Conditional Neuronal Nitric Oxide Synthase Overexpression Impairs Myocardial Contractility

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Abstract—The role of the neuronal NO synthase (nNOS or NOS1) enzyme in the control of cardiac function still remains unclear. Results from nNOS"−/−" mice or from pharmacological inhibition of nNOS are contradictory and do not pay tribute to the fact that probably spatial confinement of the nNOS enzyme is of major importance. We hypothesize that the close proximity of nNOS and certain effector molecules like L-type Ca2+-channels has an impact on myocardial contractility. To test this, we generated a new transgenic mouse model allowing conditional, myocardial specific nNOS overexpression. Western blot analysis of transgenic nNOS overexpression showed a 6-fold increase in nNOS protein expression compared with noninduced littermates (n=12; P<0.01). Measuring of total NOS activity by conversion of [3H]-l-arginine to [3H]-l-citrulline showed a 30% increase in nNOS overexpressing mice (n=18; P<0.05). After a 2 week induction, nNOS overexpression mice showed reduced myocardial contractility. In vivo examinations of the nNOS overexpressing mice revealed a 17±3% decrease of +dp/dtmax compared with noninduced mice (P<0.05). Likewise, ejection fraction was reduced significantly (42% versus 65%; n=15; P<0.05). Interestingly, immunoprecipitation experiments indicated interaction of nNOS with SR Ca2+-ATPase and additionally with L-type Ca2+-channels in nNOS overexpressing animals. Accordingly, in adult isolated cardiac myocytes, Ical density was significantly decreased in the nNOS overexpressing cells. Intracellular Ca2+-transients and fractional shortening in cardiomyocytes were also clearly impaired in nNOS overexpressing mice versus noninduced littermates. In conclusion, conditional myocardial specific overexpression of nNOS in a transgenic animal model reduced myocardial contractility. We suggest that nNOS might suppress the function of L-type Ca2+-channels and in turn reduces Ca2+-transients which accounts for the negative inotropic effect. (Circ Res. 2007;100:e32-e44.)

Key Words: nNOS ■ contractility ■ excitation ■ contraction coupling ■ conditional overexpression

Several studies have demonstrated neuronal NO synthase (nNOS) protein expression within cardiac myocytes. Specifically, nNOS has been localized to the sarcolemma and the sarcoplasmatic reticulum (SR), where it has been shown to be in close proximity to the SR Ca2+-release channel (RyR2) and the SR Ca2+-ATPase. However, the impact of nNOS on myocardial contractility remains largely controversial. Results from nNOS"−/−" mice and from pharmacological inhibition of nNOS provided insights into the role of nNOS in the cardiovascular system. But these approaches suffer from incomplete nNOS blockade and did not take into account a possible translocation of nNOS to specific subcellular sites. Some authors have shown, that inhibition of nNOS activity, via gene disruption or by pharmacological inhibition, enhanced basal contractility. In the latter study, the positive inotropic effects of nNOS inhibition or gene disruption were related to the effects of nNOS on intracellular Ca2+-handling. Both Ical and SR Ca2+-load were enhanced when nNOS was disabled. In contrast, others did not demonstrate any difference in basal contractility of left ventricular myocytes between nNOS"−/−" and C57BL/6 control mice and did not observe any alterations in Ca2+-transient amplitudes or SR Ca2+-load.

Similarly, nNOS has been shown to enhance, inhibit or not to affect SR Ca2+-release by the RyR2. The controversy continues in animal models of myocardial infarction. Dawson et al demonstrated that nNOS protects against adverse postinfarction remodeling and that infarcted nNOS"−/−" mice responded to dobutamine with a significant decrease in LV systolic function. Of note, and contrary to the findings of

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Dawson, Bellan et al\textsuperscript{13} found an increase in $\beta$-AR response after preferential nNOS inhibition in infarcted rats. They initially described an upregulation of cardiomyocyte nNOS.

To contribute the understanding of nNOS in the myocardium and to investigate whether there are direct actions of nNOS in the myocyte, independently of autonomic effects, we here introduce a new transgenic mouse model for conditional overexpression of nNOS. We hypothesize that overexpression of nNOS influences myocardial performance in mice mainly by affecting EC coupling and myocardial contractility.

**Materials and Methods**

**Transgenic Mouse Model**

To generate transgenic mice the Tet-Off system (BD Biosciences, Heidelberg, Germany) was used.\textsuperscript{14,15} For this system 2 different mouse strains were used. First strain encodes for the regulatory protein tTA (tetracycline-controlled transactivator) under control of the $\alpha$-MHC promoter (FVB/N-TgN\[MHCATTA\], JAXmice, Jackson Laboratory, Maine). The second strain contains the gene of interest (nNOS) under control of the tetracycline-responsive element (TRE).

To establish this strain, the nNOS\textsubscript{cm} cDNA was cloned into the pTRE-6xHXN vector (BD Bioscience). Importantly, this isoform contains exon 2, encoding for the nNOS PDZ-domain. Offspring mice were screened for founders by PCR. Two different primer pairs were used for genotyping. By the Tet-Off primer pair, Tet-Off-forward: 5'-GTC AGT CGA GTG CAC AGT TT-3' and Tet-Off-reverse: 5'-CAA ATG TTT CTG TGG TG-3', a 200bp fragment within the tTA-region was amplified. The nNOS primer pair, nNOS-reverse: 5'-GAG ATG ATC ACG GGA GGC-3' and nNOS-forward: 5'-CGG CTT GAG GAG CCA TCC-3' amplified a 270bp fragment. This product contained both a part of the nNOS sequence and the 6xHXN tag. Double transgenic mice were positive in both reactions.

**Conditional Cardiac Specific nNOS Expression**

Following CO\textsubscript{2}-induced euthanasia, hearts were rapidly excised, rinsed in phosphate buffered saline (PBS), pH 7.4 and frozen in liquid nitrogen. Frozen hearts were homogenized in RIPA-buffer (1x PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, all Sigma). Proteins were transferred electrophoretically onto nitrocellulose membrane, which immersed in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol, 0.037% SDS). Protein samples were probed with purified mouse monoclonal anti-nNOS antibody (Zymed, Cat.No 61–7000) for 1 hour followed by horseradish peroxidase-conjugated anti-mouse IgG 1:5.000 (GE Healthcare) for 1 hour. Bands were visualized by enhanced chemiluminescence (GE Healthcare). Induction of nNOS overexpression by removal of doxycycline (DOX) was performed 14 days before experiments were started. The nNOS protein expression is given as total amount of nNOS (nNOS\textsubscript{cm} + nNOS\textsubscript{at}).

**NOS Activity Assay and cGMP Assay**

Total NOS activity in hearts were determined by measuring the conversion of [H]-L-arginine to [H]-L-citrulline using the NOS Assay Kit (Stratagene). Frozen hearts were homogenized in 10x volumes of ice-cold 1x homogenization buffer using a tissue grinder. After centrifugation at 14.000g and 4°C for 5 minutes, 10 $\mu$L of the supernatant was incubated in 40 $\mu$L reaction mixture (2x reaction buffer, 10 mmol/L NADPH (Sigma), [H]-L-arginine (1$\mu$Ci/\(\mu$L) (GE Healthcare), 6 $\mu$L CaCl\textsubscript{2}, dH\textsubscript{2}O). To measure the ratio of arginine to citrulline, 400 $\mu$L elution buffer was added to each spin column and centrifuged in a microcentrifuge at full speed for 1 minute. Each eluate was transferred to scintillation vials and the radioactivity was quantified in a liquid scintillation counter. Left ventricular cGMP levels were determined with a cGMP immunoassay (R&D Systems, Wiesbaden, Germany).

**Immunostaining and Coimmunoprecipitation Experiments**

The adult isolated cells were plated on laminin-coated slides (BD Biosciences) and rinsed in 0.1 mol/L phosphate buffer (19 mmol/L Na\textsubscript{H}PO\textsubscript{4}, 80 mmol/L Na\textsubscript{H}PO\textsubscript{4}). After fixation and washing in 0.1 mol/L TB buffer (84 mmol/L Trizmamahydrochloride, 16 mmol/L Trizma Base and 0.1 mol/L TBSS (TB + 150 mmol/L Na\textsubscript{Cl}; all Sigma) the cells were incubated for 30 minutes in 2% avidin and 2% biotin (both Vector Laboratories). The polyclonal anti-nNOS antibody 1:200 (Zymed, Cat.No 61–7000) was used overnight at 4°C in 0.1 mol/L TBS, 0.075% Triton X, 1% goat serum. Next day cells were probed with biotinylated anti-rabbit IgG 1:300 and finally mounted with Vectashield (both Vector Laboratories; Linaris GmbH, Wertheim, Germany). Further antibodies were used: anti-SERCA2a (Biomol, Cat.No SA-209), anti-Ca,1,2 (alobane labs, Cat.No ACC-003), anti-PMCA4 (Dunn, Cat.No SM5057P), anti-RyR2 (Santa Cruz, Cat.No sc8170), anti-phospholamban (A01–10, Badrilla) and antiphospho-PLB (Ser16)(A10–12, Bardilla). For assessment of expression of $\beta$-MHC expression, frozen hearts were homogenized in MHC-buffer (0.3 mol/L Na\textsubscript{Cl}, 0.1 mol/L Na\textsubscript{H}PO\textsubscript{4}, 50 mmol/L Na\textsubscript{H}PO\textsubscript{4}, 10 mmol/L Na-Pyrophosphate, 1 mmol/L MgCl\textsubscript{2}, and 10 mmol/L EDTA). 500 ng of each sample were separated on 5% SDS-PAGE (3.3% glycerole, 0.5 mol/L EDTA, 1 mol/L glycine). Within the electron microscopy the polyclonal anti-nNOS antibody (Zymed, Cat.No 61–7000) was used, following a second antibody coupled to 12 nm gold particles. Tissue was fixed with 4% paraformaldehyde.

**Hemodynamic and Echocardiographic Measurements**

Echocardiography was performed on a Toshiba Power Vision 6000 system with a 15-MHz ultrasound probe under general anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% wt/vol, 6 $\mu$L/g body weight i.p.) under spontaneous respiration. 2D left-parasternal short-axis views at the level of the papillary muscles were recorded. LV end-systolic and end-diastolic areas were calculated by manual tracings of the endocardial border followed by planimetry with the NICE software package (Toshiba Medical Systems). Simultaneous translversal M-mode tracings were recorded with the cursor placed in the middle of the LV cavity. Hemodynamic measurements were performed according to published protocols under light isoflurane anesthesia and spontaneous respiration (isoflurane 1.5vol% supplemented by 0.5L of oxygen per minute). LV pressure curves were recorded after catheter placement in the LV cavity; systolic and diastolic blood pressure measurements were obtained on catheter withdrawal in the thoracic aorta. LV performance was assessed by ejection fraction (EF). LV preload was assessed by end-diastolic pressure (LVEDP, mm Hg), contractility by $+dP/dt\text{max}$ (mm Hg/sec) and $+dP/dt\text{min}$ (mm Hg/sec); the time constant of isovolumic relaxation (r, msec) was also determined.\textsuperscript{16} Dobutamine was administered IV using a jugular vein.

**Electrophysiological Recordings of $I_{Ca,L}$**

Ventricular myocytes were isolated from adult induced and noninduced mice, as described.\textsuperscript{17} Whole-cell calcium currents ($I_{Ca,L}$) were recorded at room temperature (21°C) from rod-shaped, striated cells within 4 hour of isolation. Myocytes were allowed to settle for 5 minutes before being superfused with external solution at a rate of 1 mL/min. The control superfusion solution (external) and the pipette filling solution (internal) were designed to block K\textsuperscript{+}-currents. The external solution contained 107.1 mmol/L Na\textsubscript{Cl}, 30 mmol/L Cs\textsubscript{Cl}, 1.8 mmol/L MgCl\textsubscript{2}, 1.8 mmol/L CaCl\textsubscript{2}, 4 mmol/L Na\textsubscript{H}CO\textsubscript{3}, 5 mmol/L glucose, 5 mmol/L sodium pyruvate, 10 mmol/L HEPES and 0.8 mmol/L Na\textsubscript{H}PO\textsubscript{4}\textsubscript{2}H\textsubscript{2}O, to pH 7.4 with NaOH. The internal solution contained 119.8 mmol/L Cs\textsubscript{Cl}, 4 mmol/L MgCl\textsubscript{2}, 5 mmol/L creatine phosphate disodium salt, 3.1 mmol/L Na\textsubscript{ATP}, 0.42 mmol/L Na\textsubscript{GTP}, 5 mmol/L EGTA, 62 mmol/L CaCl\textsubscript{2} and 10 mmol/L HEPES, to pH 7.2 with CsOH. Pipette resistance was 1 to 2 mol/L.
when filled with internal solution. Cell capacitance was calculated by applying a 10mV step from –50 to –40mV and integrating the current required to charge the membrane when stepping back to –50mV. Currents were recorded after compensating the cell and electrode capacitance and series resistance with an EPC-9 patch-clamp-amplifier controlled by the Pulse® software (HEKA, Lambrecht, Germany). Cells were voltage-clamped at a holding potential of –50mV, to inactivate cardiac Na\(^{+}\)/H\(^{+}\) channels, and 200ms depolarizing pulses in 5mV steps were applied. peak current was measured at 0mV.

Intracellular Ca\(^{2+}\)-Transients

Shortening and [Ca\(^{2+}\)]\(_{i}\) measurements were conducted simultaneously and performed as reported previously. Myocytes were loaded with fluo-3 by incubation with 10 \(\mu\)mol/L of the acetoxymethyl ester (AM) form of the dye for 15 minutes at room temperature in darkness. The dye was excited with a wavelength at 480 nm using a 75W xenon arc lamp (Ushio, Japan) on the stage of a Nikon Eclipse TE200-U inverted microscope. Emitted fluorescence was measured using a photomultiplier (at 535 nm; IonOptix Corp, Milton, Mass). From the raw fluorescence, \(F/F_0\) was calculated by dividing through the baseline fluorescence (\(F_0\), after subtraction of background fluorescence. Myocytes were field-stimulated (voltage 25% above threshold) at 0.5Hz and 37°C until steady-state was achieved and only those cells exhibiting stable steady-state contractions were included in the study. Cells were transilluminated by red light (\(\lambda\) 650 nm, to avoid interference with fluo-3 epifluorescence measurement), and shortening was measured using a sarcomere length detection system (IonOptix Corp, Milton, Mass). After steady-state conditions were achieved, stimulation frequency was increased stepwise from 0.5Hz to 1, 2, and 3Hz. Post-rest measurements were performed by arresting field-stimulation for 10s after steady-state contractions at 1Hz had been acquired. Caffeine-induced Ca\(^{2+}\)-tracings were assessed by local application of a 10 mmol/L caffeine pulse. In a subset of myocytes, pre-incubation was performed with the specific nNOS inhibitor L-VNIO (100 \(\mu\)mol/L, Alexis Biochemicals, Germany) and myocytes were also superfused during the whole functional measurements.

Statistics
All data are presented as mean±SEM. Statistical analyses were performed using Student’s \(t\) test or the two way ANOVA for repeated measurements where appropriate. Significance was assigned as a value of \(P<0.05\) (*) and \(P<0.01\) (**). No significant differences were given as n.s.

Results

Generation of Double Transgenic nNOS Mice
Single cell pronuclei injections with pTRE-6xHNI-nNOS (Figure 1A) produced 4 transgenic positive founders. These founders transmitted the nNOS transgene to offspring and did not exhibit any developmental abnormalities. In the absence of the \(\alpha\)MHC-tTA transgene there was no increase in nNOS expression and no prove of 6xHN of protein expression in nNOS\(^{-}\) or nNOS\(^{+/+}\) mice in the myocardium or in other tissues of the respective mice (data not shown).

nNOS Protein Is Functionally Expressed in Double Transgenic Mice
Heterozygous or homozygous pTRE-6xHNI-nNOS mice were crossed with \(\alpha\)MHC-tTA mice, and the expression of the

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**Figure 1.** A, Schematic drawing of the pTRE-6xHN-nNOS vector. The TRE is located upstream of the P\(_{\text{minCMV}}\) (minimal immediate early promoter of cytomegalovirus), which is silent in the presence of doxycycline. The tTA binds the TRE, and thereby activates transcription of the gene of interest (nNOS), in the absence of doxycycline, tTA, tetracycline-responsive transcriptional activator; TRE, tetracycline-responsive element. B, Western blot analysis of whole tissue homogenates from nNOS\(^{-}\) / \(\alpha\)MHC-tTA\(^{+}\) mice. Conditional nNOS overexpression was restricted to the myocardium. Non-induced animals (\(-\)) were kept on DOX to suppress conditional nNOS expression, (\(+\)) indicates animals that overexpressed nNOS. There was no difference concerning nNOS expression in other tissues except the heart. The nNOS antibody was immunoreactive to nNOS\(_{\text{H}}\) (~155 kDa) and to nNOS\(_{\text{L}}\) (~164 kDa), nNOS\(_{\text{H}}\) was the conditional overexpressing isoform. Mouse brain tissue was taken as control. C, Total myocardial nNOS protein expression was increased more than 6-fold in the nNOS overexpressing animals. D, Myocardial protein levels of eNOS and iNOS were not altered in nNOS overexpressing mice. GAPDH was used as loading control.
Conditional Myocardial nNOS Overexpression

A.

nNOS activity

B.

cGMP levels

Figure 2. A, In nNOS+/αMHC-tTA+ transgenic mice nNOS overexpression increased total NOS activity as measured by the conversion of [3H]-labeled L-arginine to L-citrulline. B, Conditional nNOS overexpression also resulted in an increase in cardiac cGMP concentration.

Subcellular Localization of nNOS

To determine the subcellular localization of nNOS protein in the hearts of nNOS+/αMHC-tTA+ mice, immunostaining was performed. nNOS immunoreactivity displayed a striated pattern which was similar to immunostainings for the SERCA and the L-type Ca2+-channel (supplemental Figure I of the online data supplement available at http://circres.ahajournals.org). However, to assign nNOS to SERCA or the L-type Ca2+-channel the resolution in immunohistochemical studies was not sufficient because of the close proximity of both structures. Therefore, we performed additional electron microscopic studies. In the non-induced animals nNOS was found at the sarcoplasmic reticulum whereas in the nNOS overexpressing mice nNOS was additionally localized at the sarcolemma (Figure 3A). To confirm these findings and to specifically assess the subcellular localization of nNOS, we studied protein-protein interactions between nNOS and SERCA2a or the L-type Ca2+-channel, respectively (Figure 3B). Total protein extracts from noninduced

NOS Activity: L-Citrulline Production and cGMP Assay

To quantify levels of cardiac NOS activity, we measured the conversion of [3H]-labeled L-arginine to L-citrulline. In nNOS+/αMHC-tTA- transgenic mice there was a robust increase in NOS activity after removal of DOX compared with control mice with persistent DOX diet (22±1.3 versus 29±1 μmol/L/sec., n=18, P<0.05). These data demonstrate that conditional myocardial induction of nNOS overexpression resulted in a clear increase in total NOS activity (Figure 2A).

Altered nNOS overexpression in hearts from nNOS+/αMHC-tTA- mice was also correlated with changed cardiac cGMP levels (Figure 2B). After removal of DOX, cGMP concentration in the nNOS+/αMHC-tTA- myocardium was increased compared with noninduced nNOS+/αMHC-tTA- mice (0.75±0.045pmol/mg versus 0.445±0.036pmol/mg, n=17, P<0.05).

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nNOS+/αMHC-tTA+ hearts that were immunoprecipitated with an anti-nNOS antibody exhibited immunoreactivity with SERCA2a but not with the L-type Ca2+-channel. In the nNOS overexpressing group there was coreactivity of nNOS and SERCA2a similar to the results from the noninduced nNOS+/αMHC-tTA+ hearts. However, there was additional coprecipitation of nNOS with the L-type Ca2+-channel, indicating preferentially colocalization of the overexpressed nNOS in close proximity to the L-type Ca2+-channel at the T-tubular system/sarcolemma.

Finally, immunoprecipitation with anti-PMCA4b in crude preparations revealed significant immunoreactivity with nNOS in the myocardium which was pronounced in nNOS overexpressing cells (supplemental Figure IIA of the online data supplement available at http://circres.ahajournals.org). There was no immunoreactivity detected between nNOS and the RyR2 (Figure IIB of the online data supplement).

Echocardiography and Markers of Hypertrophy

To analyze the consequences of nNOS overexpression on cardiac function we performed echocardiography of the
noninduced and nNOS overexpressing nNOS\(^{+/a}\)MHC-tTA\(^+\) mice. Removal of DOX and therefore nNOS overexpression resulted in a significant increase in LVEDD (4.1 ± 0.3 mm in nNOS overexpressing mice versus 3.1 ± 0.4 mm in noninduced mice, n = 6 for each group, P < 0.05) (Figure 4).

Similarly, LVESD was increased significantly (2.5 ± 0.1 mm in nNOS overexpressing mice versus 2.0 ± 0.2 mm in noninduced mice, n = 12 for each group, P < 0.05, data not shown). The induced nNOS overexpression also significantly increased heart/body weight ratio (12.9 ± 0.9 versus 5.6 ± 0.4 mg/g, n = 18, P < 0.01) and expression of β-myosin heavy chain (β-MHC) as shown in Figure 4B and C (12 ± 3% versus 0%, n = 12, P < 0.01, β-MHC expression is given in percentage of total MHC expression). Myocyte size as measured in isolated ventricular myocytes was increased significantly in nNOS overexpressing myocytes (4009 ± 451 μm\(^2\) versus 3356 ± 245 μm\(^2\), n = 42, P < 0.05). All changes were completely reversible when nNOS\(^{+/a}\)MHC-tTA\(^+\) mice were rescued and nNOS overexpression was stopped. Over an observation period of 6 months only one animal of each group (noninduced versus nNOS-overexpressing) died of 16 animals in the noninduced group and 20 animals in the nNOS-overexpressing group.

**Hemodynamics**

Under basal conditions, noninduced nNOS\(^{+/a}\)MHC-tTA\(^+\) mice showed a higher LV inotropy than their nNOS overexpressing littermates, as evaluated by LVEF, \(+\text{dp/dt}_{\text{max}}\) and \((\text{dp/dt}_{\text{max}})/\text{IP}\) (Table 1). The LV response to β-adrenergic stimulation with dobutamine (at 16 ng/g/min) was also significantly blunted in nNOS overexpressing mice. LV relaxation did not differ significantly under basal conditions and during dobutamine infusion between nNOS overexpressing and noninduced animals as shown by unchanged values for \(-\text{dp/dt}_{\text{max}}\) and \(\tau\) (Table).

Figure 5A and 5B show representative LV pressure-volume (pv) loops from a noninduced nNOS\(^{+/a}\)MHC-tTA\(^+\) mouse and
Measurement of L-Type Ca\(^{2+}\)-Current in Isolated Ventricular Myocytes

We performed whole cell patch-clamp measurements of L-type Ca\(^{2+}\)-currents (\(I_{\text{Ca,L}}\)) in noninduced animals and in response to nNOS overexpression. Representative current families (current/voltage relationship) are presented in Figure 6A. \(I_{\text{Ca,L}}\) density, which represents the ratio of \(I_{\text{Ca,L}}\) amplitude to cell capacitance was significantly decreased in the nNOS over-expressing cardiomyocytes (Figure 6B and C). \(I_{\text{Ca,L}}\) density was 5.9±0.6pA/pF in noninduced myocytes (n=12) and versus3.6±0.4pA/pF in nNOS overexpressing myocytes (n=13; \(P<0.05\)).

[Ca\(^{2+}\)]\(_e\), Transients and Myocyte Shortening

[Ca\(^{2+}\)]\(_e\), transients were recorded using epifluorescence microscopy in flou-3–loaded ventricular myocytes simultaneously together with myocyte shortening (Figure 7A). [Ca\(^{2+}\)]\(_e\), transients were significantly decreased in nNOS overexpressing myocytes compared with noninduced controls when stimulated at 1Hz (3.0±0.4F/F\(_0\) versus 2.2±0.2F/F\(_0\) at 1Hz, n=13 for each group, \(P<0.05\)). Addition of the specific nNOS inhibitor L-VNIO (100 μmol/L) to the nNOS overexpressing myocytes rescued the nNOS phenotype in part (2.7±0.2F/F\(_0\) at 1Hz, n=17, \(P=\)n.s. for comparison with noninduced animals). Accordingly, fractional shortening of isolated cardiomyocytes was significantly impaired in nNOS overexpressing cells (7.7±1.3% versus 3.8±0.5% at 1Hz, n=13 for each group, \(P<0.05\)). Again, L-VNIO improved contractility in nNOS overexpressing cardiomyocytes (7.3±0.8% at 1Hz, n=17, \(P<0.05\) for comparison with nNOS overexpressing animals; Figure 7B).

Relaxation time (50%) in the experiments for myocyte shortening was significantly prolonged in nNOS overexpressing myocytes (67±12 versus 45±7ms at 1Hz, n=13 for each group, \(P<0.05\)) and was accelerated in nNOS overexpressing myocytes plus L-VNIO (43±6ms at 1Hz, n=17, \(P<0.05\) for comparison with nNOS overexpressing animals). Similarly, nNOS overexpression prolonged Ca\(^{2+}\) decay (50%) (88±9 versus 76±7ms at 1Hz, n=13 for each group, \(P<0.05\)) with an acceleration caused by L-VNIO (78±6ms at 1Hz, n=17, \(P=\)n.s. for comparison with noninduced animals; Figure 7B).

Post-rest behavior of isolated cardiomyocytes measured as fractional shortening and Ca\(^{2+}\)-transients (Figure 7C) and Ca\(^{2+}\)-traces after 10 mmol/L caffeine application (Figure 7D) as a measure of SR Ca\(^{2+}\)-content demonstrated a significantly reduced SR Ca\(^{2+}\)-content in nNOS overexpressing animals compared with noninduced cardiomyocytes (Figure 7E).

Figure 6A. Hemodynamic Parameters in Noninduced and nNOS Overexpressing Mice

<table>
<thead>
<tr>
<th>Dobutamine</th>
<th>Noninduced</th>
<th>nNOS Overexpressing</th>
<th>Noninduced</th>
<th>nNOS Overexpressing</th>
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<tr>
<td>HR, [bpm]</td>
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<td>dp/dt/IP, [sec(^{-1})]</td>
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<td>LVEDP</td>
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Dobutamine infusion was at a rate of 16 ng/g/min. Abbreviations as in Materials and Methods.* (\(P<0.05\)) for comparisons between noninduced and nNOS overexpressing nNOS\(^{-}\)/\(\alpha\)MHC-tTA\(^{+}\) mice; †, (\(P<0.05\)) for comparisons between basal values and during dobutamine infusion.
We also investigated whether conditional nNOS protein overexpression was associated with alterations in the expression level of other proteins with relevance to Ca\(^{2+}\)-cycling. LTCC protein expression, SERCA2a, the NCX and phospholamban expression were not changed (supplemental Figure III). However, phosphorylation of phospholamban at the Ser16 site was significantly decreased in nNOS overexpressing myocytes (Figure 8).

**Discussion**

The use of pharmacological nNOS inhibitors and the genetic deletion of nNOS has helped to decipher the impact of nNOS on EC coupling and contractility.\(^{19,20}\) However, the impact of subcellular localization may not has been taken into account in these approaches. Accordingly, although the effect of nNOS on cardiac contraction has been extensively studied in recent years, a consensus remains elusive.

Here, we generated and investigated a new transgenic mouse model with conditional myocardial overexpression of nNOS based on the Tet-Off system. In this model overexpression of nNOS deteriorated cardiac function as evidenced by a decrease in cardiac contractility parameters. Heart/body weight ratios and molecular markers of heart failure were also increased in the nNOS overexpressing animals compared with littermate controls. Localization of nNOS in the noninduced nNOS\(^{-/-}\)/αMHC-tTA\(^{-/-}\) mice was mainly restricted to the SR, specifically to the SERCA2a. Otherwise in the nNOS overexpressing animals we found additional nNOS immunoreactivity at the sarcolemma and we were able to demonstrate coreactivity with the L-type Ca\(^{2+}\)-channel. Similarly, Bendall et al\(^{13}\) already described a translocation of nNOS from the SR to the sarcolemma where it colocalizes with caveolin-3 during the development of heart failure. However, in this study nNOS interacted with the RyR2 whereas an interaction with the SERCA2a was not described. Similar changes were also seen in humans with end stage heart failure.\(^{6}\) Nevertheless, colocalization of nNOS with SERCA2a and phospholamban was also described.\(^{4}\)

**nNOS and Cardiac Contractility**

In our study conditional overexpression of nNOS in cardiac myocytes significantly impaired myocardial contractility, although this did not translate into increased mortality. This is in line with previous studies, demonstrating that inhibition of nNOS activity by gene disruption or acute pharmacological blockade enhanced basal contractility\(^{7,8}\) in single cell preparations. An enhanced LV systolic function was also seen in vivo, where LV ejection fraction was higher in nNOS\(^{-/-}\) compared with WT littermates.\(^{8}\) Barouch et al\(^{5}\) also demonstrated higher values for LV dP/dt\(_{max}\)/IP (a left ventricular contractility parameter corrected for instantaneous pressure) in nNOS\(^{-/-}\) mice. However, similar to Khan et al\(^{9}\) these investigators did not observe differences in basal contractility in isolated myocytes of nNOS\(^{-/-}\) and C57BL/6 mice. A major
factor that regulates basal contractility might be S-nitrosylation of ion channels. In this regard S-nitrosylation was reported for the L-type Ca\(^{2+}\)-channel with subsequent inhibition\(^{21}\) and the ryanodine receptor with subsequent activation.\(^{22}\)

**nNOS and Ca\(^{2+}\)-Handling**

The mechanisms underlying the decreased contractility in nNOS overexpressing mice were investigated in detail in isolated ventricular myocytes, where we showed that the negative inotropic effect of myocardial nNOS overexpression was related to a reduced Ca\(^{2+}\)-current. \(I_{\text{Ca,L}}\) was significantly diminished in the presence of overexpressed nNOS. The 30% decreased entry of Ca\(^{2+}\) via \(I_{\text{Ca,L}}\) in nNOS overexpressing myocytes may directly contribute to the decreased contractile performance we observed. Further to this, it has been suggested that nNOS-derived NO increases the activity of Na\(^+\)-K\(^+\)-ATPase pump\(^{2,23}\) which may indirectly affect Ca\(^{2+}\)-fluxes through an action on intracellular Na\(^+\) levels and a resultant change in activity of the Na\(^+\)-Ca\(^{2+}\)-exchanger.

In agreement with our findings Sears and coworkers described an attenuation of L-type calcium currents by nNOS-derived NO.\(^{8}\) Others, however, have observed unchanged L-type currents in cardiomyocytes from nNOS-deficient mice.\(^{5}\)

Beside an attenuation of \(I_{\text{Ca,L}}\) we found a decrease in [Ca\(^{2+}\)]\(_{\text{i}}\) transient amplitudes and a reduction in contractility in iso-
Figure 7. Continued
lated adult cardiomyocytes from nNOS overexpressing mice which may be because of decreased Ca^{2+}-induced Ca^{2+}-release from the SR because of the changes observed in $I_{Ca,L}$. The effects were likely to be related to nNOS, since the specific nNOS inhibitor L-VNIO reversed these findings. Similarly, nNOS overexpression reduced SR Ca^{2+}-content which could be reversed by L-VNIO. In line with our findings a greater SR Ca^{2+}-load and increased [Ca^{2+}], transients in nNOS−/− myocytes were observed. In contrast, Khan et al saw no difference in [Ca^{2+}], transient amplitudes or the SR Ca^{2+}-load in nNOS+/− myocytes compared with C57BL/6 and Barouch et al also failed to demonstrate a difference in cell shortening or Ca^{2+}-transients during β-AR stimulation in nNOS+/− myocytes.

Although coimmunoprecipitation experiments from other groups indicated that nNOS may form a complex with the RyR2, we were not able to demonstrate colocalization of nNOS with the RyR2 which is suggestive of a missing effect.
of nNOS-derived NO on the Ca\(^{2+}\)-release channel. In some other work there was also no effect of NO on basal spark frequency, which is thought to be reflective of activity of the RyR2. Additionally, it has to be taken into account that an isolated effect of NO on the open probability of the RyR has only limited functional significance as such changes result in only transient effects on contraction and therefore may not explain the changes in myocardial contractility we observed.

Another complicating aspect is the observation that in our nNOS-overexpressing model the force-frequency response (FFR) was absent whereas a depressed FFR was already reported in nNOS\(^{-/-}\) mice. Ashley et al reported the complete absence of the FFR in both WT and nNOS\(^{-/-}\) mice.

In our study the SR Ca\(^{2+}\)- load (as primary determinant of the FFR) was decreased in the nNOS-overexpressing mice. This could be sufficiently explained by reduced phosphorylation of phospholamban and the resulting decrease in SERCA2a activity. Another potential explanation for reduced SR Ca\(^{2+}\)-stores could be a RyR leak. Excess NO could increase the open probability of the ryanodine receptor via S-nitrosylation. However, in this study we failed to demonstrate an interaction of the RyR2 with nNOS.

**nNOS and Inotropic Reserve**

In the present study parameters for contractility showed an attenuated response to dobutamine in vivo in nNOS overexpressing transgenic animals. Reduced myocardial contractility has been attributed to an attenuation of L-type Ca\(^{2+}\)-currents by nNOS-derived NO. As suggested by Gallo et al nNOS-derived NO could be acting via the guanylate cyclase/cyclic GMP second messenger pathway. In agreement with these findings, 8-BrcGMP has been shown to inhibit \(I_{\text{Ca,L}}\) in ferret right ventricular myocytes, and the cGMP dependent protein kinase (PKG) has been reported to cause a 40% inhibition of \(I_{\text{Ca,L}}\) in rat ventricular myocytes. Indeed, we found increased cGMP levels in the nNOS overexpressing myocytes. From the inhibitory effect of nNOS on L-type Ca\(^{2+}\)-channel currents, one would predict an attenuation of the \(\beta\)-AR response when nNOS activity is increased. In isolated cardiomyocytes, this has been consistently observed at low catecholamine concentrations, which is in favor of our findings. Again, the results at higher concentrations are divergent in vitro, with a sustained increase in contraction in one study but decreased contraction in another study. More agreement with a consistently decreased inotropic response to \(\beta\)-AR stimulation may be found in vivo.

An alternative explanation that may account for the attenuated response to \(\beta\)-AR stimulation in nNOS overexpressing mice (beside inhibition of \(I_{\text{Ca,L}}\)) might be an impact of nNOS on phosphorylation levels of phospholamban (PLN). We observed a significantly decreased phosphorylation at the Ser16 site of phospholamban when overexpressing nNOS. It is well established that \(\beta\)-adrenergic stimulation phosphorylates PLN and different studies indicated a fundamental role of PLN phosphorylation in the contractile and relaxant effects of \(\beta\)-adrenergic agents. It has been shown that PLN is phosphorylated at Ser16 by cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively). Thus, in theory one would expect increased PLN phosphorylation because of increased cGMP levels. However, PP1 (a serine/threonine phosphatase) dephosphorylates PLN at Ser16 and activation of PP1 with a resulting decrease in the phosphorylation of Ser16 residue of PLN was already observed in the presence of isoproterenol. This would explain decreased SERCA function and SR Ca\(^{2+}\)-content and give an additional reason for the blunted response to \(\beta\)-AR stimulation. Previous studies demonstrated reduced PLN protein expression in (chronic) nNOS\(^{-/-}\) mice but did not assess PLN phosphorphylation. In the setting of heart failure it has been hypothesized that nNOS exerts inhibitory effects on \(\beta\)-adrenergic responsiveness which might be maladaptive. On the other
hand nNOS translocation to the plasmamembrane and concomitant attenuation of β-adrenergic contractility could also be beneficial and prevent adverse remodelling by protecting the myocardium. In this setting nNOS would act as endogenous brake against catecholamine toxicity.

How do the results from our transgenic animal model with conditional overexpression of nNOS that is restricted to the cardiac myocyte fit into the image of nNOS that we have from genetic deletion models and pharmacological inhibition?

Presumably, subcellular localization and translocation during different disease states is of major importance for the functional effects of nNOS. In vivo studies demonstrated that nNOS was localized to the SR. During increased workload or heart failure there was a translocation to the sarcolemma. From this point of view our conditional nNOS model resembles a heart failure model with additional localization of the overexpressed nNOS at the surface membrane. Both the L-type Ca\(^{2+}\)-channel and the PMCA3 seem to be able to interact with nNOS probably via its PDZ domain that tethers the nNOS enzyme to its effector molecules. In this work, we were not able to demonstrate effects from nNOS on PMCA, at least with regard to short term regulation of contractility or beat to beat Ca\(^{2+}\)-regulation. Clearly however, we found an inhibitory effect of abundant nNOS on \(I_{\text{Ca,L}}\) density and Ca\(^{2+}\)-transient amplitudes.

Several studies demonstrated a protective role of nNOS in myocardial infarction,12,13,32 or in ischemia/reperfusion injury.21 The reason for these favorable results may not only be overexpression or (more physiologically) upregulation, but the translocation of nNOS to the plasmamembrane. There, nNOS dependent S-nitrosylation of the L-type Ca\(^{2+}\)-channel inhibited \(I_{\text{Ca,L}}\) in female mice. This reduced Ca\(^{2+}\)-load during ischemia and reperfusion and therefore consequently also reduced ischemia/reperfusion injury.22 S-Nitrosylation of the RyR2 was also described which resulted in activation of this channel22,33 although nNOS was not directly implicated. Following the translocation hypothesis, increasing the distance between nNOS and RyR2 might decrease nitrosylation levels and subsequently decrease the open probability of the RyR2. This might have beneficial effects in ischemia although in this study we were not able to observe effects of nNOS on the RyR2. The idea that nNOS signaling is spatially confined could also be applied to the nitroso-redox equilibrium.34 NO inhibits both the activity of the xanthine oxidoreductase (XOR) which is localized at the SR35 and the NADPH oxidase which is localized at the sarclemma.36 Saraiva demonstrated that mainly NO derived from nNOS inhibited XOR activity. In nNOS−/− mice the increased XOR activity was linked to higher mortality after myocardial infarction.32 Transfer of this data to heart failure models would predict increased XOR activity at the SR because of the potential translocation of nNOS from the SR to the plasmamembrane.

Additionally, recent studies37,38 support the hypothesis that there is also compartmentation of cGMP in cardiomyocytes. Therefore, the specific role of the particulate (pGC) and the soluble guanylyl cyclase (sGC) as important effector mole-


Conditional Neuronal Nitric Oxide Synthase Overexpression Impairs Myocardial Contractility

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Supplementary data

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Titel: Conditional neuronal nitric oxide synthase overexpression impairs myocardial contractility

Authors: Burkard et al.
Supplementary figure 1)

non-induced

nNOS overexpressing

merge L-Type/nNOS

merge SERCA/nNOS

L-type Ca\(^{2+}\)-channel

SERCA

SERCA

nNOS

nNOS
Supplementary figure 1 shows confocal images of adult cardiomyocytes from nNOS+/αMHC-tTA+ mice in the presence (left, non-induced) and in the absence (right, nNOS overexpressing) of DOX; nNOS protein expression is shown by red immunofluorescence. The striated pattern is suggestive of localization of nNOS at the SR or T-tubuli. SERCA2a and L-type Ca\textsuperscript{2+}-channel protein expression is shown by green immunofluorescence, respectively. In non-induced animals merge indicates overlay of nNOS and SERCA2a, in nNOS overexpressing animals merge indicates overlay of nNOS and L-type Ca\textsuperscript{2+}-channel.

Supplementary figure 2A)

Supplementary figure 2 shows coimmunoprecipitation experiments for nNOS and PMCA4b. There is an association between nNOS and PMCA4b in hearts from non-induced animals. In nNOS overexpressing animals there is a stronger immunoreactivity of nNOS with PMCA4b, suggestive of a pronounced compartmentalization of nNOS at the plasmamembrane in the nNOS overexpressing animals. IP: immunoprecipitation; IB: Immunoblotting; neg ctr: IP with an irrelevant antibody
Supplementary figure 2B shows coimmunoprecipitation experiments for nNOS and RyR2. No association between nNOS and RyR2 could be detected in heart lysates from non-induced or nNOS overexpressing animals. Control experiments and abbreviations are the same than in supplementary figure 2A.
Supplementary figure 3)

A) 

**L-type Ca^{2+}-channel (Ca1.2_\gamma subunit)**

<table>
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<th>nNOS overexpressing</th>
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n.s.
B)  

Supplementary data CIRCRESAHA/2006/146027/R1

**NCX expression**

- non-induced
- nNOS overexpressing

Bar graph showing higher NCX expression in nNOS overexpressing compared to non-induced. The difference is not significant (n.s.).

**Western Blot images**

- NCX
- GAPDH
Suppl. figure 3: Representative Western blots for proteins involved in Ca\(^{2+}\)-cycling

Expression of L-type Ca\(^{2+}\)-channels (Ca1.2, subunit) (A), NCX (B) and SERCA2a (C) was not changed in myocardium of non-induced compared to nNOS overexpressing nNOS\(^+\)/\(\alpha\)MHC-tTA\(^+\) mice.