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Original article

Restoration of mechanical and energetic function in failing aortic-banded rat hearts by gene transfer of calcium cycling proteins

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Abstract

The aim of this study was to examine whether short- and long-term gene transfer of Ca^{2+} handling proteins restore left ventricular (LV) mechanoenergetics in aortic banding-induced failing hearts. Aortic-banded rats received recombinant adenoviruses carrying sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (Banding+SERCA), parvalbumin (Banding+Parv) or β-galactosidase (Banding+βgal), or an adeno-associated virus carrying SERCA2a (Banding+AAV.SERCA) by a catheter-based technique. LV mechanoenergetic function was measured in cross-circulated hearts. "Banding", "Banding+βgal" and "Banding+saline" groups showed lower end-systolic pressure at 0.1 ml intraballoon water ($ESP_{0.1}$), higher end-diastolic pressure at 0.1 ml intraballoon water ($EDP_{0.1}$) and slower LV relaxation rate, compared with "Normal" and "Sham". However, "Banding+SERCA" and "Banding+Parv" showed high $ESP_{0.1}$, low $EDP_{0.1}$ and fast LV relaxation rate. In "Banding", "Banding+βgal" and "Banding+saline", slope of relation between cardiac oxygen consumption and systolic pressure–volume area, O_2 cost of total mechanical energy, was twice higher than normal value, whereas slope in "Baning+SERCA" and "Banding+Parv" was similar to normal value. Furthermore, O_2 cost of LV contractility in the 3 control banding groups was ~3 times higher than normal value, whereas O_2 cost of contractility in "Banding+SERCA", "Banding+AAV.SERCA" and "Banding+Parv" was as low as normal value. Thus, high O_2 costs of total mechanical energy and of LV contractility in failing hearts indicate energy wasting both in chemomechanical energy transduction and in calcium handling. Improved calcium handling by both short- and long-term overexpression of SERCA2a and parvalbumin transforms the inefficient energy utilization into a more efficient state. Therefore enhancement of calcium handling either by resequestration into the SR or by intracellular buffering improves not only mechanical but energetic function in failing hearts.

Keywords: Gene therapy; Heart failure; Energetic function; SERCA2a; Parvalbumin

1. Introduction

Calcium handling is central to the process of excitation–contraction (E-C) coupling. Sarcoplasmic reticulum (SR), which releases Ca^{2+} during contraction by a trigger of Ca^{2+} entry and takes it up during relaxation by SR-ATPase (SERCA2a) pump, plays a major role in controlling the synchronized Ca^{2+} handling in myocardial cells [1]. Removal of Ca^{2+} from the cytoplasm is governed mainly by SERCA2a, of which the activity is regulated by phospholamban, and to a lesser extent by Na^+-Ca^{2+} exchanger (NaCaX). SERCA2a protein/mRNA expression and activity were decreased in human heart failure and in animal models of heart failure

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[2,3]. This SERCA2a reduction results in abnormal Ca^{2+} handling, which causes a prolongation of Ca^{2+} transient, an increase in diastolic intracellular Ca^{2+}, a decrease in systolic intracellular Ca^{2+} and a reduced SR Ca^{2+} content. Therefore, abnormal Ca^{2+} handling by the SERCA2a reduction contributes to the systolic and diastolic dysfunction in failing hearts [4,5].

Our group [6–11] has previously shown that overexpression of SERCA2a, ablation of phospholamban or overexpression of parvalbumin by adenoviral gene transfer modify intracellular Ca^{2+} handling and modulate physiological mechanical performance in normal, senescent, aortic banding-induced failing and ischemia/reperfusion-injured rat whole hearts. In addition, global cardiac gene transfer of SERCA2a improved survival and energetic state shown as phosphocreatine/ATP ratio in failing rat hearts induced by aortic banding [9] and reduced ventricular arrhythmias in the model rat of ischemia followed by reperfusion [10]. More recently, SERCA2a overexpression improved the energy utilization in special reference to myocardial oxygen consumption in diabetic failing hearts [12].

The aim of this study was to examine whether restoring calcium cycling by either re-sequestering the elevated intracellular calcium back into the SR (through SERCA2a gene transfer) or by buffering the elevated intracellular calcium (through parvalbumin gene transfer) would improve the left ventricular (LV) mechanical and energetic functions in the aortic-banded rats, especially in terms of oxygen cost of LV contractility.

2. Materials and methods

2.1. Recombinant adenoviral vectors

Recombinant adenoviral vectors were used with cytomegalovirus-driven expression cassettes for SERCA2a, parvalbumin or β-galactosidase with a second cassette in each adenovirus containing green fluorescent protein substituted for E1 by means of homologous recombination. Adenoviral SERCA2a (Ad.SERCA), adenoviral parvalbumin (Ad.Parv) and adenoviral β-galactosidase (Ad.βgal) had concentrations of 9×10^{10} pfu/ml, 10×10^{10} pfu/ml and 8×10^{10} pfu/ml, respectively, with a particle/pfu ratio of 10:1. Wild-type adenovirus contamination was excluded by the absence of PCR-detectable E1 sequences.

2.2. Recombinant adeno-associated virus (AAV) vectors

The AAV1 vector used in this study was manufactured using standard calcium phosphate transfection methods in 293 cells. Three plasmids were used, one containing helper functions from adenovirus, one containing the AAV rep2 and cap1 genes, and the third containing the vector genome. Vector production, harvest, purification and testing were as previously described [13].

2.3. Animals

All animal experiments in this study were performed with the approval of the Animal Care Committee of Massachusetts General Hospital and in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Five-week-old male Wistar rats (Charles River, Mass; body weight (BW), 100 to 120 g) were anesthetized with intraperitoneal (i.p.) Ketamine–Xyla-Jet mixture and placed on a ventilator. An incision on the right side of chest was made, exposing the aortic root, and a tantalum clip with an internal diameter of 0.58 mm (Weck Hemoclip) was placed on the ascending aorta. Animals in the sham group underwent a similar procedure without insertion of a clip. Twenty-four to twenty-six weeks later, about 90 % of the animals thus operated survived (aortic banding, 89%; sham-operation, 93%) and were randomized into 8 groups:

(1) six normal control rats (Normal);
(2) six uninfected sham-operated rats (Sham);
(3) six uninfected aortic-banded rats (Banding);
(4) five aortic-banded rats infected with Ad.βgal (Banding + βgal);
(5) five aortic-banded rats injected with saline (Banding + saline);
(6) six aortic-banded rats infected with Ad.SERCA (Banding + SERCA);
(7) five aortic-banded rats infected with Ad.Parv (Banding + Parv);
(8) three aortic-banded rats infected with AAV1 carrying SERCA2a (Banding + AAV.SERCA).

2.4. Cardiac gene delivery

The in vivo cardiac gene transfer has been described previously in detail by our group [14]. Briefly, after the rats were anesthetized and a thoracotomy performed, a 22-guage catheter containing 200 μl of adenovirus and 50 μl adenosine (3 mg/ml) was advanced from the apex of the left ventricle to the aortic root. The aorta and main pulmonary artery were clamped for 30 seconds distal to the site of the catheter and the solution injected, then the chest was closed, and the animals were allowed to recover. In groups (4)–(8), 60–67% of the rats thus operated survived until LV mechanical and energetic studies (Banding + βgal, 60%; Banding + saline, 63%; Banding + SERCA, 62%; Banding + Parv, 64%; Banding + AAV.SERCA; 67%). The rats underwent LV mechanical and energetic studies 2–3 days after the injection of the adenovirus or saline and 45 days after the AAV injection.

2.5. LV mechanical and energetic studies

2.5.1. Surgical preparations

The LV mechanical and energetic studies were performed on the excised, cross-circulated rat heart preparations. The surgical preparations have been described previously in detail [15]. Briefly, in each experiment, 2 male 500 to 650 g Wistar rats (blood supplier and metabolic supporter) and 1 heart donor rat were anesthetized with pentobarbital sodium (50 mg/kg, ip) and intubated and heparinized (1000 U, iv). In the beating heart, cross-circulated and maintained at 37 °C, a thin latex balloon,

which was connected to a pressure transducer for measuring LV pressure (LVP), was inserted into the LV. Systolic unstressed volume (V_0=0.08 ml for normal or sham-operated hearts and V_0=0.06 ml for aortic-banded hearts) should be determined as the volume at which peak isovolumic pressure was zero. Heart rate was maintained constant at 300 beats/min by right atrium electrical pacing. Systemic arterial blood pressure of the support rat served as coronary perfusion pressure (diastolic–systolic, 80–120 mmHg; mean, about 90 mmHg). Arterial pH, Po_2 and Pco_2 of the support rat and perfused blood were maintained within their physiological ranges by supplemental oxygen and sodium bicarbonate throughout the experiment.

2.5.2. Calculation for oxygen consumption

Cardiac VO_2 was obtained as the product of coronary flow and arteriovenous O_2 content difference. The right ventricular (RV) component of total VO_2, which is considered constant irrespective of LV volume, was calculated by multiplying biventricular VO_2 under LV volume-unloading with the weight ratio of RV/RV+LV. The RV VO_2 was subtracted from the total VO_2 to yield LV VO_2.

2.5.3. Experimental protocol

LVP, LV VO_2 and systolic pressure–volume area (PVA) data were obtained at five different LV volumes, changed with a step of 0.025 ml, without any inotropic interventions (control volume run). After control volume run, Ca^{2+} inotropism run was performed at a midrange LV volume (mLVV) by intracoronary infusion of 1% $CaCl_2$ solution. In some experiments, dobutamine (78 μM) inotropism run was also performed after Ca^{2+} inotropism run. The steady state of heart was reached 2–3 min after change of LV volume and 4 min after change of the infusion rate. Cardiac arrest was induced by intracoronary infusion of 1 M KCl (12 ml/h) to obtain VO_2 for basal metabolism. In each steady state, the data sampling, performed at 500 Hz for 2 s, was repeated 3 times at intervals of 0.5 min.

2.6. Data analysis

2.6.1. PVA calculation

The best-fit end-systolic/diastolic pressure–volume relations (ESPVR/EDPVR) were obtained by fitting the data with the exponential functions [12]. PVA was defined as the pressure–volume area circumscribed by the curvilinear ESPVR, EDPVR and the systolic portion of the ventricular pressure–volume trajectory.

2.6.2. VO_2 for Ca^{2+} handling in E-C coupling during inotropic run

During Ca^{2+}/dobutamine inotropic run at a mLVV, ESP–volume relations at different infusion rates were obtained as a best-fit exponential function curve. We calculated PVA at mLVV (PVA_{mLVV}) by integrating each ESPVR from V_0 up to mLVV. We then obtained the two composite VO_2–PVA_{mLVV} data points. Next, the lines including each VO_2–PVA_{mLVV} data point were drawn in parallel to control VO_2–PVA relation. VO_2-intercept value (PVA-independent VO_2) at each infusion rate was thus obtained. VO_2 for Ca^{2+} handling in E-C coupling was obtained by subtracting basal metabolic VO_2 per beat, measured in KCl-induced arrest hearts, from PVA-independent VO_2.

2.6.3. LV contractility

Equivalent maximal elastance (eEmax), an index for LV contractility, was obtained by calculating ESP–volume ratio of the specific virtual triangle, which is energetically equivalent to the real PVA_{mLVV} [16].

2.6.4. Oxygen cost of LV contractility

The oxygen cost of LV contractility was obtained as the slope of the linear relation between VO_2 for Ca^{2+} handling in E-C coupling and eEmax during Ca^{2+}/dobutamine inotropism run. This slope is considered an index quantifying Ca^{2+} handling VO_2 per unit LV contractility change.

2.6.5. Logistic time constant

To evaluate the LV relaxation rate, we analyzed LV isovolumic relaxation pressure–time curves at mLVV by using the logistic time constant (T_L) derived from a logistic model.

2.7. Western blot for SERCA2a protein

Lysates from hearts were matched for protein concentration and then separated by SDS–PAGE and transferred to nitrocellulose membranes. Blots were incubated with SERCA2a antibodies (Affinity Bioreagents, CA) followed by detection with enhanced chemiluminescence.

2.8. Statistics

Data are presented as mean±SD. Multiple comparisons were performed by ANOVA with STATVIEW (Abacus Concepts, Barkeley, CA). Statistical significance was accepted at the level of $P<0.05$.

3. Results

3.1. Characterization of animals

There was no statistical difference in BW among the 7 groups (Table 1). LV/BW ratio and LV+RV/BW ratio, as well as LV weight and LV+RV weight, in 5 aortic banding groups were significantly higher than those of “Normal” and “Sham” groups, while RV/BW ratio, as well as RV weight, was not different among 7 groups (Table 1). Thus, the rats, which underwent an aortic banding for 24–26 weeks, had a significant increase in LV mass normalized to BW, and a cross-section of the aortic-banded hearts showed apparent LV concentric hypertrophy (data not shown).

3.2. LV mechanics

Fig. 1A shows representative control ESPVRs and EDPVRs in “Sham”, “Banding” and “Banding+SERCA” hearts.

Table 1
Morphometric analyses

Group	*n*	BW (g)	LV (g)	RV (g)	LV+RV (g)	LV/BW ($\times 10^{-3}$)	RV/BW ($\times 10^{-3}$)	LV+RV/BW ($\times 10^{-3}$)
Normal	6	598±34	1.014±0.076	0.288±0.022	1.301±0.084	1.69±0.08	0.48±0.05	2.18±0.11
Sham	6	627±53	1.091±0.115	0.297±0.049	1.388±0.159	1.74±0.06	0.47±0.05	2.21±0.10
Banding	6	614±53	1.351±0.127[a, b]	0.302±0.025	1.653±0.143[a]	2.21±0.24[a, b]	0.50±0.07	2.71±0.30[a, b]
Banding+βgal	5	607±65	1.312±0.176[a, b]	0.306±0.042	1.618±0.207[a]	2.16±0.14[a, b]	0.51±0.05	2.66±0.16[a, b]
Banding+saline	5	648±59	1.488±0.164[a, b]	0.312±0.021	1.799±0.160[a, b]	2.29±0.12[a, b]	0.49±0.07	2.78±0.13[a, b]
Banding+SERCA	6	580±46	1.317±0.138[a, b]	0.325±0.067	1.642±0.195[a]	2.28±0.28[a, b]	0.57±0.15	2.85±0.43[a, b]
Banding+Parv	5	554±52	1.277±0.115[a, b]	0.288±0.023	1.565±0.135[a]	2.32±0.24[a, b]	0.52±0.05	2.84±0.29[a, b]

All data are shown as mean±SD. BW, body weight; LV, left ventricle; RV, right ventricle; *n*, number of hearts.
[a] $P<0.05$ compared to "Normal" group.
[b] $P<0.05$ compared to "Sham" group.

Curvilinear ESPVRs and EDPVRs of "Banding+βgal" and "Banding+saline" hearts were similar to those of "Banding" hearts (data not shown). ESPVR of "Banding+SERCA" and "Banding+Parv" hearts was shifted upward compared with ESPVR of "Banding" hearts. Summarized data of LV mechanics are shown in Table 2. The volume intercepts of ESPVR and EDPVR, V_0 and V_u, in 5 aortic banding groups were significantly smaller than those of "Normal" and "Sham" groups, resulting from LV concentric hypertrophy. In "Banding" hearts, the end-systolic pressure observed at 0.1 ml of intraballoon water volume ($ESP_{0.1}$) was lower (130±27 mmHg), and end-diastolic pressure observed at 0.1 ml of intraballoon water volume ($EDP_{0.1}$) was higher (22.1±6.2 mmHg), compared with "Normal" hearts ($ESP_{0.1}$,188±15 mmHg; $EDP_{0.1}$, 9.8±4.0 mmHg). However, in "Banding+SERCA" group, like in "Banding+Parv" group, $ESP_{0.1}$ was increased over 200 mmHg and $EDP_{0.1}$ was decreased to 12.4±2.7 mmHg, although $ESP_{0.1}$ in "Banding+βgal" and "Banding+saline" groups was as low as that of "Banding" group. "Banding+βgal" group showed a higher $EDP_{0.1}$, but without significant difference, compared with "Banding+SERCA" group. Two of "Banding+βgal" hearts had lower $EDP_{0.1}$ (12.3 and 12.6 mmHg), shorter logistic time constant, T_L (13.0 and 14.2 msec) and higher eEmax at mLVV (3538 and 3005 mmHg ml^{-1} g), compared with the rest of "Banding+βgal" hearts. Thus, in the two hearts, the systolic/diastolic cardiac function was not much deteriorated. The systolic/diastolic function was dependent on the levels of SERCA2a protein expression or SERCA2a activity [17,18]. In this study, therefore, the degree of cardiac dysfunction appears somewhat

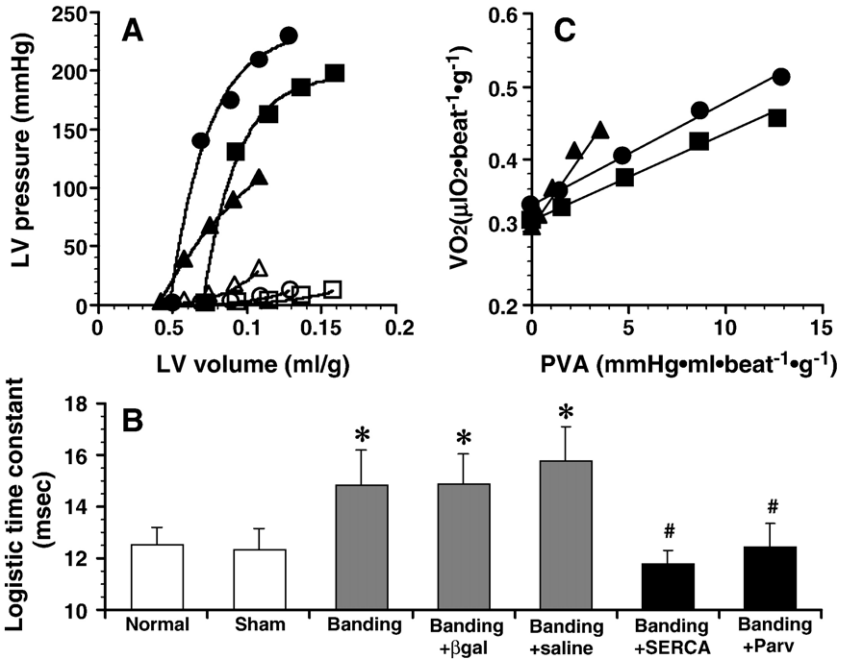

Fig. 1. (A) End-systolic volume relation (closed symbols) and end-diastolic pressure–volume relation (open symbols) in "Sham" (■ and □), "Banding" (▲ and △) and "Banding+SERCA" (● and ○) hearts. Both relations were obtained by control volume-loading runs where balloon water was changed from 0 to 0.1 ml by 0.025 ml. (B) Comparison of logistic time constants among 7 groups. Logistic time constants were obtained from a best-fit logistic curve of pressure–time curve during isovolumic relaxation at midrange LV volume (0.05 ml of balloon water volume). *$P<0.05$ compared to "Normal" and "Sham" groups. #$P<0.05$ compared to "Banding", "Banding+βgal" and "Banding+saline" groups. $n=6$ in "Normal", $n=6$ in "Sham", $n=6$ in "Banding", $n=5$ in "Banding+βgal", $n=5$ in "Banding+saline", $n=6$ in "Banding+SERCA", $n=5$ in "Banding+Parv". (C) Linear relations between myocardial oxygen consumption per beat (VO_2) and systolic pressure–volume area (PVA) in "Sham" (■), "Banding" (▲) and "Banding+SERCA" (●) hearts.

Table 2
Variables of LV mechanics

Group	n	ESPVR				EDPVR			
		A (mmHg)	B (1/ml)	V_0 (ml/g)	$ESP_{0.1}$ (mmHg)	A' (mmHg)	B' (1/ml)	V_u (ml/g)	$EDP_{0.1}$ (mmHg)
Normal	6	221±20	13.2±5.4	0.079±0.007	188±15	0.35±0.40	28.1±11.2	0.079±0.007	9.8±4.0
Sham	6	191±20	45.9±21.6	0.074±0.008	188±15	1.44±1.07	28.0±6.5	0.074±0.008	13.4±4.7
Banding	6	177±45	25.3±17.4	0.045±0.005 [a, b]	130±27 [a, b]	0.93±0.35	43.4±5.5	0.045±0.005 [a, b]	22.1±6.2 [a]
Banding+βgal	5	132±24 [a, b]	57.0±26.8 [a]	0.047±0.007 [a, b]	128±27 [a, b]	2.12±2.79	34.0±12.6	0.047±0.007 [a, b]	15.1±3.0
Banding+saline	5	155±22 [a]	27.3±16.3	0.040±0.005 [a, b]	118±19 [a, b]	2.45±1.74	37.4±7.4	0.040±0.005 [a, b]	24.8±9.8 [a, b]
Banding+SERCA	6	206±46 [d]	65.6±30.1 [a]	0.046±0.005 [a, b]	210±46 [c, d, e]	2.45±3.56	33.6±12.2	0.046±0.005 [a, b]	12.4±2.7 [c, e]
Banding+Parv	5	238±39 [d, e]	47.6±18.3	0.047±0.004 [a, b]	232±31 [c, d, e]	1.83±1.13	33.7±10.9	0.047±0.004 [a, b]	16.7±3.8

All data are shown as mean±SD. ESPVR, end-systolic pressure–volume relation; A and B are parameters in the equation $ESP=A\{1-\exp[-B(V-V_0)]\}$; V_0, volume intercept of end-systolic pressure–volume relation; $ESP_{0.1}$, end-systolic pressure observed at maximum left ventricular volume (0.1 ml of balloon water volume); EDPVR, end-diastolic pressure–volume relation; A' and B' are parameters in the equation $EDP=A'\{\exp[B'(V-V_u)]-1\}$; V_u, volume intercept of end-diastolic pressure–volume relation; $EDP_{0.1}$, end-diastolic pressure observed at maximum left ventricular volume (0.1 ml of balloon water volume); n, number of hearts.
[a] $P<0.05$ compared to "Normal" group.
[b] $P<0.05$ compared to "Sham" group.
[c] $P<0.05$ compared to "Banding" group.
[d] $P<0.05$ compared to "Banding+βgal" group.
[e] $P<0.05$ compared to "Banding+saline" group.

different among the aortic-banded animals because of the different levels of down-regulated SERCA2a expression. In all groups, moreover, T_L was obtained from LV isovolumic relaxation pressure–time curves at mLVV. T_L in "Banding" group was significantly longer than that of "Normal" and "Sham" groups ($P<0.05$). In "Banding+SERCA" and "Banding+Parv" groups, however, T_L was decreased to T_L level observed in "Normal" and "Sham" groups, although T_L in "Banding+βgal" and "Banding+saline" groups remained as long as that of "Banding" group.

3.3. LV energetics; VO_2–PVA relations

Fig. 1C shows representative control VO_2–PVA relations in "Sham", "Banding" and "Banding+SERCA" hearts. "Banding" heart showed a linear VO_2–PVA relation with steeper slope compared with "Sham" and "Banding+SERCA" hearts. Summarized data of LV energetics are shown in Table 3. There was no significant difference in the VO_2 intercept of VO_2–PVA relation among 7 groups. On the other hand, the slope in "Banding" group was about twice as high as that of "Normal" and "Sham" groups. However, the slope in "Banding+SERCA" and "Banding+Parv" groups was similar to the slope found in "Normal" and "Sham" groups, although the slope in "Banding+βgal" and "Banding+saline" groups remained high. In five aortic banding groups, moreover, the minute VO_2 for basal metabolism and for Ca^{2+} handling in E-C coupling, which are both components of the VO_2 intercept of VO_2–PVA relation, were not significantly different from those of "Normal" and "Sham" groups.

3.4. ESP_{mLVV} in response to calcium

Table 4 shows changes in end-systolic pressure observed at a mLVV (ESP_{mLVV}) in response to Ca^{2+} infusion. ESP_{mLVV} prior to Ca^{2+} infusion in "Banding+SERCA" and "Banding+ Parv"

Table 3
Variables of LV energetics

Group	n	VO_2–PVA relation		VO_2 per minute ($\mu l\ O_2 \cdot min^{-1}\ g^{-1}$)	
		Slope ($\times 10^{-2}\ \mu l\ O_2 \cdot mmHg^{-1} \cdot ml^{-1}$)	VO_2 intercept ($\mu l\ O_2 \cdot beat^{-1} \cdot g^{-1}$)	Basal metabolism	E-C coupling
Normal	6	1.13±0.29	0.303±0.043	29.0±3.2	60.3±13.4
Sham	6	1.26±0.24	0.348±0.026	29.6±3.4	71.8±5.3
Banding	6	2.61±0.82 [a, b]	0.364±0.040	33.8±3.1	72.4±11.8
Banding+βgal	5	2.19±0.25 [a, b]	0.310±0.044	35.1±3.4	56.7±13.5
Banding+saline	5	2.11±0.40 [a, b]	0.304±0.036	28.6±3.3	62.7±11.8
Banding+SERCA	6	1.09±0.23 [c, d, e]	0.377±0.064	33.4±2.7	78.6±17.9
Banding+Parv	5	1.37±0.34 [c, d, e]	0.350±0.032	32.0±4.1	69.4±7.1

All data are shown as mean±SD. VO_2, myocardial oxygen consumption per beat; PVA, systolic pressure–volume area; E-C, excitation–contraction; n, number of hearts. VO_2 per minute for basal metabolism was measured in the hearts under KCl-induced arrest. VO_2 per minute for E-C coupling was obtained by subtracting VO_2 per minute for basal metabolism from VO_2 per minute for mechanically unloaded (i.e., free of balloon water) contraction.
[a] $P<0.05$ compared to "Normal" group.
[b] $P<0.05$ compared to "Sham" group.
[c] $P<0.05$ compared to "Banding" group.
[d] $P<0.05$ compared to "Banding+βgal" group.
[e] $P<0.05$ compared to "Banding+saline" group.

Table 4
ESP at midrange left ventricular volume in response to Ca^{2+} infusion

Group	*n*	ESP_{mLVV} before Ca^{2+} infusion (mmHg)	ESP_{mLVV} during Ca^{2+} infusion (mmHg)	Increase in ESP_{mLVV} (mmHg)
Normal	6	114.2±26.0	168.2±24.8	54.0±18.1
Sham	6	144.5±22.7	199.8±21.7	55.3±12.2
Banding	6	86.1±26.7	98.8±28.9[a, b]	12.7±4.6[a, b]
Banding+βgal	5	94.6±26.8	115.0±34.8[a, b]	20.3±9.8[a, b]
Banding+saline	5	91.1±21.5	114.7±18.6[b]	23.6±7.1[a, b]
Banding+SERCA	6	154.1±58.2[c, d, e]	198.8±62.6[c, d, e]	44.7±14.7[c, d, e]
Banding+Parv	5	170.6±26.1[c, d, e]	231.8±34.1[a, c, d, e]	61.2±11.2[c, d, e]

All data are shown as mean±SD.
ESP_{mLVV}, end-systolic pressure observed at midrange left ventricular volume (0.05 ml of balloon volume); *n*, number of hearts.
ESP_{mLVV} during Ca^{2+} infusion was obtained in a steady state of hearts after intracoronary infusion of 1% $CaCl_2$ at a 6 ml/h infusion rate for at least 4 min.
[a] $P<0.05$ compared to "Normal" group.
[b] $P<0.05$ compared to "Sham" group.
[c] $P<0.05$ compared to "Banding" group.
[d] $P<0.05$ compared to "Banding+βgal" group.
[e] $P<0.05$ compared to "Banding+saline" group.

groups were significantly higher than those in "Banding", "Banding+βgal" and "Banding+saline" groups. ESP_{mLVV} was gradually increased as the infusion rate of Ca^{2+} solution was increased step-wise from 2 to 6 ml/h. In "Banding+ SERCA" and "Banding+Parv" groups, the maximal increase in ESP_{mLVV} in response to Ca^{2+} solution (6 ml/h) was similar to those in "Normal" and "Sham" groups and was much higher than those in "Banding", "Banding+βgal" and "Banding+ saline" groups. In some experiments, we infused dobutamine solution into the coronary perfusion tubing at 2–4 ml/h of infusion rate after the Ca^{2+} infusion. The changes in ESP_{mLVV} in response to dobutamine were similar to those to Ca^{2+} in all groups (data not shown).

3.5. LV contractility

The eEmax at mLVV, an index of LV contractility, in "Banding+SERCA" and "Banding+Parv" groups was significantly higher than that in "Normal", "Sham" and 3 other control aortic banding groups (Fig. 2). In the preliminary experiments, 3 aortic-banded rats underwent LV mechanical and energetic studies 45 days after recombinant AAV.SERCA transfer. As shown in Fig. 2, "Banding+AAV.SERCA" group showed the high LV contractility similar to that found in "Banding+ SERCA" and "Banding+Parv" groups. On the other hand, "Banding", "Banding+βgal" and Banding+saline groups showed the lower LV contractility.

3.6. LV energetics; oxygen cost of LV contractility

Fig. 3A shows representative relations between VO_2 for Ca^{2+} handling in E-C coupling and eEmax at mLVV during Ca^{2+} inotropism run in "Sham", "Banding" and "Banding+SERCA" hearts. These 3 distinct linear relations had the different slopes, which mean the different oxygen costs of LV contractility. In some experiments, the intracoronary dobutamine infusion was

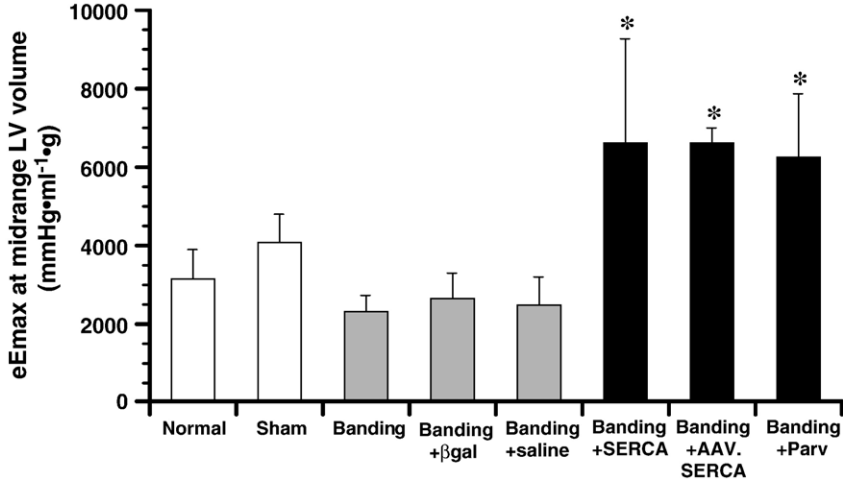

Fig. 2. Comparison of eEmax at midrange LV volume, an index of LV contractility, among all groups. *$P<0.05$ compared to "Normal", "Sham", "Banding", "Banding +βgal" and "Banding+saline" groups. $n=6$ in "Normal", $n=6$ in "Sham", $n=6$ in "Banding", $n=5$ in "Banding+βgal", $n=5$ in "Banding+saline", $n=6$ in "Banding+SERCA", $n=3$ in "Banding+AAV.SERCA", $n=5$ in "Banding+Parv".

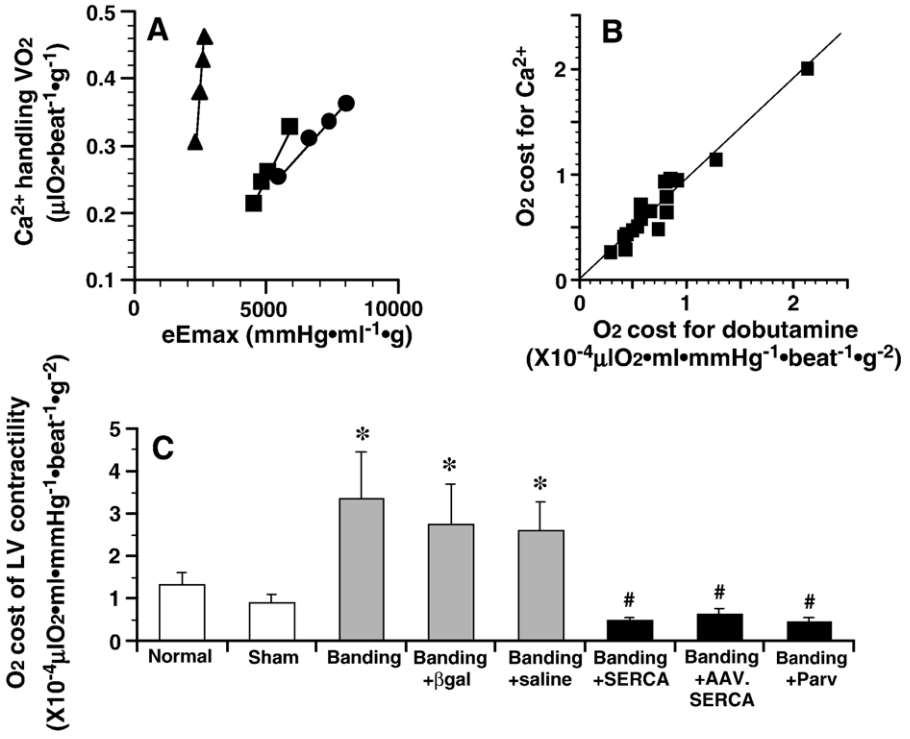

Fig. 3. (A) Linear relations between Ca^{2+} handling VO_2 and eEmax at midrange LV volume during Ca^{2+} inotropic run in "Sham" (■), "Banding" (▲) and "Banding+SERCA" (●) hearts. Slope of these linear relations means oxygen cost of LV contractility. (B) Correlation between O_2 costs for Ca^{2+} and for dobutamine. In 17 heart preparations, dobutamine (78 μM) inotropic run was performed 1h after Ca^{2+} inotropic run. An equation of regression line is $f(x)=0.93\times+1.8\times10^{-6}$, $r=0.96$. (C) Comparison of oxygen costs of LV contractility among all groups. $^{*}P<0.05$ compared to "Normal" and "Sham" groups. $^{\#}P<0.05$ compared to "Banding", "Banding+βgal" and "Banding+saline" groups. $n=6$ in "Normal", $n=6$ in "Sham", $n=6$ in "Banding", $n=5$ in "Banding+βgal", $n=5$ in "Banding+saline", $n=6$ in "Banding+SERCA", $n=3$ in "Banding+AAV.SERCA", $n=5$ in "Banding+Parv".

performed after the Ca^{2+} infusion in the same heart preparation. There was a fairly good correlation between the oxygen costs of LV contractility in response to Ca^{2+} and to dobutamine (Fig. 3B). The oxygen cost of LV contractility for Ca^{2+} in "Banding" group was about 3 times as high as that in "Normal" and "Sham" groups (Fig. 3C). In "Banding+SERCA", "Banding+AAV. SERCA" and "Banding+Parv" groups, the oxygen costs of LV contractility were as low as those in "Normal" and "Sham" groups, but in "Banding+βgal and "Banding+saline" groups the oxygen costs remained as high as that of "Banding" group.

3.7. SERCA2a and parvalbumin protein expression

We examined the protein expression of SERCA2a in the heart preparations used for the analysis of mechanical and energetic function. As shown in Fig. 4, there is a decrease in SERCA2a expression in the failing hearts compared to sham. The overexpression of SERCA2a either by adenovirus or AAV1 restores SERCA2a protein levels in the failing hearts. Gene transfer of parvalbumin induces a robust expression in the hearts infected.

4. Discussion

In this study, using a unique technique for measuring LV mechanoenergetics in the cross-circulated heart preparations, we found that cardiac gene transfer of Ca^{2+} handling proteins (SERCA2a and parvalbumin) can restore not only mechanical but also energetic function in pressure overload-induced failing hearts.

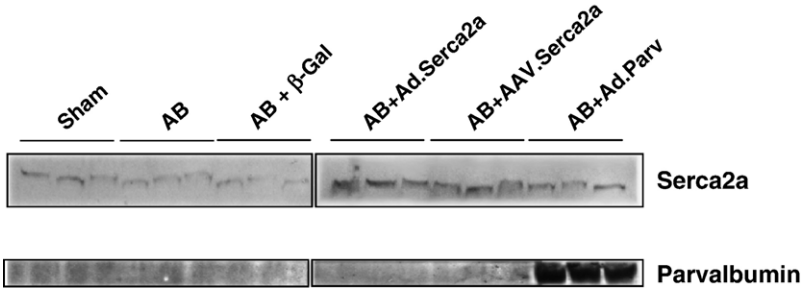

Fig. 4. Immunoblots of SERCA2a and parvalbumin from left ventricular tissues of various groups.

4.1. Mechanical function and gene transfer

In the failing rats, we found a downward shift of ESPVR, a decreased $ESP_{0.1}$ and deceased eEmax at mLVV compared with "Normal" and "Sham" rats. Furthermore, the logistic time constant of isovolumic relaxation and $EDP_{0.1}$ were found to be increased in these failing rats. Thus, the aortic-banded rats used in this study showed both LV systolic and diastolic dysfunction. The central mechanisms for systolic dysfunction in this rodent model of heart failure have been reported to be: decreased SERCA2a expression [17], increased sarcolemmal NaCaX expression [19] and the transition of cardiac myosin isoform components from V_1 with higher ATPase activity to V_3 with lower ATPase activity [20]. The decreased SERCA2a and/or the increased NaCaX can reduce the Ca^{2+} content in the SR and limit the Ca^{2+} release from the SR, leading to diastolic increases in calcium and general intracellular calcium overload [19]. V_1 is known to be associated with an increased shortening velocity of the cardiac fibers and V_3 with a slower shortening velocity [21], although in aortic-banded rats, the improved contractile function by clenbuterol, a selective β_2-adrenergic agonist, was not related to changes in myosin isoform components [3]. On the other hand, the central mechanisms that cause diastolic dysfunction in this rodent model of heart failure are due to decreased SERCA2a expression and increase in cardiac stiffness due to enhanced collagen and fibrosis [3]. However, in this study the contractile dysfunction found in the "Banding" hearts (ESPVR, $ESP_{0.1}$, $EDP_{0.1}$, and time constant of isovolumic relaxation) could be completely corrected by SERCA2a gene transfer, indicating that the decreased SERCA2a expression plays a key role in both systolic and diastolic dysfunction. Furthermore, similar correction of contractile dysfunction could be accomplished by parvalbumin gene transfer as reported in normal and aged rats [11,22]. In senescent rat cardiomyocytes, introduction of parvalbumin, that functions as a Ca^{2+} sink due to its Ca^{2+} affinity and enhances relaxation in skeletal muscle, increased both the rate of Ca^{2+} transient decay and the rate of myocyte re-lengthening [23]. This in vitro study provides the cellular mechanism for improved diastolic function by parvalbumin gene transfer in the present in vivo study. On the other hand, the possible mechanism for improved systolic function by the parvalbumin gene transfer is as follows; the parvalbumin-induced decline in intracellular free Ca^{2+} during relaxation attenuates the intracellular Ca^{2+} overload and may improve the impaired Ca^{2+} handling in the present failing hearts. Finally, the prominent eEmax at mLVV could be induced by the SERCA2a or parvalbumin gene transfer in the aortic-banded hearts. The high LV contractility, as indicated by high $ESP_{0.1}$ and high eEmax at mLVV, seems due mainly to both the hypertrophied LV free wall and the enhanced Ca^{2+} handling by overexpression of the SERCA2a or parvalbumin.

In the failing hearts, the increase in ESP_{mLVV} in response to Ca^{2+} infusion was much lower than that of "Normal" and "Sham" hearts. This blunted inotropic response to Ca^{2+} is most likely caused by multiple factors including abnormal Ca^{2+} handling, deficient production of cyclic AMP [24], along with a decrease in energy reserve via a deficient creatine kinase reaction [25]. Both SERCA2a and parvalbumin gene transfer was capable of completely reversing the inotropic response to Ca^{2+} (i.e., contractile reserve), indicating that in the aortic-banded hearts the abnormal Ca^{2+} handling is a central cause of the lowered contractile reserve and regardless how calcium is decreased intracellularly, there is a beneficial response.

4.2. LV energetic function and gene transfer

In the failing hearts, the slope of the VO_2–PVA relation, which is an index of the O_2 cost of PVA, i.e., the O_2 cost of total mechanical energy of LV contraction [26], was about twice as high as that of "Normal" and "Sham" hearts. A similar steeper slope of the VO_2–PVA relation was reported in hyperthyroid rabbit hearts which actually have increased V_1/V_3 ratio [27]. In contrast, in hypothyroid [15] and diabetic [12] rat hearts with decreased V_1/V_3 ratio, the slope remained unchanged. Thus, the inconsistent relationship between the slope and the V_1/V_3 ratio suggests that a change in myosin isoform components, i.e., a change in myosin ATPase activity, does not directly affect the contractile efficiency, which is the reciprocal of slope of the VO_2–PVA relation [26]. The contractile efficiency reflects the chemo-mechanical energy transduction efficiency of the contractile machinery. Therefore, in the aortic-banded hearts, the high O_2 cost of total mechanical energy, shown by the steeper slope of the VO_2–PVA relation, indicates that energy wasting in the chemo-mechanical energy transduction is present. In the pressure overload-induced hypertrophied myocardium, the slope of relation between VO_2 and isometric developed tension was increased [28,29], and the increased VO_2 was due to the increased nonphosphorylating (state 4) mitochondrial respiration linked to Ca^{2+} transport [28], while in volume overload-induced hypertrophied myocardium, the slope was not different from that of normal myocardium [30]. Therefore, It seems likely that in the aortic-banded hearts, the increased slope of the VO_2–PVA relation is attributable to the increased VO_2 induced by the increased nonphosphorylating mitochondrial respiration. This high slope could be completely improved by gene transfer of SERCA2a or parvalbumin. We have speculated on one possible mechanism for this; the overexpression of SERCA2a or parvalbumin restores the increased cytosolic free Ca^{2+} to normal levels and improves the enhanced Ca^{2+} transport to mitochondria, leading to the normal nonphosphorylating respiration of mitochondria. On the other hand, the VO_2–intercept (i.e., PVA-independent VO_2) of the VO_2–PVA relation, which reflects VO_2 for non-mechanical works consisting of Ca^{2+} handling during E-C coupling and basal metabolism [26], did not differ among all the groups studied. Furthermore, there was no significant difference in minute VO_2 for Ca^{2+} handling and basal metabolism among all the groups, showing that these minute VO_2 are affected neither by cardiac hypertrophy nor by adenoviral gene transfer. In addition, the unchanged VO_2 for Ca^{2+} handling found in the aortic-banded hearts suggests that SERCA2a, albeit down-regulated, can exert its function nor-

mally because of a lower level of cytosolic free Ca^{2+} during diastole in steady state of mechanically unloaded and Ca^{2+} unloaded hearts.

The most important finding of this study is that the O_2 cost of LV contractility, defined as the slope of relation between Ca^{2+} handling VO_2 and eEmax, was increased in the failing hearts, and this increased O_2 cost could be restored to normal levels by short-term and long-term gene transfer of SERCA2a or parvalbumin. The O_2 cost of LV contractility is thought to indicate the energy cost of Ca^{2+} handling during E-C coupling in failing hearts where the Ca^{2+} responsiveness of myofilaments remains unchanged [31]. There seems three possible mechanisms for the increased O_2 cost of LV contractility, which means the energy wasting in Ca^{2+} handling, in the aortic-banded hearts. First, the molar coupling ratio of calcium to ATP in SERCA2a was decreased due to increased Ca^{2+} permeability of the SR membrane [32]. Such SERCA2a dysfunction would need more ATP to lower cytosolic Ca^{2+} during relaxation. Second, abnormal Ca^{2+} leak through ryanodine receptor (RyR), caused by RyR conformational change due to a partial loss of RyR-bound FK506-binding protein, was found in a canine model of heart failure [33]. This Ca^{2+} leak via RyR will elevate basal cytosolic Ca^{2+} levels during diastole, leading to the futile Ca^{2+} handling. However, the above-mentioned mechanisms cannot fully explain the increased O_2 cost of LV contractility because of the down-regulated SERCA2a expression in our aortic-banded hearts. Finally, cardiac NaCaX expression was reported to be up-regulated in heart failure [19,34,35]. Although NaCaX consumes no ATP to remove cytosolic Ca^{2+} in exchange with influx Na^+ on the basis of a stoichiometry of $3Na^+$:$1Ca^{2+}$, the influx Na^+ will be pumped out by Na^+-K^+-ATPase with a stoichiometry of $3Na^+$:$2K^+$:1ATP, resulting in the net stoichiometry of $1Ca^{2+}$:1ATP [36]. On the other hand, SERCA2a removes cytosolic Ca^{2+} on the basis of a stoichiometry of $2Ca^{2+}$:1ATP. Therefore, the Ca^{2+} extrusion via NaCaX leads to twice increase in energy expenditure compared with the Ca^{2+} uptake by SERCA2a. Thus, it seems most likely that the energy wasting in Ca^{2+} handling is induced mainly by the enhanced NaCaX activity. Nevertheless, overexpression of SERCA2a or parvalbumin was capable of restoring the high O_2 cost of LV contractility to normal levels in the aortic-banded hearts. The enhanced expression of SERCA2a or parvalbumin appears to induce the decrease in the cytosolic free Ca^{2+} level during diastole and thereby the decrease in Ca^{2+} extrusion via NaCaX, leading to the decrease in the energy cost of Ca^{2+} handling, namely, the decrease in the O_2 cost of LV contractility.

4.3. Therapeutic implications

In patients with congestive heart failure (CHF), inotropic agents improve contractility and hemodynamics in the short term. However, they increase VO_2, and subsequently induce an energetic imbalance, followed by arrhythmias and worsening survival. Therefore, long-term inotropic interventions have been shown to increase morbidity and mortality in patients with CHF [37]. In this study, gene transfer of SERCA2a or parvalbumin was found to improve the energy wasting both in chemomechanical energy transduction and in Ca^{2+} handling during E-C coupling in pressure-overload failing hearts. In a previous study [9], our group had already shown improvement in mortality and energy reserve (measured by NMR) in the same model of heart failure following short-term gene transfer of SERCA2a. Therefore, targeting Ca^{2+} handling in CHF may provide great advantage of efficient energy utilization over standard inotropic agents.

4.4. Limitations of the study and future directions

In the present failing hearts, correction of the energetic dysfunction by SERCA2a overexpression was accomplished, in a long-term transgene expression system of AAV1-based vectors as well as in a short-term transgene expression system of adenoviral vectors. To further confirm this finding, large animal studies are also required to verify these provocative results, because the contribution of SERCA2a to the lowering of cytosolic Ca^{2+} levels varies between rodents and large mammals. In humans, about 75% of the Ca^{2+} is removed by SERCA2a, whereas in rodents 92% is removed by SERCA2a [1]. However, overexpression of SERCA2a was capable of improving contractile function in failing human cardiomyocytes [38]. Therefore, SERCA2a overexpression is expected to be useful for improving LV energetic function in failing human hearts. Taken together, large animal studies using AAV vectors are indispensable for leading to clinical trials of gene therapy for CHF.

4.5. Conclusions

High O_2 costs of total mechanical energy and of LV contractility in the aortic-banded hearts indicate the energy wasting both in chemomechanical energy transduction and in Ca^{2+} handling during E-C coupling. The gene transfer of Ca^{2+} handling proteins (SERCA2a and parvalbumin) was capable of correcting both the high O_2 costs. Thus, the gene transfer of the Ca^{2+} handling proteins transforms the inefficient energy utilization into a more efficient state and improves not only mechanical but also energetic function in pressure-overload failing hearts. Gene transfer of Ca^{2+} handling proteins may be a potential therapeutic strategy with the benefit of efficient energy utilization.

Acknowledgments

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