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Gene transfer of a phospholamban-targeted antibody improves calcium handling and cardiac function in heart failure

Thomas Dieterle[1], Markus Meyer, Yusu Gu, Darrell D. Belke, Eric Swanson, Mitsuo Iwatate[2], John Hollander, Kirk L. Peterson, John Ross Jr., Wolfgang H. Dillmann*

Department of Medicine, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0618, USA

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Abstract

Background: Abnormalities of intracellular calcium handling are widely recognized as a common hallmark of heart failure in animal models and humans. Modifying the interaction of phospholamban (PLB) with the sarcoplasmic reticulum ATPase (SERCA) by PLB mutants improves cardiac function but may also lead to heart failure. In this study we describe the in vivo effects of a new approach to modify the PLB–SERCA interaction using a recombinant, intracellularly expressed chicken-antibody derived protein (PLADP) targeting the cytoplasmic domain of PLB in the cardiomyopathic BIO 14.6 hamster.

Methods and results: In vivo gene transfer was performed in 12–14-week-old BIO 14.6 cardiomyopathic hamsters using intracoronary delivery of adenovirus containing the PLADP or the β-galactosidase (LacZ) gene (8×10^9 PFU per animal). A third group was injected with saline (Sham). Echocardiography was performed before and, together with hemodynamic measurements, repeated 4–5 days after gene transfer. Indo-1 calcium transients and myocyte contractility were measured in isolated cardiomyocytes from the BIO 14.6 hamster transfected with the PLADP. Gene expression (LacZ) was found in 54±15% of cells throughout the heart without any signs of myocardial inflammation. Echocardiographic and hemodynamic indices of left ventricular function were significantly increased after gene transfer with the PLADP, compared to controls. Measurements of myocyte contractility and calcium transients in isolated cardiac myocytes from BIO 14.6 hamsters revealed improved intracellular calcium handling and contractility.

Conclusion: In vivo adenoviral gene transfer with the PLADP resulted in short-term intracellular expression of a PLADP, improving LV function and enhancing myocardial contractility in the failing cardiomyopathic hamster heart. The PLADP enhanced contractility and cardiac function by improving intracellular calcium handling. Expression of antibody-derived protein represents a new approach to modify protein–protein interactions at the cellular level.

Keywords: Gene transfer; Phospholamban; SERCA; Phospholamban antibody; Cardiomyopathic hamster

1. Introduction

Abnormalities of intracellular calcium handling are widely recognized as a common hallmark of heart failure in humans and animal models [1]. The sarcoplasmic reticulum (SR), an intracellular membrane system which stores calcium in cardiac cells, plays a predominant role in cardiac excitation–contraction coupling and cardiac contractility. An intracellular calcium gradient is maintained by the cardiac SR calcium ATPase (SERCA), which is mainly modulated through its accessory phosphoprotein phospholamban (PLB, [2]). The magnitude of SR calcium released is a function of the quantity of calcium stored in the SR [3] and the development of contractile force depends on the amount of cytosolic calcium available [4].

* Corresponding author. Tel.: +1 858 534 3348; fax: +1 858 534 1626.
E-mail address: wdillmann@uscd.edu (W.H. Dillmann).
[1] Current address: University Hospital Basel, Division of Cardiology, Basel, Switzerland.
[2] Current address: University of Yamaguchi, Division of Cardiology, Yamaguchi, Japan.

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Inhibition of SERCA can be relieved by cyclic-AMP mediated phosphorylation of PLB, one of the mechanisms by which β-adrenergic stimulation increases contractility [5]. Complete ablation of PLB as the most extreme example of modification of PLB–SERCA interaction greatly enhances contractility of the heart under physiological and pathological conditions [6,7]. It could be shown in several cardiomyopathic states that the effect of PLB on contractility may even be exaggerated by a decrease in SERCA expression and a reduction in the phosphorylated state of PLB [8–10]. These studies suggest that modification of the PLB–SERCA interaction may be an attractive target for therapeutic interventions to increase cardiac contractility.

However, ablation of PLB as the most extreme modification of PLB–SERCA interaction is impractical for therapeutic purposes and has recently even been identified as a cause for dilated cardiomyopathy in humans [11]. Moreover, PLB mutations not directly influencing the PLB–SERCA interaction may indirectly contribute to the development of heart failure [12].

On the other hand, two recent studies have shown beneficial long-term effects of gene transfer of a mutant PLB on cardiac function in the BIO 14.6 hamster [13] and in the myocardial infarct rat [14]. These studies suggest that interventions involving the PLB–SERCA system may be a safe and promising approach to the therapy of heart failure.

A novel approach has been proposed to modify PLB–SERCA interaction using antibodies directed against PLB [15]. These antibodies bind to the cytoplasmic portion of PLB and thereby enhance SERCA function in vitro. However, these antibodies can only be used in isolated SR vesicles and are impracticable for in vivo therapeutic use. One way to overcome this limitation is to express an antibody or an antibody-derived protein targeting PLB in the cardiac myocyte. If feasible in vivo the principle of intracellular expression of antibodies targeting intracellular proteins would offer the possibility to alter protein–protein interactions at the cellular level and eventually cellular function.

In the present study, we tested the feasibility of modifying PLB–SERCA interaction in vivo using this approach as an example of modifying protein–protein interaction at the cellular level. We describe the effects on cardiac function in vivo of a recombinant single chain antibody, developed from avian heavy and light chain IgY chains, that specifically targets the cytoplasmic portion of PLB which we named PLB-antibody derived protein (PLADP). In vivo cardiac transfer of the PLADP gene was performed in the BIO 14.6 cardiomyopathic hamster, a well-characterized model of progressive heart failure [16,17], using a highly efficient method recently developed in our laboratory [18]. In addition, we directly demonstrated the effects of the PLADP on intracellular calcium handling and contractility in isolated cardiac myocytes of the BIO 14.6 hamster after in vivo gene transfer.

2. Methods

2.1. Animals

BIO 14.6 male cardiomyopathic hamsters (12–14 weeks old) were obtained from BIO Breeders (Fitchburg, MA, USA). The hamsters were maintained at 20±2 °C, 55±20% humidity, with 12/12-h light/dark cycles and had free access to food and water. All animal protocols were approved by the University of California San Diego Animal Subjects Committee, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Development of the phospholamban-antibody derived protein (PLADP)

Chickens were immunized with a synthetic peptide containing the amino acid sequence 3–19 of human phospholamban (PLB) that spans most of the cytoplasmic domain of PLB including the phosphorylation sites Ser16 and Thr17. Bone marrow and spleen from the chicken were harvested, mRNA isolated and RT-PCR used to amplify the genes for the antibody variable regions of both IgY light and heavy chains. PCR products were combined and PCR overlap extended into a single PCR product. When expressed as a protein this construct contains randomly combined light and heavy chain variable region representing the IgY repertoire of the chicken connected by a short linker. These genes were cloned into a phagemid expression vector, resulting in a fusion of the single chain antibody product to the filamentous phage coat protein III. To select for PLB-binders the phages were incubated in polystyrol tubes coated with BSA cross-linked to PLB peptide.

After six rounds of panning the selected library was subcloned into a bacterial expression plasmid which introduced a His-tag and a hemagglutinin (HA)-tag. The C-terminal HA-tag was used to identify protein expression on Western blots. Proteins from 20 independent clones were expressed and DNA sequences determined for 5 of the best PLB-binders. Four of them demonstrated complete sequence identity (Fig. 1).

2.3. Adenoviral vector construct

PLADP DNA or nuclear encoding β-galactosidase (LacZ) DNA signal sequences or green fluorescent protein (GFP) DNA were inserted into an AdV shuttle vector PacCMV.pApL. Replication-deficient recombinant AdV-5 vectors were generated as previously described [19]. Gene expression was driven by a CMV promoter.

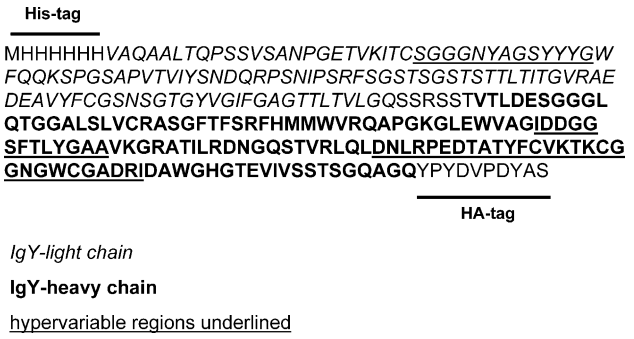

Fig. 1. Protein sequence of the PLADP.

2.4. In vivo transfection methods

Animals were randomly assigned to in vivo gene transfer either with Ad5-CMV-PLADP or Ad5-CMV-LacZ or intracoronary injection of saline (Sham operation) using a method modified from Ikeda et al. [18].

Animals were anaesthetized with pentobarbital (75 mg/kg BW), intubated and ventilated with room air. Via a small anterior thoracotomy ligatures were looped around the aorta and pulmonary artery and threaded through occluder tubes. The right carotid artery was cannulated for measurement of arterial pressure and performing injections with the tip of the catheter placed above the aortic valve.

The animals were cooled down until a core temperature of 19–21 °C and a heart rate of 60–80/min was reached. The pulmonary artery and aorta were occluded and cardioplegic solution (CP, in mM: NaCl 110, KCl 20, $CaCl_2$ 1.2, $MgCl_2$ 16, and $NaHCO_3$ 10, total volume 200 μl) was bolus-injected into the aorta. This was immediately followed by a second injection of CP plus histamine 20 mM (total volume 250 μl). After 3 min a third injection of CP (in mM: NaCl 110, KCl 10, $CaCl_2$ 1.2, $MgCl_2$ 16, and $NaHCO_3$ 10) together with 10 mM histamine and the virus suspension (8×10^9 PFU per animal) or normal saline was performed (total volume 300 μl).

Forty-five seconds later both snares were released and an intraaortic infusion of dobutamine (10 μg/kg/min) started. When the arterial pressure reached about 50 mm Hg, the animals were placed on a heating pad (42 °C), the chest closed and intrathoracic air evacuated. Animals were extubated upon spontaneous breathing and closely observed until fully awake.

To achieve co-transfection of PLADP and GFP for measurements of isolated cardiac myocyte calcium transients and contractility, five 10 μl aliquots (50 μl total) consisting of buffer and viral vectors (Ad5-CMV-LacZ together with Ad5-CMV-GFP or Ad5-CMV-PLADP together with Ad5-CMV-GFP (ratio 10:1)) were injected into the free wall of the left ventricle (LV) in a further set of experiments. GFP served as a marker for myocyte transfection. Myocytes isolated from hearts injected with Ad5-CMV-GFP alone served as control.

Overall mortality of both procedures was between 10% and 15%. Mortality in the PLADP group was below 10%.

2.5. Estimation of transfection efficiency with the hypothermia–cardioplegia method

Four to 5 days after gene transfer animals were euthanized with an overdose of pentobarbital. The heart was rapidly removed, flushed with phosphate-buffered saline containing 0.3 M KCl, transversely sliced into 3 pieces and quick frozen in Tissue-Tek O.C.T. compound (Sakura Finetek USA Inc., Torrance, CA, USA). Sections (10 μm) from basal, mid and LV slices were used for LacZ staining [20]. Efficiency of LacZ transfection was estimated as the percentage of positive myocyte nuclei using a steorological point-counting method.

2.6. Transthoracic echocardiography

Echocardiography was performed on anaesthetized (pentobarbital 37.5 mg/kg BW) spontaneously breathing animals using an Agilent Sonos 5500 system (Agilent, Andover, MA, USA) and a 15 MHz transducer before and after gene transfer. LV dimensions and function were measured as described previously [18].

2.7. Hemodynamic measurements

Hemodynamic evaluation was performed under general anaesthesia (pentobarbital 75 mg/kg BW) with the animals connected to a ventilator. Following bilateral vagotomy, a micromanometer catheter (Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the ascending aorta and then LV to measure phasic and mean pressures. All physiologic signals were acquired using WINDAQ (DATAQ Inc., Akron, OH, USA), A-D converted at 2000 samples/s. Signal post-processing for calculation of peak systolic LV pressure, end-diastolic LV pressure, the maximum (dP/dt_{max}) and minimum (dP/dt_{min}) derivative of LV pressure and tau, the mono-exponential rate constant of relaxation, was performed using HeartBeat software developed for our laboratory.

2.8. Measurement of sarcoplasmic reticulum calcium uptake in whole heart homogenates

Measurement of sarcoplasmic reticulum calcium uptake was performed in whole heart homogenates according to a previously described method [21].

2.9. Measurement of Ca^{2+} transient and myocyte shortening

Hamster myocytes were isolated using a method modified from Belke et al. [22] and plated on laminin-coated cover slips. For measurement of Ca^{2+} transients cells were incubated with 10 μM Indo-1/AM (Molecular Probes,

Table 1
Baseline characteristics of the BIO 14.6 hamsters

Parameter	PLADP	LacZ	Sham	*P*
n	8	8	8	n.s.
Age (days)	90±3	91±3	88±2	n.s.
Body weight (grams)	95±4	103±3	94±4	n.s.
Heart rate (bpm)	419±21	447±7	434±21	n.s.
End-diastolic LV diameter (mm)	5.4±0.0	5.4±0.1	5.4±0.1	n.s.
Posterior wall thickness (mm)	0.9±0.0	0.9±0.0	0.9±0.0	n.s.
Fractional shortening (%)	26.5±0.9	25.4±0.4	26.9±1.1	n.s.
Velocity of circumferential fiber shortening (circ/s)	4.50±0.23	4.47±0.11	4.85±0.24	n.s.

No differences were found between the groups that subsequently underwent surgical intervention (n.s.=not significant).

Eugene, OR) for 30 min as previously described [23]. The cells were superfused with buffer containing (in mM, modified from Ref. [24]) NaCl 137, KCl 5.4, $MgSO_4$ 1.2, NaH_2PO_4 1.2, HEPES 5, glucose 5.6, pyruvate 0.9, and $CaCl_2$ 1.0 and stimulated to contract (0.3 Hz) using platinum electrodes in a Biophysica (Sparks, MD) coverslip chamber. Fluorescence measurements were performed on a Solamere Technology Group fluorescence measurement system (Solamere Technology Group, Salt Lake City, UT) at room temperature as previously described [25]. Incubation of cardiac myocytes with Indo-1 did not *significantly* alter myocyte shortening (data not shown). Measurements of cell contraction were performed in cells not incubated with Indo-1/AM at room temperature using a video edge motion detector (Crescent Electronics, Sandy, UT) interfaced to a Pulnix TM-640 video camera (Pulnix, Sunnyvale, CA) attached to a Nikon Diaphot microscope (Nikon Inc., Melville, NY) as previously described [24,25]. Data were collected at a sample rate of 20 Hz using MacLab/8e (ADInstruments, Mountain View, CA) on a Macintosh computer and analysed off-line.

2.10. Statistics

All values are expressed as mean±SEM. Statistical analysis was performed using GB-Stat Version 6.0 for Windows (Dynamic Microsystems Inc., Silver Spring, MD, USA). Differences between two groups were compared with Student's *t*-test or Mann–Whitney's *U*-test. Differences between the three gene transfer groups were analysed by one-way ANOVA or, for the analysis of serial echocardiographic measurements, by a two-way repeated measures ANOVA. Post hoc comparisons were performed using the Student–Newman–Keuls test. A value of $p<0.05$ was considered to be statistically significant.

3. Results

3.1. Animal characteristics

The baseline characteristics of the animals that underwent the hypothermia–cardioplegia gene transfer are given in Table 1. Treatment groups were well matched for age, body weight, heart rate and echocardiographic parameters. Compared to normal hamsters, BIO 14.6 hamsters used in our experiments had marked left ventricular (LV) dilation, LV wall thinning and markedly reduced LV function similar to echocardiographic parameters recently published [16].

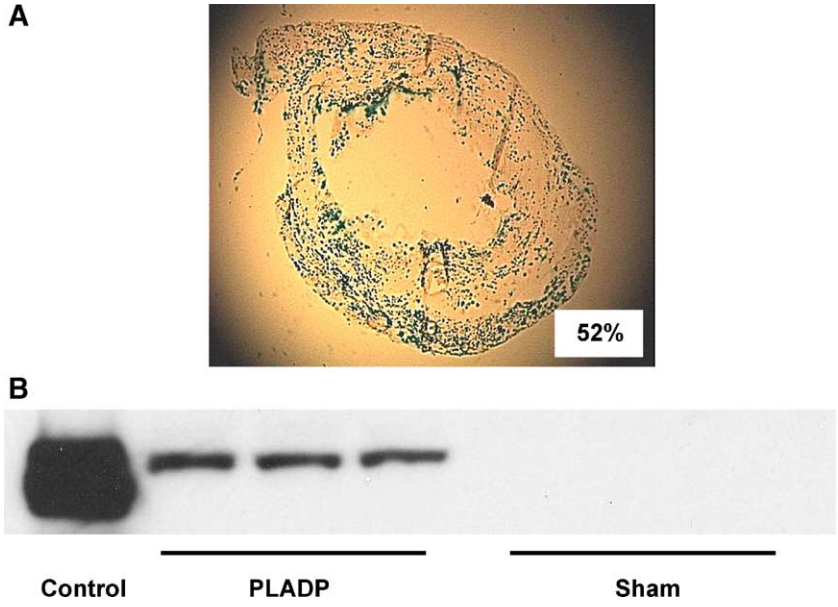

Fig. 2. A. Representative LacZ staining from the left ventricle after gene transfer using the hypothermia–cardioplegia method. The transfection rate in this example was 52%. B. Representative immunoblot showing the expression of the PLADP (protein derived from whole heart homogenates) using an antibody directed to the hemagglutinin (HA)-tag. The positive control was derived from H9C2 cells transfected with AdV-CMV-PLADP in vitro. Fifty micrograms of protein were loaded per lane.

3.2. Efficiency of gene transfer

Gene expression using Ad5-CMV-LacZ using the hypothermia–cardioplegia method was found throughout the LV (Fig. 2A). The average transfection rate was 54% (37–74%). No signs of inflammation were detected in either of the gene transfer groups.

Immunoblots revealed gene expression of Ad5-CMV-PLADP in all PLADP-treated animals (Fig. 2B).

3.3. Echocardiography (Fig. 3)

Echocardiographic analysis of LV function was performed before and 4–5 days after gene transfer (Fig. 3). LV end-diastolic diameter was not affected by gene transfer with PLADP or the Sham intervention; in the LacZ group we found slight LV dilation (increased end-diastolic diameter) compared to baseline and the other groups. Posterior wall thickness was not affected by either of the treatments.

Fractional shortening (FS) and the velocity of circumferential fiber shortening (V_{cf}) were not changed in the LacZ and the Sham group. However, in the PLADP group we observed a significant increase, both compared to baseline and the other groups, of FS from 26.5±0.9% to 30.5±0.8% and of V_{cf} from 4.50±0.23 circ/s to 5.82±0.24 circ/s.

3.4. Hemodynamics (Fig. 4)

Hemodynamic analysis was performed 4–5 days after the intervention. Heart rate did not differ between groups (Fig. 4). Peak systolic LV pressure was significantly higher in the PLADP group compared to the LacZ and Sham group. No significant changes were observed for end-diastolic LV pressure in the PLADP-treated animals.

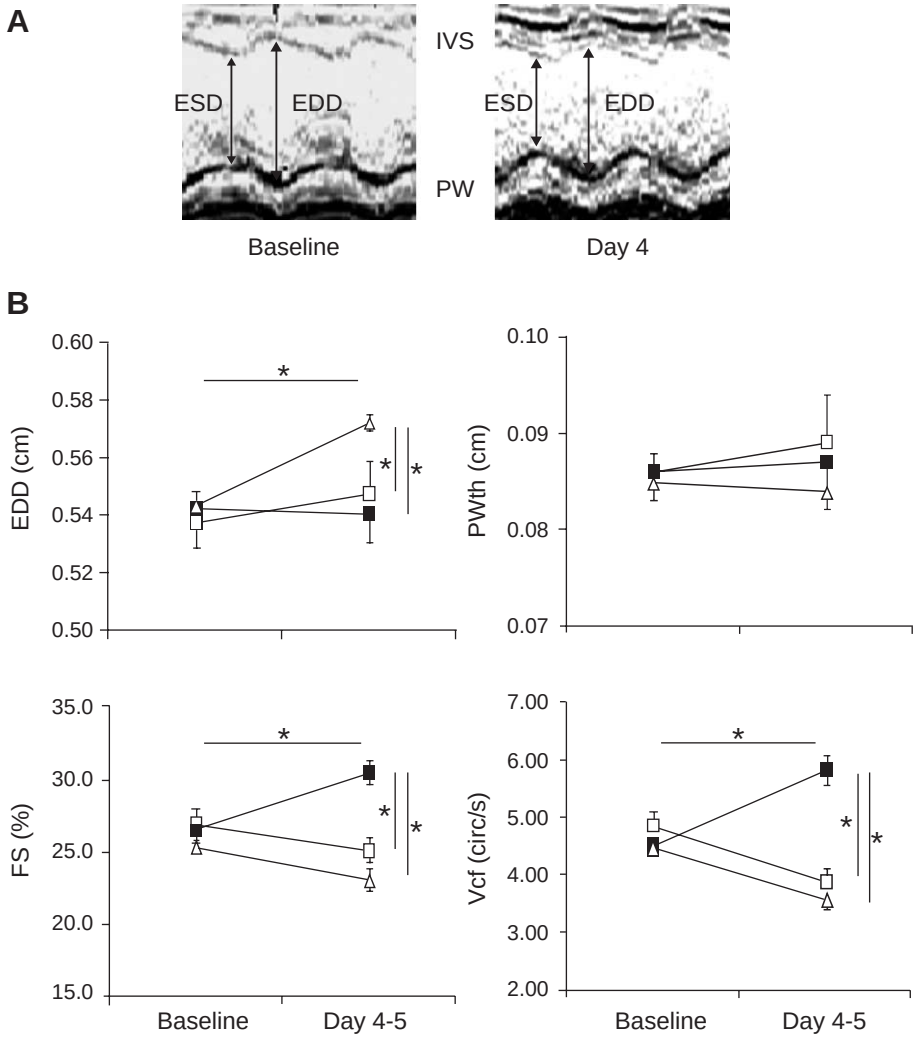

Fig. 3. A. Representative echocardiographic recording obtained before and 4 days after gene transfer. Fractional shortening in this example increased from 28.9% to 34.2%. (EDD=end-diastolic left-ventricular diameter; ESD=end-systolic left-ventricular diameter; IVS=intra-ventricular septum; and PW=left-ventricular posterior wall.) B. Echocardiographic left ventricular dimensions and function before (baseline) and 4–5 days after gene transfer of AdV-CMV-PLADP (■), AdV-CMV-LacZ (□), or Sham intervention (△) ($n=8$ per group, *$p<0.05$.

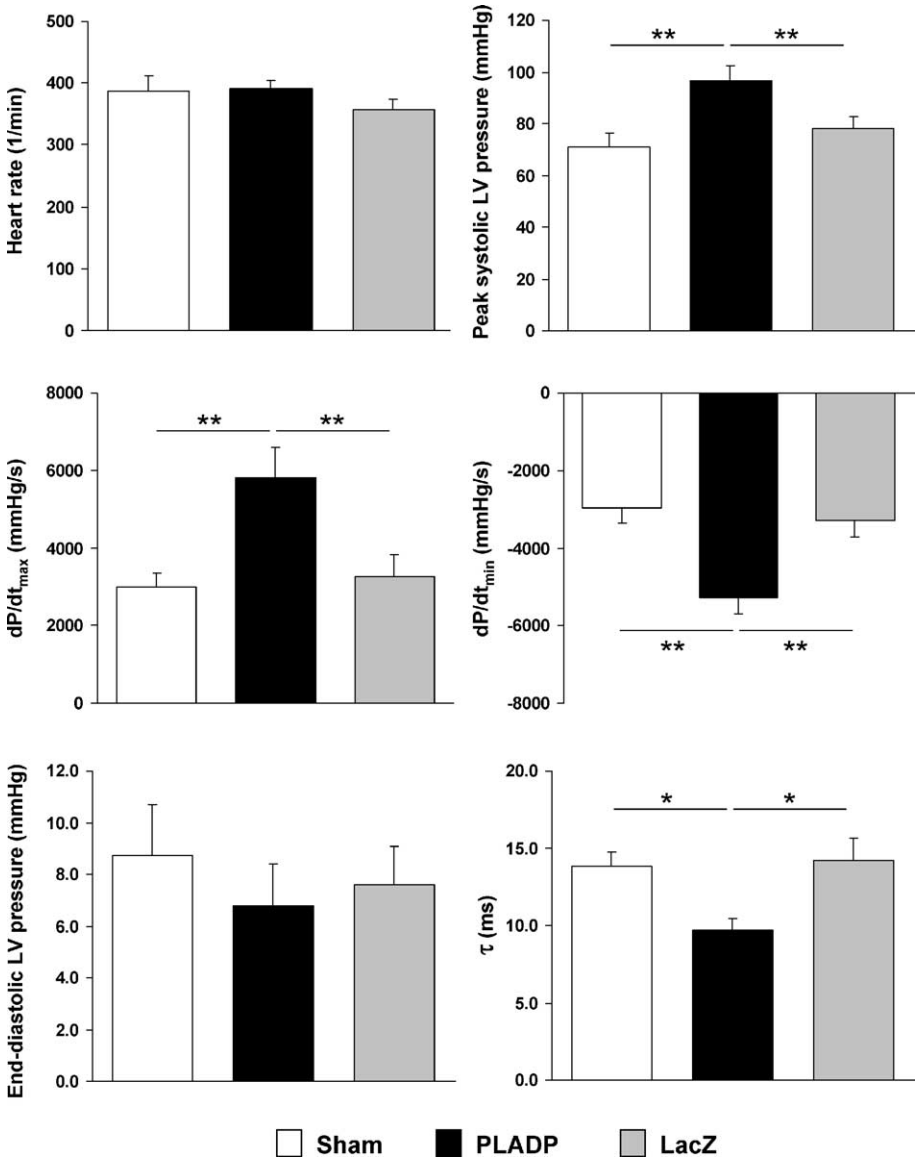

Fig. 4. Left ventricular hemodynamic measurements obtained 4–5 days after gene transfer ($n=8$ per group, $*p<0.05$, $**p<0.01$, dP/dt_{min}, dP/dt_{max}=minimum, maximum derivative of left ventricular pressure, and τ=mono-exponential rate constant of relaxation).

The maximum and minimum derivatives of LV pressure dP/dt_{max} and dP/dt_{min} in the PLADP-treated group were both significantly enhanced compared to the LacZ and the Sham groups.

The time constant of isovolumic relaxation τ was significantly reduced in the PLADP group compared to the Sham and LacZ group.

3.5. Phospholamban and SERCA levels (Fig. 5)

Because we expressed an antibody against PLB, part of the beneficial effects on cardiac function might be explained by increased intracellular degradation of PLB. However, the quantitative analysis of immunoblots to examine PLB monomer, PLB pentamer and total PLB expression levels did not reveal significant differences between the PLADP and the Sham group. No significant differences between the PLADP and Sham group were found for SERCA expression levels (Fig. 5).

3.6. Sarcoplasmic reticulum calcium uptake in whole heart homogenates

To demonstrate that the effects of the PLADP in vivo can in principle be due to the interaction with PLB we measured oxalate-supported SR calcium uptake in whole heart homogenates following in vivo gene therapy. SR calcium uptake was significantly higher in whole heart homogenates after in vivo gene therapy with the PLADP compared to homogenates obtained from control hearts (198±18 vs. 130±17 nmol Ca^{2+}/μg protein/min, mean±SEM, $n=4$ per group, $p<0.05$).

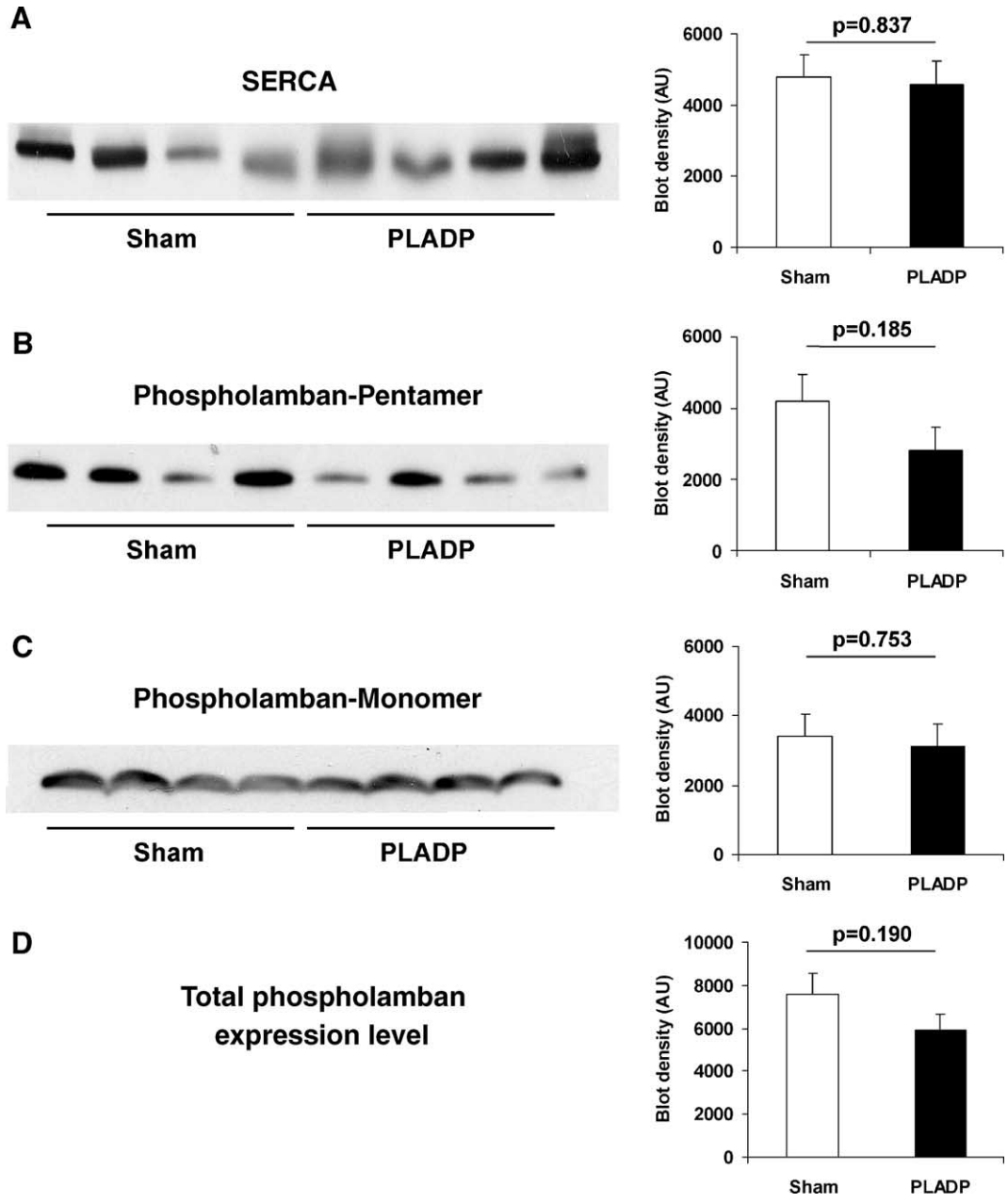

Fig. 5. Immunoblots showing SERCA (A), phospholamban pentamer (B), phospholamban monomer (C) and total phospholamban (D) levels. Data are given as mean ± SEM ($n = 8$ per group). Measurements of blot density did not reveal differences in the expression of these proteins.

3.7. Intracellular Indo-1 calcium transients and myocyte shortening

To further define the effects of the PLADP on cardiac function we performed measurements of Indo-1 calcium transients and myocyte contractility in isolated cardiomyocytes from the BIO 14.6 hamster co-transfected with Ad5-CMV-PLADP and Ad5-CMV-GFP.

To test whether co-transfection in vivo is feasible, hearts were co-transfected in a first step in vivo with Ad5-CMV-LacZ and Ad5-CMV-GFP using direct intramyocardial injection of the vectors. Co-expression of LacZ and GFP was demonstrated in 95% of GFP expressing myocytes (Fig. 6A).

For the analysis of the effects of PLADP hearts were co-transfected with Ad5-CMV-PLADP and Ad5-CMV-GFP. Measurements of myocyte shortening and Indo-1 calcium transients were performed on GFP-expressing myocytes and compared to cardiac myocytes from hearts transfected with Ad5-CMV-GFP. Data were obtained from 3 to 4 animals per group.

While diastolic calcium levels were unchanged, peak systolic calcium levels were significantly increased during systole in PLADP-transfected myocytes compared to

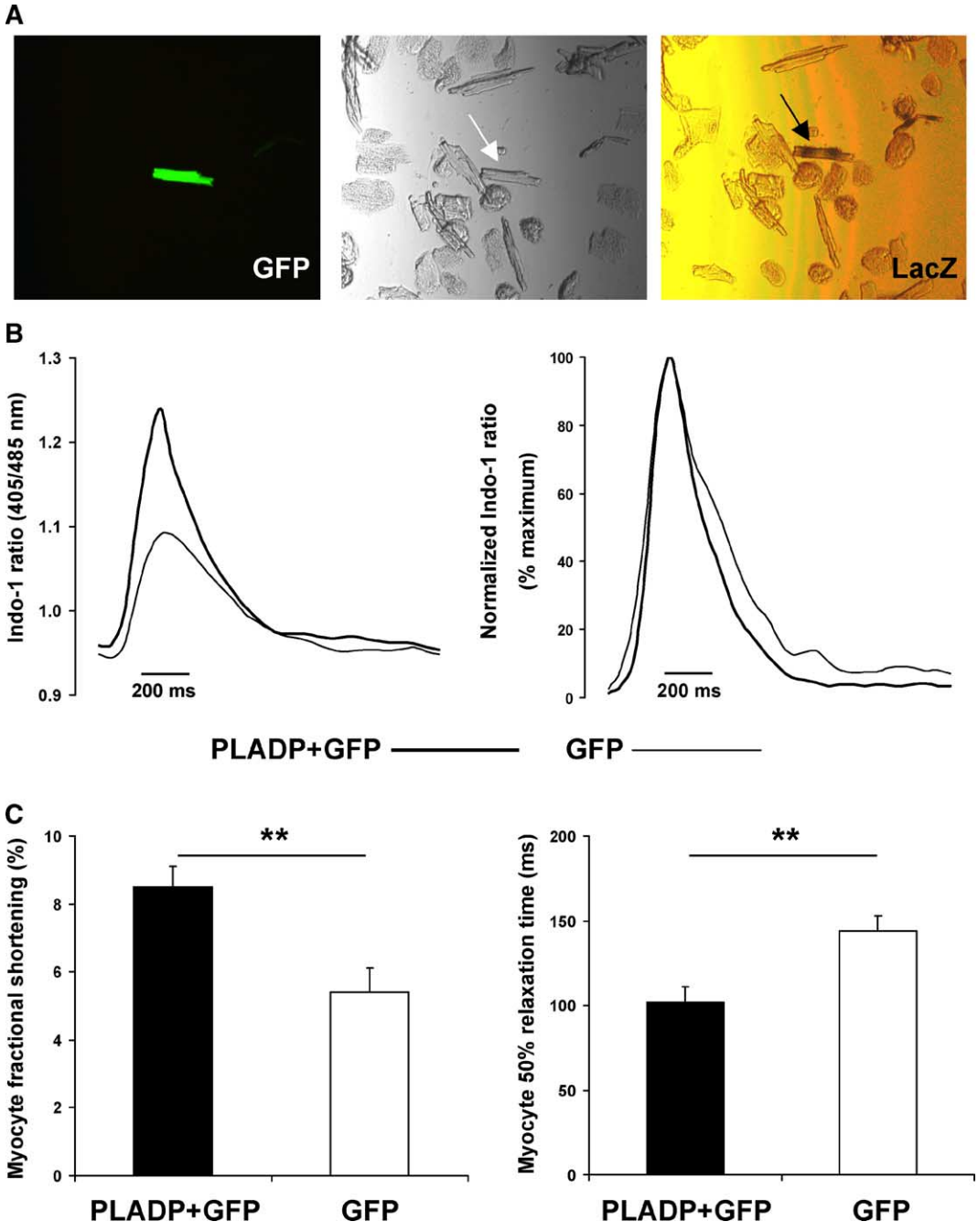

Fig. 6. A. Example of co-expression of GFP and LacZ in a myocyte co-transfected with AdV-CMV-GFP and AdV-CMV-LacZ in the BIO 14.6 hamster. Vectors were administered in vivo at a ratio of 1 : 10 with AdV-CMV-GFP being the minor part. B. Signal-averaged and normalized Indo-1 ratios measured in isolated cardiac myocytes (PLADP/GFP: $n=72$; GFP: $n=51$). Peak systolic calcium was increased and the calcium transient significantly shortened by the PLADP. C. Myocyte shortening and myocyte 50% relaxation time measured in isolated cardiac myocytes (PLADP/GFP: $n=50$; GFP: $n=43$). Shortening in myocytes transfected was significantly increased and myocyte 50% relaxation time significantly shortened with PLADP compared to control myocytes (**$p<0.01$).

control (Fig. 6B, left panel). The time to the maximum of the calcium transient was not affected by the gene transfer, but the calcium decay was significantly accelerated compared to the control myocytes (Fig. 6B, right panel). The functional analysis showed that contractility in isolated myocytes co-transfected with the PLADP and GFP as well as the myocyte 50% relaxation time were significantly improved compared to myocytes transfected with GFP alone (Fig. 6C).

To test whether improvement of contractility observed at room temperature is preserved at 37 °C and physiological heart rates (400 bpm), maximum (dP/dt_{max}) and minimum (dP/dt_{min}) derivatives of left ventricular pressure and developed pressure (DP) were measured in isolated Lan-

gendorff-perfused hearts following in vivo gene therapy with the PLADP. Hearts were perfused with a HEPES buffered Krebs–Henseleit (pH 7.4) solution and function recorded at 37 °C followed by reduction of water bath temperature and recording of function at 25 °C. The HEPES buffered Krebs–Henseleit solution was bubbled continuously with 100% oxygen.

All parameters were significantly improved by the PLADP compared to control conditions both at 25 °C (dP/dt_{max}: 1174±114 vs. 774±56 mm Hg/s; dP/dt_{min}: 793±37 vs. 633±35 mm Hg/s; DP 35±6 vs. 20±4 mm Hg; PLADP vs. control) and 37 °C (dP/dt_{max}: 3209±100 vs. 2077±360 mm Hg/s; dP/dt_{min}: 2015±141 vs. 1172±167 mm Hg/s; DP 77±2 vs. 49±9 mm Hg; PLADP vs. control).

4. Discussion

The purpose of this study was to test the feasibility of modifying PLB–SERCA interaction in vivo using a newly developed chicken antibody-derived protein mimicking phosphorylation of phospholamban (PLADP) in the BIO 14.6 hamster, a well-characterized model for rapidly progressive dilated cardiomyopathy. We demonstrated that the hypothermia–cardioplegia gene transfer of the PLADP is feasible at a stage of established heart failure and could achieve sufficient efficiency. Furthermore, we demonstrated that expression of PLADP in cardiac myocytes results in improved cardiac function with enhanced contractility and relaxation in vivo and improved contractility in isolated cardiac myocytes from the BIO 14.6 hamster. Analysis of calcium transients revealed that peak systolic calcium concentration and the reuptake of calcium into the sarcoplasmic reticulum (SR) were enhanced.

4.1. Phospholamban and the regulation of contractility

Contraction of the heart is initiated by release of calcium into the cytoplasm from an internal storage pool in the SR through the ryanodine receptor and cardiac relaxation is initiated by muscle-specific SR calcium ATPase-2 (SERCA) that sequesters calcium back into the SR. Thus, the activity of SERCA affects both cardiac relaxation and SR calcium loading [26].

Phospholamban (PLB) plays a crucial role in the physiological regulation of SERCA and hence cardiac contractility. As an inhibitor of SERCA, PLB reduces diastolic calcium uptake into the SR and hence the amount of calcium available for the next contraction. Cyclic-AMP-mediated phosphorylation of PLB can relieve this inhibition of SERCA.

It has been shown that in models of cardiomyopathy the inhibitory effect of PLB on cardiac contractility can be exaggerated by a decrease in SERCA expression relative to PLB and a reduction in the phosphorylated state of PLB [8–10]. On the other hand, complete ablation of PLB, representing the most extreme form of PLB modification, enhances contractility of the heart under normal [6] and pathophysiological conditions [7]. Modifying the interaction of PLB and SERCA therefore appears to be an attractive therapeutic goal for the treatment of heart failure.

Several approaches to modify the PLB–SERCA interaction have been tested. These included gene transfer of SERCA2 in rats exhibiting heart failure after ascending aortic constriction [27,28], the use of antisense PLB–RNA [29,30] and overexpression of a mutant form of PLB [30] in isolated neonatal rat or adult rabbit cardiac myocytes.

The latter approach was recently questioned by the finding of an L39 stop mutation in PLB in patients with hereditary lethal dilated cardiomyopathy, representing a possible long-term adverse effect of complete functional PLB ablation [11]. Also, it was shown that heart failure due to excessive hypertrophy could not be prevented by complete PLB ablation even though dysfunction in isolated cardiomyocytes from the hearts of these transgenic animals was rescued [31]. Not only functional PLB ablation but also mutations of PLB may lead to human heart failure as was shown with a missense mutation at residue 9 of human PLB [12] and with other mutations towards the region of protein interaction with SERCA in transgenic mice [32].

However, two recent studies have shown that a long-term treatment using gene transfer of a mutant PLB in established heart failure is feasible, safe and efficient [13,14]. Thus, though inborn PLB mutations may be deleterious, targeting the PLB–SERCA interaction seems to be a promising approach to the treatment of heart failure.

4.2. Antibody against phospholamban: a new approach

In this study we applied a new approach to modify the PLB–SERCA interaction not involving PLB ablation or PLB mutants which has evolved from previous observations that antibodies directed against PLB enhance SR calcium uptake in isolated SR vesicles [10,15]. A major disadvantage of these antibodies is that they cannot be used therapeutically in vivo. To overcome this problem, a recombinant single chain antibody specifically targeting the cytoplasmic portion of PLB was engineered that can be expressed in the cardiac myocyte via an adenoviral vector delivery system. To test whether this approach is feasible and can be potentially used for the treatment of heart failure, we performed gene transfer studies in the BIO 14.6 hamster.

4.3. Gene transfer

Our results demonstrate that adenoviral gene transfer using the hypothermia–cardioplegia method [18] at a progressed stage of heart failure is feasible with low mortality and no signs of inflammatory response. We achieved a transfection efficiency of greater than 50%. This expression rate is lower than that observed in an initial study exploring this method [18], most likely due to the

progressive cardiac fibrosis observed in the BIO 14.6 hamster at older ages [33].

4.4. Improvement of cardiac function with the PLADP

The most important finding of our study was a significant improvement of left ventricular (LV) function by gene transfer with the PLADP while no significant changes were found in the LacZ and Sham group. Echocardiographic LV fractional shortening and velocity of circumferential fiber shortening observed after PLADP gene transfer were similar to values observed in 10-week-old animals [16], indicating a substantial reversal of the progression of heart failure in these animals. The echocardiographic findings were further supported by the in vivo hemodynamic data indicating improved LV contractility and relaxation and the finding of an increased shortening in cardiac myocytes isolated from BIO 14.6 hamster hearts transfected with the PLADP. Increased peak systolic intracellular calcium concentration as well as a higher rate of calcium reuptake into the SR indicate an improved intracellular calcium handling as the reason for improved contractility in the PLADP-treated animals. Together with findings from in vitro fluorescence resonance energy transfer (FRET) experiments with the PLADP published recently [34], we conclude that the improvement of contractility by the PLADP is due to a reduced inhibitory effect of PLB on SERCA2a, leading to increased calcium uptake into the SR, higher SR calcium loading and enhanced SR calcium release. Although we cannot completely exclude interactions of the PLADP with other proteins, the functional data together with unchanged levels of PLB and SERCA point to a conformational change of PLB by the PLADP with reduced inhibitory effect on SERCA as the main reason for improvement of intracellular calcium handling and myocyte contractility.

In summary, we have achieved efficient gene transfer using a hypothermia–cardioplegia approach in established heart failure with acceptable mortality. We have also shown a beneficial effect on cardiac function by modifying the inhibitory effect of PLB on SERCA through expression of a recombinant antibody against PLB in cardiomyocytes of the BIO 14.6 hamster. To our knowledge, the PLADP represents the first example of intracellular expression of an antibody-derived protein in cardiac myocytes in vivo. Our results demonstrate the feasibility of targeting intracellular proteins in cardiac myocytes to study and alter cardiac function, which may provide a useful experimental tool. Longer-term studies will be needed to assess the potential of this approach as a future therapy to improve cardiac function in heart failure.

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