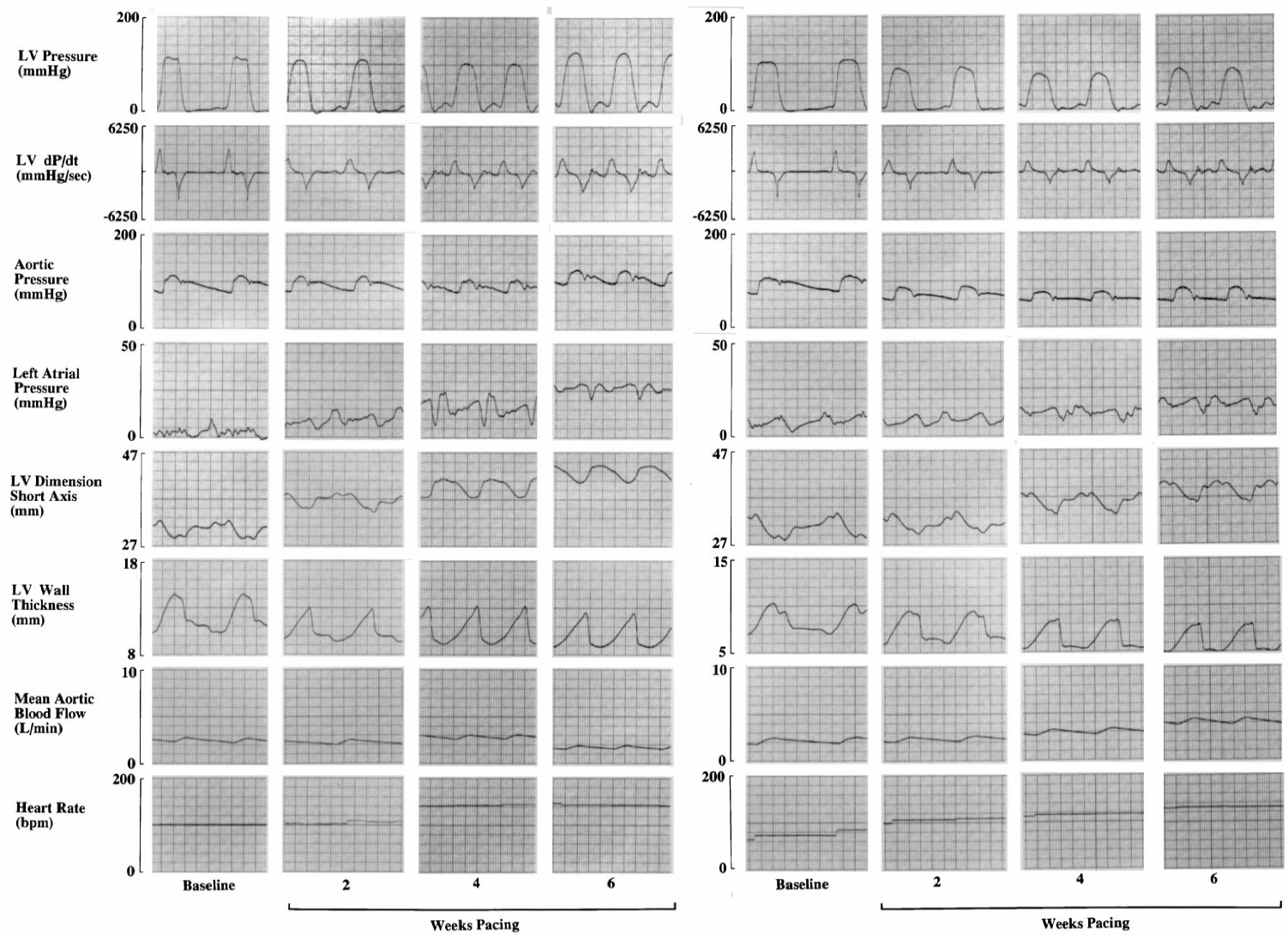

Fig. 1. Representative waveforms of left ventricular (LV) pressure, LV d*P*/d*t*, aortic pressure, left atrial pressure, LV dimension, LV wall thickness, mean aortic blood flow and heart rate from a conscious dog treated with either vehicle (left) or the $ET_{A/B}$ receptor antagonist (right) before and after 2–6 weeks of pacing. Note that the main effects of the $ET_{A/B}$ receptor antagonist were a decrease in aortic pressure and an increase in mean aortic blood flow compared to the vehicle.

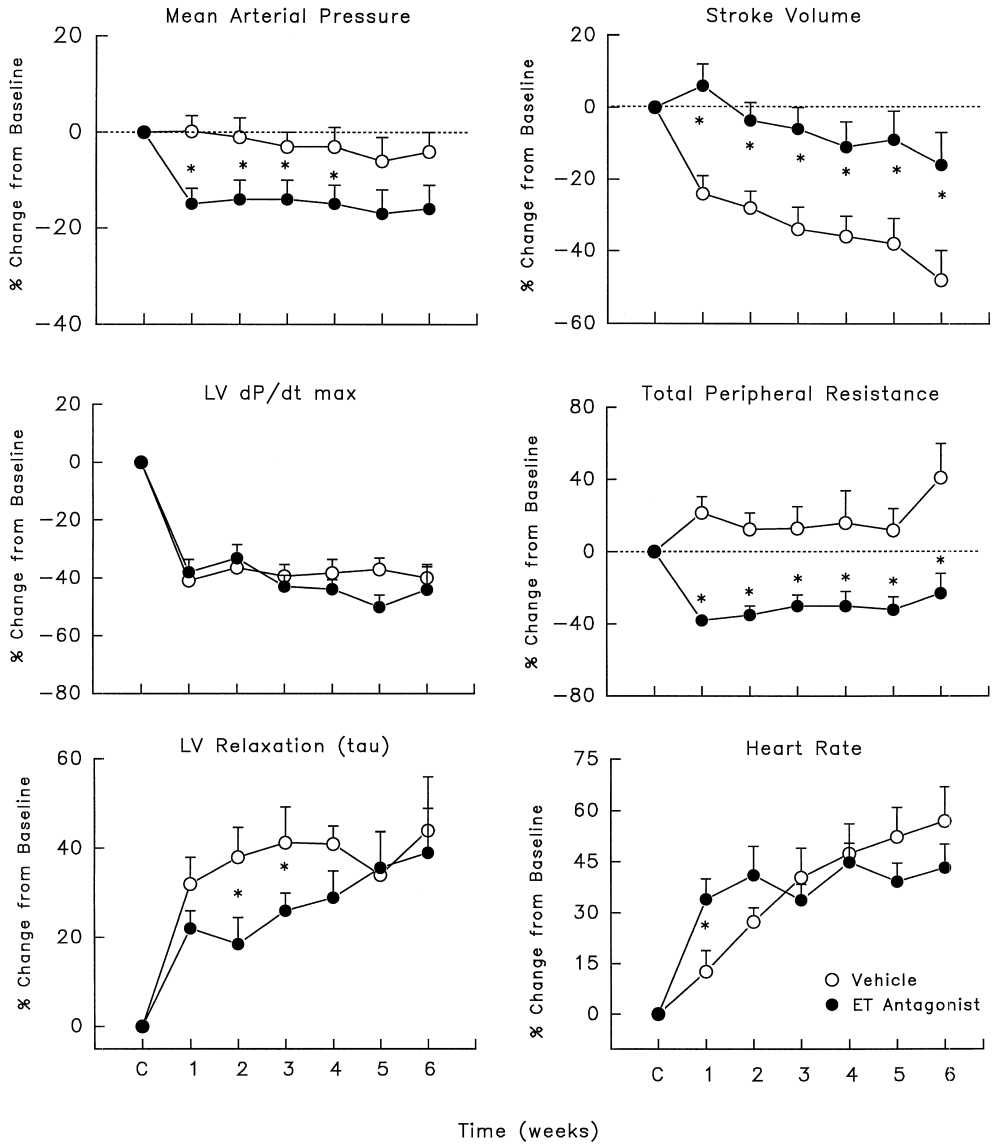

Fig. 2. Effects of $ET_{A/B}$ receptor antagonist on mean arterial pressure, LV dP/dt, LV relaxation (tau), stroke volume, total peripheral resistance and heart rate in conscious dogs during the development of heart failure. Values are % changes from baseline (C) levels. The $ET_{A/B}$ receptor antagonist decreased mean arterial pressure and total peripheral resistance. It also attenuated the increased tau and prevented the decrease in stroke volume compared to the vehicle. Heart rate was increased initially slightly more in the $ET_{A/B}$ receptor antagonist-treated group compared to the vehicle. * $P<0.05$ vs. vehicle-treated group.

antagonist compared to the vehicle-treated group after 6 weeks of pacing. The changes in tau and LV end-diastolic stress were also less ($P<0.05$) in the $ET_{A/B}$ antagonist-treated group than in the vehicle-treated group after 2 weeks of pacing. LV systolic wall thickening and LV end-systolic stress tended to be less, but not statistically significant in the group treated with the $ET_{A/B}$ antagonist. The mean coronary blood flows for the two groups were similar until after 4 weeks of pacing when it started to decline in the vehicle-treated group, but not in the group treated with $ET_{A/B}$ antagonist. The difference between these two groups, however, did not reach statistical significance. During heart failure development, heart rate initially increased more and then slightly less in the $ET_{A/B}$ antagonist-treated group compared to the vehicle-treated group, but again there were no statistically significant differences for this parameter between the two groups.

Fig. 4 shows the P–D loops and relationship between Vcfc and LV end-systolic wall stress during the development of heart failure in the groups treated with either vehicle or $ET_{A/B}$ antagonist. After heart failure, the P–D loops were shifted similarly to right between the two groups (top). The LV end-diastolic pressure–diameter was 233±30 and 696±62 mmHg/mm in the vehicle-treated group, and 231±30 and 583±94 mmHg/mm in the $ET_{A/B}$ antagonist-treated group before and after heart failure, respectively. The LV end-systolic pressure–diameter was 3031±120 and 3606±173 mmHg/mm in the vehicle-treated group, and 3347±182 and 3337±243 mmHg/mm in the $ET_{A/B}$ antagonist-treated group before and after

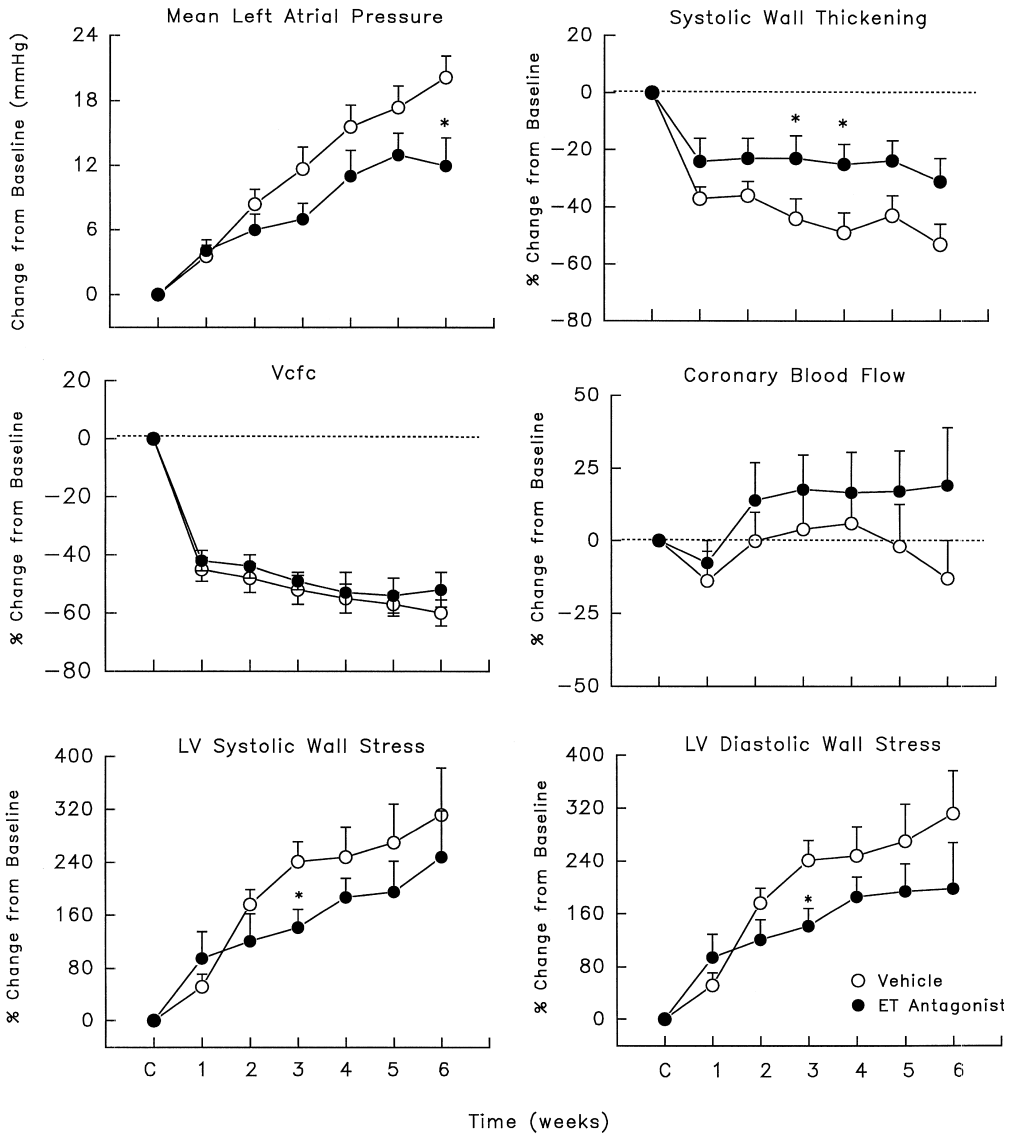

Fig. 3. Effects of $ET_{A/B}$ receptor antagonist on mean left atrial pressure, Vcfc, LV systolic wall stress, systolic wall thickening, coronary blood flow and LV diastolic wall stress in conscious dogs during the development of heart failure. Values are % changes from baseline (C) levels for all indices, except for the left atrial pressure. The $ET_{A/B}$ receptor antagonist attenuated the increased mean left atrial pressure, LV systolic and diastolic wall stress compared to the vehicle. Systolic wall thickening was decreased less in the $ET_{A/B}$ receptor antagonist-treated group than in the vehicle-treated group. At 6 weeks of pacing, coronary blood flow tended to be decreased in the vehicle-treated group, while in the $ET_{A/B}$ receptor antagonist-treated group, coronary blood flow was maintained. * $P<0.05$ vs. vehicle-treated group.

heart failure, respectively. Also, there was no significant difference either in the slope or in the *y*-intercept between these two groups (bottom).

3.2. Effects of $ET_{A/B}$ antagonist on inotropic response to β-adrenergic receptor challenge

The basal hemodynamics and responses to dobutamine (10 mg/kg/min, i.v.) and NE (0.4 mg/kg/min, i.v.) before and after 2 and 4 weeks of pacing in the $ET_{A/B}$ antagonist-treated and vehicle-treated groups are shown in Tables 3 and 4, respectively. Following 2 and 4 weeks of pacing, LV d*P*/d*t* responses to both agents were markedly attenuated compared to baseline. However, the changes in LV systolic pressure, LV d*P*/d*t*, mean arterial pressure and heart rate induced by each of these agents were similar in the groups treated with $ET_{A/B}$ antagonist or vehicle.

3.3. Effects of $ET_{A/B}$ antagonist on plasma NO levels and isolated coronary artery vasoactivity

NO levels at baseline from either arterial blood or coronary sinus blood were similar for the groups treated with $ET_{A/B}$ antagonist or vehicle (Fig. 5). During the development of heart failure, the NO level from the coronary sinus was initially increased and later decreased

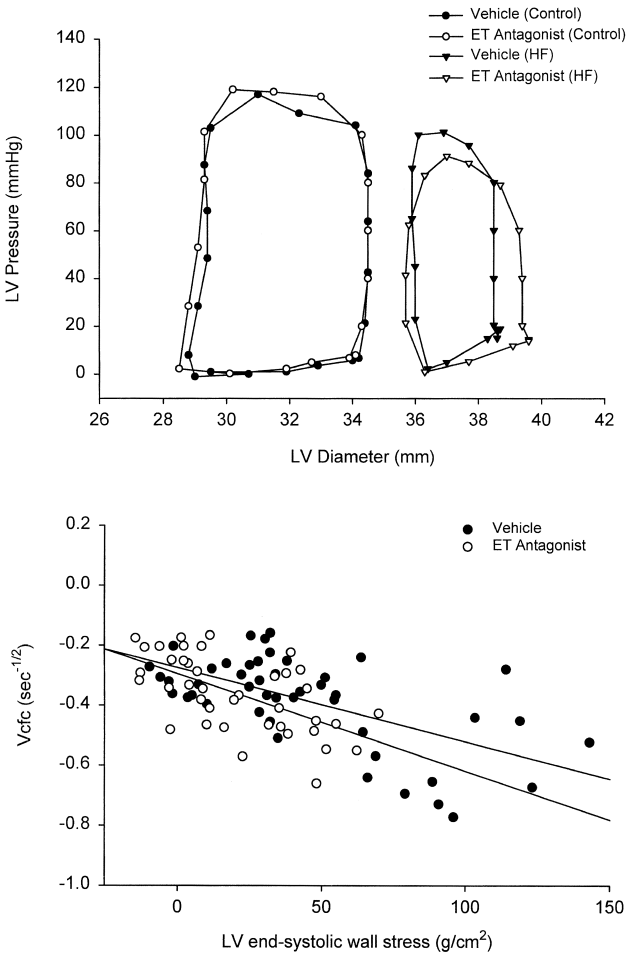

Fig. 4. LV pressure–diameter (P–D) loops (top) and relationship between Vcfc and LV end-systolic wall stress in the $ET_{A/B}$ receptor antagonist-treated and vehicle-treated groups during the development of heart failure (bottom). The P–D loops were shifted similarly to the right after heart failure in the two groups. Also, the slope of this relationship was not significantly different for these two groups.

in the vehicle-treated group. In contrast, treatment with $ET_{A/B}$ antagonist resulted in progressively increasing NO levels. After 6 weeks of pacing, there was a significant ($P<0.05$) difference between the two groups.

Relaxation activity of coronary arteries isolated from control dogs and dogs with heart failure (6 weeks of pacing) treated with either vehicle or $ET_{A/B}$ antagonist are shown in Fig. 6. After heart failure, coronary relaxation responses to acetylcholine at a concentration of 1 and 10 μM were significantly ($P<0.01$) attenuated compared to non-heart failure controls. However, the coronary artery rings from the dogs treated with $ET_{A/B}$ antagonist for 6 weeks displayed significantly ($P<0.01$) enhanced vascular relaxation compared to those from the vehicle-treated heart failure dogs. Coronary relaxation responses to acidified $NaNO_2$ were similar among all three groups.

3.4. Effects of $ET_{A/B}$ antagonist on cardiac SR Ca^{2+} transport and SR protein expression

The maximal Ca^{2+} uptake rate in normal cardiac SR was 1.82±0.10 μmol/mg/min at *p*Ca 4.75. The maximal rate of SR Ca^{2+} uptake for vehicle-treated dogs occurred at *p*Ca 5.0 with a rate of 1.34±0.07 μmol Ca^{2+}/mg/min which was significantly less ($P<0.01$) than the rate in normal SR at *p*Ca 5.0 (1.66±0.03). For the $ET_{A/B}$ antagonist-treated group, SR Ca^{2+} uptake was maximal at *p*Ca 5.0 with a rate of 1.60±0.06 μmol Ca^{2+}/mg/min which was significantly greater than vehicle-treated dogs ($P<0.02$) as shown in Fig. 7 (top). The ratio of maximal Ca^{2+} uptake rates from *p*Ca 6.25 to *p*Ca 4.75 was 0.75±0.03 for vehicle-treated/normal compared to 0.86±0.02 for $ET_{A/B}$ antagonist-treated/normal ($P<0.01$). Fig. 7 (bottom) shows the SERCA2a ^{125}I counts from the normal, vehicle-, and $ET_{A/B}$ antagonist-treated dogs. For

Table 3
Effects of dobutamine (10 μg/kg/min, i.v.) on LV function in conscious dogs before and during heart failure development[a]

	Control		2 weeks pacing		4 weeks pacing	
	Baseline	Change	Baseline	Change	Baseline	Change
LV systolic pressure (mmHg)						
Vehicle ($n=8$)	115±5	+18±4*	104±2	+11±3*	109±3	+8±3*
$ET_{A/B}$ antagonist ($n=7$)	110±2	+24±3*	100±3	+10±2*	91±4#	+9±2*
LV dP/dt (mmHg/s)						
Vehicle ($n=8$)	2875±155	+1938±127*	1836±62	+1082±171*†	1836±122	+961±145*†
$ET_{A/B}$ antagonist ($n=7$)	2821±185	+2170±299*	1982±57	+1045±149*†	1571±66	+848±132*†
Mean arterial pressure (mmHg)						
Vehicle ($n=8$)	92±4	+9±3*	89±2	+2±3	94±3	+1±2
$ET_{A/B}$ antagonist ($n=7$)	88±2	+13±4*	83±2	+3±3	76±2#	+3±4
Heart rate (beats/min)						
Vehicle ($n=8$)	82±6	+6±5	98±3	+6±5	114±5	+4±5
$ET_{A/B}$ antagonist ($n=7$)	86±4	+4±5	118±8	+2±6	117±8	+5±5

[a] Values are mean±S.E.; *n*: number of animals; * $P<0.05$ vs. baseline; # $P<0.05$ vs. vehicle; † $P<0.05$ vs. control.

Table 4
Effects of norepinephrine (0.4 μg/kg/min, i.v.) on LV function in conscious dogs before and during heart failure development[a]

	Control		2 week pacing		4 week pacing	
	Baseline	Change	Baseline	Change	Baseline	Change
LV systolic pressure (mmHg)						
Vehicle (n=8)	116±4	+53±6*	104±3	+41±8*	106±3	+37±4*
$ET_{A/B}$ antagonist (n=7)	111±3	+51±8*	99±3	+43±6*	93±3	+27±2*#
LV dP/dt (mmHg/s)						
Vehicle (n=8)	3031±139	+2578±207*	1834±53	+1703±338*†	1891±119	+1445±380*†
$ET_{A/B}$ antagonist (n=7)	2857±214	+2446±320*	1938±87	+1438±192*†	1589±81	+1054±149*†
Mean arterial pressure (mmHg)						
Vehicle (n=8)	92±4	+42±6*	87±2	+43±8*	92±3	+34±4*
$ET_{A/B}$ antagonist (n=7)	90±3	+48±7*	83±3	+44±8*	77±2#	+25±6*
Heart rate (beats/min)						
Vehicle (n=8)	80±5	−3±4	91±5	+4±7	110±4	−4±7
$ET_{A/B}$ antagonist (n=7)	86±6	−5±8	116±8#	−20±10	116±7	−23±9*

[a] Values are mean±S.E.; n: number of animals; * $P<0.05$ vs. baseline; # $P<0.05$, vs. vehicle; † $P<0.05$ vs. control.

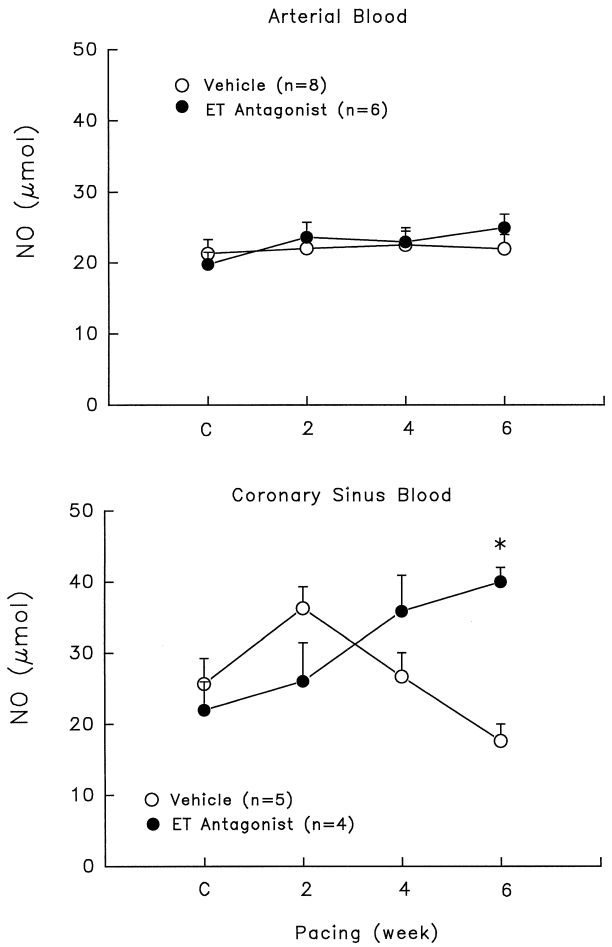

Fig. 5. Plasma levels of NO (μmol/l) from arterial blood (top) and coronary sinus blood (bottom) at baseline (C) and after 2, 4 and 6 weeks of pacing in conscious dogs. NO levels in the coronary sinus were slightly increased after 2 weeks of pacing and then decreased below baseline after 6 weeks of pacing in the vehicle-treated group. In contrast, the NO levels gradually increased during the 6-week pacing period in the $ET_{A/B}$ receptor antagonist-treated group. * $P<0.05$ vs. vehicle-treated group.

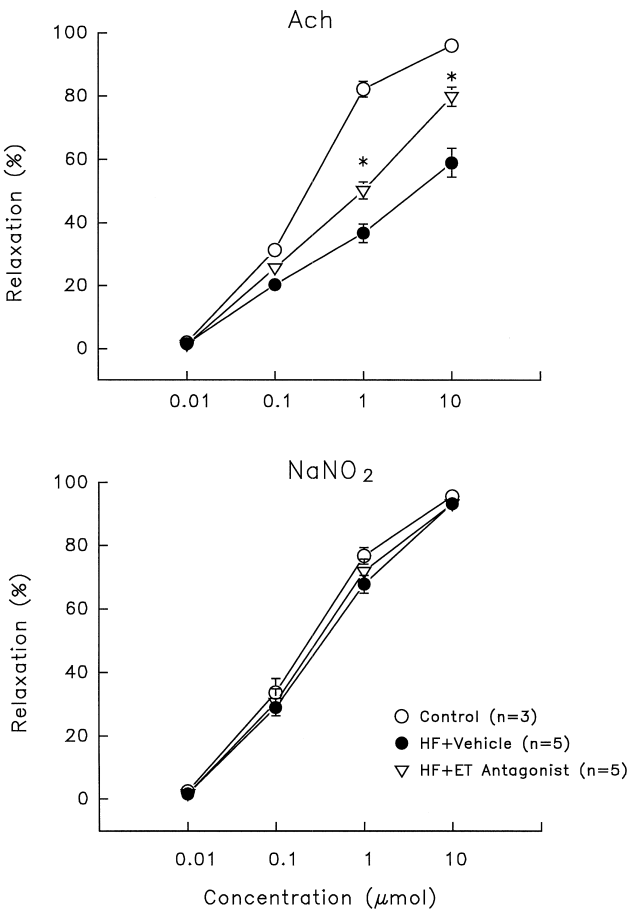

Fig. 6. Dose–response effects of acetylcholine (Ach) and $NaNO_2$ in coronary artery rings isolated from control non-heart failure dogs, and dogs with heart failure treated with vehicle or $ET_{A/B}$ receptor antagonist. Note that the relaxation response to Ach was attenuated in the heart failure group treated with vehicle. However, in the heart failure group treated with $ET_{A/B}$ receptor antagonist, the attenuated response was improved. * $P<0.01$ vs. heart failure group treated with vehicle.

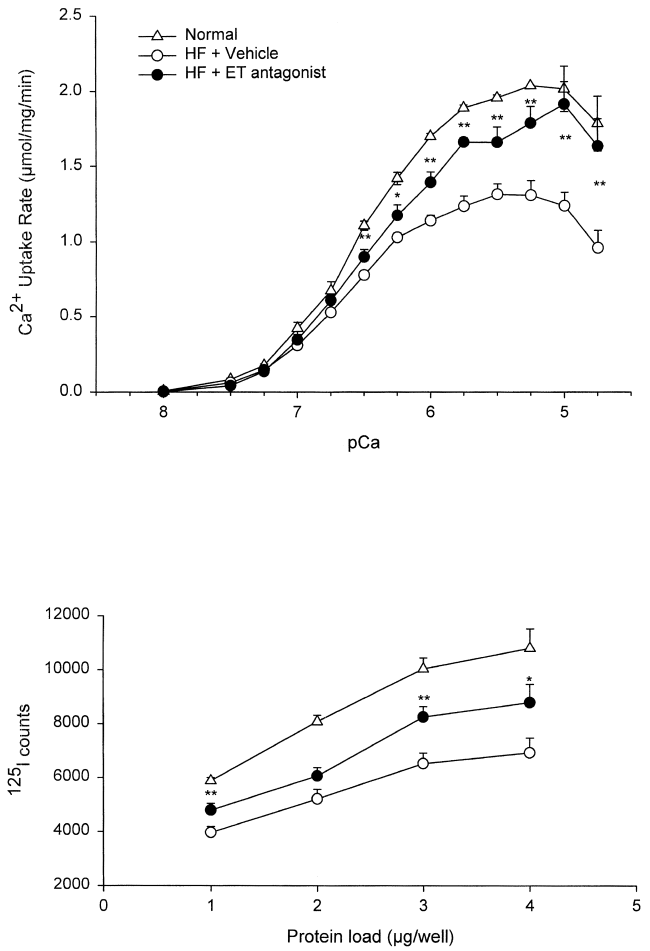

Fig. 7. Ca^{2+} uptake rates in cardiac SR (Top) and levels of ^{125}I labeling of SERCA2a protein in cardiac SR (Bottom) from normal dogs and failing dogs treated with vehicle or $ET_{A/B}$ antagonist. Each point represents the mean of duplicate runs at each protein load. Note that Ca^{2+} transport was improved in the $ET_{A/B}$ antagonist-treated group compared to the vehicle. There was a greater level of protein expression in the $ET_{A/B}$ antagonist-treated dogs compared to the vehicle. * $P<0.05$ vs. vehicle-treated groups.

all protein loads, SERCA2a expression was significantly less ($P<0.05$) in vehicle-treated dogs than in normal dogs. With $ET_{A/B}$ antagonist treatment, SERCA2a levels were significantly greater ($P<0.05$) than the vehicle but still less than the normal tissue. The ratio of SERCA 2a for vehicle-treated/normal was 0.68 ± 0.03 compared to 0.81 ± 0.02 ($P<0.01$) for $ET_{A/B}$ antagonist-treated/normal. Similar results were obtained for the Ca^{2+} binding protein calsequestrin where the ratio for vehicle-treated/normal was 0.63 ± 0.03 compared with 0.77 ± 0.04 ($P<0.05$) for $ET_{A/B}$ antagonist-treated/normal. For phospholamban expression, there was a significant reduction in the ratio of vehicle-treated/normal protein (0.71 ± 0.03) but for $ET_{A/B}$ antagonist-treated hearts the ratio was 1.08 ± 0.04 which was significantly greater than vehicle ($P<0.01$) but similar to normal.

4. Discussion

The present investigation has demonstrated, for the first time, that chronic treatment with an $ET_{A/B}$ receptor antagonist prevents the decline of coronary NO levels in conscious animals during the progression of congestive heart failure induced by rapid ventricular pacing. Additionally, the impairment in endothelium-dependent coronary vasorelaxation that occurs at the end stage of congestive heart failure was improved significantly by the $ET_{A/B}$ receptor antagonist treatment. The major favorable hemodynamic effects of the treatment were (1) a reduced vascular resistance, as reflected by increased cardiac output and mainly due to the improvement of stroke volume, associated with moderately decreased mean arterial pressure; (2) an improved LV diastolic dysfunction at the early stage of heart failure, as determined by an attenuated rise in LV isovolumetric relaxation time constant, tau; and (3) an attenuation of the increased LV end-diastolic pressure, left atrial pressure, LV systolic and diastolic stress during the development of heart failure. In addition, reductions in intracellular Ca^{2+} regulation that are evident at the end stage of heart failure are attenuated by chronic $ET_{A/B}$ receptor antagonist administration. However, the present investigation also showed that neither myocardial contractile function, at rest or in response to inotropic challenges, nor progressive dilation of the left ventricle were altered by chronic treatment with the $ET_{A/B}$ receptor antagonist during a 6-week period of rapid pacing.

Increasing evidence indicates there is a coronary endothelial dysfunction in heart failure that is associated with a reduced NO production [20–23]. In the current study, coronary NO production in vehicle-treated dogs, measured from blood collected from a coronary sinus catheter, was slightly increased soon after rapid pacing was initiated, possibly due to compensatory mechanisms, but then progressively decreased at the late stage of heart failure. This decreased NO production was clearly prevented by chronic administration of the $ET_{A/B}$ receptor antagonist. In connection with this finding, we found that the coronary vasorelaxant response to acetylcholine, an endothelium-dependent vasodilator, was improved in the treated group compared to the vehicle. However, the coronary artery relaxation responses to acidified $NaNO_2$, an NO donor, were identical among the heart failure animals treated with the $ET_{A/B}$ receptor antagonist or vehicle, as well as non-heart failure animals, suggesting that the reduction in endogenous NO production is primarily responsible for the coronary dysfunction during heart failure. This observation is consistent with the previous finding that a decrease in endothelial NO production during heart failure is the primary cause of the coronary dysfunction [21]. In addition, it is known that NO plays a critical role in regulation of mitochondrial respiration [24,25], which leads to an increase in myocardial oxygen consumption without a change in ATP synthesis, thus decreasing myocardial

metabolic efficiency [26]. Consequently, it is conceivable that preservation of the coronary NO levels through the chronic inhibition of $ET_{A/B}$ receptors would have long-term beneficial effects on the efficiency of LV performance in congestive heart failure.

Hasenfuss et al. [27] have demonstrated a strong correlation between the severity of heart failure and reductions in the Ca^{2+} handling proteins SERCA2a and phospholamban. A reduction in SERCA2a would be expected to prolong the time required for Ca^{2+} sequestration leading to increases in LV end-diastolic pressure. Recently, reductions of approximately 28% in each of these proteins were correlated with a prolongation of cytosolic Ca^{2+} removal in myocytes isolated from dogs subjected to 3–4 weeks of pacing [28], which is consistent with the present finding. Six weeks of pacing in vehicle-treated dogs in the present study was associated with a 32% reduction in SERCA2a and a 29% reduction in phospholamban expression, which were associated with reduced rates of SR Ca^{2+} sequestration. However, in dogs treated with the $ET_{A/B}$ receptor antagonist, the reductions in SERCA2a, phospholamban and calsequestrin expression were attenuated significantly improving rates of Ca^{2+} transport. The attenuation of the loss in function with $ET_{A/B}$ receptor antagonist treatment may be a consequence of both an indirect hemodynamic effect as well as a direct antagonism of the effects of ET-1, which has been shown to have direct negative effects on intracellular Ca^{2+} regulation leading to a prolongation of relaxation [29], as well as, the reduction in the expression of SERCA2a [30].

Several prior studies in animals and patients with heart failure have shown that administration of selective ET_A or $ET_{A/B}$ receptor antagonists enhanced cardiac output and decreased systemic vascular resistance [6,12,14,17,18,31], reduced LV end-diastolic pressure, pulmonary wedged pressure or right atrial pressure [6,12,18,31], and improved LV relaxation [12,18]. These cardiac and systemic hemodynamic effects were similar to what was observed in the present study. Furthermore, several previous studies also reported that administration of ET receptor antagonists enhanced myocardial contractile function [12,13,16,18], while other studies found either no effect [17] or a decrease in myocardial contractile function [11]. It is difficult to reconcile these contradictory results because of the different models, treatment regimens, and parameters measured in these prior studies. The unique feature of the present study was that the prolonged chronic effects of an $ET_{A/B}$ receptor antagonist on myocardial contractile function were examined in conscious animals with direct measurements of LV dP/dt, Vcfc and fractional shortening using chronically implanted instrumentation, not only at rest, but also in response to β-adrenergic receptor stimulation to assess myocardial contractile reserve during the different stages of heart failure. Our results show no significant differences in these indices between the $ET_{A/B}$ receptor antagonist-treated and vehicle-treated groups suggesting that in intact, conscious dogs during the development of heart failure, chronic inhibition of both ET_A and ET_B receptors for 6 weeks does not affect myocardial contractile performance. However, since loading conditions were altered in the $ET_{A/B}$ receptor antagonist-treated group, we further compared the relationship between Vcfc and LV end-systolic wall stress, an index of afterload, and LV dP/dt corrected for the change in the end-diastolic diameter (EDD), an index of preload. These results show that the inverse relationships were similar and there was no difference in LV $dP/dt/EDD$ for the two groups, further indicating a lack of effect of $ET_{A/B}$ receptor antagonist treatment on cardiac contractile function. Although previous studies have shown that ET-1 directly inhibits myocardial contractile function of isolated myocytes [13,29], the different findings between these studies and the present investigation could be due to different treatment regimens and preparations.

Whether or not chronic inhibition of ET receptors has any impact on the regression of ventricular dilation during the development of heart failure is unclear. To date, all observations of changes in LV dimension with administration of ET receptor antagonists have been made in either anesthetized or tranquilized conditions using non-invasive echocardiography measurements [13,16,17]. This methodology, particularly used in animal studies, has multiple limitations which add to the difficulty in reconciling the inconsistent results among the previous studies, even when a similar animal model and same ET receptor subtype antagonists were used [13,17]. As mentioned above, the potential problems in the these prior studies have been avoided in the present investigation. Using chronically implanted instrumentation on the myocardium, allowing direct measurement of LV dimension, as well as, LV pressure–diameter loops in conscious dogs, clearly, the LV pressure–diameter loops were similarly shifted to the right after heart failure in the ET receptor antagonist-treated and vehicle-treated groups. In addition, we did not observe any differences in wall thickness between these two groups during the development of heart failure. The rapid pacing model used in the present investigation, however, is characterized by a progressively deteriorating dilated cardiomyopathy within a relatively short period of time, rather than by a prolonged, stable, ischemia-induced dilated cardiomyopathy. Therefore, we cannot exclude any potential long-term effects of $ET_{A/B}$ receptor antagonism on ventricular remodeling.

Another limitation of the pacing-induced heart failure model is that cardiac output and total peripheral resistance, in fact, were well-maintained until the late stage of heart failure, partly due to powerful compensation via the marked increase in heart rate that occurs in the canine species [32]. Consequently, stroke volume is reduced substantially. Because of this particular intricacy, the effects of $ET_{A/B}$ receptor antagonism observed in the present study should be considered as an improvement in

or a prevention of the decline in stroke volume, rather than simply as an increase in cardiac output above or a decrease in vascular resistance below the baseline levels.

In the present study, heart rate was increased initially more and then slightly less in the $ET_{A/B}$ antagonist-treated group compared to the vehicle-treated group, although these changes were not significantly different between the two groups. It is conceivable that since administration of the $ET_{A/B}$ receptor antagonist was initiated when the rapid pacing was started, the profound vasodilatory effect of the ET antagonist that occurred early during the period of rapid pacing stimulated the intact baroreflex system, causing the moderate increase in heart rate. When advanced heart failure was evident, i.e. after 3–6 weeks of rapid pacing, the loss of the correlation between heart rate and vascular tone could be explained by impaired baroreflex function.

In conclusion, in conscious dogs during the development of heart failure chronic inhibition of $ET_{A/B}$ receptors does not affect resting myocardial contractile function nor contractile reserve, but reduces vascular resistance and improves LV diastolic function**.** At the late stage of heart failure with $ET_{A/B}$ receptor antagonist treatment, reduction in intracellular Ca^{2+} regulation by the SR is attenuated and coronary vasoactivity is improved, most likely due to the preservation of myocardial nitric oxide levels.

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