GENETIC INHIBITION OF CALCINEURIN INDUCES DIASTOLIC DYSFUNCTION IN MICE WITH CHRONIC PRESSURE OVERLOAD

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Short title: Calcineurin inhibition and diastolic dysfunction

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ABSTRACT

Background*:** Calcineurin is a calcium/calmodulin-dependent protein phosphatase that induces myocardial growth in response to several physiological and pathological stimuli. Calcineurin inhibition, induced either via cyclosporine or genetically, can decrease myocardial hypertrophy secondary to pressure overload, without affecting left ventricular (LV) systolic function. Since hypertrophy can also affect LV diastolic function, the goal of this study was to examine the effects of chronic pressure overload (2 wks aortic banding) in transgenic (Tg) mice overexpressing Zaki-4β (TgZ), a specific endogenous inhibitor of calcineurin, on LV diastolic function. ***Methods and Results: As expected, in the TgZ mice with calcineurin inhibitor overexpression, aortic banding reduced the degree of LV hypertrophy, as assessed by LV/body weight (3.5±0.1) compared with non-Tg (NTg) mice (4.6±0.2). LV systolic function remained compensated in both groups with pressure overload. However, the LVED Stress/LVEDD ratio, an index of diastolic stiffness and T½ and isovolumic relaxation time, two indices of isovolumic relaxation, increased significantly more in TgZ mice with aortic banding. Protein levels of phosphorylated phospholamban (PS16), SERCA2a, phosphorylated ryanodine receptor and the Na^{+}/Ca^{2+} exchanger were also reduced significantly, $p<0.05$, in the banded TgZ mice. ***Conclusions***: As expected, genetic calcineurin inhibition inhibited the development of LV hypertrophy with chronic pressure overload, but also induced LV diastolic dysfunction, as reflected by both impaired isovolumic relaxation and increased myocardial stiffness. Thus, genetic calcineurin inhibition reveals a new mechanism regulating LV diastolic function.

Keywords: hypertrophy, Diastole, Hemodynamics

INTRODUCTION

Left ventricular (LV) hypertrophy (H) is the major compensatory mechanism in response to pressure or volume overload in the heart. There are few adverse effects of mild to moderate LVH, but more severe LVH is thought to be deleterious because of limitations in subendocardial coronary reserve (12, 13, 27) and alterations in LV function, particularly in isovolumic relaxation. Since the calcineurin pathway is thought to be a key mechanism mediating the development of cardiac hypertrophy, it is not surprising that several studies have suggested that inhibition of this pathway could be an important approach for clinical therapy (4, 14, 18, 20, 24). However the prior work in this field has not determined if this therapeutic approach might invoke the precise adverse effects the therapy is designed to correct, e.g., alterations in LV diastolic function.

Calcineurin is a Ca^{2+}/calmodulin activated serine/threonine phosphatase, that is activated by sustained elevations in intracellular Ca^{2+} and which plays a significant role in cardiac hypertrophy as a sensing molecule that links alterations with Ca^{2+} handling and the genetic program of hypertrophic growth (18). The hypertrophy process is initiated by dephosphorylation of transcription factors of the NFAT (nuclear factor of activated T cells) family. It is presumed that translocation of activated NFAT3 to the nuclei leads to genetic reprogramming and initiation of the hypertrophic transcriptional response. Cardiac specific overexpression of calcineurin in transgenic mice was shown to induce profound hypertrophy characterized by a 2 to 4-fold increase in the heart size, which rapidly progresses to dilated heart failure (23, 24, 28). On the other hand calcineurin inhibition blunted hypertrophy, further demonstrating involvement of calcineurin in this process. In this sense, several studies have shown that pharmacological (15,

24, 26) or genetic calcineurin inhibition (10, 20) can regress hypertrophy, without affecting LV systolic function. However, the extent to which inhibiting calcineurin affects LV diastolic function is not established, despite its importance in LVH (7), and its contribution to the development of cardiac failure (1).

Therefore, to address the effects of inhibition of LVH by calcineurin on LV diastolic function, it would be more desirable to inhibit the calcineurin pathway genetically. To this end, genetic inhibition of calcineurin was achieved using Tg mice that over-expressed ZAKI 4β (TgZ) (also designated as MCIP2 or DSCR1L1), a specific endogenous calcineurin inhibitor (17, 21). Accordingly, the objective of this study was to assess the effects of genetic inhibition of calcineurin on the development of LV hypertrophy, on LV systolic and LV diastolic function and on Ca^{2+} regulatory proteins in TgZ and NTg mice, with chronic pressure overload due to aortic stenosis. Diastolic function was assessed both on isovolumic relaxation and myocardial stiffness.

MATERIALS AND METHODS

Transgenic mice:

TgZ mice were generated in FVB background using the α-myosin heavy chain promoter (courtesy, Dr. J. Robbins, University of Cincinnati) to achieve cardiac specific expression.

Experimental Groups:

Four experimental groups were performed: non-Tg (NTg) and TgZ sham and NTg and TgZ with 2 weeks of transverse aortic banding. n=8-18 in each group. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School. The transverse thoracic aorta between the innominate artery and left common carotid artery was constricted using a 30-gauge needle and a 7-0 nylon suture with the aid of a dissecting microscope and under anesthesia (11, 22, 32-34). After removal of the needle, the aorta remained constricted. Aortic constriction was performed using a mixture of ip ketamine 0.065 mg/g, xylazine 0.013 mg/g, and acepromazine 0.002 mg/g for anesthesia.

Cardiac catheterization

2 weeks after aortic banding, closed-chest catheterization was performed. Two high-fidelity catheter tip transducers (1.4F, Millar) were used; one was inserted into the right carotid artery and carefully advanced to the LV, the other into the left femoral artery and abdominal aorta, respectively. The pressures in the LV and abdominal aorta were measured simultaneously to calculate the pressure gradient. LV+dP/dt was used as an isovolumic index of systolic function. After the hemodynamic study the mice were then killed and the heart and lungs were

dissected and weighed. Half of the LV tissue was frozen in liquid nitrogen and the other half was fixed in 10% formalin.

Echocardiography

Mice were anesthetized using 12 µl/g body weight of 2.5% filtered avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (VisualSonics Vevo770 Ultrasound Imaging System). A 13-MHz linear ultrasound transducer was used. We took 2D-guided motion mode measurements of LV internal diameter from more than 3 beats and averaged the measurements. LVEDD was measured at the time of the apparent maximal LVEDD while LVESD was measured at the time of the most anterior systolic excursion of the posterior wall. Ejection fraction (EF) was also calculated and used as an ejective index of systolic function.

Indices of Diastolic Function

Diastolic function was assessed by indices derived from the curve of left ventricular pressure and dimensions, and also using echocardiography - Doppler data.

End-diastolic LV global circumferential wall stress was calculated using a cylindrical model:

Stress= 1.36*[(LVSP* LVEDD)/2 LVEDWT)]

The LV end-diastolic stress/diameter ratio was assessed to measure diastolic stiffness.

T½, an isovolumic relaxation index was calculated from the left ventricular pressure curve.

The E wave, A wave, E wave/A wave ratio, and the duration of the isovolumic relaxation time (IVRT), were estimated by the Doppler-echo study.

Histology

In each group, hearts were separated and processed for histological analysis. They were cut and stained with hematoxylin and eosine and picrosirius red. The interstitial collagen concentration in the septum and left ventricle free wall was assessed in the slides stained with picrosirius red technique. Digital image software (Image Pro-Plus 5.0) bound to a Leitz light microscope was used, with a ×10 objective, counting a total of 50 fields per heart. The percentage of collagen in each assessed region was calculated by adding the areas corresponding to collagen, divided by the addition of all the areas corresponding to myocytes plus the areas of collagen tissue

Western Blot Analysis

Western blots were performed for phospholamban (PLB), phospho-PLB(Ser16) (PS16), SERCA2a, phospho-ryanodine receptor (Ser2808) (p-ryanodine receptor), Na^{+}/Ca^{2+} exchanger and protein phosphatase 1 (PP1). Other non contractile proteins like vinculin, paxillin, desmin, tallin and beta tubulin were also evaluated using total protein lysates prepared from homogenized LV of hearts. Membrane fractions were separated from total lysates by centrifugation at 100,000 g for 45 min. The membrane pellet was then suspended in extraction buffer. The proteins were separated on 8% SDS-PAGE, transferred to nitrocellulose and probed with antibody. Secondary antibody was goat anti-rabbit coupled to horseradish peroxidase. The blots were developed with ECL, scanned and band densities was measured and expressed as arbitrary units. PKA activity was determined by StressXpress® PKA Kinase Activity Assay kit.

NFAT assays

NFAT-luciferase reporter mice (Tg-NFAT-Luc) generated on an FVB background (30). Heart homogenates (10 μg) from NFAT-luciferase transgenic mice were lysed in Reporter lysis buffer

(Promega) and analyzed for luciferase activity. The luciferase activity was measured using the luciferase assay system (Promega) and OPTOCOMP I (MGM instruments).

Statistical analysis

All data are presented as mean ± SE. For statistical analysis of data from multiple groups, ANOVA was used and comparisons between groups were made using Student-Newman-Keuls test, $p<0.05$ was taken as a minimal level of significance.

RESULTS

After 2 weeks of aortic stenosis the LV - aortic pressure gradient was similar in the NTg mice and TgZ mice (97±5 vs 90±4 mmHg). However, the LV hypertrophy, LV weight/body weight ratio (mg/g), was greater in the NTg animals (4.6±0.2) as compared with TgZ (3.5±0.1) ($p<0.05$). Table 1 shows systolic function and hemodynamic data. LV + dP/dtmax and LVEF were not reduced in both banded groups, demonstrating preserved LV systolic function. LVEDD was not increased in either group.

Although LVEDD was similar in both groups, LV end diastolic pressure (LVEDP) rose significantly more, $p<0.05$, in the TgZ group with aortic banding than in the NTg group (10.1 ± 2.13 vs 3.7 ± 0.8 mmHg), resulting in increased LVED wall stress and increased myocardial stiffness, as reflected by the LVED stress/LVEDD ratio and the E/A wave ratio, a Doppler index of diastolic function (Figure 1). T½, an isovolumic relaxation index derived from the LV pressure curve, and IVRT, an isovolumic relaxation index derived from Doppler, both also consistently increased in the TgZ group with and without aortic banding (Figure 2). These data suggest that calcineurin genetic inhibition caused diastolic dysfunction that affected not only myocardial stiffness but isovolumic relaxation as well.

The total collagen did not change significantly with aortic banding in either group. The non contractile proteins vinculin, paxillin, desmin, tallin and beta tubulin were measured by western blotting and levels were not different in the two groups (data not shown). PLB phosphorylation, reflected by the ratio of PS16 to PLB, was decreased, $p<0.05$, in aortic banded TgZ mice. Protein levels for SERCA2a, the Na^{+}/Ca^{2+} exchanger and phosphorylated ryanodine receptor were also decreased in aortic banded TgZ mice (Figure 3). As noted in Table 2, protein

levels for SERCA2a, the ratio of PS16 to PLB and the Na^+/Ca^{2+} exchanger were also significantly reduced in sham operated TgZ mice. In addition, PP1 expression and protein kinase A (PKA) activity, which could be involved in PLB phosphorylation, were examined in the two groups. As shown in Figure 3, PP1 was increased significantly, $p<0.05$, in TgZ mice with aortic banding, but PKA activity showed no difference between the two groups.

Transcriptional activity of NFAT is one of the most reliable indicators of the calcineurin activity in the heart *in vivo* (30). In order to evaluate the effect of ZAKI-4β upon the calcineurin activity *in vivo* hearts, TgZ mice were crossed with Tg-NFAT-Luc mice, which harbor an NFAT-Luc reporter gene, and then the mice were subjected to aortic banding for 2 weeks. Aortic banding-induced increases in NFAT-Luc activity were significantly attenuated in TgZ-NFAT-Luc mice compared to Tg-NFAT-Luc mice, suggesting that calcineurin activity is significantly attenuated in the presence of ZAKI-4β (Figure 4).

DISCUSSION

In the present study we evaluated the effects of 2 weeks of chronic pressure overload induced by aortic banding in mice with transgenic inhibition of calcineurin on LVH, and systolic and diastolic function. Calcineurin is a major mechanism mediating the development of LVH (10, 15, 20, 23, 24, 26, 28) and inhibition of calcineurin has been shown to reduce LVH without affecting LV systolic function (10, 20). Our study confirmed these results, demonstrating significantly less hypertrophy in the banded TgZ mice without compromise of LV systolic function. Although LVH is one of the major causes of LV diastolic function leading to diastolic heart failure (1, 7), few studies have examined the role of calcineurin on LV diastolic function. Surprisingly, despite reduced hypertrophy, which should protect against diastolic dysfunction and the preservation of LV systolic function in the banded transgenic mice with inhibition of calcineurin, both components of LV diastolic function were compromised severely, i.e., relaxation was impaired and myocardial stiffness was significantly increased. The increase in myocardial stiffness was characterized by significant increases in LV end diastolic pressure, LV end-diastolic wall stress, without an increase in LV end-diastolic dimensions. Mitral inflow E/A ratio, an indirect index of stiffness, was also increased with banding in TgZ mice. The impairment of isovolumic relaxation was also measured by T ½ using a high fidelity micromanometer. The impaired diastolic function with pressure overload due to calcineurin inhibition has not been observed previously. Yamamoto et al. (31) found that both an angiotensin II type I receptor antagonist and calcineurin inhibition protected against the development of LV hypertrophy, but the angiotensin II type I receptor antagonist also reduced myocardial stiffness in hypertensive rats with LVH, whereas the calcineurin inhibitor did not exhibit this positive effect on LV diastolic function. Semeniuk, et al.

(23) found that inhibition of LVH with cyclosporine in mice with overexpressed calcineurin in the heart did not reverse either LV systolic or diastolic dysfunction. The lack of effect could have been due to the inability to inhibit all of the effects of the overexpressed calcineurin, since cyclosporine was administered later, or because of the toxic effects of cyclosporine (15).

It is conceivable that the impaired LV diastolic function observed after 2 weeks of banding in the TgZ group could reflect more rapid LV systolic dysfunction and development of heart failure with banding in this group. This was not likely since systolic LV function was almost identical in the two groups. Furthermore, we followed a subgroup of banded NTg and TgZ mice (n=4-5/group) for 4 weeks and found that LV systolic function declined similarly in the NTg and TgZ groups, e.g., LVEF (61±0.8 vs 61±1.2%). Thus, LV systolic function was maintained in both groups after 2 weeks aortic banding, and began to decline by similar amounts after 4 weeks aortic banding.

The sarcoplasmic reticulum (SR) includes SERCA2a (Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase), a Ca^{2+} ATPase. These proteins regulate Ca^{2+} dynamics, through Ca^{2+} release during contraction and Ca^{2+} sequestration during relaxation. During depolarization, a small amount of Ca^{2+} enters the cardiac cell through the L-type Ca^{2+} channels, which causes a localized increase in Ca^{2+} between the sarcolemma and the SR, which activates the Ca^{2+}-release channel or ryanodine receptors to release Ca^{2+} into the cytosol. This process is termed Ca^{2+}-induced- Ca^{2+}-release and is involved in excitation–contraction coupling. Relaxation is initiated by the uptake of Ca^{2+} into the sarcoplasmic reticulum by Ca^{2+}-ATPase and SERCA2a, which are under regulation of phospholamban, and to a lesser extent by the Na^{+}/Ca^{2+} exchanger.

Although it is known that calcineurin affects Ca^{2+} handling and regulation of SERCA and PLB (5, 16, 18, 19, 25), less is known how these proteins may regulate diastolic function with

inhibition of calcineurin, most likely as noted above. In the current investigation we found that the impairment in isovolumic relaxation was associated with a decrease in PLB phosphorylation, as well as in the SERCA2a, ryanodine receptor phosphorylation, and the Na^{+}/Ca^{2+} exchanger, which could be involved in the mechanism for the impaired relaxation. These findings were also detected in the TgZ sham operated mice, which are consistent with the diastolic dysfunction found even in the absence of aortic banding. Interestingly, the data from Chu et al. (2), demonstrating increased PLB phosphorylation and SERCA protein levels in mice overexpressing calcineurin activity, are consistent with our data demonstrating reduced PLB phosphorylation and SERCA protein levels with calcineurin inhibition. In the present study we also observed a reduction in ryanodine receptor phosphorylation and in the Na^{+}/Ca^{2+} exchanger protein levels. Thus, based on our data, we speculate that the regulation of diastolic Ca^{2+} by calcineurin may result from a complex interplay among the different proteins involved in controlling Ca^{2+} in cardiac cells. Previous studies also demonstrated that PP1 (6, 8, 9) and PKA (3, 29) are involved in PLB phosphorylation. Our data indicate that a reduction in PLB phosphorylation may be due to an upregulation of PP1, but PKA is not involved in PLB phosphorylation.

Although increased collagen can affect LV diastolic stiffness, we did not find an increase in collagen or other non-contractile proteins (data not shown) in the TgZ mice compared with NTg mice with aortic banding.

In summary, in the present investigation we have found that TgZ mice, which inhibit calcineurin, develop less hypertrophy with chronic pressure overload with preserved LV systolic function, but with a clear impairment of diastolic function as reflected by isovolumic relaxation impairment and increased myocardial stiffness. This suggests that the inhibition of calcineurin in hearts subjected to pressure overload could represent a new mechanism for diastolic dysfunction

276 resulting in impaired relaxation and increased myocardial stiffness, despite its concomitant
277 action to reduce LVH.
278

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FIGURE LEGENDS

Figure 1. LVED stress, LVED stress/LVEDD, and the E/A wave ratio, are shown in NTg and TgZ mice with and without (sham) aortic banding. In the transgenic group with aortic banding the 2 indices of LV diastolic function was impaired, whereas LV diastolic dimensions did not change, resulting in increases in LV diastolic stress and diastolic stiffness. *$p<0.05$ TgZ with aortic banding vs NTg with aortic banding.

Figure 2. T½ and IVRT are shown in NTg and TgZ mice with and without (sham) aortic banding. In the transgenic group with aortic banding the 2 indices show an impairment of LV isovolumic relaxation. *$p<0.05$ TgZ with aortic banding vs NTg with aortic banding.

Figure 3. The ratio of PS16 to PLB, SERCA2a, p-ryanodine receptor, and the Na^{+}/Ca^{2+} exchanger are shown. There is a decrease in all the Ca^{2+} handling proteins in the TgZ group with aortic banding. PP1 was increased significantly in the TgZ group with aortic banding. There was no difference in PKA activity between the two groups. *$p<0.05$ TgZ with aortic banding vs NTg with aortic banding; n=4-5/group

Figure 4. NFAT-Luciferase reporter mice (Tg-NFAT-Luc) were crossed with TgZ. Tg-NFAT-Luc and TgZ-NFAT-Luc (bigenic) mice were subjected to either sham or transverse aortic banding. The luciferase activity was measured from heart homogenates and normalized by the protein content. Values are mean ± SEM. n=4/group.

Table 1. LV Systolic Function and Hemodynamics

	NTg Sham	TgZ Sham	NTg Aortic Banded	TgZ Aortic Banded
LVEF (%)	70 ± 1.1	72 ± 0.7	69 ± 0.8	67 ± 0.9
LV dP/dt (mmHg/Sec)	7756 ± 443	8000 ± 328	9659 ± 714	8798 ± 518
LVSP (mmHg)	92±2.3	91±2.3	168±5.5†	163±3.9†
MAP (mmHg)	77 ± 2.0	76 ± 1.9	59 ± 3.4†	63 ± 1.9†
HR (b/min)	445 ± 16	470 ± 10	472 ± 20	493 ± 22
LVEDD (mm)	3.56±0.07	3.64±0.04	3.56±0.04†	3.58±0.04
LV/BW	2.9±0.0	2.7±0.1	4.6±0.2†	3.5±0.1*†
Lung weight /BW	5.2±0.2	5.9±0.1	7.8±0.4†	6.7±0.2

EF: Ejection fraction; MAP: mean arterial pressure; HR: heart rate; BW: body weight.
*$p<0.05$ TgZ Banded vs NTg Banded; $^{\dagger}p<0.05$ Aortic Banded vs. Sham. n=10-18

Table 2. Calcium handling proteins

	NTg Sham	TgZ Sham
PS16/PLB	2.2 ± 0.3	0.6 ± 0.1†
SERCA2a	9.0 ± 1.4	3.2 ± 0.6†
Phospho-ryanodine receptor	6.5 ± 1.2	4.1 ± 1.4
Na^+/Ca^{2+} exchanger	5.6 ± 0.6	2.4 ± 0.2†

Values are arbitrary units. N=5-6/group.
†$p<0.05$ vs Sham.

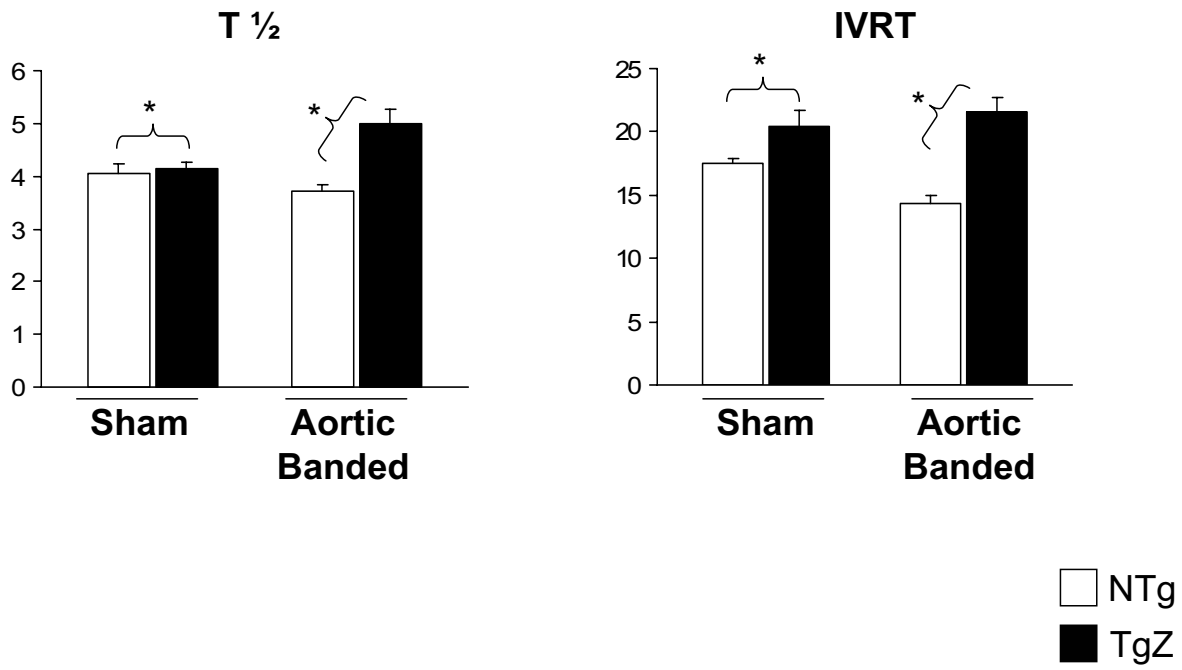
T ½
6
5
4
3
2
1
0
*
*
Sham
Aortic Banded
IVRT
25
20
15
10
5
0
*
*
Sham
Aortic Banded
NTg
TgZ

Figure 1

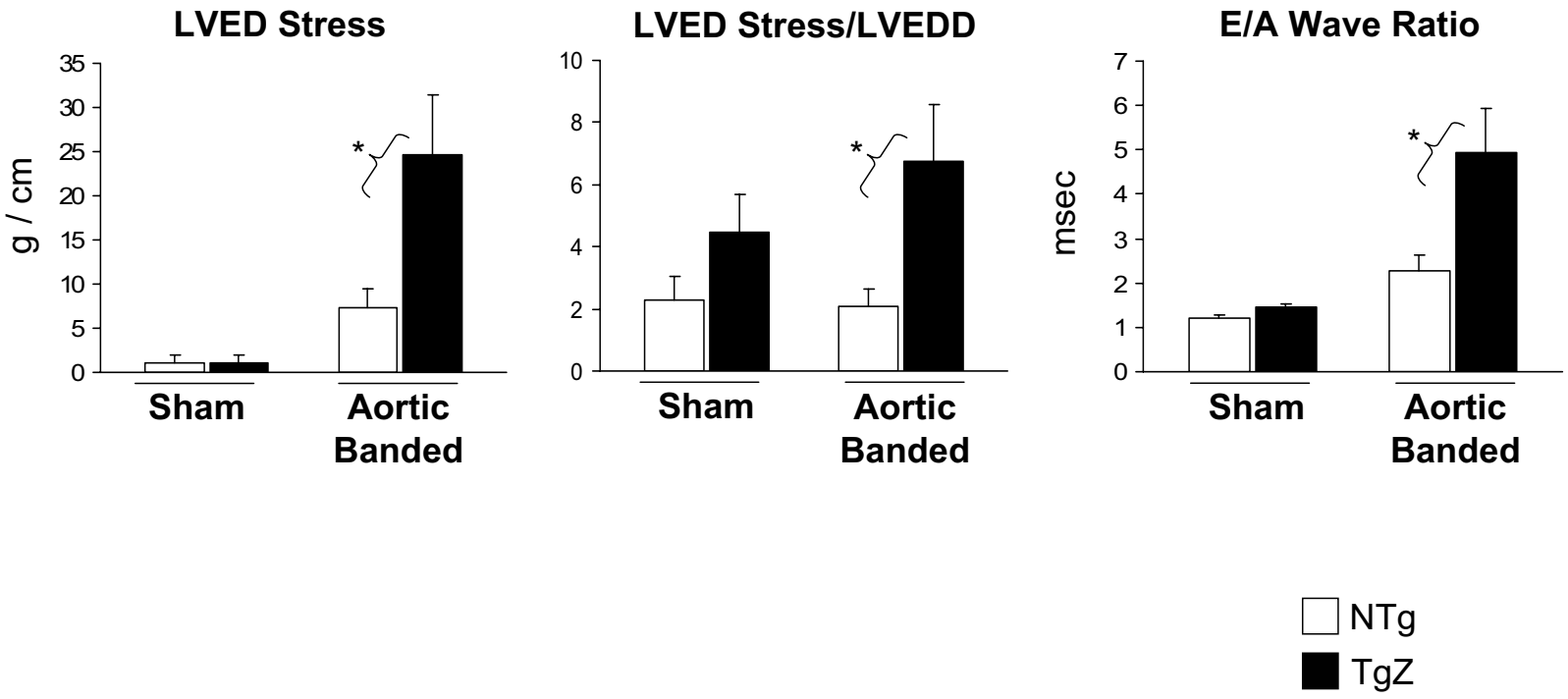
LVED Stress
g / cm
35
30
25
20
15
10
5
0
*
Sham
Aortic Banded
LVED Stress/LVEDD
10
8
6
4
2
0
*
Sham
Aortic Banded
E/A Wave Ratio
msec
7
6
5
4
3
2
1
0
*
Sham
Aortic Banded
NTg
TgZ

Figure 2

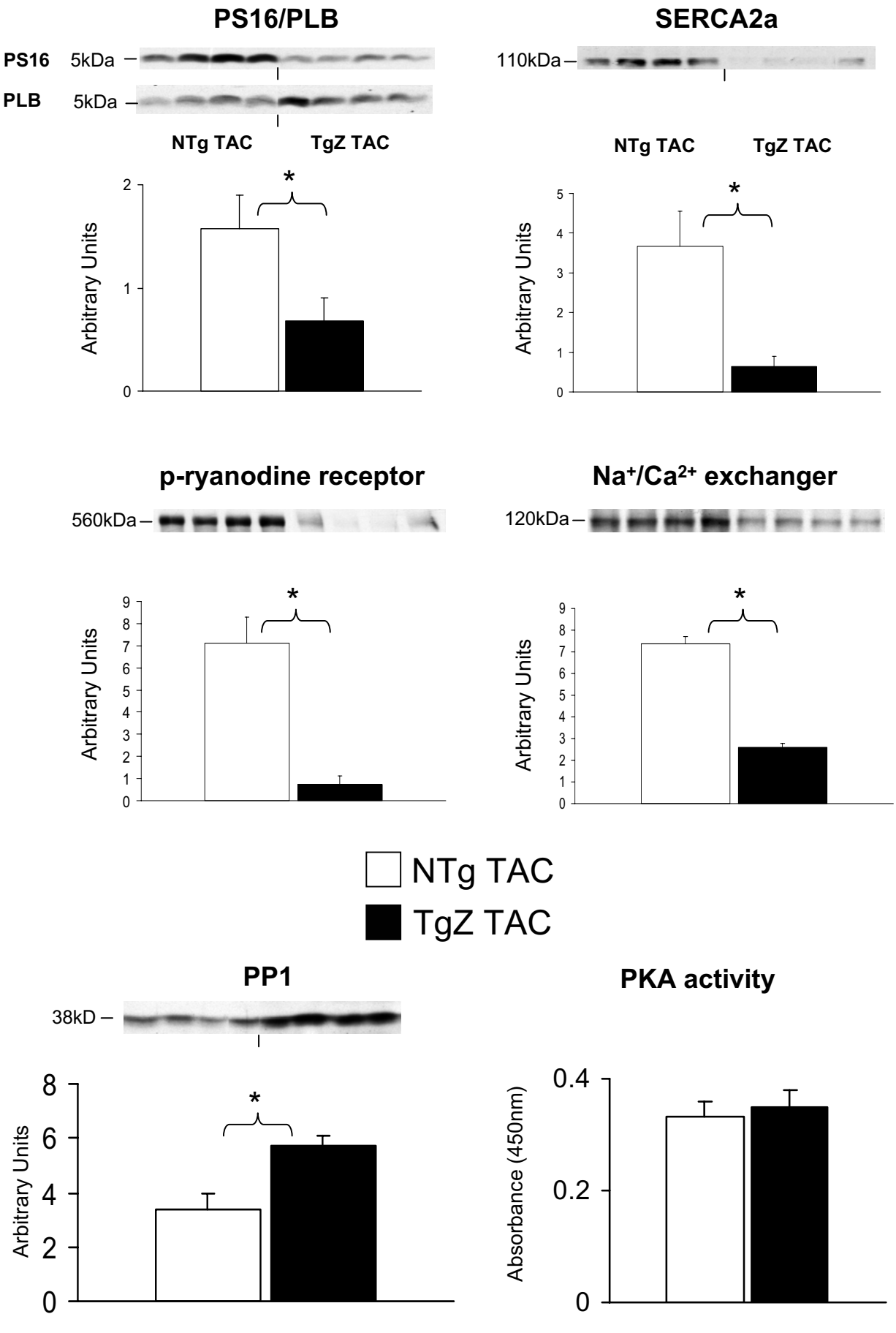
PS16/PLB
PS16
5kDa
PLB
5kDa
NTg TAC
TgZ TAC
Arbitrary Units
SERCA2a
110kDa
NTg TAC
TgZ TAC
Arbitrary Units
p-ryanodine receptor
560kDa
Arbitrary Units
Na+/Ca2+ exchanger
120kDa
Arbitrary Units
NTg TAC
TgZ TAC
PP1
38kD
Arbitrary Units
PKA activity
Absorbance (450nm)

Figure 3

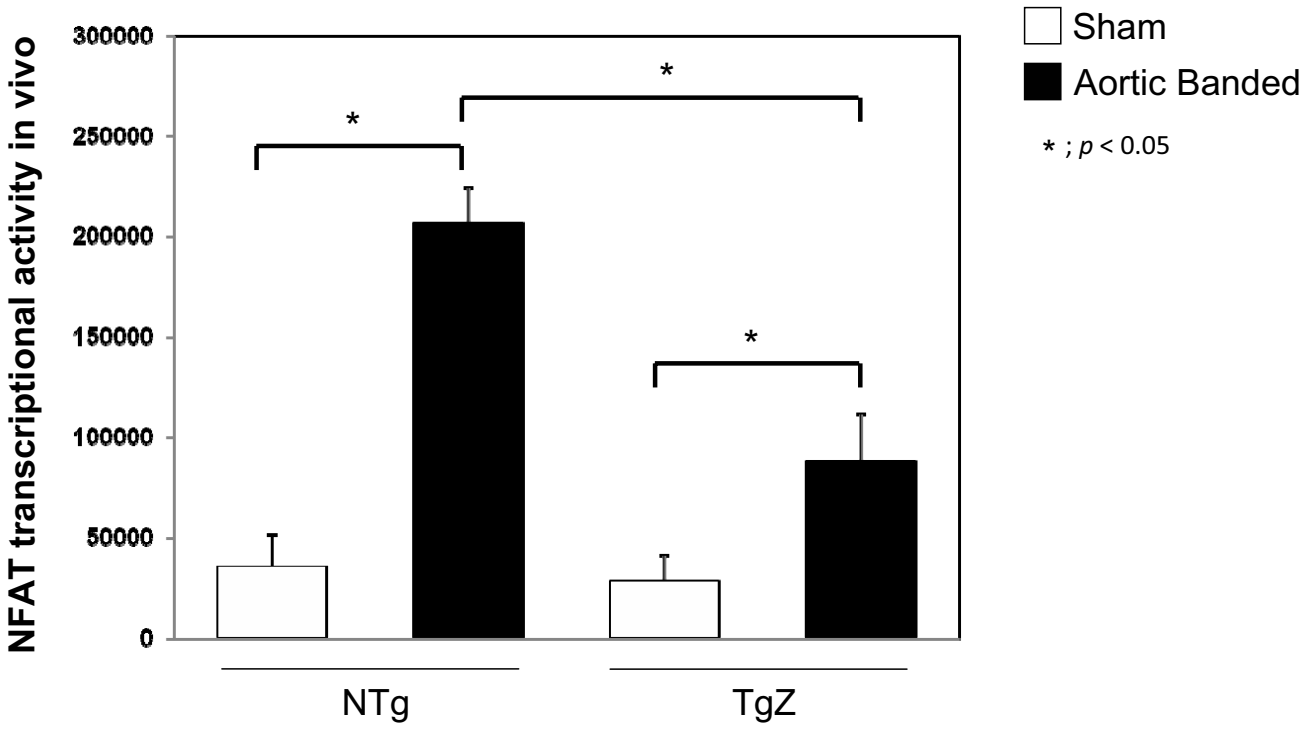
NFAT transcriptional activity in vivo
300000
250000
200000
150000
100000
50000
0
*
*
*
NTg
TgZ
Sham
Aortic Banded
* ; p < 0.05

Figure 4