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Cardiomyocyte Overexpression of Neuronal Nitric Oxide Synthase Delays Transition Toward Heart Failure in Response to Pressure Overload by Preserving Calcium Cycling

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Background—Defects in cardiomyocyte Ca^{2+} cycling are a signature feature of heart failure (HF) that occurs in response to sustained hemodynamic overload, and they largely account for contractile dysfunction. Neuronal nitric oxide synthase (NOS1) influences myocyte excitation-contraction coupling through modulation of Ca^{2+} cycling, but the potential relevance of this in HF is unknown.

Methods and Results—We generated a transgenic mouse with conditional, cardiomyocyte-specific NOS1 overexpression (double-transgenic [DT]) and studied cardiac remodeling, myocardial Ca^{2+} handling, and contractility in DT and control mice subjected to transverse aortic constriction (TAC). After TAC, control mice developed eccentric hypertrophy with evolution toward HF as revealed by a significantly reduced fractional shortening. In contrast, DT mice developed a greater increase in wall thickness ($P<0.0001$ versus control+TAC) and less left ventricular dilatation than control+TAC mice ($P<0.0001$ for both end-systolic and end-diastolic dimensions). Thus, DT mice displayed concentric hypertrophy with fully preserved fractional shortening ($43.7\pm0.6\%$ versus $30.3\pm2.6\%$ in control+TAC mice, $P<0.05$). Isolated cardiomyocytes from DT+TAC mice had greater shortening, intracellular Ca^{2+} transients, and sarcoplasmic reticulum Ca^{2+} load ($P<0.05$ versus control+TAC for all parameters). These effects could be explained, at least in part, through modulation of phospholamban phosphorylation status.

Conclusions—Cardiomyocyte NOS1 may be a useful target against cardiac deterioration during chronic pressure-overload–induced HF through modulation of calcium cycling. (***Circulation***. 2008;117:3187-3198.)

Key Words: hypertrophy ■ nitric oxide synthase ■ calcium ■ heart failure ■ remodeling

A primary event in the pathogenesis of heart failure (HF) induced by hemodynamic overload is cardiomyocyte hypertrophy. During chronic pressure overload, hypertrophy progresses to a decompensated phase with cardiac dilation and contractile impairment.[1–4] The prevention of this deterioration is a major therapeutic goal. Recent studies suggest that a critical factor causing cardiac decompensation after pressure overload may be the failure to preserve myocardial inotropy.

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Defects in cardiomyocyte sarcoplasmic reticulum (SR) Ca^{2+} cycling are a signature feature of experimental and human HF that largely accounts for contractile dysfunction.[4,5]

A prominent abnormality is a decrease in SR Ca^{2+} reentry, which is predominantly governed by the activity of SR calcium ATPase2 (SERCA2a) and controlled by its inhibitory protein, phospholamban (PLB).[6,7] Increasing SR Ca^{2+} reuptake by stimulating SERCA2a has been proposed as an approach to improving cardiomyocyte function in the failing myocardium.[8,9]

Previous studies using mice with global deficiency of neuronal nitric oxide synthase (NOS1 knockout mice, $NOS1^{-/-}$) suggested that NOS1 influences several aspects of myocardial excitation-contraction coupling, including sarcolemmal Ca^{2+} influx through L-type channels, SR Ca^{2+} content, and SR Ca^{2+} release. NOS1 is located at dyadic sarcolemmal-SR junctions, where it is in close proximity to both L-type channels and SR Ca^{2+} release channels (ryanodine receptors [RyR2]),[10] and it has been suggested that it may act physiologically to optimize excitation-contraction coupling.[11–14] However, the impact of NOS1 on myocardial contractility and Ca^{2+} cycling remains controversial, and the potential relevance of this mechanism in HF is unknown. Nevertheless, it is intriguing that NOS1 expression and activity are significantly increased in the hypertrophic[15] or failing[16,17] rat myocardium and in failing human hearts.[18] Moreover, NOS1 gene deletion is associated with more severe cardiac remodeling and functional deterioration in murine models of myocardial infarction, which suggests that cardiomyocyte NOS1 may play an active role in the myocardial stress response.[19,20] In the present study, we tested the hypothesis that NOS1 overexpression within cardiomyocytes may be beneficial during chronic hemodynamic overload by favorably modulating Ca^{2+} cycling and myocardial contractility.

Methods

Generation of Transgenic Mice

The human NOS1 (*h*NOS1, α-isoform) coding sequence was used to generate the *h*NOS1 conditional model. The fragment of *h*NOS1 that included the full-length coding sequence was blunt-ended and inserted into the EcoRV site located between the tetO promoter and the rabbit β-globin polyadenylation sequence of the bidirectional tet-inducible pBI4 vector, which resulted in the tetO-*h*NOS1 construct. Double-transgenic (DT) mice were obtained by crossing the tetO-*h*NOS1 mice with the α-MHCtTA transactivator mouse strain (kindly provided by G.I. Fishman, New York University School of Medicine, New York), which allows conditional, cardiac-specific transgene expression as early as embryonic day 9.5 (E9.5). In all experiments, male and age-matched nontransgenic littermates were used for comparison with DT mice. The present study was conducted in accordance with institutional guidelines and those formulated by the European Community for experimental animal use (L358-86/609/EEC).

Determination of NOS Activity

Ca^{2+}-dependent activity was determined from myocardial homogenates by [^{3}H]-L-arginine to [^{3}H]-L-citrulline conversion, according to the manufacturer's instructions (nitric oxide assay kit, Calbiochem, San Diego, Calif). Assays for specific NOS1 activity were performed in the presence of the preferential NOS1 inhibitor, vinyl-L-*N*-5-(1-imino-3-butenyl)-L-ornithine (L-VNIO; 0.1 μmol/L; Axxora LLC, San Diego, Calif).

Immunoblots of Cardiac Lysates

Immunoblots and coimmunoprecipitations assays were performed on homogenates of ventricular tissue with anti-NOS1, anti-NOS3, anti-VEGF (vascular endothelial growth factor), anti-SERCA2a from Santa Cruz Biotechnology Inc (Santa Cruz, Calif); anti-VEGF receptor 2 (Flk-1) from US Biological (Marblehead, Mass); anti-phospho-PLB (Ser16 and Thr17) from Badrilla Ltd (Leeds, United Kingdom); anti-phosphorylated glycogen synthase kinase (GSK)-3β and anti-GSK-3β from Cell Signaling (Danvers, Mass); anti-GAPDH from Chemicon International (Temecula, Calif); anti-PLB, anti-NOS1, and anti-RyR2 from Affinity Bioreagents (Neshanic Station, NJ); and anti-calmodulin from Zymed Laboratories, Inc (South San Francisco, Calif).

Left Ventricular Pressure Overload and Echocardiography

Transverse aortic constriction (TAC) surgery and echocardiography were performed with protocols blinded with respect to genotype.

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA extraction and a reverse-transcription reaction were performed, as described previously. Details of primer sequences are listed in the online-only Data Supplement.

Ca^{2+} Imaging and Cellular Electrophysiology

Single ventricular myocytes were prepared by enzymatic dissociation. Myocytes were loaded with the cell-permeant Ca^{2+} fluorescent dye fluo-3 AM. Confocal images were acquired in the line-scan mode. L-type Ca^{2+} current was measured with the whole-cell patch-clamp technique.

Statistical Analysis

A detailed Methods section can be found in the online-only Data Supplement. Results are expressed as mean±SEM. One-way ANOVA followed by a post hoc Bonferroni t test was used in Figure 1. The interaction between genotype and the response to pressure overload was tested with a 2-way ANOVA followed by a post hoc Dunnett's test (Figures 2 through 8). Results were considered significant if $P<0.05$.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Conditional Expression of Human NOS1 in the Heart of Transgenic Mice

Reverse-transcription polymerase chain reaction, Western blot analysis, and immunostaining confirmed that human NOS1 (*h*NOS1) was expressed only in hearts from DT mice and not in doxycycline-treated DT mice, which shows that the system is not leaky and that *h*NOS1 is not expressed in monotransgenic tetO-*h*NOS1 (Figure 1A through 1E). Cardiac extracts from DT mice exhibited markedly greater NOS1 activity than tetO-*h*NOS1 and doxycycline-treated mice. NOS1 overexpression was not associated with any change in NOS3 protein (Figure 1F). The tetO-*h*NOS1, wild-type littermates, and doxycycline-treated DT mice were phenotypically similar, had similar survival rates and echocardiographic parameters, and were used interchangeably as controls for the following experiments (control; not shown). Basal cardiac morphology was unaltered, but cardiac function assessed by echocardiographic fractional shortening was increased significantly in DT compared with control mice (43.6±1.2% versus 40.6±0.3%, $P<0.05$).
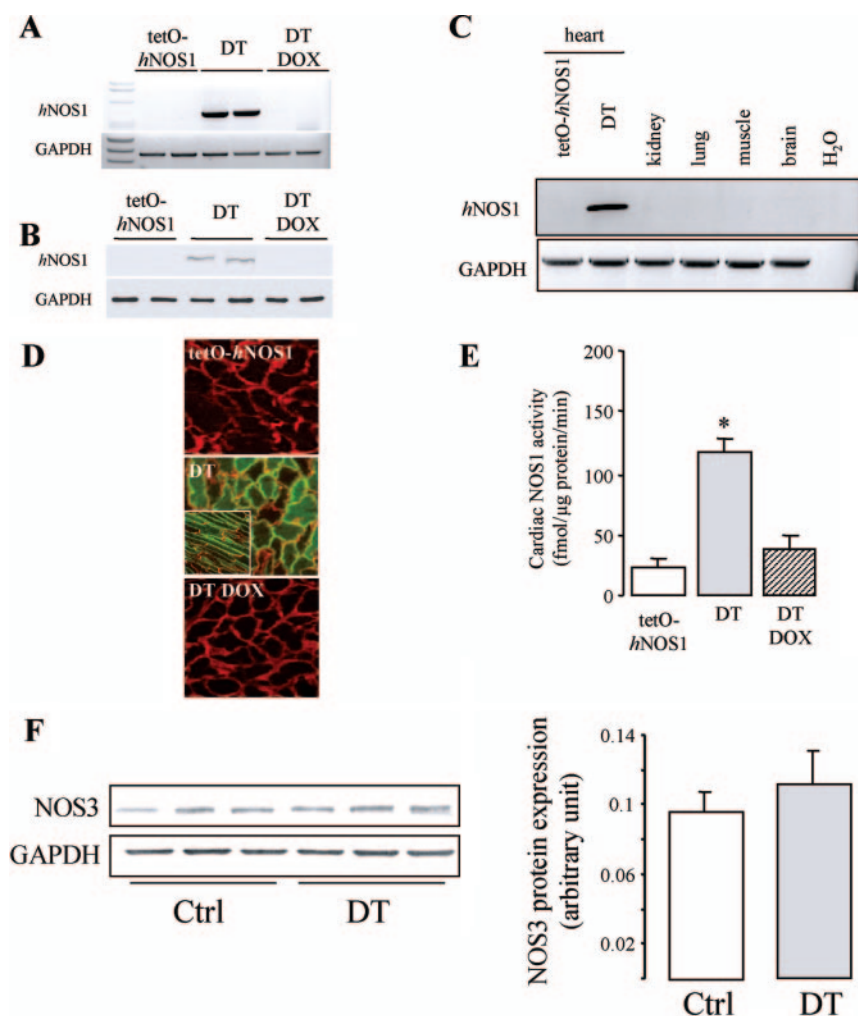
Figure 1. Conditional cardiac *h*NOS1 overexpression. Cardiac *h*NOS1 mRNA (A) and protein (B), as determined by reverse-transcription polymerase chain reaction and immunoblot, were expressed in heart of 2-month-old DT mice only (1 mouse per lane). No expression was detectable in monotransgenic littermate (tetO-*h*NOS1), which indicates that control of the transgene is not leaky. Expression of *h*NOS1 in DT mice was prevented by doxycycline treatment (DOX, 1 g/kg) for 3 weeks. C, *h*NOS1 expression, as determined by reverse-transcription polymerase chain reaction, is restricted to heart of DT mice. Expression of the transgene is absent in kidney, lung, muscle, and brain. D, Representative dual labeling of LV sections for vinculin (red) and *h*NOS1 (green) in tetO-*h*NOS1, DT, and DOX-treated mice, demonstrating increased abundance of the transgene in numerous cardiomyocytes of DT mice only. Inset represents longitudinal section of cardiomyocytes. E, NOS1 activities based on L-citrulline formation. NOS1 activity was determined with the NOS1 inhibitor L-VNIO. Note the 5-fold increase in NOS1 activity in DT only, this effect being reversed after administration of DOX for 3 weeks. F, Immunoblots demonstrating that cardiomyocyte overexpression of NOS1 did not induce any change in NOS3 protein expression. *$P < 0.05$ vs basal. Ctrl indicates control.

## Effect of TAC on NOS1 Expression, Activity, and Localization

After TAC, both cardiac endogenous NOS1 and exogenous transgenic *h*NOS1 expression increased significantly, as described previously (Figure 2A).[21] TAC induced a 3-fold increase in specific NOS1 activity in both groups (22.3 ± 6.9 in control versus 72.1 ± 14.6 fmol · μg protein⁻¹ · min⁻¹ in control+TAC (Ctrl+TAC) mice, $P < 0.01$; 117.3 ± 11.1 in DT versus 363 ± 32.9 fmol · μg protein⁻¹ · min⁻¹ in DT+TAC mice, $P < 0.01$; n=5 to 7 per group). In DT+TAC mice treated for 3 weeks with doxycycline before surgery, cardiac NOS1 activity returned to values observed in Ctrl+TAC mice (81.1 ± 11.1 fmol · μg protein⁻¹ · min⁻¹, P=NS versus Ctrl+TAC). To more specifically assess the subcellular localization of both endogenous NOS1 and transgenic *h*NOS1, we studied protein-protein interactions between NOS1 and caveolin-3 (Cav-3) or RyR2. Both antibodies coprecipitated NOS1 in myocardial extracts from control and DT mice (Figure 2B), but the NOS1 signal intensity was increased significantly in DT hearts (0.335 ± 0.048 arbitrary units [AU] in wild-type versus 0.523 ± 0.37 AU in DT for NOS1/Cav-3 complexes, $P < 0.05$; 0.0193 ± 0.012 AU in control versus 0.497 ± 0.036 AU in DT for RyR2/NOS1 complexes, $P < 0.05$; n=3 to 5 per group). In contrast to the HF

situation during which NOS1 is translocated to caveolae through interactions with Cav-3, pressure overload did not alter the amount of the NOS1/Cav-3 complex.[21] Immunoprecipitation with anti-RyR2 revealed a decreased immunoreactivity with endogenous NOS1 in Ctrl+TAC (0.193 ± 0.012 AU in wild-type versus 0.13 ± 0.08 AU in Ctrl+TAC, $P < 0.05$). In contrast, the amount of RyR2/NOS1 complexes was maintained in DT+TAC mice.

Cardiomyocyte NOS1 Overexpression Protects Against Maladaptive Hypertrophy After TAC

Six weeks after TAC, control mice showed a 50% increase in heart weight–to–tibia length ratio compared with sham controls; however, DT mice displayed a further 20% increase in cardiac hypertrophy compared with Ctrl+TAC mice, an effect not seen in doxycycline-treated DT+TAC mice. A similar difference in cardiomyocyte cross-sectional areas was also confirmed by morphometric analysis of LV sections (Figure 2D).

Transthoracic echocardiography demonstrated that control mice subjected to TAC developed a modest increase in wall thickness and significant chamber enlargement, characterized by increased LV end-diastolic and end-systolic dimensions (Figure 3). The eccentric hypertrophy and unfavorable geometric adaptation to sustained pressure overload were accom-
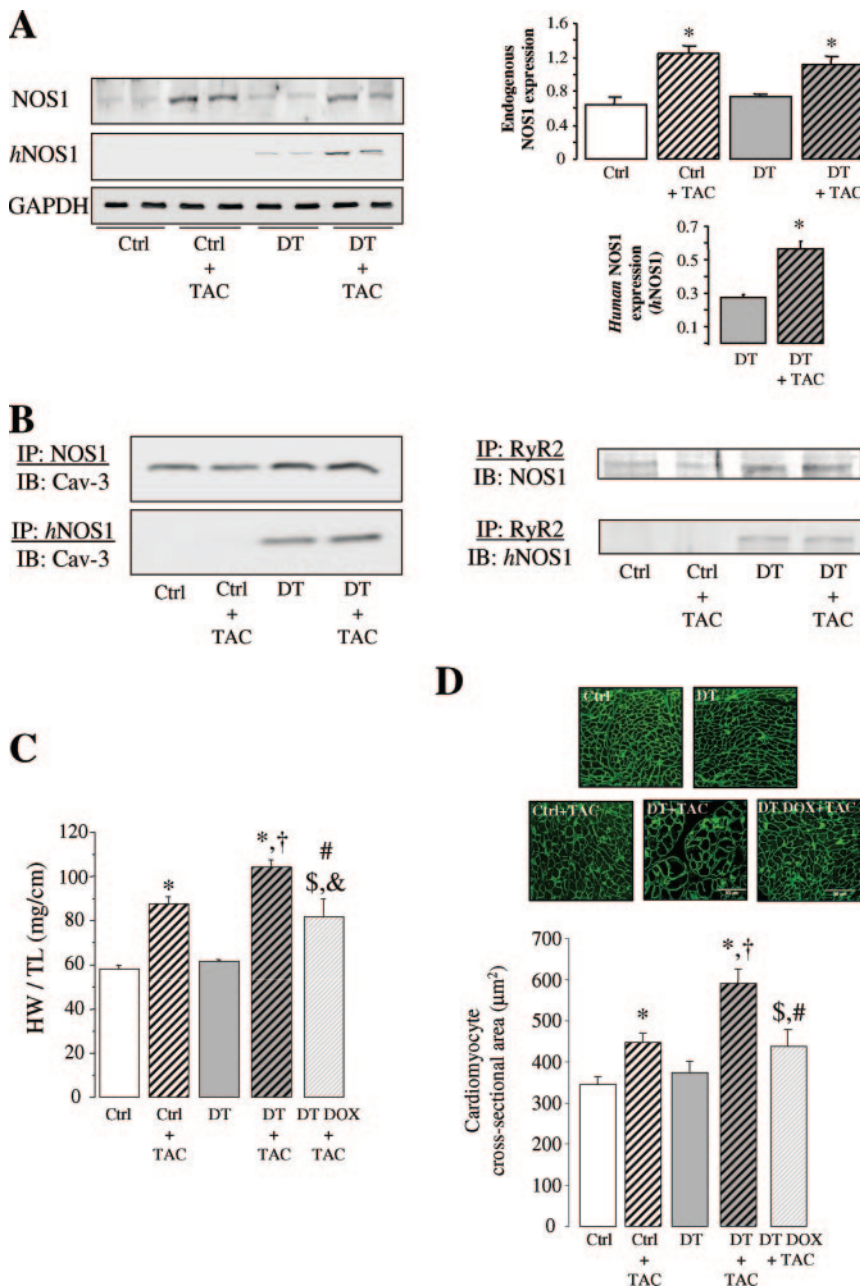
Figure 2. A, Western blot and densitometric data reflecting endogenous NOS1 and exogenous human NOS1 (*h*NOS1) protein expression in control (Ctrl) and DT LV homogenates in response to pressure overload. B, Association between endogenous NOS1 and *h*NOS with either Cav-3 or RyR2. LV lysates or crude particulate preparations were immunoprecipitated with anti-NOS1 polyclonal antibody or anti-RyR2 polyclonal antibody. Immunoprecipitates (IPs) were immunoblotted (IB) for Cav-3 or NOS1. C, Mean data for heart weight–to–tibia length ratio (n≥5 per group). D, Representative immunostaining of heart sections with anti-Cav-3 and summary quantification of cardiomyocyte cross-sectional area (n=6 to 8 per group). *$P<0.05$ vs basal; †$P<0.05$ vs Ctrl+TAC; #$P<0.05$ vs DT+TAC; $$P<0.05$ vs DT; &$P<0.05$ vs Ctrl.
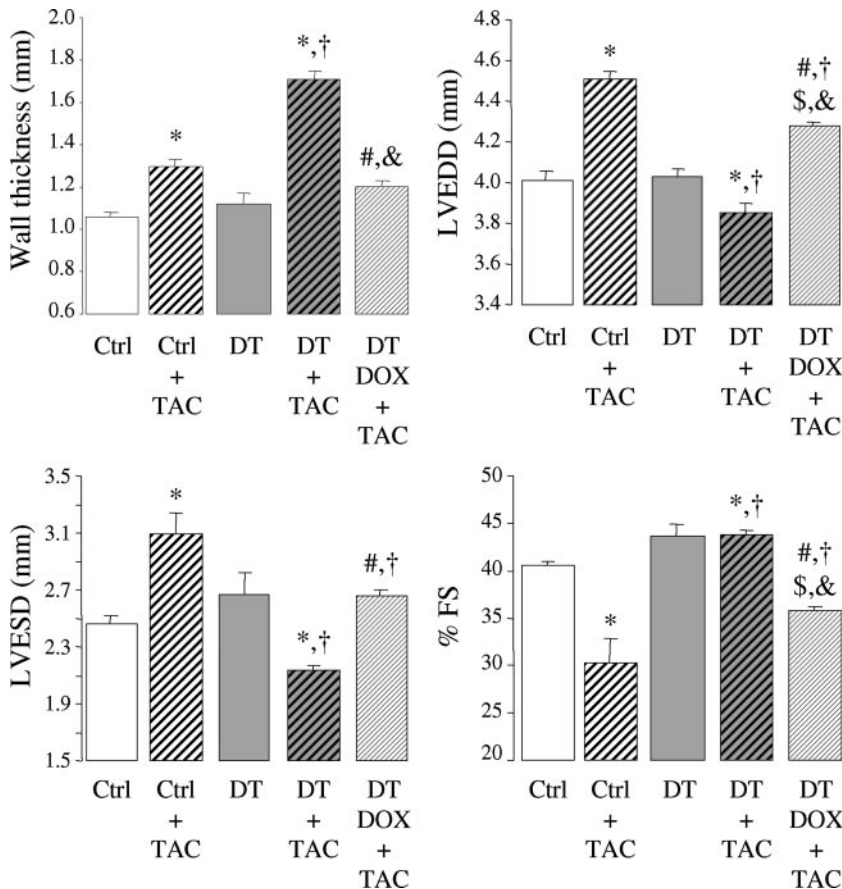
panied by a significantly decreased fractional shortening compared with control sham-operated mice ($30.3\pm2.6\%$ versus $40.6\pm0.3\%$, $P<0.001$). In contrast, DT mice developed a greater increase in wall thickness ($P<0.01$ versus Ctrl+TAC) but had reduced LV dimensions compared with Ctrl+TAC mice ($P<0.01$ for both LV end-diastolic and end-systolic dimensions). Thus, DT mice displayed concentric hypertrophy and fully preserved fractional shortening after TAC. Conversely, doxycycline-treated mice subjected to TAC displayed cardiac remodeling and function similar to the Ctrl+TAC group. Taken together, these data indicate that NOS1 overexpression maintains concentric hypertrophic remodeling and prevents the onset of HF.

In addition to biomechanical stress, several neurohumoral factors are also potent hypertrophic agents. To investigate whether NOS1 affects hypertrophy in the absence of hemodynamic overload, mice were treated chronically (14 days) with the β-adrenergic agonist isoproterenol ($30\ mg \cdot kg^{-1} \cdot d^{-1}$). No difference was observed in blood pressure between groups. Isoproterenol induced similar hypertrophy in control and DT mice, as assessed by heart weight–to–tibia length ratio and cardiomyocyte cross-sectional area (Data Supplement Figure I).

Markers of Adaptive Hypertrophy in DT Mice After TAC

The concentric LV remodeling and preserved contractile function in DT mice suggested that cardiomyocyte NOS1 overexpression could be associated with an enhanced adaptive hypertrophic program. In contrast to adaptive hypertrophy,

Figure 3. Summary data from M-mode echocardiography in conscious animals (n=6 to 10 per group) demonstrating dilated hypertrophy with decreased fractional shortening in control (Ctrl)+TAC but concentric hypertrophy with preserved fractional shortening in DT+TAC hearts. Wall thickness further increases in DT mice in response to TAC. LV end-diastolic (LVEDD) and end-systolic (LVESD) dimensions and fractional shortening differed markedly between the genotypes. *$P<0.05$ vs basal; †$P<0.05$ vs Ctrl+TAC; #$P<0.05$ vs DT+TAC; $$P<0.05$ vs DT; &$P<0.05$ vs Ctrl.

pathological hypertrophy is consistently associated with the reexpression of fetal genes. As expected, TAC robustly increased mRNA levels of atrial natriuretic factor, brain natriuretic peptide, β-myosin heavy chain, and skeletal muscle (SK)-actin in control mice (Figure 4A). In marked contrast, none of these genes were upregulated in DT mice. Interstitial cardiac fibrosis assessed by Sirius red staining revealed a similar increase in control and DT mice (Figure 4B).

Pressure-overload–induced maladaptive cardiac hypertrophy is also associated with a reduction in capillary density. As expected, TAC significantly decreased capillary density in control mice (Figure 5A). However, cardiomyocyte NOS1 overexpression prevented the TAC-induced decrease in capillary density, with DT+TAC hearts displaying a normal capillary density compared with Ctrl+TAC hearts despite their greater extent of hypertrophy. Similar results were obtained when we studied the capillary-to-cardiomyocyte ratio (data not shown). We next investigated a major angiogenic growth factor/receptor system, VEGF/VEGF receptor 2 (Flk-1). TAC was associated with a significant increase in VEGF protein expression in control mice, but Flk-1 protein expression was decreased significantly, which could explain the observed decrease in capillary density (Figure 5B). In contrast, DT mice displayed a further significant increase in VEGF protein expression compared with Ctrl+TAC mice, together with an increased level of Flk-1 receptor (Figure 5B).

Effects of NOS1 Overexpression on Myocyte Contractility, Ca²⁺ Cycling, and Ca²⁺ Handling Proteins

To examine whether NOS1 may be protective through modulation of Ca²⁺ cycling, we next examined intracellular Ca²⁺ and contraction in isolated cardiomyocytes from control and DT mice subjected to TAC or sham surgery. Figure 6A shows confocal line-scan images of steady state [Ca²⁺]ᵢ transients. Peak amplitude of [Ca²⁺]ᵢ transient was greater in DT myocytes than in control myocytes (by 26%, $P=0.005$) in the sham groups and was associated with increased cell shortening (by 43%, $P<0.05$; Figure 6B). The time to peak of the Ca²⁺ transients was significantly lower in DT myocytes than in control (20.6 ± 1.1 versus 27.7 ± 2.0 ms, $P<0.05$). The [Ca²⁺]ᵢ transient also declined more rapidly in DT myocytes than in control, but SR Ca²⁺ content estimated after rapid caffeine application was similar in the 2 groups. We thus considered that the increased Ca²⁺ transients in DT myocytes despite a similar SR Ca²⁺ content might result from modulation of L-type Ca²⁺ current (I_{CaL}), the trigger of SR Ca²⁺ release. Indeed, the density of I_{CaL} tested at different potentials to obtain current-voltage relation curves was significantly higher, in the −20- to 40-mV voltage range, in DT compared with control cells (Figure 7A). These results suggest that increased myocyte contraction in NOS1 transgenic mice is driven by greater Ca²⁺ entry via L-type Ca²⁺ channels.

After TAC in control mice, the peak Ca²⁺ transient was decreased significantly together with a prolonged transient

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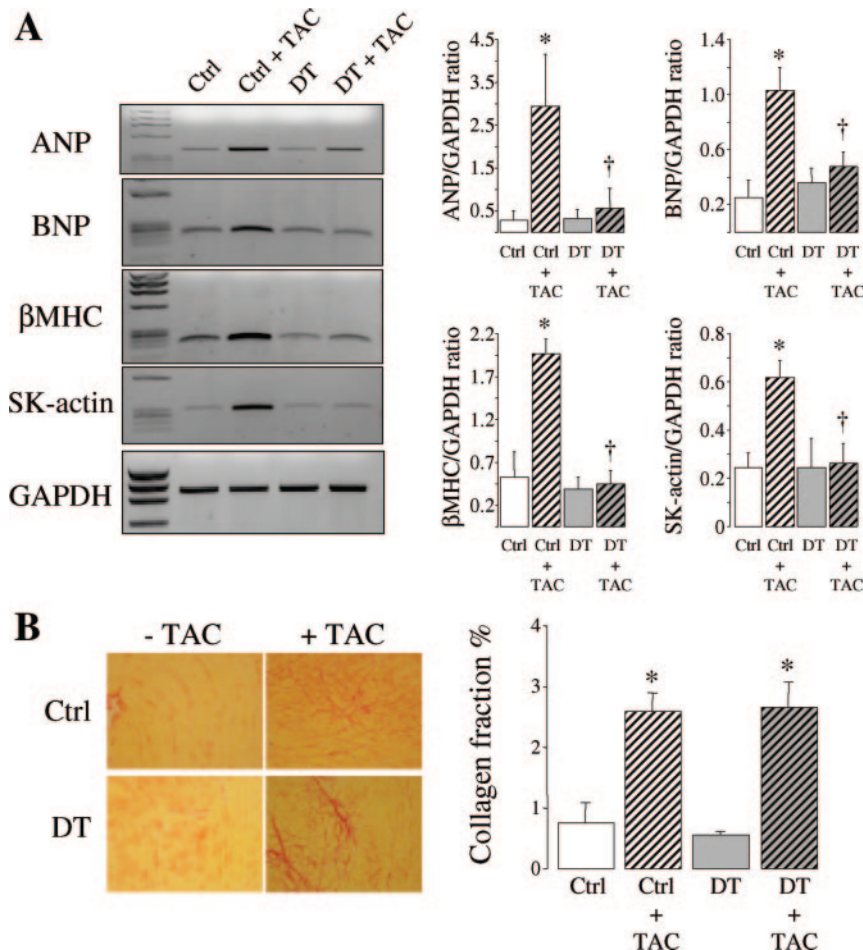
Figure 4. A, Representative semiquantitative reverse-transcription polymerase chain reaction and average data for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (βMHC), skeletal muscle (SK)-actin, and GAPDH transcripts. B, Red Sirius staining of hearts sections and average histograms for collagen fraction. n=6 per group. *$P<0.05$ vs basal, †$P<0.05$ vs Ctrl+TAC.

decline and a reduction in myocyte shortening (Figure 6B; $P<0.05$). This was associated with an increase in time to peak Ca^{2+} transient from 27.7 ± 2.0 to 33.5 ± 1.5 ms ($P<0.05$). There was no significant change in I_{CaL} density (peak I_{Ca} normalized to cell capacitance; Figure 7B), but the reduction in cell shortening was attributable to a decrease in SR Ca^{2+} load, as assessed by the maximal caffeine-induced Ca^{2+} transient (Figure 6B). Remarkably, cardiomyocyte NOS1 overexpression fully rescued this phenotype after pressure overload. As such, DT mice subjected to TAC showed no significant reduction in Ca^{2+} transient amplitude or SR Ca^{2+} load and no delay in transient decline compared with DT sham. Time to peak Ca^{2+} transient was also reduced significantly compared with Ctrl+TAC (26.4 ± 1.4 versus 33.5 ± 1.5 ms, $P<0.05$). In line with this, cell shortening was fully preserved after TAC. Thus, both SR Ca^{2+} load and Ca^{2+} transient are preserved in NOS1 transgenic mice subjected to TAC, in marked contrast to Ctrl+TAC mice.

Immunoblot analysis was used to determine whether NOS1 overexpression was associated with alterations in the protein abundance of PLB, SERCA2a, and phosphorylated PLB. Despite no change in SR load and PLB/SERCA2a ratio in cardiomyocytes from DT mice, phosphorylation of PLB was increased significantly at both the Ser16 and Thr17 sites, which could account for the shorter Ca^{2+} transient decay (Figure 8A through 8C). After TAC,

there was a significant increase in the PLB/SERCA2a ratio in control mice due to increased PLB protein expression, a change that is associated with depressed contractile reserve in the maladaptive hypertrophied heart. In addition, although PLB phosphorylation at the Ser16 site was significantly greater in control banded animals, the PLB-Ser16/PLB ratio was significantly reduced in Ctrl+TAC mice. In contrast, the PLB/SERCA2a ratio was unaltered in DT+TAC mice. Furthermore, in response to TAC, PLB phosphorylation was further increased in DT mice at both sites compared with Ctrl+TAC, which resulted in increased PLB-Ser16/PLB and PLB-Thr17/PLB ratios.

Potential Ca^{2+}-Dependent Signaling Pathway Involved in NOS1-Mediated Cardiac Hypertrophy

Because cardiomyocyte NOS1 was found to modulate intracellular Ca^{2+} cycling, we tested whether NOS1-mediated adaptive cardiac hypertrophy might involve activation of the Ca^{2+}-dependent serine/threonine protein-phosphatase calcineurin. In control mice, TAC increased calcineurin protein expression and decreased the activity of another prominent signaling cascade involved in cardiomyocyte hypertrophy, GSK-3β, as shown by an increase in the ratio of phosphorylated to total GSK-3β protein (Data Supplement Figure IIA). In agreement with our previous morphological and remodeling results, TAC induced a further and significant increase in both calcineurin expression and phosphorylated GSK-3β/
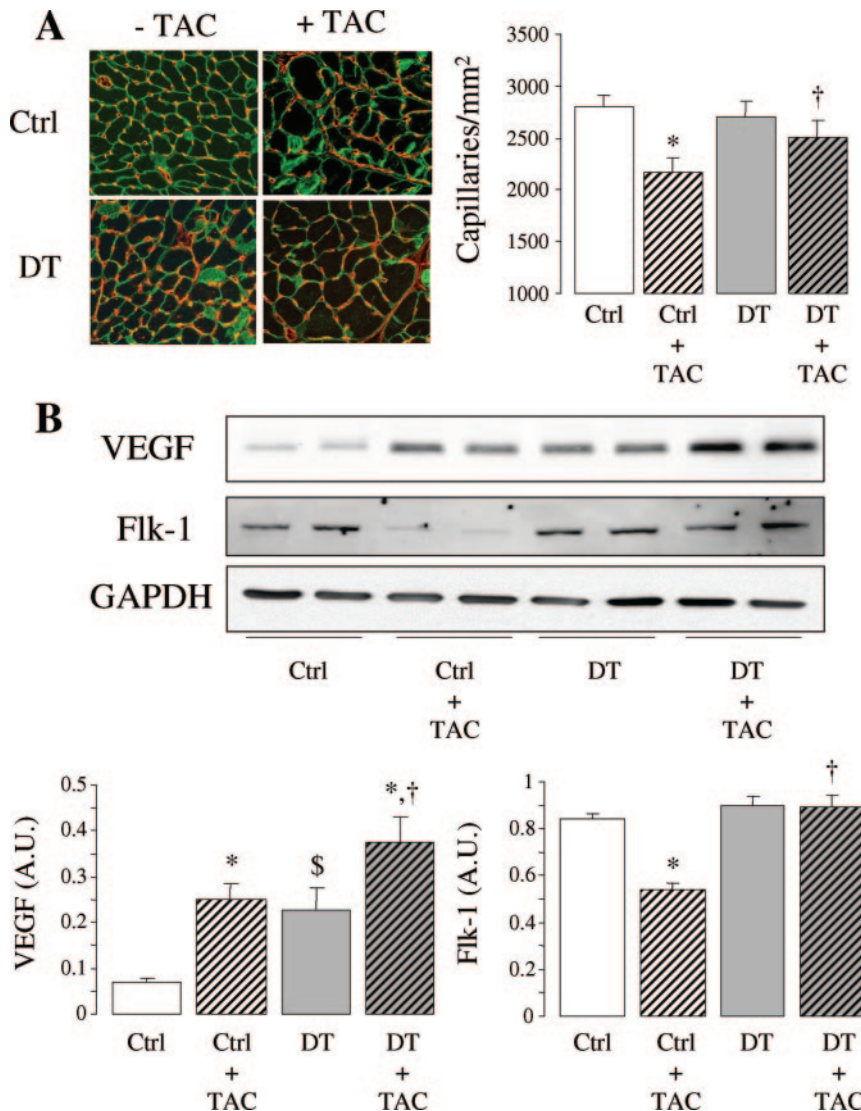
Figure 5. A, Representative immunostaining of heart sections for capillary density. Capillaries in LV sections are identified by anti-Cav-1α antibody (red) and cardiomyocytes with anti-Cav-3 (green). B, Representative immunoblots showing VEGF and Flk-1 expression in Ctrl and DT LVs and the response to TAC. *$P<0.05$ vs basal; †$P<0.05$ vs Ctrl+TAC; $$P<0.05$ vs DT+TAC.

GSK-3β ratio in DT mice ($P<0.05$ for both versus Ctrl+TAC). To assess calcineurin activity, we performed immunoprecipitation experiments between calmodulin and calcineurin in heart homogenates from the different groups, as described previously (Data Supplement Figure IIB).[22] These experiments showed that there was a greater increase in calcineurin association with calmodulin after TAC in DT than in control mice.
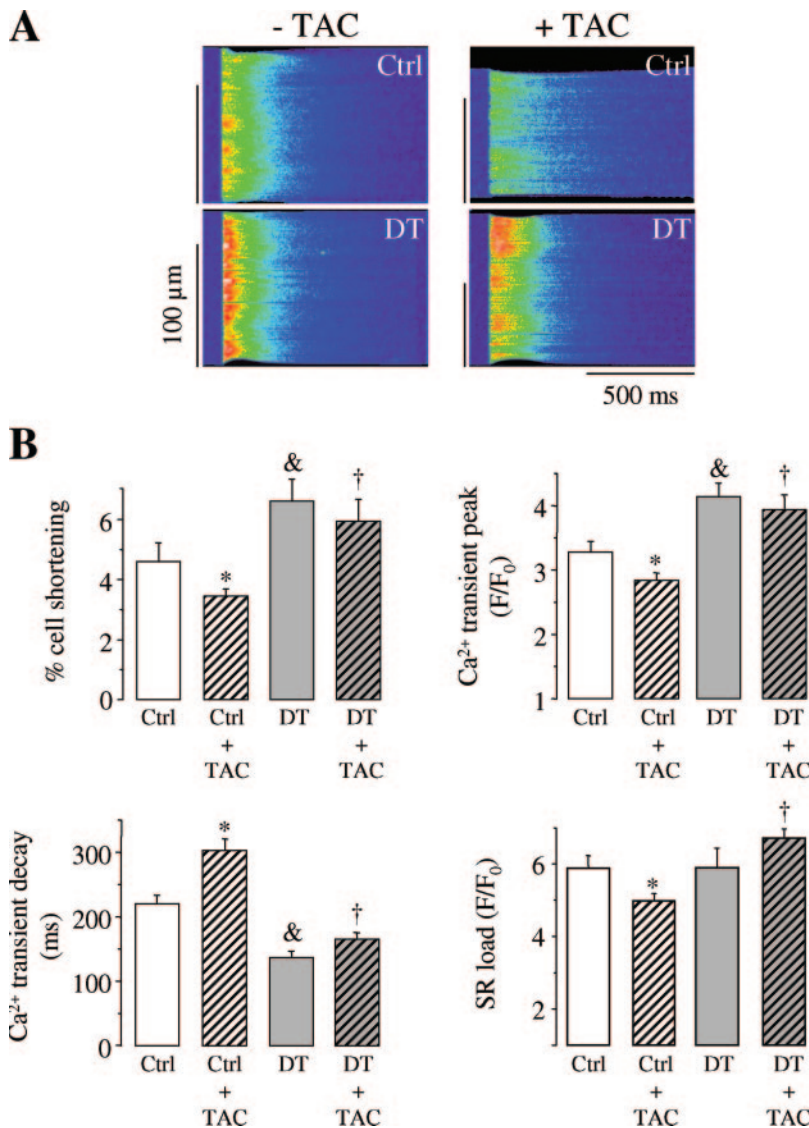
Discussion

The major new finding of the present study is that a chronic increase in cardiomyocyte NOS1 protein expression is associated with enhanced cardiac contractility and may be protective against the development of maladaptive cardiac hypertrophy in the face of increased hemodynamic load. To the best of our knowledge, this is the first report to demonstrate the efficacy of selective NOS1 targeting in the setting of hypertrophy and transition to HF. Mechanistically, the beneficial effects of NOS1 overexpression may be associated with preserved SR Ca²⁺ cycling, which involves Ca²⁺ load in particular, through modulation of PLB expression and phosphorylation levels.

NOS1 Regulation of Basal Myocyte Contractility and Calcium Handling

A major new paradigm in NO biology has emerged in the last few years to suggest that NOS1-derived NO plays an important role in the physiological regulation of myocardial contraction and Ca²⁺ fluxes. In the present study, we demonstrated that NOS1-overexpressing mice showed a modest but significant increase in LV contractile function in vivo compared with control mice. Isolated ventricular myocyte studies showed that cardiomyocytes overexpressing NOS1 have greater basal cell shortening, higher Ca²⁺ transients, and faster relaxation than control littermates.

Several proteins involved in excitation-contraction coupling are potential targets for NOS1 modulation, and Xu and coworkers[10] suggested that NOS1 inhibits the activity of SERCA2a. Inhibition of SERCA2a would be expected to slow the speed of relaxation and rate of decay of the [Ca²⁺]$_i$ transient in DT myocytes, because Ca²⁺ reuptake into the SR would occur more slowly. However, we found that the [Ca²⁺]$_i$ transient declined much more rapidly in DT myocytes than in control myocytes. Furthermore, SR Ca²⁺ content estimated after rapid caffeine

Figure 6. Overexpression of NOS1 in the cardiomyocyte rescues cardiac contractile dysfunction in response to pressure overload. A, Representative line-scan images of fluo-3 fluorescence recorded in 1 control (Ctrl; top) and 1 DT (bottom) myocyte field stimulated at 1 Hz. B, Bar graphs corresponding to cell shortening, the amplitude $[Ca^{2+}]_i$ transient (measured as peak F/F_0), $[Ca^{2+}]_i$ transient rate of decay, and pooled amplitude histogram of $[Ca^{2+}]_i$ transients induced by 10 mmol/L caffeine (n=20 to 35 cells from 4 animals in each group). *$P<0.05$ vs basal; †$P<0.05$ vs Ctrl+TAC; $$P<0.05$ vs DT; &$P<0.05$ vs wild-type.

application was similar in the 2 groups. One potential explanation would be that NOS1 influences SR Ca^{2+} uptake by stimulating phosphorylation of PLB. Although the PLB/SERCA2a ratio was unchanged in DT myocytes, NOS1 overexpression induced an increase of PLB phosphorylation, which would stimulate PLB dissociation from SERCA2a and lead to increased SR Ca^{2+} reuptake. Similarly, it has been demonstrated recently that both relaxation and the rate of $[Ca^{2+}]i$ transient decay were significantly prolonged in LV myocytes from NOS1$^{-/-}$ mice. Disabling the SR abolished the differences in both parameters, which suggests that impaired SR Ca^{2+} reuptake may account for the slower relaxation in NOS1$^{-/-}$ mice. In line with these findings, disruption of NOS1 decreased PLB phosphorylation.[23]

A potential alternative target for NOS1 is RyR2, which has variably been shown to be activated[24,25] or inhibited[26] by NO. We found that the time to peak $[Ca^{2+}]_i$ was reduced significantly in myocytes overexpressing NOS1, which could also explain why SR load was unchanged despite the increase in Ca^{2+} transient. Although we did not assess whether NOS1 overexpression alters the level of RyR2 protein expression,

coimmunoprecipitation experiments demonstrated that the level of NOS1 interacting with RyR2 was increased in DT mice. All these findings are in keeping with the notion that RyR2 activity could be activated in DT myocytes, as described recently.[27] NOS1 from the SR may also affect the L-type Ca^{2+} channel, and consistent with the findings of increased contractility in myocytes from DT mice, we demonstrated that there was an increased Ca^{2+} current in cardiomyocytes from DT mice.

The present results are, however, in sharp contrast with previous studies demonstrating that myocytes from NOS1$^{-/-}$ mice had a greater basal cell shortening, associated with a greater Ca^{2+} influx through L-type Ca^{2+} channels and a larger SR Ca^{2+} content.[12,13] In contrast, others have observed different effects using the same mice, with no differences in basal contractility and unchanged L-type Ca^{2+} currents but decreased cardiomyocyte contractility and calcium transients at higher pacing rates in isolated cardiomyocytes.[11,14] Recently, conditional myocardium-specific NOS1 overexpression was associated with a decrease in Ca^{2+} current density, $[Ca^{2+}]_i$ transient amplitude, SR Ca^{2+} load, and cell shortening.[28] Surprisingly,

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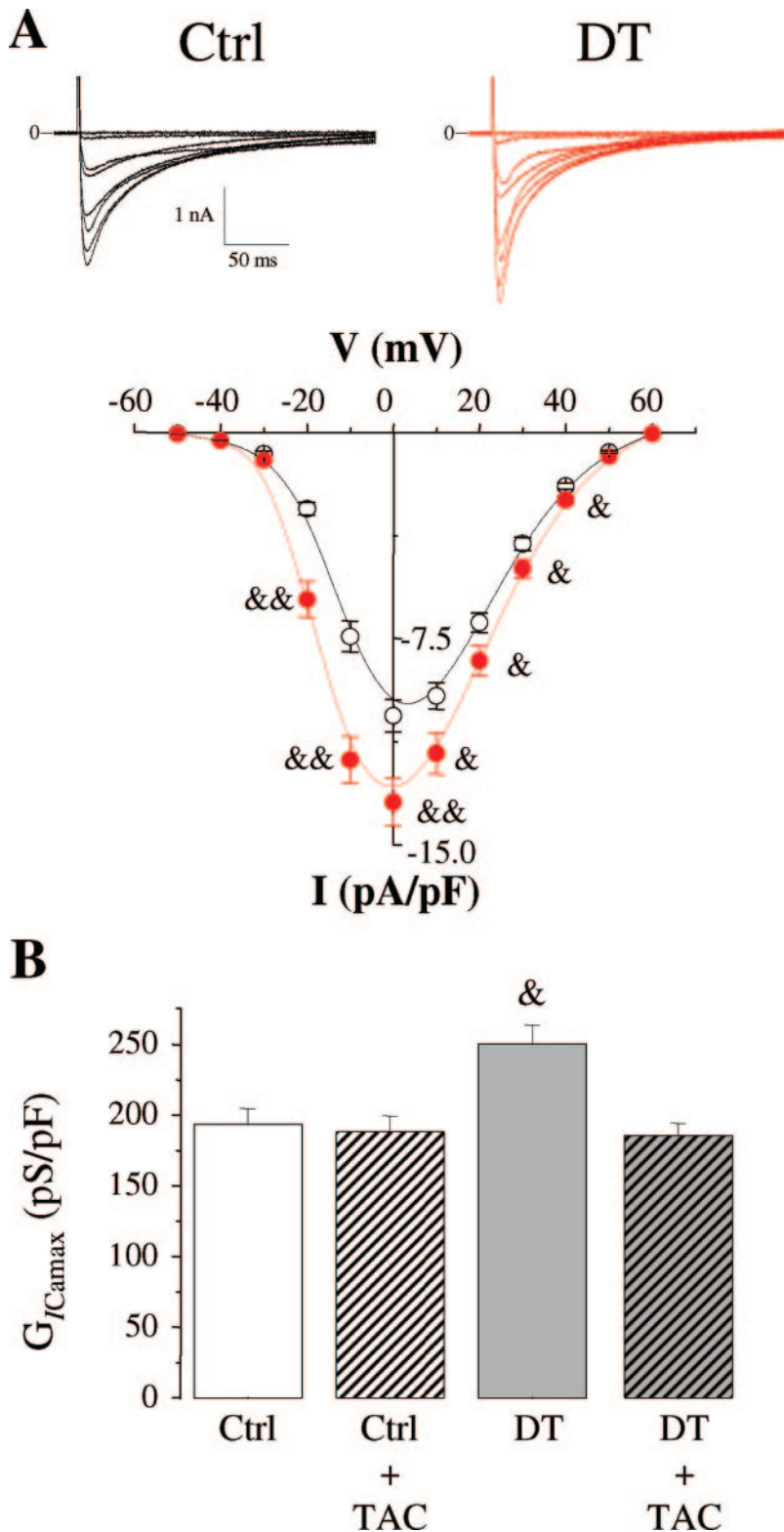
Figure 7. A, Representative I_{CaL} recording and plots of average I_{CaL} density vs membrane potential obtained from cardiomyocytes from control (Ctrl) and DT mice. B, Bar graphs corresponding to pooled maximal conductance of I_{CaL} (n=15 to 20 cells from 4 animals in each group). &$P < 0.05$ vs Ctrl.

those authors[28] demonstrated that relaxation time (50%) in the experiments for myocyte shortening was significantly prolonged in NOS1-overexpressing mice, a result similar to that obtained with NOS1$^{-/-}$ mice.[12] Given that these authors used the same isoform of NOS1 (the α-isoform) to generate their mice as used in the present study, potential explanations for the opposite results could be the background of the mice used or the

subcellular localization of the NOS1 transgene. As described previously, we found that NOS1 was both localized at the SR, where it physically interacted with RyR2, and at the sarcolemma, where it interacted with Cav-3. However, Burkard and coworkers[28] demonstrated interactions of NOS1 with SERCA2a and the L-type Ca²⁺ channel but were unable to find any interaction with RyR2.
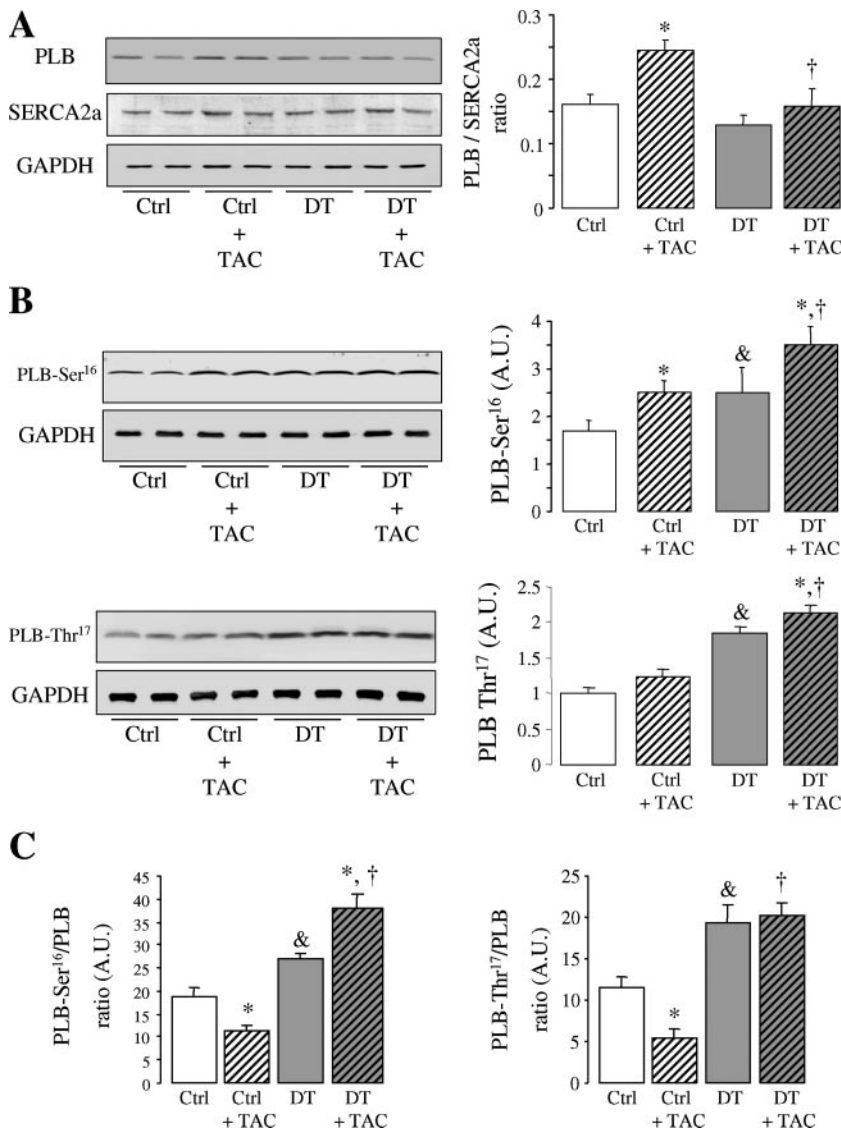
Figure 8. A, Representative Western blots showing the response of PLB and SERCA2a protein expression to TAC in both genotypes and quantification results for the ratio PLB/SERCA2a (n=6 to 8 per group). Relative quantification demonstrated a higher PLB/SERCA2a ratio in Ctrl+TAC, which could account for the decrease in calcium SR load and decrease in cardiomyocyte contractility. Cardiomyocyte overexpression of NOS1 normalizes this ratio, which could account for the preserved function in DT+TAC. B, Western blot analysis and corresponding data of PLB phosphorylation state with site-specific antibodies (Ser16- and Thr17-site phosphorylation). C, PLB-Ser16 and PLB-Thr17 phosphorylation state-specific band intensity normalized to PLB abundance. &$P<0.05$ vs Ctrl; *$P<0.05$ vs basal; †$P<0.05$ vs Ctrl+TAC.

NOS1 Overexpression Prevents Cardiac Dysfunction and Delays HF in Response to Pressure Overload

During the last few years, emerging data suggest that NOS1 may have important functions in the pathophysiology of adverse cardiac remodeling. Here, we provide the first evidence that NOS1-overexpressing hearts undergo further hypertrophy, retaining concentric LV remodeling and full contractile function, whereas wild-type mice showed pronounced chamber dilation and impaired contractility in response to chronic pressure overload. Consistent with an adaptive role for myocardial NOS1 overexpression, recent studies found that NOS1^{-/-} mice developed more pronounced LV dilation after myocardial infarction than control mice.[19,20] In contrast, NOS1 overexpression was recently associated with an increase in cardiac hypertrophy and a failing cardiac phenotype, because these mice are characterized by increased LV dilation associated with decreased LV ejection fraction.[28] However, the subcellular mechanisms underlying these in vivo findings were not elucidated.

Sustained cardiac hypertrophy typically progresses to cardiac dilatation and failure, conditions associated with sub-

stantial morbidity and mortality. Although hypertrophy per se has been proposed as an early therapeutic target, the blockade of the growth response is not always beneficial,[29–31] and importantly, inhibition of the growth response is not absolutely required to ameliorate cardiac dysfunction.[32,33] Recent studies have suggested that the critical factor causing cardiac decompensation after pressure overload may be the failure to preserve calcium homeostasis and thus myocardial inotropy. Indeed, defects in SR Ca^{2+} cycling are a highly conserved signature feature of experimental and human HF, and the most notable abnormalities are a decrease in Ca^{2+} reentry into the SR, which is predominantly governed by the activity of SERCA2a and dysregulated Ca^{2+} release through RyR2. In both cases, the SR Ca^{2+} level is diminished in failing hearts, which creates defects in both systolic and diastolic function. In many forms of human and experimental HF, the downregulation of SERCA2a expression level, impairment in the SERCA2a/PLB ratio, and a decrease in phosphorylated PLB have been documented. In general, genetic manipulations that improve Ca^{2+} cycling either by SERCA2a gene therapy or PLB ablation have been shown to be beneficial in preventing

cardiac dysfunction in both transgenic and surgically induced HF models.[34,35] The present results in NOS1 transgenic mice are consistent with the idea that NOS1 may prevent adverse remodeling and cardiac deterioration by preserving calcium cycling and contractile function even though the extent of hypertrophy is increased in response to pressure overload. The improvement in contraction appears to be mainly attributable to higher SR Ca²⁺ loading in NOS1-overexpressing hearts, as a consequence of a preserved PLB/SERCA2a ratio and increased phosphorylated PLB level compared with control mice. A similar result was recently described by Chohan and coworkers,[36] who demonstrated that L-arginine administration prevented contractile dysfunction in ischemic reperfused hearts, which was associated with a recovery of SR function and SR regulation by PLB phosphorylation. This effect was mainly attributable to myocyte NOS1. In agreement with our findings, SERCA2a overexpression has been shown to be protective against the pressure-overload–induced decrease in contractile function, maintaining contractile properties and Ca²⁺ homeostasis in cardiac myocytes despite significant hypertrophy.[36–38] It is of interest that the hypertrophic response was even more pronounced in banded transgenic animals than in controls.

In conclusion, we demonstrated that conditional transgenic overexpression of NOS1 may enhance basal cardiac contractility, as well as play a significant role in maintaining cardiac contractility and calcium handling during chronic pressure overload. These findings strongly support the hypothesis that cardiomyocyte NOS1 might play an important role in cardiac protection and suggest that NOS1 could represent a new means of targeting Ca²⁺ regulatory proteins in the setting of chronic pressure-overload–induced HF.

Disclosures
None.

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CLINICAL PERSPECTIVE

Sustained cardiac hypertrophy typically progress to cardiac dilatation and failure, conditions associated with substantial morbidity and mortality. The prevention of this deterioration is a major therapeutic goal. Recent studies have suggested that a critical factor causing cardiac decompensation after pressure overload may be the failure to preserve calcium homeostasis and thus myocardial inotropy. It is well established that the neuronal (NOS1) isoform of nitric oxide synthase facilitates Ca^{2+} handling that is central to excitation-contraction coupling. However, the impact of NOS1 on myocardial contractility and Ca^{2+} cycling remains controversial, and the potential relevance of this mechanism in HF is unknown. Here, we generated a transgenic mouse with conditional, cardiomyocyte-specific NOS1 overexpression (DT) and studied cardiac remodeling, myocardial Ca^{2+} handling, and contractility in DT and control mice subjected to transverse aortic constriction (TAC). After TAC, control mice developed eccentric hypertrophy with evolution toward HF as revealed by a significantly reduced fractional shortening. In contrast, after TAC, DT mice developed a greater increase in wall thickness and less left ventricular dilatation. Thus, DT mice displayed concentric hypertrophy with fully preserved fractional shortening. The improvement in contraction appears to be due mainly to greater sarcoplasmic reticulum Ca^{2+} loading in the NOS1-overexpressing DT hearts. This enhanced Ca^{2+} sarcoplasmic reticulum loading was a consequence of a preserved ratio of phospholamban to sarcoplasmic reticulum Ca^{2+} ATPase2a and an increased level of phosphorylated phospholamban. These findings support the hypothesis that cardiomyocyte NOS1 plays an important role in cardiac protection. They also suggest that NOS1 could represent a new means of targeting Ca^{2+} regulatory proteins in the setting of chronic pressure-overload HF.