

# Bradycardic Therapy Improves Left Ventricular Function and Remodeling in Dogs with Coronary Embolization-Induced Chronic Heart Failure

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## ABSTRACT

Both  $\beta$ -adrenergic blockade and bradycardia may contribute to the therapeutic effect of  $\beta$ -blockers in chronic heart failure (CHF). This study tested the relative importance of bradycardia by comparing cilobradine (Cilo), a sinus node inhibitor, with a  $\beta$ -blocker, metoprolol (Meto), in an established canine model of CHF. Dogs were chronically instrumented for hemodynamic and left ventricular (LV) volume measurements. CHF was created by daily coronary embolization via a chronically implanted coronary (left anterior descending coronary artery) catheter. After establishment of CHF, control ( $n = 6$ ), Meto (30 mg/day,  $n = 5$ ), Cilo (low) (1 mg/kg/day,  $n = 5$ ), or Cilo (high) (3 mg/kg/day,  $n = 5$ ) was given orally for 12 weeks. Systemic hemodynamics, echocardiography, and pressure volume analysis were measured at baseline, at CHF, and 3 months after treatment in an awake state. Protein levels of cardiac sarcoplasmic retic-

ulum calcium-ATPase (SERCA2a), ryanodine receptor (RyR2), and  $\text{Na}^+\text{-Ca}^{2+}$  exchanger (NCX1) were measured by Western blot. RyR2 protein kinase A (PKA) phosphorylation was determined by back-phosphorylation. After 12 weeks, Meto and Cilo (high and low) produced similar bradycardic effects, accompanied by a significantly improved LV dP/dt versus control [Meto,  $2602 \pm 70$ ; Cilo (low),  $2517 \pm 45$ ; Cilo (high),  $2579 \pm 78$ ; control,  $1922 \pm 115$  mm Hg/s;  $p < 0.05$ ]. Both Meto and Cilo (high) normalized protein levels of SERCA2a and NCX1 and reversed PKA hyperphosphorylation of RyR2, in contrast to controls. High-dose cilobradine effectively produced bradycardia and improved cardiac function after CHF, comparable with metoprolol. Restored protein levels of SERCA2a and improved function of RyR2 may be important mechanisms associated with cilobradine therapy.

$\beta$ -Adrenergic blocker therapy reduces morbidity and mortality in patients with chronic heart failure (CHF) (Packer et al., 1996). A number of molecular mechanisms have been

proposed to account for these benefits, including restoration of  $\beta$ -receptor density and signal transduction (Heilbrunn et al., 1989), normalization of abnormal phosphorylation status (Reiken et al., 2001), improved calcium channel function (Reiken et al., 2003), and correction of neurohormonal imbalance (Bristow, 1984). The role of  $\beta$ -blocker-induced bradycardia in improving left ventricular (LV) dysfunction in CHF has also been investigated. Nagatsu et al. (2000) reported that if  $\beta$ -blocker-induced bradycardia was prevented by cardiac pacing in a canine model of CHF, no improvement in LV function would be seen, highlighting the importance of heart rate

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**ABBREVIATIONS:** CHF, chronic heart failure; LV, left ventricular; RyR2, ryanodine receptor; Meto, metoprolol; Cilo, cilobradine; IVC, inferior vena cava; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; PV, pressure-volume; MAP, mean arterial pressure; EF, ejection fraction; EDV, end-diastolic volume;  $\alpha$ , chamber stiffness;  $\beta$ , end-diastolic pressure-volume relationship scaling constant; SERCA2a, cardiac sarcoplasmic reticulum calcium-ATPase; NCX1,  $\text{Na}^+\text{-Ca}^{2+}$  exchanger; PAGE, polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline with 0.1% Tween 20; PKA, protein kinase A;  $\tau$ , isovolemic relaxation constant; CIBIS, Cardiac Insufficiency Bisoprolol Study.

reduction in CHF therapy. Prevention of tachycardia may contribute to clinical benefit after  $\beta$ -blockade because tachycardia can be both a cause and secondary effect of progressive CHF (Packer et al., 1986). These studies suggest a role for bradycardic therapy in the treatment of CHF (Lechat, 2003).

However,  $\beta$ -blocker therapy poses special challenges in the heart failure population, mainly due to its effects on systolic function. Certain populations of patients, such as those with reactive airway disease, conduction system disease, or hemodynamic compromise, may not tolerate therapy. This has led to the need for dose titration and limitations on its clinical use. An ideal agent for these patients can produce the same physiologic effects as a  $\beta$ -blocker without the negative effects of  $\beta$ -receptor blockade. Accordingly, the purpose of this study was to test whether cilobradine, a sinus node inhibitor, produces beneficial effects on cardiac function in a canine model of embolization-induced heart failure similar to  $\beta$ -adrenergic blocker therapy. Levels of calcium-handling proteins and phosphorylation status of the ryanodine receptor (RyR2), shown to normalize with  $\beta$ -blockade (Reiken, 2001, 2003), were also examined as potential mechanisms of benefit.

## Materials and Methods

**Study Design.** Multiple daily coronary embolizations were used in 21 mongrel dogs of both sexes (22–27 kg) to create CHF. After a stable CHF state was maintained for 7 days, each animal was assigned to one of four groups: 1) control, who received normal saline ( $n = 6$ ); 2) metoprolol (Meto;  $n = 5$ , 30 mg/day p.o.) (Novartis Pharmaceuticals, East Hanover, NJ); 3) low-dose cilobradine (Cilo) (Boehringer Ingelheim, Ingelheim, Germany) ( $n = 5$ , 1 mg/kg/day p.o.); and 4) high-dose Cilo ( $n = 5$ , 3 mg/kg/day p.o.). Hemodynamic measurements and echocardiograms were performed before CHF, after an established stable CHF, and 12 weeks after treatment with cilobradine, metoprolol, or saline. At the end of 12 weeks, the animals were sacrificed, and LV myocardial tissue from the nonembolized region was harvested for assessment of calcium-handling protein levels and phosphorylation of RyR2. This study was approved by the institutional Animal Care and Use Committee of Columbia University and conforms with the *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1996).

**Surgical Preparation.** On the day of surgery, each animal (22–25 kg) was premedicated with 3 to 5 mg/kg Telazol i.m. (tiletamine hydrochloride and zolazepam hydrochloride; Wyeth, Madison, NJ), induced with 15 to 20 mg/kg sodium thiopental i.v. (Abbott Laboratories, Abbott Park, IL), and ventilated on isoflurane at 1.5 to 2.5% (Abbott Laboratories). The instrumentation surgery began with a left thoracotomy between the fourth and fifth intercostal space to expose the heart. A Konigsberg solid state pressure gauge (6.5; Konigsberg Instruments Inc., Pasadena, CA) was placed through the LV apex for measurement of LV pressure. Fluid-filled catheters were placed in the ascending aorta and the LV for measurement of aortic pressure and calibration of the Konigsberg pressure gauge, respectively. A pneumatic cuff was placed around the inferior vena cava (IVC) to vary loading conditions postoperatively. The left anterior descending coronary artery was isolated, and a thin silastic cannula was inserted through an arteriotomy for microsphere embolization administration. In addition, six sonomicrometric crystals (Sonometrics Corp., London, Canada) were placed on the anterior, posterior, apex, base, septal, and free walls of the LV. These surgical procedures and cardiac instrumentations have been used in our laboratory previously (He et al., 2005).

**Coronary Embolization-Induced Heart Failure.** This model has been described previously in detail (Knecht et al., 1997). In brief, CHF was produced by daily intracoronary microembolization with

25,000 to 50,000 (90–120  $\mu$ m in diameter) polymer bead injections through the previously implanted left anterior descending coronary artery cannula. Embolization proceeded until left ventricular end-diastolic pressure (LVEDP) reached 15 mm Hg, and LV dP/dt was less than 2400 mm Hg/s. Embolization was halted at this time, and hemodynamics and echocardiography were repeated in an awake state after an additional 7 days to confirm the stability of the CHF state.

**Hemodynamics and Echocardiography.** Hemodynamics and echocardiography were measured as described previously in an awake state (He et al., 2005). In brief, on the day of each experiment, the dog was brought to the laboratory and placed on the table. All measurements were taken after the dogs were quiet for an average of 60 min and accustomed to the laboratory. Arterial pressure was measured by attaching the previously implanted catheters to P231D strain-gauge transducers (Statham Instruments, Inc., Oxnard, CA). Left ventricular systolic pressure (LVSP) was measured with the previously implanted solid-state pressure gauges, which were calibrated in vitro against an electronic signal of known size and cross-calibrated in vivo with measurements of pressure from the LV catheter. Inflating the previously placed pneumatic cuff transiently occluded the IVC for construction of LV pressure-volume (PV) relationships. Mean arterial pressure (MAP) was determined on-line by use of 3-Hz averaging filters (DA26; Medtron Engineering, Olivenhain, CA). Continuous electrocardiographic data were obtained using leads placed on the skin. Data were recorded on an eight-channel thermal writing chart recorder (30-V8808-10; Gould Electronics, East Rutherford, NJ), and periods of interest were digitized (Gateway 2000 486 computer equipped with an analog-to-digital conversion system; Sonometrics Corp.) for off-line analysis. Heart rate was determined manually from the electrocardiographic tracing over a 60-s period and averaged over three readings during a 2-h period. Echocardiograms were performed using a Hewlett Packard Sonos 5500 (Hewlett Packard, Palo Alto, CA) at baseline, CHF state, and every 4 weeks after CHF in an awake state. Echocardiograms were analyzed off-line by a blinded observer by quantifying short axis end-diastolic and end-systolic area at mid-papillary levels with a 3.5-MHz transducer. Ejection fraction (EF) was similarly calculated as end-diastolic volume (EDV) minus end-systolic volume, divided by EDV ( $[(EDV - \text{end-systolic volume})/EDV \times 100]$ ).

**Pressure-Volume Loop Analysis.** LV pressure and LV volume were measured at baseline, CHF state, and every 4 weeks after device implantation via previously implanted solid state pressure gauges in the LV and sonomicrometric crystals in an awake state, as described previously (He et al., 2005). In brief, LV PV relationship loops were measured at rest and during a transient preload reduction induced by IVC occlusion. LV volumes ( $LV_{vol}$ ) were estimated from sonomicrometrically measured dimensions assuming an ellipsoid model:  $LV_{vol} = \pi AP \times SF \times AB/6$ , where AP is anterior-posterior, SF is septal-free wall, and AB is apex-base dimensions. The end-systolic pressure-volume relationship was assessed using a linear approach:  $E_{es} = P_{es}/(V_{es} - V_0)$ , where the slope [end-systolic elastance ( $E_{es}$ )] defines contractility, and  $V_0$  defines its volume-axis intercept. The end-diastolic pressure-volume relationship was determined by applying nonlinear regression analysis to the end-diastolic pressure and volume points ( $P_{ed}$  and  $V_{ed}$ , respectively) during a rapid IVC occlusion via pneumatic cuff inflation. These data were fit to the following equation:  $P_{ed} = \beta e^{\alpha V_{ed}}$ , where  $\alpha$  is a chamber stiffness constant, and  $\beta$  is a scaling constant. Each steady state measurement was calculated and averaged over 10 cardiac cycles using custom software (MATLAB, version 6.5; The MathWorks, Inc., Natick, MA).

**Protein Isolation and Western Analysis for Calcium-Handling Proteins.** Three calcium-handling protein levels of myocardium were examined: sarcoplasmic reticulum calcium-ATPase (SERCA2a),  $Na^+$ - $Ca^{2+}$  exchanger (NCX1), and RyR2. In brief, lysates of LV tissue samples from nonembolization zone were made by sonication of approximately 150 mg of each sample in a 7-fold volume

of lysis buffer (20 mM Na-HEPES, 4 mM EGTA, 1 mM dithiothreitol, pH 7.4) in the presence of proteinase inhibitors (0.1 mM leupeptin and 0.3 mM phenylmethylsulfonyl fluoride) for 1 min on ice. The protein concentration was determined using bovine serum albumin as a standard. Samples (50  $\mu$ g) denatured at 95°C were size-fractionated using SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. To determine the levels of SERCA2a, NCX1, and tubulin, SDS-PAGE was performed using 7.5% separating and 5% stacking gels; for RyR2, 5% separating and 4% stacking gels were used. Electrophoresis was performed in a Miniprotean II cell (Bio-Rad Ltd., Hercules, CA) followed by transfer of proteins (4°C for 2 h at 100 V) onto nitrocellulose in a mini trans-blot transfer cell (Bio-Rad Ltd.) filled with transfer buffer (25 mM Tris-HCl, pH 8.3, 150 mM glycine, and 20% methanol).

Blots were blocked overnight at 4°C in 5% nonfat milk diluted in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, and 150 mM NaCl) with 0.1% Tween 20 (TBS-T). Blots were then incubated with primary antibody diluted in TBS-T [anti-SERCA2a, 1:8000 mouse monoclonal antibody (Novocastra Laboratories Ltd, Benton Lane, UK); anti-NCX1, 1:5000 rabbit antiserum (Swant Co., Bellinzona, Switzerland); anti-RyR2, 1:4000 mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY); and anti-tubulin, 1:6000 mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO)] for 2 h at room temperature. After washing in TBS-T, blots were incubated in the presence of a peroxidase-labeled secondary antibody (GE Healthcare, Piscataway, NJ) diluted 1:10000 for 1 h at room temperature. Blots were washed again and incubated with ECL reagent (GE Healthcare) for 1 min, followed by autoradiography.

Optical densities of protein signals on X-ray films were determined by a laser scanning densitometer (Molecular Dynamics, Vernon Hills, IL) for quantification of protein levels. All three protein levels were expressed relative to tubulin.

The phosphorylation status of RyR2 was also examined. Myocardial homogenates were prepared, RyR2 was immunoprecipitated, and protein kinase A (PKA) phosphorylation was determined as described previously (Reiken et al., 2003). In brief, 1 g of LV tissue was homogenized in 1.0 ml of a buffer containing 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM NaF, 1.0 mM  $\text{Na}_3\text{VO}_4$ , and 1.0 mM dithiothreitol and protease inhibitors. Samples were centrifuged (3000g for 10 min), and the supernatant was centrifuged (8000g for 10 min). RyR2 was immunoprecipitated from samples by incubating 500  $\mu$ g of homogenate with anti-RyR antibody (2  $\mu$ l of 5029 Ab) in 0.5 ml of modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM  $\text{Na}_3\text{VO}_4$ , and 0.5% Triton X-100 and protease inhibitors) overnight at 4°C. The samples were incubated with Protein A Sepharose beads (GE Healthcare) at 4°C for 1 h, after which the beads were washed three times with radioimmunoprecipitation assay. Samples were heated to 95°C and size-fractionated by PAGE. Immunoblots were developed using either an anti-RyR (5029, 1:5000 dilution) or a phosphospecific Ab that recognizes PKA phosphorylated RyR2 (P2809, 1:5000) (Reiken et al., 2003). Band densities were quantified by densitometry.

**Statistics.** Values are reported as mean  $\pm$  S.E. A one-way analysis of variance with post hoc Bonferroni analysis was used for all comparisons of continuous variables between groups. A paired Student's *t* test was used only to compare measurements obtained at baseline, CHF state, and after 12 weeks within one cohort.  $p < 0.05$  was considered statistically significant. Analysis was performed using SPSS version 11.5 (Chicago, IL).

## Results

**Hemodynamics.** The hemodynamics at baseline, at CHF, and after 12 weeks of treatment are summarized in Table 1. Representative steady-state PV loops are shown in Fig. 1. After  $6.0 \pm 1.5$  weeks of coronary microembolization, PV loops in all groups shifted rightward. Treatment with Meto,

TABLE 1

Hemodynamics

All data are mean  $\pm$  S.E.M.

	Baseline	CHF	12 Weeks
LVSP (mm Hg)			
Control	127 $\pm$ 7	110 $\pm$ 3 <sup>a</sup>	115 $\pm$ 5
Meto	124 $\pm$ 2	108 $\pm$ 4 <sup>a</sup>	121 $\pm$ 3 <sup>b</sup>
Cilo (low)	130 $\pm$ 3	113 $\pm$ 3 <sup>a</sup>	121 $\pm$ 4 <sup>b</sup>
Cilo (high)	125 $\pm$ 3	102 $\pm$ 3 <sup>a</sup>	120 $\pm$ 1 <sup>b</sup>
LVEDP (mm Hg)			
Control	9.5 $\pm$ 0.6	22.5 $\pm$ 1.4 <sup>a</sup>	20.2 $\pm$ 3.1 <sup>a</sup>
Meto	9.2 $\pm$ 1.4	20.0 $\pm$ 1.4 <sup>a</sup>	15.2 $\pm$ 2.9
Cilo (low)	10.2 $\pm$ 0.9	22.8 $\pm$ 3.4 <sup>a</sup>	18.8 $\pm$ 1.2 <sup>a</sup>
Cilo (high)	13.0 $\pm$ 2.1	21.0 $\pm$ 1.2 <sup>a</sup>	16.0 $\pm$ 3.8
MAP (mm Hg)			
Control	103 $\pm$ 5	88 $\pm$ 3 <sup>a</sup>	89 $\pm$ 5 <sup>a</sup>
Meto	104 $\pm$ 3	91 $\pm$ 1 <sup>a</sup>	94 $\pm$ 1
Cilo (low)	104 $\pm$ 2	92 $\pm$ 2 <sup>a</sup>	98 $\pm$ 3
Cilo (high)	100 $\pm$ 3	84 $\pm$ 2 <sup>a</sup>	101 $\pm$ 2 <sup>bc</sup>

<sup>a</sup>  $p < 0.05$  vs. baseline.<sup>b</sup>  $p < 0.05$  vs. CHF.<sup>c</sup>  $p < 0.05$  vs. control.

Cilo (low), and Cilo (high) shifted PV loops back toward baseline, in contrast to PV loops from control animals, which shifted rightwards slightly over 12 weeks. Cilo (high and low) and Meto effectively decreased heart rate, compared with control animals (Fig. 2A). LV dp/dt was uniformly improved after 12 weeks of treatment in Meto and Cilo (both low and high) groups after significant reductions with coronary embolization (Fig. 2B). A significant increase in MAP was seen after 12 weeks in Cilo (high) compared with control ( $p < 0.05$ ) (Table 1).

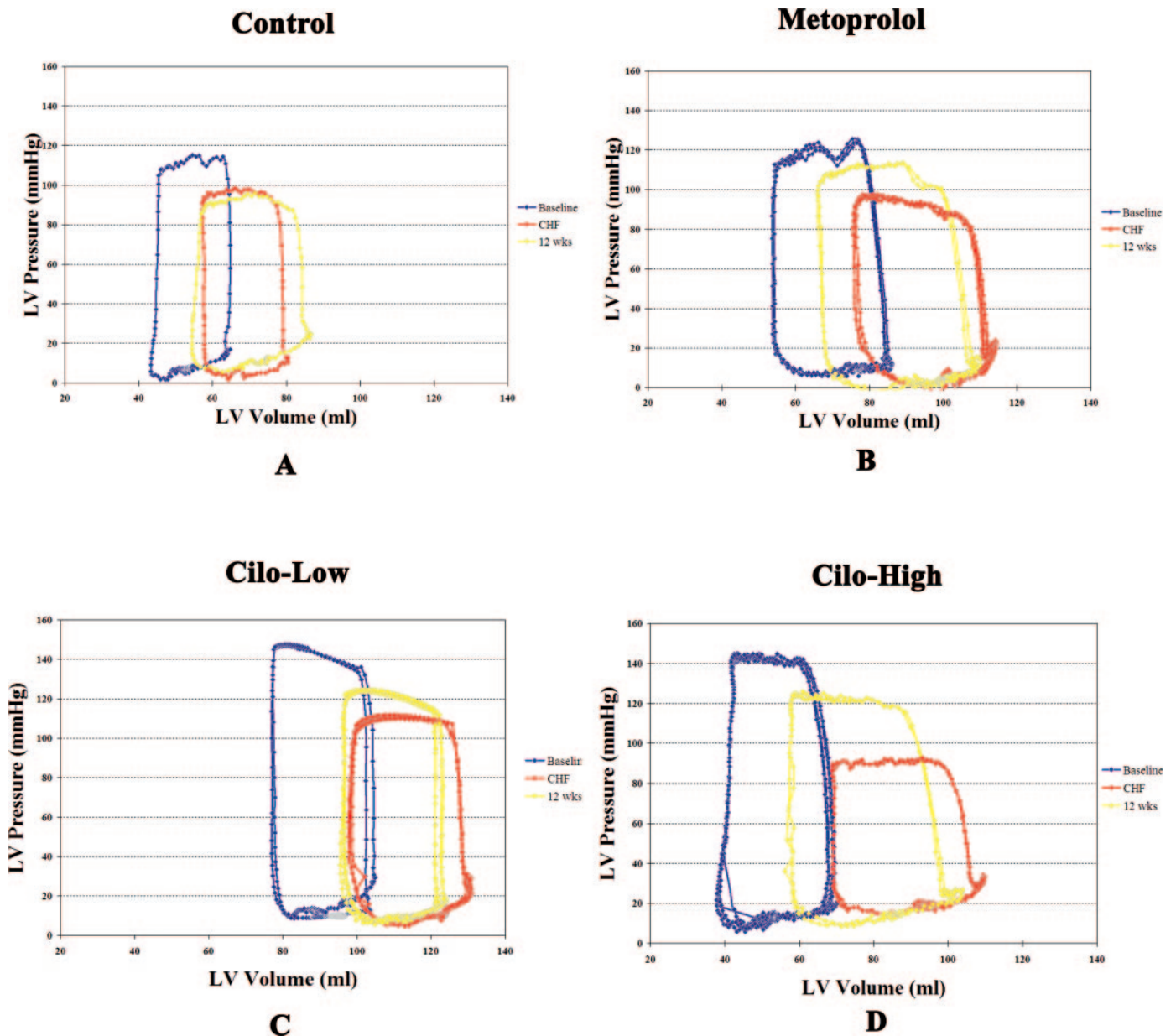
**Echocardiography.** Echocardiographic measurements are presented in Table 2. All animals experienced an equal and significant reduction in EF after embolization. EF recovered significantly in Meto animals versus control group, and trends toward improvements were seen in Cilo (low and high), as seen in Fig. 2C. It is important to note that LV end-diastolic diameter remained constant in metoprolol and Cilo (high-dose) animals, versus a significant increase in control animals and a trend toward increase in Cilo (low-dose) animals.

**Pressure-Volume Analysis.** Representative steady-state PV loops are shown in Fig. 1. A summary of the PV loop analysis is shown in Table 3. Trends to improvement in  $E_{es}$ , a rate-independent measure of systolic function, after embolization were seen in the Cilo (low and high) groups, compared with metoprolol and control animals. No significant change in chamber stiffness ( $\alpha$ ) or the isovolemic relaxation constant ( $\tau$ ) was seen, demonstrating that diastolic function was not affected by cilobradine or metoprolol therapy in the present study.

**Alterations in Calcium-Handling Proteins.** Western blots of SERCA2a, NCX1, and RyR2 are shown in Fig. 3A. The SERCA2a protein level was significantly reduced and NCX1 markedly increased in the CHF state. These levels of SERCA2a and NCX1 proteins were restored to levels comparable with normal animals in both Meto- and Cilo (high)-treated animals (Fig. 3B).

Although the total RyR2 protein levels were not changed in CHF or after treatment with either metoprolol or cilobradine, PKA phosphorylation of immunoprecipitated RyR2 was significantly increased in chronic failing hearts (Fig. 4). Metoprolol and Cilo (high) treatment restored PKA phosphoryla-





**Fig. 1.** A, representative PV loops at baseline, CHF, and after 12 weeks of control. B, treatment with metoprolol. C, cilobradine, low dose (Cilo-Low). D, cilobradine, high dose (Cilo-High).

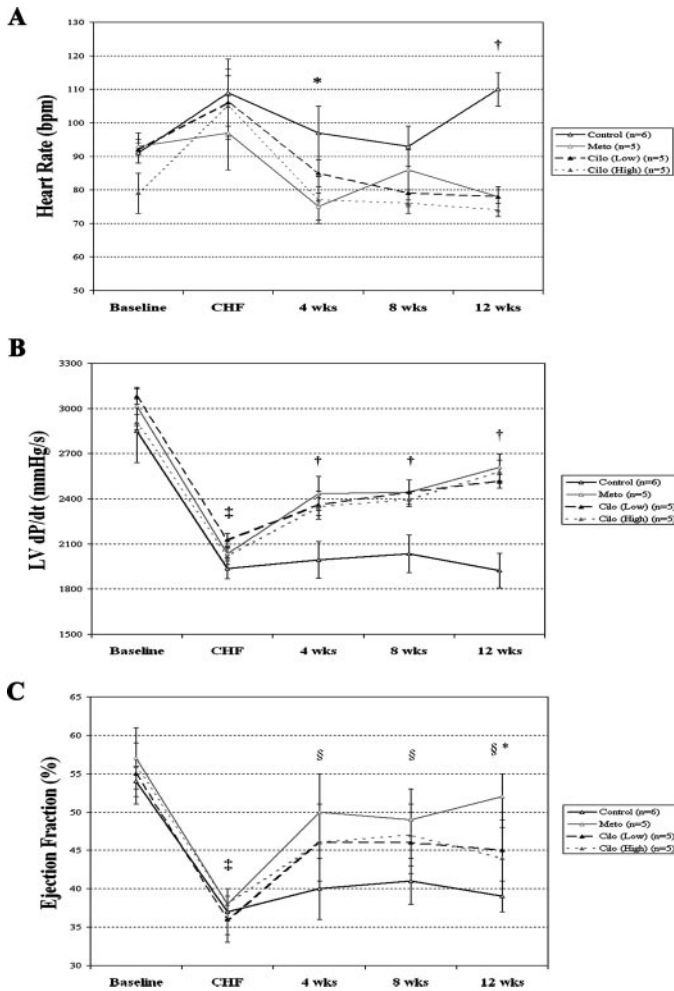
tion of RyR2 in failing hearts to the levels seen in normal hearts.

## Discussion

$\beta$ -Blockade has been shown to prevent the progression of heart failure (Colucci et al., 1996), improve cardiac performance (Sabbah et al., 1994), and promote reverse remodeling in failing hearts (Sabbah et al., 1994). Heart rate reduction has been proposed as an important component of benefit after long-term  $\beta$ -blockade therapy. In the present study, we tested whether bradycardic therapy can improve CHF and evaluated the effects of a bradycardic agent with no inotropic actions, cilobradine, on cardiac performance and calcium handling in dogs with coronary embolization-induced CHF. The primary findings of this study were: 1) cilobradine reduced heart rate at both low and high doses comparable with metoprolol, 2) systolic function was significantly improved

after treatment with cilobradine, and 3) myocardial calcium regulation was partially restored with cilobradine therapy, similar to metoprolol. These results suggest that bradycardic therapy without negative inotropic effects, such as seen with cilobradine, may provide benefit for heart failure patients with systolic dysfunction.

The rationale for heart rate reduction after ischemic injury rests on the intrinsic effect of lowering the heart rate to improve myocardial oxygen consumption imbalance, as well as long-term effect on the autonomic control of heart rate. A greater heart rate reduction and lower baseline resting heart rates were both associated with improved survival in the Cardiac Insufficiency Bisoprolol Study (CIBIS) II (CIBIS II Investigators, 1999), similar to findings in the United States Carvedilol Study (Packer et al., 1996). Patients after myocardial infarction also suffer from autonomic dysregulation of heart rate (Osculati et al., 1990), contributing to higher rates



**Fig. 2.** Heart rate (A), LV dp/dt (B), and ejection fraction (C) before and after treatment. \*, Meto versus control,  $p < 0.05$ . †, Meto, Cilo (low), Cilo (high) versus control, all  $p < 0.05$ . ‡, control, Meto, Cilo (low), Cilo (high) versus baseline, all  $p < 0.05$ . §, Meto, Cilo (low), Cilo (high) versus CHF, all  $p < 0.05$ .

**TABLE 2**

**Echocardiography**

LVESD, left ventricular end systolic diameter; LVEDD, left ventricular end-diastolic diameter. All data are mean  $\pm$  S.E.M.

	Baseline	CHF	12 Weeks
<b>LVESD (cm)</b>			
Control	2.96 $\pm$ 0.20	3.39 $\pm$ 0.14 <sup>a</sup>	3.56 $\pm$ 0.16 <sup>a</sup>
Meto	2.73 $\pm$ 0.18	3.10 $\pm$ 0.17 <sup>a</sup>	3.25 $\pm$ 0.23 <sup>a</sup>
Cilo (low)	3.15 $\pm$ 0.09	3.25 $\pm$ 0.14	3.20 $\pm$ 0.19
Cilo (high)	3.00 $\pm$ 0.21	3.20 $\pm$ 0.16 <sup>a</sup>	3.20 $\pm$ 0.16
<b>LVEDD (cm)</b>			
Control	4.69 $\pm$ 0.19	4.75 $\pm$ 0.12	4.99 $\pm$ 0.14 <sup>ab</sup>
Meto	4.38 $\pm$ 0.19	4.54 $\pm$ 0.18	4.58 $\pm$ 0.13
Cilo (low)	4.70 $\pm$ 0.15	4.64 $\pm$ 0.26	4.92 $\pm$ 0.34
Cilo (high)	4.61 $\pm$ 0.14	4.67 $\pm$ 0.19	4.69 $\pm$ 0.16

<sup>a</sup>  $p < 0.05$  vs. baseline.

<sup>b</sup>  $p < 0.05$  vs. CHF.

of mortality and ventricular arrhythmias (ATRAMI Investigators, 1998). As reported by Nagatsu et al. (2000), dogs treated with  $\beta$ -blockade alone (and subsequently lower heart rates) were found to have enhanced contractility compared with  $\beta$ -blockade plus pacing (with higher heart rates). However, both nonpacing and pacing arms received  $\beta$ -blocker treatment, and the conclusions regarding the sole effect of

**TABLE 3**

**Pressure-volume relationships**

All data are mean  $\pm$  S.E.M.

	Baseline	CHF	12 Weeks
<b><math>E_{es}</math> (mm Hg/ml)</b>			
Control	7.20 $\pm$ 0.99	4.66 $\pm$ 0.83 <sup>a</sup>	4.06 $\pm$ 1.24 <sup>ab</sup>
Meto	8.80 $\pm$ 1.26	5.27 $\pm$ 1.42	4.21 $\pm$ 0.92 <sup>ab</sup>
Cilo (low)	5.05 $\pm$ 1.45	2.29 $\pm$ 0.51	3.42 $\pm$ 1.06
Cilo (high)	7.35 $\pm$ 1.36	3.56 $\pm$ 0.89 <sup>a</sup>	4.30 $\pm$ 0.79
<b><math>V_0</math> (ml)</b>			
Control	38.6 $\pm$ 5.6	45.2 $\pm$ 10.1	48.0 $\pm$ 10.0
Meto	37.7 $\pm$ 7.8	35.3 $\pm$ 10.0	34.0 $\pm$ 12.2
Cilo (low)	36.6 $\pm$ 13.7	24.5 $\pm$ 18.2	55.1 $\pm$ 7.4
Cilo (high)	22.7 $\pm$ 4.1	26.2 $\pm$ 10.5	29.1 $\pm$ 12.8
<b><math>\alpha</math></b>			
Control	0.045 $\pm$ 0.014	0.040 $\pm$ 0.008	0.063 $\pm$ 0.020
Meto	0.022 $\pm$ 0.004	0.060 $\pm$ 0.031	0.029 $\pm$ 0.009
Cilo (low)	0.047 $\pm$ 0.021	0.018 $\pm$ 0.002	0.016 $\pm$ 0.008
Cilo (high)	0.033 $\pm$ 0.010	0.047 $\pm$ 0.021	0.027 $\pm$ 0.006
<b><math>\beta</math></b>			
Control	2.98 $\pm$ 2.10	1.54 $\pm$ 1.34	0.63 $\pm$ 0.46
Meto	4.23 $\pm$ 1.14	2.30 $\pm$ 1.36	4.17 $\pm$ 1.86
Cilo (low)	2.03 $\pm$ 1.66	2.77 $\pm$ 0.68	8.06 $\pm$ 4.32
Cilo (high)	4.77 $\pm$ 3.38	2.56 $\pm$ 1.58	5.05 $\pm$ 3.83
<b><math>\tau</math> (ms)</b>			
Control	19 $\pm$ 5	28 $\pm$ 6	40 $\pm$ 8
Meto	28 $\pm$ 8	34 $\pm$ 7	23 $\pm$ 7
Cilo (low)	34 $\pm$ 9	48 $\pm$ 12	36 $\pm$ 9
Cilo (high)	36 $\pm$ 5	39 $\pm$ 3	37 $\pm$ 5

$E_{es}$ , end-systolic elastance;  $V_0$ , volume-axis intercept;  $\alpha$ , chamber stiffness constant;  $\beta$ , scaling constant;  $\tau$ , isovolemic relaxation constant.

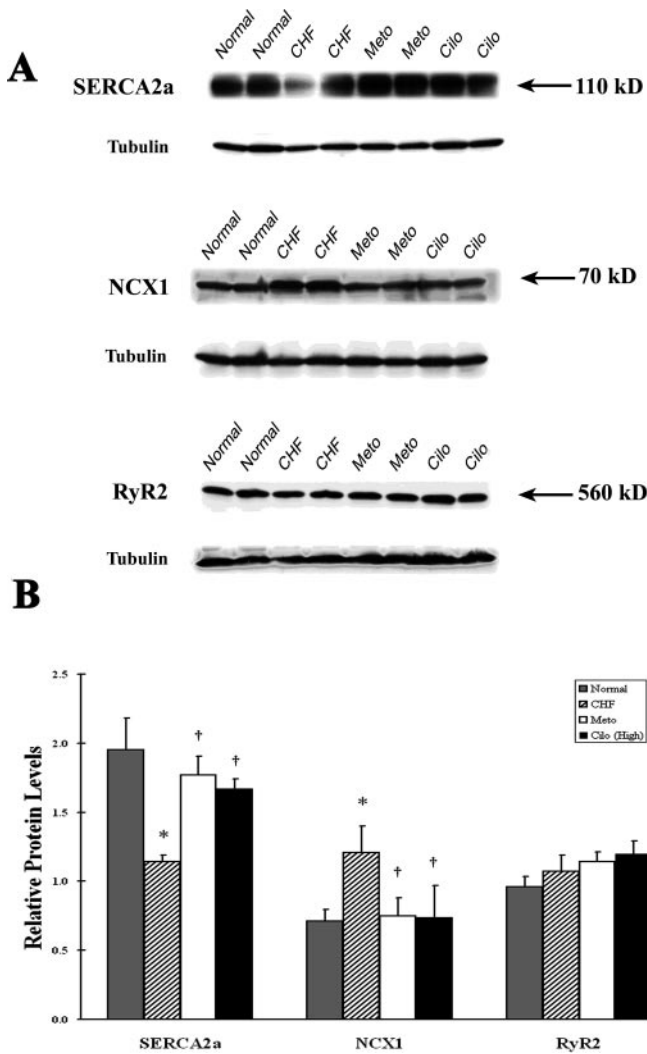
<sup>a</sup>  $p < 0.05$  vs. baseline.

<sup>b</sup>  $p < 0.05$  vs. CHF.

bradycardia without  $\beta$ -adrenergic blockade on functional improvement remain limited.

Sinus node inhibitors act to directly inhibit  $I_f$  currents (cardiac pacemaker current) passing through hyperpolarization-activated cyclic nucleotide-gated channels, serving to shift the resting potential closer to the maximal diastolic voltage without altering other phases of the action potential (Noble, 1984; Van Bogaert and Pittoors, 2003). The most extensively studied sinus node inhibitor, zatebradine, has been shown to reduce the incidence of ventricular fibrillation (Hu et al., 2003), reduce infarct size (Hu et al., 2003), and enhance arterial baroreflex sensitivity, implying higher central and peripheral vagal (autonomic) tone (Kruger et al., 2000). However, despite lowering early mortality and decreasing infarct size as reported by Hu et al. (2003) in a rat model, zatebradine induced LV remodeling with concurrent increased neurohormonal turnover, suggestive of continued sympathetic stimulation. Likewise, zatebradine produced disappointing results in the treatment of stable angina in patients, failing to improve time to moderate angina in two separate double-blinded clinical trials (Frishman et al., 1995; Glasser et al., 1997).

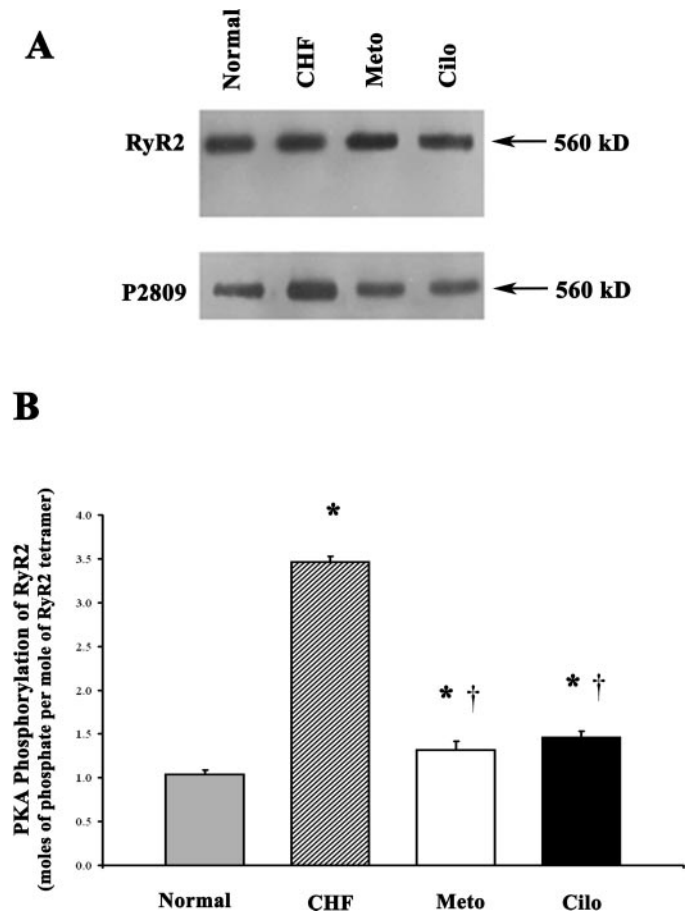
We hypothesized that cilobradine might produce differing results from zatebradine, based on two key physical properties. Cilobradine produces a use and dose-dependent blockade of the  $I_f$  current that is both stronger (82 versus 36%, respectively) and faster than zatebradine (Van Bogaert and Pittoors, 2003). As shown elegantly by Van Bogaert and Pittoors (2003), cilobradine and its racemic mixture also allow unblocking to occur at a much slower rate than with zatebradine, probably due to a higher association rate for the binding site in the hyperpolarization-activated cyclic nucleotide-gated channel. Early animal studies with cilobradine support our hypothesis. In a study reported by Schmitze-



**Fig. 3.** SERCA2, sarcolemmal NCX1, and RyR2 protein levels were quantified and normalized to tubulin at baseline (normal), CHF, and after Meto and Cilo high-dose treatment. A, images of Western blot analysis. B, significant reduction in SERCA2a after CHF and restoration of SERCA2a levels after Meto and Cilo. Both Meto and Cilo therapy produced a significant reduction in NCX1 versus CHF (B). \*,  $p < 0.05$  versus normal; †,  $p < 0.05$  versus CHF. Values reflect mean  $\pm$  S.D.

Spanke et al. (2004), cilobradine was administered after 60 min of coronary occlusion followed by 30 min of reperfusion in rabbits; in this model, cilobradine reduced heart rate by 30% and significantly reduced myocardial infarct size.

The present experiment utilizes a sinus node inhibitor, cilobradine, to address whether bradycardia as a primary therapeutic endpoint can be used to improve left ventricular function after ischemic injury. In our study, cilobradine effectively reduced heart rate and produced hemodynamic effects similar to metoprolol. Systolic function, as measured by LV dP/dt, was significantly improved after cilobradine, and trends to improvement in EF and end-systolic elastance were evident in all cilobradine-treated animals. A significant increase in mean arterial pressure was seen after high-dose cilobradine treatment, and increases in LV systolic pressure were evident in all cilobradine treatment groups after CHF, in contrast to control animals. These results compare favorably with the effects of metoprolol on systolic function although to a lesser degree with cilobradine, as shown in pre-



**Fig. 4.** Phosphorylation of the RyR2 was measured at normal, in failing hearts (placebo group, CHF), and after treatment with high-dose Meto and Cilo at 12 weeks. A, immunoblots probed for both total RyR2 (using 5029 Ab) and PKA phosphorylated RyR2 with phosphospecific antibody (anti-RyR2-2809P that recognizes RyR2 PKA phosphorylated on Ser<sup>2809</sup>). B, significant increase in RyR2 phosphorylation (moles of phosphate per mole of RyR2 tetramer) was seen after CHF (\*,  $p < 0.05$  versus normal), and both Meto and Cilo therapy restored levels to normal (†,  $p < 0.05$  versus CHF). Values reflect mean  $\pm$  S.D.

vious studies as well as our study, in which increases in MAP and EF were evident after metoprolol (Sabbah et al., 1994; Nagatsu et al., 2000). Most importantly, cilobradine therapy affected ventricular remodeling similar to metoprolol and prevented further increases in ventricular size compared with control animals after CHF. These results are in direct contrast to the effect of chronic zatebradine in rats (Hu et al., 2003), which produced a significant increase in ventricular dimensions after 8 weeks of therapy. Animals treated with metoprolol and high-dose cilobradine, as hypothesized, demonstrated the greatest improvements in ventricular function. As suggested by previous investigators (Lechat, 2003), the correction of myocardial consumption imbalance in heart failure through heart rate reduction and impairment of the sympathetic response with high-dose cilobradine therapy probably led to a sustained decrease in heart size and improved contractility in these animals. Although low-dose cilobradine produced a significant decrease in resting heart rate, low-dose therapy did not affect ventricular size or change end-systolic elastance. It is apparent from these results that sinus node therapy efficacy is dose-dependent, and important cellular actions mediated by  $I_b$ , such as heart rate



regulation after stress (Steiber et al., 2006) do not occur at low doses, accounting for crucial differences in remodeling that are independent of heart rate reduction alone. Finally, the added benefit of adrenergic blockade with  $\beta$ -blocker therapy must play an important role in inhibiting neurohormonal activation seen in CHF; although inotropy may be decreased upon initiation, chronic  $\beta$ -blocker use clearly resulted in the largest improvements after CHF in this study in both pressure-volume and echocardiographic indices of systolic function. A wide range of effects of  $\beta$ -blockade have been documented, including actions on vascular, renal, and inflammatory pathways. As such, it remains to be seen whether hemodynamic regulation, such as solely heart rate reduction with sinus node inhibitors, can provide the same degree of reverse remodeling and, ultimately, long-term mortality reduction as  $\beta$ -blockade. Prospective clinical trials will be required to address this important issue. Finally, we feel that the degree of heart failure at the CHF state was equally severe among all treatment groups, based on changes in EF, LV dP/dt, LVSP, and LVEDP.

The cellular mechanism examined in this experiment is the effect of heart rate reduction on calcium ( $\text{Ca}^{2+}$ ) handling and function. Abnormalities in cellular  $\text{Ca}^{2+}$  handling have consistently been implicated in progressive hemodynamic deterioration (Gwathmey et al., 1987) and are now considered a fundamental component of heart failure. Precise mechanisms have focused on the role of  $\text{Ca}^{2+}$  resequestration into the sarcoplasmic reticulum by SERCA2a and  $\text{Ca}^{2+}$  efflux via NCX1, resulting in low levels of  $\text{Ca}^{2+}$  release in CHF (Houser et al., 2000). Although still not completely understood from a functional standpoint, it is clear that increased NCX1 levels contribute to altered  $\text{Ca}^{2+}$  homeostasis in CHF, likely in balance with SERCA2a levels, because lowered SERCA2/NCX levels lead to reduced sarcoplasmic reticulum  $\text{Ca}^{2+}$  loading, decreased decay of the  $\text{Ca}^{2+}$  transient, and resultant negative contractility (Hasenfaus et al., 1999). After induction of CHF through coronary embolization in our study, significant decreases in SERCA2a and significant increases in NCX1 were apparent, consistent with previous literature (Gwathmey et al., 1999). After therapy with cilobradine and metoprolol, SERCA2a levels were restored compared with controls, and both metoprolol and cilobradine therapy provided significant reductions in NCX1 levels, suggestive of improved  $\text{Ca}^{2+}$  handling similar to chronic  $\beta$ -blockade therapy (Gwathmey et al., 1999). Although no changes in protein levels of RyR2 were seen, phosphorylation of the RyR2, which causes defective channel function through dissociation of the regulatory protein FKBP12.6 (Marx et al., 2000), was seen in CHF and was reversed with chronic  $\beta$ -blockade and cilobradine therapy, also consistent with prior literature (Reiken et al., 2003). It is noted that improvement in heart failure after therapy may have accounted for changes in calcium handling proteins rather than the direct actions of bradycardia, which were unable to be determined from the present study. Nevertheless, restoration of  $\text{Ca}^{2+}$  homeostasis may be an important component of heart rate reduction with cilobradine therapy and may help to partially explain functional improvement.

The limitations of the current experiment must be acknowledged. Although no change was found in prior experiment in sympathetic neurohormones after zatebradine therapy (Hu et al., 2003), serum neurohormonal analysis was not

performed in the current study and could corroborate our functional improvements with biochemical data. A complete metabolic analysis, including myocardial enzymes, LDH isoenzymes, and creatinine, would also aid in understanding the effect of cilobradine on long-term markers of energy parameters. Measurements of LV mass were not taken at the time of echocardiography; thus, we are unable to determine the degree, if any, of LV hypertrophy induced. Concurrently, wall stress was not measured.

In conclusion, high-dose cilobradine, a potent sinus node inhibitor that blocks pacemaker  $I_f$  currents, effectively reduced heart rate comparable with metoprolol in dogs with coronary embolization-induced CHF. Bradycardia in both cilobradine- and metoprolol-treated animals was accompanied by significant improvements in left ventricular contractile function, and restoration of calcium handling proteins was evident after both metoprolol and cilobradine treatment. These findings suggest a rationale for bradycardic therapy in the treatment of severe systolic dysfunction and justify further research into clinical application in humans unable to tolerate  $\beta$ -blocker therapy.

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