Genetic inhibition of calcineurin induces diastolic dysfunction in mice with chronic pressure overload

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Calcineurin is a Ca2+/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 wk aortic banding) in transgenic (Tg) mice overexpressing Zaki-4 (TgZ), a specific endogenous inhibitor of calcineurin, on LV diastolic function. As expected, in the TgZ mice with calcineurin inhibitor overexpression, aortic banding reduced the degree of LV hypertrophy, but more severe LVH is thought to be deleterious because of limitations in subendocardial coronary reserve and diastolic function. Therefore, to address the effects of the inhibition of LVH by calcineurin on LV diastolic function, it would be more desirable to inhibit the calcineurin pathway genetically. To this end, the genetic inhibition of calcineurin was achieved using Tg mice that overexpressed ZAKI-4B (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 the genetic inhibition of calcineurin on the development of LVH, on LV systolic and LV diastolic function, and on Ca2+ 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

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

Experimental groups. Four experimental groups were performed: non-Tg (NTg) and TgZ sham-operated mice and NTg and TgZ mice with 2 wk 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 intraperitoneally using a mixture of ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g) for anesthesia.

Cardiac catheterization. Two weeks after aortic banding, closed-chest catheterization was performed. Two high-fidelity catheter tip transducers (1.4 Fr, Millar) were used: one was inserted into the right carotid artery and carefully advanced to the LV, and 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. The first derivative of LV pressure was used as an isovolumic index of systolic function. After the hemodynamic study, the mice were then euthanized 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 µg/kg body wt of 2.5% filtered Avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions). A 13-MHz linear ultrasound transducer was used. We took two-dimensional-guided motion mode measurements of LV internal diameter from more than three beats and averaged the measurements. The LV end-diastolic (LVED) dimension (LVEDD) was measured at the time of the apparent maximal LVEDD, whereas LV end-systolic dimension was measured at the time of the anterior systolic excursion of the posterior wall. Ejection fraction was also calculated and used as an ejective index of systolic function. After the hemodynamic study, the mice were then euthanized 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.

Indexes of diastolic function. Diastolic function was assessed by indexes derived from the curve of LV 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[(LVEDP/LVEDWT)²], where LVEDP is LV end-diastolic pressure and LVEDWT is LVED wall thickness.

The LVED stress-to-diameter ratio was assessed to measure diastolic stiffness.

LV pressure half-time (T1/2), an isovolumic relaxation index, was calculated from the LV pressure curve.

Histology. In each group, the hearts were separated and processed for histological analysis. They were cut and stained with hematoxylin and eosin and picrosirius red. The interstitial collagen concentration in the septal and LV free walls was assessed in the slides stained with the picrosirius red plus technique. Digital image software (Image Pro Plus 5.0) bound to a Leitz light microscope was used with a ×20 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), sarco(endo)plasmic reticulum Ca²⁺-ATPase 2a (SERCA2a), phospho-ryanodine receptor (Ser2808), Na⁺/Ca²⁺ exchanger, and protein phosphatase 1 (PP1).

RESULTS

After 2 wk of aortic stenosis, the LV aortic pressure gradient was similar in the NTg and TgZ mice (97 ± 5 vs. 90 ± 4 mmHg). However, the LVH, LV weight-to-body weight ratio (mg/g), was greater in the NTg animals (4.6 ± 0.2) compared with TgZ (3.5 ± 0.1) (P < 0.05). Table 1 shows systolic function and hemodynamic data. The maximal first derivative of LV pressure and LV ejection fraction were not reduced in both banded groups, demonstrating a preserved LV systolic function. LVEDD was not increased in either group.

Although LVEDD was similar in both groups, LV pressure 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 an increased LVEDD wall stress and increased myocardial stiffness, as reflected by the LVED stress-to-LVEDD ratio and the E-wave-to-A-wave ratio, a Doppler index of diastolic function (Fig. 1). T1/2, an isovolumic relaxation index derived from the LV pressure curve, and isovolumic relaxation time, an isovolumic relaxation index derived from Doppler, both also consistently increased in the TgZ group with and without aortic banding (Fig. 2).

Table 1. LV systolic function and hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>NTg Sham</th>
<th>TgZ Sham</th>
<th>NTg Aortic Banded</th>
<th>TgZ Aortic Banded</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEF, %</td>
<td>70 ± 1.1</td>
<td>72 ± 0.7</td>
<td>69 ± 0.8</td>
<td>67 ± 0.9</td>
</tr>
<tr>
<td>LV dp/dt, mmHg/s</td>
<td>7.756 ± 2.44</td>
<td>8.000 ± 0.32</td>
<td>9.659 ± 0.714</td>
<td>8.798 ± 0.518</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>92 ± 2.3</td>
<td>91 ± 2.3</td>
<td>128 ± 5.51</td>
<td>163 ± 3.9†</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>77 ± 2.0</td>
<td>76 ± 1.9</td>
<td>59 ± 3.41</td>
<td>63 ± 3.1†</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>445 ± 16</td>
<td>470 ± 10</td>
<td>472 ± 20</td>
<td>493 ± 22</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.56 ± 0.07</td>
<td>3.64 ± 0.04</td>
<td>3.56 ± 0.04†</td>
<td>3.58 ± 0.04</td>
</tr>
<tr>
<td>LV/BW, mm</td>
<td>2.9 ± 0.0</td>
<td>2.7 ± 0.1</td>
<td>4.6 ± 0.21</td>
<td>3.5 ± 0.1†</td>
</tr>
<tr>
<td>Lung weight/BW</td>
<td>5.2 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>7.8 ± 0.47</td>
<td>6.7 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10–18 mice. LV, left ventricular; NTg, nontransgenic; TgZ, transgenic mice overexpressing Zaki-4β; LVEF, LV ejection fraction; dp/dt, first derivative of LV pressure; LVSP, LV systolic pressure; MAP, mean arterial pressure; HR, heart rate; LVEDD, LV end-diastolic dimension; BW, body weight. *P < 0.05, TgZ banded vs. NTg banded; †P < 0.05, aortic banded vs. sham operated.
suggest that calcineurin genetic inhibition caused diastolic dysfunction which affected not only myocardial stiffness but isovolumic relaxation as well.

The total collagen did not change significantly with aortic banding in either group. The noncontractile proteins vinculin, paxillin, desmin, tallin, and β-tubulin were measured by Western blot analysis, and the 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. The protein levels for SERCA2a, the Na\(^+\)/Ca\(^{2+}\) exchanger, and phosphorylated ryanodine receptor were also decreased in aortic-banded TgZ mice (Fig. 3). As noted in Table 2, the 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 PKA activity, which could be involved in PLB phosphorylation, were examined in the two groups. As shown in Fig. 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). To evaluate the effect of ZAKI-4 upon the calcineurin activity in vivo hearts, TgZ mice were crossed with Tg-NFAT-Luc mice, which harbor an NFAT-Luc reporter gene, and the mice were then subjected to aortic banding for 2 wk. Aortic banding-induced increases in NFAT-Luc activity were significantly attenuated in TgZ-NFAT-Luc mice compared with Tg-NFAT-Luc mice, suggesting that calcineurin activity is significantly attenuated in the presence of ZAKI-4 (Fig. 4).

**DISCUSSION**

In the present study we evaluated the effects of 2 wk of chronic pressure overload induced by aortic banding in mice with Tg 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 the 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 Tg mice without a 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 Tg 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 LVED pressure and LVED wall stress.
Fig. 3. The ratio of PS16 to phospholamban (PLB), sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2a (SERCA2a), phospho-ryanodine receptor (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. Protein phosphatase 1 (PP1) was increased significantly in the TgZ group with aortic banding. There was no difference in PKA activity between the 2 groups. *\(P < 0.05\), TgZ with aortic banding vs. NTg with aortic banding (\(n = 4\) to 5/group). TAC, transverse aortic constriction.
Table 2. $Ca^{2+}$-handling proteins

<table>
<thead>
<tr>
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<th>NTg Sham</th>
<th>TgZ Sham</th>
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<tbody>
<tr>
<td>PS16/phospholamban</td>
<td>2.2±0.3</td>
<td>0.6±0.1*</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>9.0±1.4</td>
<td>3.2±0.6*</td>
</tr>
<tr>
<td>Phospho-ryanodine receptor</td>
<td>6.5±1.2</td>
<td>4.1±1.4</td>
</tr>
<tr>
<td>Na*/Ca2+ exchanger</td>
<td>5.6±0.6</td>
<td>2.4±0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in arbitrary units); *p < 0.05 vs. sham operated.

Table 3. NFAT-Luciferase Reporter Assay

<table>
<thead>
<tr>
<th></th>
<th>NTg Sham</th>
<th>TgZ Sham</th>
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<tbody>
<tr>
<td>Luc activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg-NFAT-Luc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TgZ-NFAT-Luc</td>
<td></td>
<td></td>
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Values are means ± SE; n = 4/group.

Aortic banding and began to decline by similar amounts after 4 wk systolic function was maintained in both groups after 2 wk.

Ca2+ channels, which causes a localized increase in Ca2+ release during contraction and Ca2+ sequestration during relaxation. During depolarization, a small amount of Ca2+ enters the cardiac cell through the L-type Ca2+ channels, which causes a localized increase in Ca2+ between the sarcolemma and the sarcoplasmic reticulum, which activates the Ca2+-release channel or ryanodine receptors to release Ca2+ into the cytosol. This process is termed “Ca2+-induced Ca2+ release” and is involved in excitation-contraction coupling. Relaxation is initiated by the uptake of Ca2+ into the sarcoplasmic reticulum by Ca2+-ATPase and SERCA2a, which are under the regulation of PLB, and to a lesser extent by the Na+/Ca2+ exchanger.

Although it is known that calcineurin affects Ca2+ handling and the regulation of SERCA and PLB (5, 16, 18, 19, 25), less is known how these proteins may regulate diastolic function with inhibition of calcineurin. 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+/Ca2+ 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+/Ca2+ exchanger protein levels. Thus, based on our data, we speculate that the regulation of diastolic Ca2+ by calcineurin may result from a complex interplay among the different proteins involved in controlling Ca2+ 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 noncontractile 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. We cannot rule out 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 resulting in an impaired relaxation and an increased collagen content in the LV wall.

![Fig. 4. Nuclear factor of activated T cells (NFAT)-luciferase reporter mice](http://ajpheart.physiology.org/)

Fig. 4. Nuclear factor of activated T cells (NFAT)-luciferase reporter mice (Tg-NFAT-Luc) were crossed with TgZ mice. Tg-NFAT-Luc and TgZ-NFAT-Luc (Bigenic) mice were subjected to either sham operation or transverse aortic banding. The Luc activity was measured from heart homogenates and normalized by the protein content. Values are means ± SE; n = 4/group.
increased myocardial stiffness, despite its concomitant action to reduce LVH.

REFERENCES


