Original Article

Right Ventricular Beneficial Effects of Intracoronary *SERCA*$_{2a}$ Gene Transfer in an Experimental Model of Heart Failure

(heart failure / pharmacogenomics / calcium metabolism)

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Abstract. *SERCA*$_{2a}$ gene transfer ameliorates heart failure pathologic processes in left ventricular myocardium. We sought to assess the simultaneous molecular changes that occur in the right ventricle. Sprague-Dawley rats underwent aortic banding and were followed by echocardiography for development of heart failure. After a decrease in fractional shortening of 25 % from baseline, intracoronary injection of *adenoviral-SERCA*$_{2a}$ or *adenoviral-β-galactosidase* was performed. Successful gene transfer was confirmed by immunoblotting. Rats were randomly euthanized on post-operative day 7 or 21. Protein analysis including right ventricular levels of SERCA$_{2a}$, βARK1, inflammatory mediators (IL-1, IL-6 and TNF-α), apoptotic markers (Bax, Bak and Bcl-2) and MAPK (Jnk, p38 and Erk) was performed. *Adenoviral-SERCA*$_{2a}$-treated animals showed increased right ventricular expression of SERCA$_{2a}$ compared with controls. Decreased levels of inflammatory markers were also demonstrated in this group. Expression of pro-apoptotic markers was similarly improved. Levels of MAPK were increased compared with the control group. These differences were most significant 7 days after gene transfer, but the majority of these changes persisted at 21 days. These results suggest that attenuation of pathologic mechanisms of calcium cycling, inflammation and apoptosis also occur in the right ventricular myocardium after *SERCA*$_{2a}$ gene transfer during heart failure. These findings support a therapeutic role for genetic manipulation of this pathway in patients with right ventricular or biventricular failure.

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Abbreviations: ANOVA – analysis of variance, βARK1 – beta-adrenergic receptor kinase, CMV – cytomegalovirus, FS – fractional shortening, MAPK – mitogen-activated protein kinase, SERCA$_{2a}$ – SR Ca^{2+} ATPase, SR – sarcoplasmic reticulum.

Introduction

Profound alterations in calcium cycling pathways have been described in the setting of heart failure (Gwathmey et al., 1987). Trabeculae and isolated cardiac cells from failing hearts have three characteristic functional abnormalities: an increase in diastolic Ca^{2+}, an increase in the time course of Ca^{2+} transients, and a decrease in sarcoplasmic reticulum (SR) Ca^{2+} release (Morgan et al., 1990; Davies et al., 1995; Gwathmey et al., 1995). Because SR plays a central role in controlling Ca^{2+} movements in myocardial cells during excitation-contraction coupling, numerous studies have been performed examining the expression and function of the SR Ca^{2+} ATPase (SERCA$_{2a}$) (Arai et al., 1994; Cheng et al., 1996; Yue, 1997). A reduction in the ATPase activity and Ca^{2+} uptake has been documented in many models of heart failure, including the failing human ventricle (Schwartz and Mercadier, 1996). This reduction in activity is generally associated with reductions in SERCA$_{2a}$ mRNA and protein. These results suggest that abnormal Ca^{2+} handling in failing hearts is caused in part by a decrease in *SERCA*$_{2a}$ gene expression and enzyme activity (Miyamoto et al., 2000).

Several recent studies have shown that increased SR Ca^{2+} ATPase expression improves Ca^{2+} cycling and left ventricular myocardial function. Transgenic mice overexpressing *SERCA*$_{2a}$ showed improved cardiac function and Ca^{2+} handling (He et al., 1997; Baker et al., 1998). In neonatal rat cardiomyocytes with normal and depressed *SERCA*$_{2a}$ expression, adenovirus-mediated transfer of *SERCA*$_{2a}$ resulted in enhanced SR Ca^{2+} uptake and accelerated decay of Ca^{2+} transients (Giordano et al., 1997; Hajjar et al., 1997). Furthermore, catheter-based transfection with adenovirus encoding *SERCA*$_{2a}$ restored left ventricular cardiac function in rats in transition to heart failure (Miyamoto et al., 2000; del Monte et al., 2001). We have recently replicated these findings in our laboratory in an experimental rat model of pressure

overload heart failure (Gupta et al., 2008). In human cardiomyocytes isolated from end-stage failing hearts, adenovirus-mediated augmented expression of *SERCA$_{2a}$* resulted in improved contractility and calcium handling (del Monte et al., 1999).

However, most of these research studies were focused on changes occurring in the left ventricular myocardium. The concomitant molecular changes occurring in the right ventricle in this experimental model are unknown. We have recently characterized this animal model of pressure overload hypertrophy (Molina et al., 2009a). Following aortic constriction, transition from compensated to decompensated left ventricular hypertrophy occurred between 22 and 26 weeks in most animals. The transition to heart failure was associated with markedly decreased left ventricular systolic and diastolic function, echocardiographic signs of left ventricular remodelling, impaired exercise capacity, up-regulation in profiles of systemic and local inflammation and volume overload, and abnormalities in pathways of calcium metabolism (decreased expression of *SERCA$_{2a}$*) and beta-adrenergic receptor signalling. Concomitant changes occurring in the right ventricle are less well understood. However, we have recently shown that progression of left ventricular failure in this experimental model is also associated with worsening right ventricular systolic and diastolic function, and increased expression of markers of local inflammation, apoptosis and extracellular matrix remodelling (Molina et al, 2009b).

In the present study, we analysed the effects of intracoronary adenoviral gene transfer of *SERCA$_{2a}$* upon the right ventricular myocardium in this animal model of pressure overload hypertrophy with transition to heart failure. We specifically investigated changes related to expression of SERCA$_{2a}$, beta-adrenergic receptor kinase 1 (βARK1), inflammatory mediators, apoptosis and activation of mitogen-activated protein kinases (MAPKs). Our working hypothesis was that intracoronary delivery of *adenoviral-SERCA$_{2a}$* in this experimental model of heart failure is associated with right ventricular beneficial effects as previously demonstrated for the left ventricular myocardium.

Material and Methods

All animals received humane care in compliance with institutional guidelines and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, D.C., 1996). The study was conducted in accordance with the animal care and use guidelines of the Institutional Animal Care and Use Committee (IACUC) of Temple University.

Experimental Model for Heart Failure

Induction of Heart Failure. Following appropriate acclimatization, male Sprague-Dawley rats (150–200 g, Harlan, Indianapolis, IN) were anaesthetized with pentobarbital (50 mg/kg, intraperitoneal), endotracheally intubated with a 16-Gauge Insyte® Autoguard intravenous catheter (BD Medical Systems, Sandy, UT), and mechanically ventilated (Harvard rodent ventilator model # 683, tidal volume 2 ml, 90 breaths per min, 21% oxygen, positive end-expiratory pressure of 3 mmHg). Following shaving and sterile prep and drape of the chest, an upper median sternotomy was performed. With cephalad retraction of the thymus gland, the aortic arch was visualized and a small titanium clip with an internal diameter of 0.6 mm (Ligaclip® Extra, Ethicon Endo-Surgery, Inc., Cincinnati, OH) was applied across the aorta proximal to the right brachiocephalic artery. The sternum and subcutaneous layers were closed with 4-0 chromic gut suture (Ethicon, Inc., Somerville, NJ). Ventilation was then gradually weaned. A dose of buprenorphine (0.1–0.5 mg/kg, subcutaneously) was administered at the time of closure. Animals were observed every 15 min until they recovered from anaesthesia. After recovery, animals were returned to individual cages. Animals were monitored hourly for four hours post-operatively. Twelve hours after surgery, the animals were reassessed to determine their level of discomfort by watching for lethargy or decrease in activity. Animals received additional buprenorphine (0.1–0.5 mg/kg, subcutaneously) every 12 h if needed.

Follow-up and Randomization

While lightly anaesthetized (0.2 l/min of oxygen/isoflurane mixture via facial mask), all animals underwent baseline echocardiography 15 weeks after clip placement. Transthoracic M-mode and 2D echocardiography was performed with a HP Sonos 5500 Imaging System (Hewlett-Packard, Palo Alto, CA) using a 4 MHz transducer. Images were standardized to the mid-papillary short axis view of the left ventricle and fractional shortening (FS) was recorded. Starting at 15 weeks after aortic clip placement, echocardiography was performed weekly. Following detection of an absolute decline in left ventricular FS of at least 25 % compared to baseline (relative 50% reduction), animals were randomized to one of two groups: *SERCA$_{2a}$* gene delivery (*adenoviral-SERCA$_{2a}$*, N = 8) or *β-galactosidase* gene delivery (*adenoviral-βGal* or control, N = 10). Within these treatment groups, animals were randomly assigned to be euthanized on day 7 or 21 following gene delivery.

Adenovirus Replication

Replication-deficient first-generation (E1, E3 deleted) adenoviral vectors containing the *SERCA$_{2a}$* transgene driven by the cytomegalovirus (CMV) promoter were kindly donated by the laboratory of Dr. S. M. Periyasamy (Medical College of Ohio, Toledo, OH). Replication-deficient first-generation (E1, E3 deleted) adenoviral vectors containing the *β-galactosidase* gene driven by the CMV promoter were graciously donated by the laboratory of Dr. Walter J. Koch (Thomas Jefferson University, Philadelphia, PA). Detailed methods for adenoviral propagation are described elsewhere (Croyle et al.,

1998). Briefly, adenoviral constructs were amplified in human embryonic kidney cells (293 cells). Ultrafiltration in 20 to 80% sucrose gradients was then performed for viral purification. Viral concentration was determined by ultraviolet spectrophotometric analysis at 260 nm. Solutions were subsequently diluted to 1×10^{10} particles/ml, and stored in a -80 °C freezer.

Gene Delivery

Within 48 h of detection of > 25% decline in FS from baseline and subsequent randomization, animals were anaesthetized as described above and a full median sternotomy was performed. The pericardium was opened and a 5-0 prolene (Ethicon, Inc., Somerville, NJ) retraction suture was placed at the left ventricular apex. The aorta and main pulmonary artery were together encircled with a 4-0 prolene (Ethicon) and a tourniquet was loosely applied. Two hundred µl of adenoviral solution (see above) was pre-warmed to 37 °C and mixed with 50 µl of adenosine (3 mg/ml, Fujisawa Healthcare, Inc., Deerfield, IL) just prior to injection. A sterile 20-gauge 1.16-inch intravenous catheter (BD Insyte® Autoguard, BD Medical Systems, Sandy, UT) was advanced from the left ventricular apex to the aortic root. Immediately following tightening of the tourniquet and occlusion of the aorta and pulmonary artery, the solution was injected. Coronary delivery was confirmed by observation of temporary bradycardia and epicardial blanching. Total time of aortic and pulmonary artery occlusion was 20 s. Following injection, recovery of the baseline heart rate and haemostasis, the sternum and subcutaneous layers were closed with 4-0 chromic gut suture (Ethicon). Ventilation was gradually weaned, and animals were transferred back to their cages after recovery. Postoperative analgesia was administered as described above.

Protein Assay

Protein analysis including right ventricular myocardial levels of $SERCA_{2a}$, βARK1**,** inflammatory tissue mediators (IL-1, IL-6 and TNF-α), apoptotic markers (Bax, Bak and Bcl-2), and MAPKs (Jnk, Erk and p38) was performed. We adhered to standard protein assay techniques, which have been published previously (Morgan, 1991; Bristow, 2000). Homogenization of tissue occurred in 1× PBS lysis buffer containing 2% SDS (Fisher), 1% Igepal CA-630 (Sigma Chemical Co., St. Louis, MO), 0.5% deoxycholate (Sigma), proteinase inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 5 µg/ml pepstatin A, 1 mmol/l phenylmethylsulfonyl fluoride, and 10 µg/ml calpain inhibitor I and II), and 5 mmol/l EDTA (Kubo et al., 2001). Total protein content was obtained by assaying the supernatant mixed with SDS sample buffer (2% SDS), 1 mmol/l Tris-Cl (pH 6.8), 100 mmol/l DTT, 20% glycerol, and 0.02% bromophenol blue) and stored at -70 °C (Jayasankar et al., 2003).

Western Blot Analysis

Each sample underwent electrophoresis on a 15% SDS-polyacrylamide gel after 30–60 min of denaturation at 37 °C. Proteins were then transferred to Immobilon-P (Millipore, Billerica, MA) by use of wet transfer apparatus. To prevent non-specific binding, the membrane was blocked using 5% non-fat dry milk in Tris-buffered saline (0.1% Tween 20, 137 mmol/l NaCl, 2.7 mmol/l KCl, and 25 mmol/l Tris base, pH 7.6). Antigens of interest were probed with the following polyclonal antibodies: β-Gal, $SERCA_{2a}$, βARK1, IL-1, IL-6, TNF-α, Bax, Bak, Bcl-2, Jnk, Erk, and p38 (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed by enhanced chemiluminescence assay (NEN Life Science, Boston, MA). The Immobilon-P membrane was stained (coomassie blue), and the actin band was identified, thus confirming equal loading conditions. Films were scanned (Epson Expression 636, Epson, Long Beach, CA), and band intensities were quantified with densitometric analysis by the use of NIH Image 1.62f program. All targeted bands were normalized to cardiac actin.

Statistical Analysis

All data are presented as mean ± standard deviation. Between-group differences in frequency data were analysed using Fisher's exact test. Differences in continuous variables were analysed using independent *t*-tests. Analysis of variance (ANOVA) with the least significant difference (LSD) procedure was used. After ANOVA, a Bonferroni post-hoc analysis was used for adjustment for multiple comparisons**.** The SAS v8.1 (SAS Institute Inc, Cary, NC) was used for statistical analysis. A P value of 0.05 or less was considered to be statistically significant.

Results

Most animals underwent transgene injection between 26 and 28 weeks following aortic banding. Adequate gene delivery was confirmed by right ventricular β-Gal immunoblotting. Fig. 1 shows comparable levels of β-galactosidase protein expression between right and left ventricles at 7 and 21 days following gene transfer of adenoviral-βGal.

$SERCA_{2a}$ protein levels are shown in Fig. 2. As heart failure progressed after gene delivery, $SERCA_{2a}$ levels decreased from day 7 to day 21 in both groups. However, $SERCA_{2a}$ levels were significantly higher in the *adenoviral-$SERCA_{2a}$*-treated group compared with the control group at each time point.

βARK1 levels were found to increase as heart failure progressed in both groups. However, significantly lower levels were demonstrated in the *adenoviral-$SERCA_{2a}$*-treated group at 21 days after gene delivery compared with the control group (1.673 ± 0.024 vs. 1.792 ± 0.039, $P < 0.05$).

Tissue levels of inflammatory markers are shown in Table 1 (IL-1 and IL-6) and Fig. 3 (TNF-α). As heart failure progressed, both groups demonstrated increased levels of markers of tissue inflammation. However, ani-

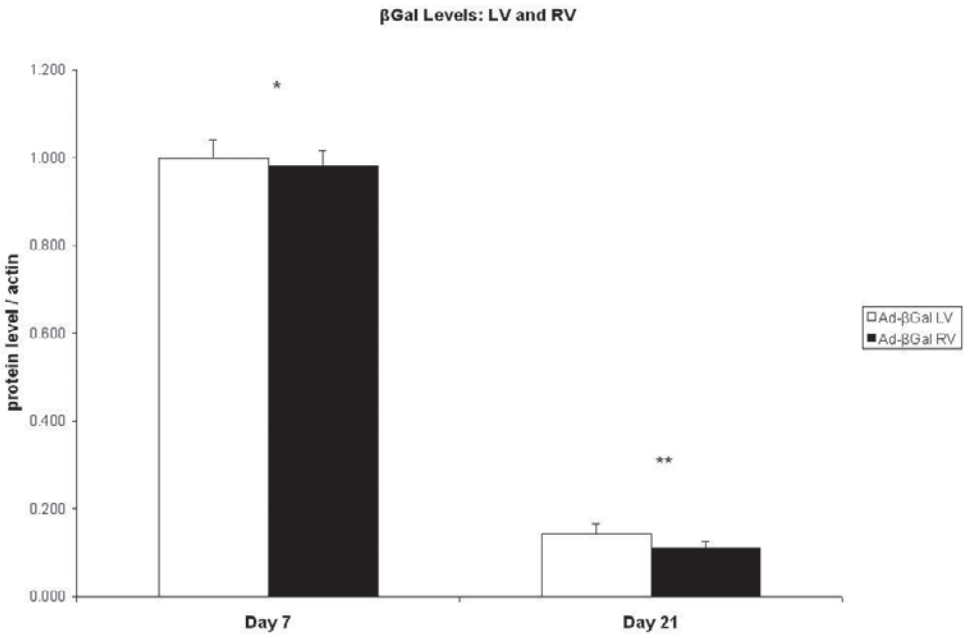

Fig. 1. Immunoblotting revealing no significant differences in the expression of β-galactosidase between left and right ventricles at 7 and 21 days after intracoronary delivery of *adenoviral-βGal* (* P = 0.42, ** P = 0.27)

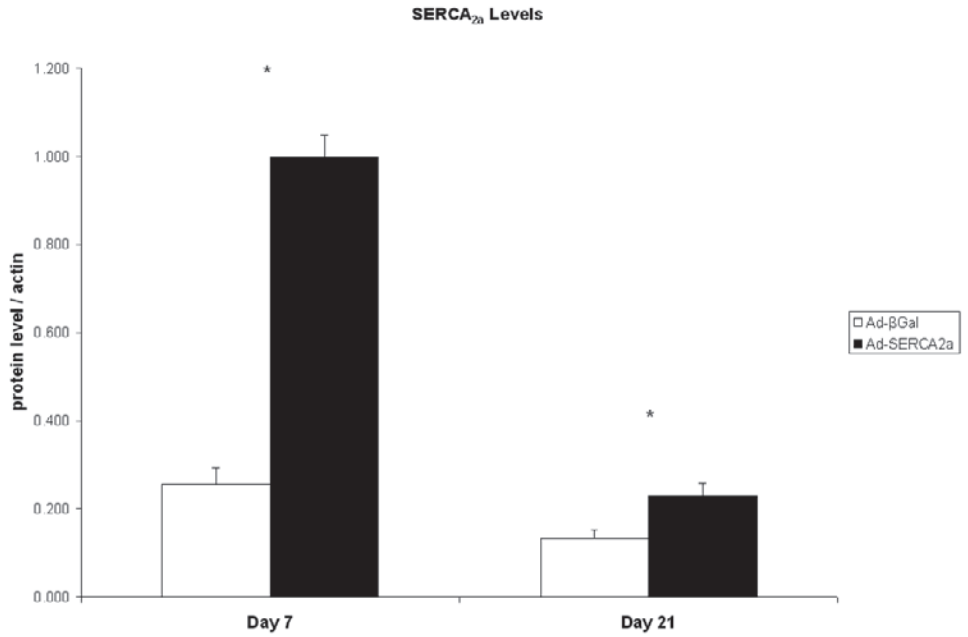

Fig. 2. Right ventricular levels of $SERCA_{2a}$ after *adenoviral-βGal* vs. *aden oviral-SERCA*$_{2a}$ gene transfer (* P < 0.05)

mals treated with *adenoviral-SERCA*$_{2a}$ had significantly decreased levels of these markers compared with *adenoviral-βGal*-treated animals. These differences were present on days 7 and 21 following treatment.

The pro-apoptotic marker Bax was found to be significantly decreased at 21 days in the *adenoviral-SERCA*$_{2a}$ group (Fig. 4). Bak, another pro-apoptotic marker, and the anti-apoptotic factor Bcl-2 were not found to be significantly different at any time point (Table 1). However, there was a trend towards decreased Bak levels in the *adenoviral-SERCA*$_{2a}$ group.

Figure 5 shows representative western blot images of β-galactosidase, $SERCA_{2a}$, TNF-α, and Bax right ventricular expression 7 days following gene delivery.

Among the MAP kinases, Jnk levels were found to be significantly higher in the *adenoviral-SERCA*$_{2a}$ group. Erk and p38 were also found to be increased in the *adenoviral-SERCA*$_{2a}$ group compared with controls on

Table 1. Right ventricular levels of inflammatory markers, apoptotic factors, and MAP kinases following intracoronary gene transfer

MARKER (level/actin)	Ad-βGal Day 7	Ad-SERCA$_{2a}$ Day 7	Ad-βGal Day 21	Ad-SERCA$_{2a}$ Day 21
IL-1	1.201 ± 0.044	1.026 ± 0.030 *	1.510 ± 0.051	1.378 ± 0.039*
IL-6	1.193 ± 0.039	1.110 ± 0.121 *	1.603 ± 0.052	1.454 ± 0.066 *
Bak	0.789 ± 0.022	0.731 ± 0.080	1.008 ± 0.041	0.958 ± 0.044
Bcl-2	1.244 ± 0.021	1.168 ± 0.042	1.443 ± 0.101	1418 ± 0.077
Jnk	0.401 ± 0.034	0.824 ± 0.090 *	0.225 ± 0.022	0.377 ± 0.017 *
Erk	0.921 ± 0.043	1.102 ± 0.170 *	0.960 ± 0.031	0.974 ± 0.041
p38	0.456 ± 0.019	0.535 ± 0.053 *	0.499 ± 0.015	0.507 ± 0.032

* P < 0.05 for comparison of Ad-SERCA$_{2a}$ vs. Ad-βGal

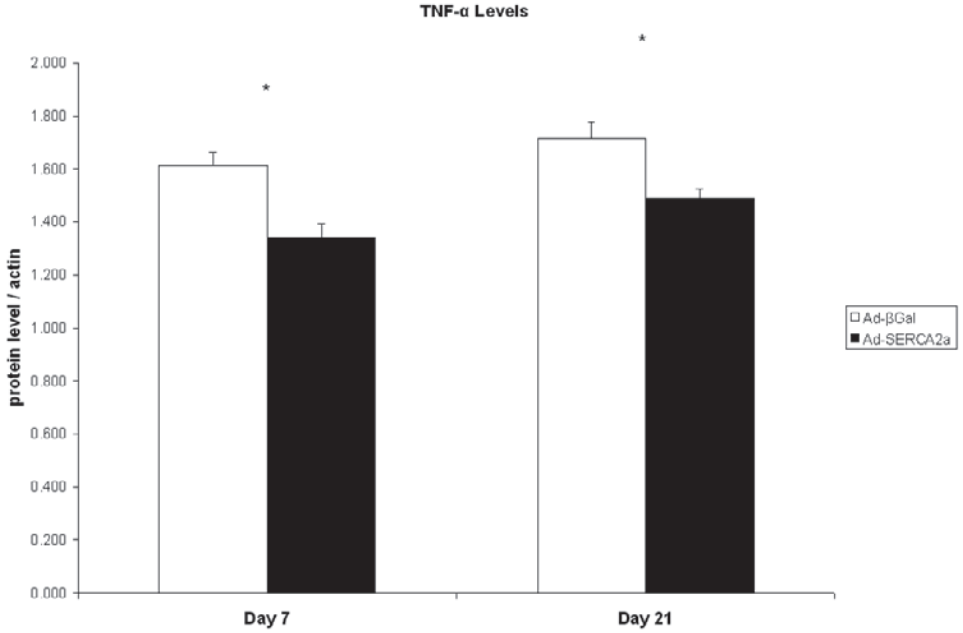

Fig. 3. Right ventricular levels of TNF-α after *adenoviral-βGal* vs. *adenoviral-SERCA$_{2a}$* gene transfer (* P < 0.05)

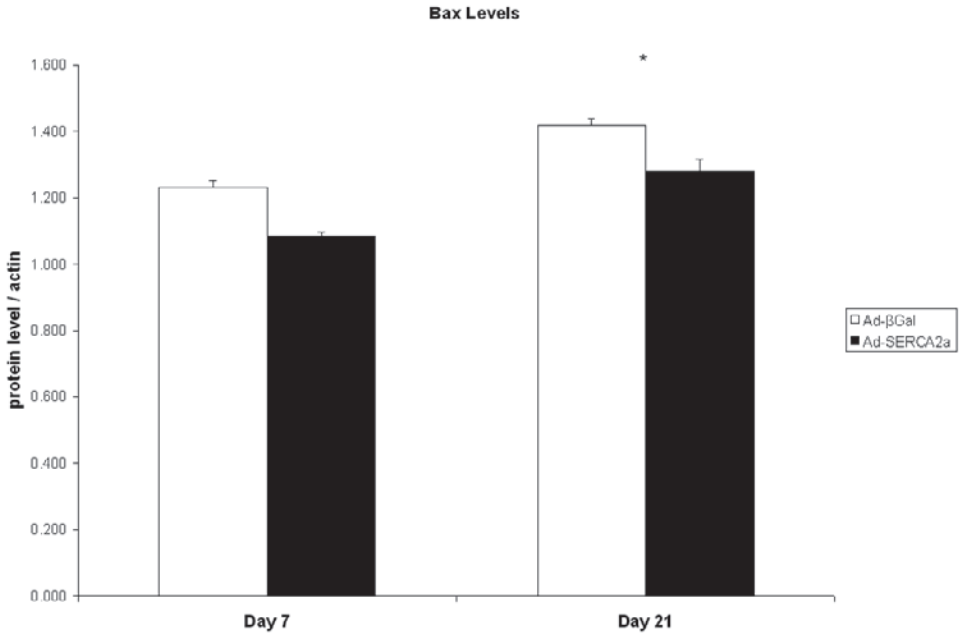

Fig. 4. Right ventricular levels of the pro-apoptotic factor Bax after *adenoviral-βGal* vs. *adenoviral-SERCA$_{2a}$* gene transfer (* P < 0.05)

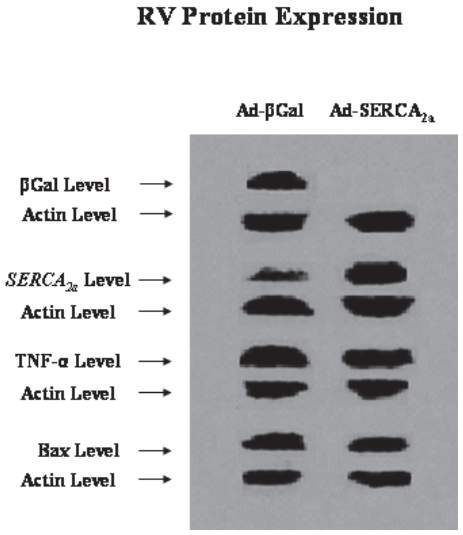

Fig. 5. Representative western blot images of β-galactosidase, $SERCA_{2a}$, TNF-α, and Bax right ventricular expression 7 days following gene delivery

days 7 and 21 after gene delivery, but these changes reached statistical significance only on day 7 (Table 1).

Discussion

In the present study, we observed that some of the pathologic mechanisms of left ventricular failure in this model of pressure overload hypertrophy also occurred in the right ventricle: decreasing levels of $SERCA_{2a}$, increasing expression of the beta-adrenergic receptor kinase, and rising levels of inflammatory makers and pro-apoptotic factors. *Adenoviral-$SERCA_{2a}$* gene transfer was associated with an amelioration of some of these pathological mechanisms.

Effective right ventricular adenoviral-mediated gene transfer in this study was confirmed by analysis of tissue levels of this enzyme by western blot. We observed comparable expression levels between left and right ventricular myocardium after intracoronary delivery. This adenoviral technique has been found to be a reliable strategy for gene transfer in previous studies (Miyamoto et al., 2000, del Monte et al., 2001, 2002).

Animals receiving *adenoviral-$SERCA_{2a}$* showed markedly increased expression of $SERCA_{2a}$. We found a 5-fold increase in $SERCA_{2a}$ expression at 7 days. This represents a higher increment than previously reported for left ventricular myocardium (Miyamoto et al., 2000). This effect persisted at 21 days, although at a much lower magnitude. Animals receiving *adenoviral-$SERCA_{2a}$* also exhibited significantly lower levels of βARK1. This finding suggests decreased homologous desensitization of the beta-adrenergic receptor system after treatment in this model of heart failure.

It has been well demonstrated that congestive heart failure is associated with increased levels of circulating inflammatory cytokines (Levine et al., 1990; Matsumori et al., 1994; Aukrust et al., 1999). In this model of heart failure induced by aortic banding, we found increasing right ventricular levels of IL-1, IL-6, and TNF-α – all potent pro-inflammatory agents. After intracoronary delivery of *adenoviral-$SERCA_{2a}$*, however, we observed an amelioration of right ventricular tissue levels of these mediators. These beneficial effects were present at 7 and 21 days following gene delivery, and suggest an improvement of the inflammatory profile that has been associated with progression to uncompensated heart failure.

Several studies have suggested that apoptosis may contribute to the decline in ventricular function and progression of heart failure in ischaemic and idiopathic cardiomyopathy (Kang and Izumo, 2000; Latif et al., 2000). In this study, we found improvements in right ventricular myocardial levels of the pro-apoptotic factor Bax in animals receiving *adenoviral-$SERCA_{2a}$* compared with animals receiving *adenoviral-βGal*. These findings may be associated with a decreased rate of cardiomyocyte loss. Moreover, increased levels of TNF-α is one of the most important stimuli associated with apoptosis (Feuerstein and Young, 2000; Latif et al., 2000). Interestingly, TNF-α levels were found to be significantly decreased in the *adenoviral-$SERCA_{2a}$* treatment group compared with controls, as discussed above.

MAP kinases play a significant role in the intracellular signal transduction. Jnk, Erk and p38 have been demonstrated to participate in the regulation of cell growth and cell death in the myocardium. In a similar model of severe pressure overload in rats, a previous study has shown increased levels of Jnk, Erk and p38 at the time of transition to heart failure (Sopontammarak et al., 2005). Furthermore, Jnk, Erk and p38 have been found to be up-regulated in patients with ischaemic and dilated cardiomyopathy (Cook et al., 1999). In this study, we have found decreased levels of Jnk and relatively stable levels of Erk and p38 as heart failure progressed in both groups. Interestingly, animals treated with *adenoviral-$SERCA_{2a}$* exhibited a significant rise in Jnk, Erk and p38 expression at 7 days. This finding persisted at 21 days for Jnk only. There is evidence that suggests that a stage-specific differential activation of MAP kinases exists in this model of heart failure (Hayashida et al., 2001). It is difficult to draw conclusions from our results until the regulatory mechanisms of MAP kinase expression are better characterized.

This study has several important limitations. First, the degree of right ventricular dysfunction in this model of left ventricular pressure overload has not been well characterized. Although we have recently shown progressive worsening of systolic and diastolic right ventricular function (Molina et al., 2009b), this experimental animal model primarily represents a left ventricular failure model. Furthermore, it is difficult to differentiate improvements in right ventricular function and gene profiling determined by right ventricular $SERCA_{2a}$ overexpression from secondary changes due to improvement of left ventricular function and diminished phenotypic severity. Second, we have not obtained right ventricular

haemodynamic data and geometric data in order to demonstrate improved right ventricular function and remodelling after SERCA$_{2a}$ modulation. Third, we have not included an untreated control group, which would help to determine whether gene expression is influenced by the reactive inflammatory changes that follow adenoviral infection.

In summary, our findings suggest that adenoviral vector-mediated genetic modulation using *SERCA$_{2a}$* ameliorated several right ventricular pathophysiological mechanisms of heart failure including decreased SERCA$_{2a}$ protein levels, increased expression of βARK1, and abnormal profiles of inflammation and apoptosis. These beneficial effects were seen as early as 7 days following injection and lasted at least 21 days. These findings support a potential therapeutic role for genetic manipulation of these pathways in patients with right ventricular or biventricular failure.

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