Conditional Overexpression of Neuronal Nitric Oxide Synthase Is Cardioprotective in Ischemia/Reperfusion

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Background—We previously demonstrated that conditional overexpression of neuronal nitric oxide synthase (nNOS) inhibited L-type Ca\(^{2+}\) channels and decreased myocardial contractility. However, nNOS has multiple targets within the cardiac myocyte. We now hypothesize that nNOS overexpression is cardioprotective after ischemia/reperfusion because of inhibition of mitochondrial function and a reduction in reactive oxygen species generation.

Methods and Results—Ischemia/reperfusion injury in wild-type mice resulted in nNOS accumulation in the mitochondria. Similarly, transgenic nNOS overexpression caused nNOS abundance in mitochondria. nNOS translocation into the mitochondria was dependent on heat shock protein 90. Ischemia/reperfusion experiments in isolated hearts showed a cardioprotective effect of nNOS overexpression. Infarct size in vivo was also significantly reduced. nNOS overexpression also caused a significant increase in mitochondrial nitrite levels accompanied by a decrease of cytochrome c oxidase activity. Accordingly, O\(_2\) consumption in isolated heart muscle strips was decreased in nNOS-overexpressing nNOS\(^{+}/\alpha\)MHC-tTA\(^{+}\) mice already under resting conditions. Additionally, we found that the reactive oxygen species concentration was significantly decreased in hearts of nNOS-overexpressing nNOS\(^{+}/\alpha\)MHC-tTA\(^{+}\) mice compared with noninduced nNOS\(^{+}/\alpha\)MHC-tTA\(^{+}\) animals.

Conclusion—We demonstrated that conditional transgenic overexpression of nNOS resulted in myocardial protection after ischemia/reperfusion injury. Besides a reduction in reactive oxygen species generation, this might be caused by nitrite-mediated inhibition of mitochondrial function, which reduced myocardial oxygen consumption already under baseline conditions. (Circulation. 2010;122:1588-1603.)

Key Words: ischemia • mitochondria • nitric oxide synthase • reperfusion

Several studies in recent years have investigated the effects of neuronal nitric oxide synthase (nNOS) exerted on cardiomyocytes, with special emphasis on the intracellular localization of nNOS. However, results from pharmacological inhibition and genetic deletion or from conditional overexpression of nNOS brought divergent results. Interestingly, despite the fact that current studies demonstrated discrepant findings in terms of myocardial contractility and Ca\(^{2+}\) homeostasis, it seems consistent that nNOS is upregulated and is cardioprotective in different models of heart disease.\(^1-3\) In this context, different targets of nNOS relevant in Ca\(^{2+}\) cycling, eg, RyR2,\(^4\) L-type Ca\(^{2+}\) channel,\(^5\) SERCA2a,\(^6\) and PLN,\(^7\) were identified in the past. Additionally, cardioprotective effects have also been ascribed to exert on the cardiac xanthine oxidoreductase (XOR). nNOS suppresses the activity of the cardiac XOR, which is one of the major sources of O\(_2^-\) production in the heart. It has been shown that this targeted inhibition maintains the balance between the production of reactive nitrogen species and reactive oxygen species (ROS)\(^3,8,9\) in cardiac tissues. nNOS, also found in mitochondria,\(^10,11\) inhibits the mitochondrial respiratory chain, resulting in inhibition of ATP production. NO reversibly binds to the oxygen binding site of cytochrome c oxidase, reacting either with the oxidized copper to give inhibitory nitrite or with the reduced heme, resulting in reversible inhibition in competition with oxygen.\(^12\) However, it has also been suggested that NO derived from nNOS does not directly contribute to the inhibition of mitochondrial respiration but rather represents an antioxidant system by inhibiting XOR activity.\(^8\)

Clinical Perspective on p 1603

To elucidate the impact of abundant nNOS on cardiomyocyte function, we assessed the direct and subcellular effects of conditional overexpression of nNOS after ischemia/reperfu-
sion injury. We hypothesized that nNOS acts cardioprotective via a decrease in superoxide formation and differential effects on mitochondrial respiration.

Methods

Animal Model

In brief, the induction of nNOS overexpression by removal of doxycycline was started a minimum of 10 days before any experiment. Animals of both genders were used. The mice used for experiments were <3 months of age. Myocardial nNOS protein expression was increased >6-fold in the overexpressing animals (627±13% versus 100±7%; n=19; P<0.01) as reported earlier. For more details, see the online-only Data Supplement.

Infarct Size Measurement After Ischemia/Reperfusion

Infarct size measurements and ischemia/reperfusion experiments were performed as recently described. After triphenyl tetrazolium chloride staining, viable myocardium stains red and the infarcted areas appear pale. For more details, see the online-only Data Supplement.

In Vitro Ischemia/Reperfusion Experiments With Isolated Hearts

Mice were anesthetized by injection of pentobarbital intraperitoneally. Retrograde perfusion of the heart was started in the Langendorff mode at 37°C at a constant coronary perfusion pressure of 100 mm Hg. Oxygenated Krebs-Henseleit buffer was used for perfusion. A water-filled balloon was inserted into the left ventricle and fixed by a ligature. The balloon was connected to a Statham P23Db pressure transducer (Gould Statham Instruments) for continuous measurement of left ventricular pressure. Hearts were subjected to 20 minutes of ischemia followed by reperfusion. The specific nNOS inhibitor S-methyl-l-thiocitrulline acetate salt (SMTC) was administered at a final concentration of 0.125 mg/kg.

Electron Microscopy With Immunogold Labeling

In brief, for electron microscopy, the excised hearts were fixed in 4% paraformaldehyde and embedded in LR White (Sigma, Munich, Germany). For detection of the transgene nNOS, a polyclonal anti-fxHN antibody 1:50 (BD Biosciences, San Jose, Calif) was used, followed by a secondary antibody coupled to 12-nm gold particles. For detection of all nNOS in electron microscopy, a polyclonal anti-nNOS antibody 1:50 (Zymed) was used, followed by a second antibody that was coupled to 6-nm gold particles; simultaneously, a monoclonal anti–cytochrome c oxidase antibody 1:200 (MitoSciences) was used, followed by a second antibody that was coupled to 12-nm gold particles. For more details, see the online-only Data Supplement.

Immunofluorescence Staining of Isolated Adult Cardiac Myocytes

The isolated adult cardiac myocytes were plated on laminin-coated slides. After fixation, cells were incubated in 2% avidin and 2% biotin. The polyclonal anti-nNOS antibody 1:50 (Zymed) was used overnight. The next day, cells were probed with biotinylated anti-rabbit IgG 1:300 followed by avidin D fluorescence antibody 1:300. The monoclonal anti–cytochrome c 1:100 antibody also was used (MitoSciences).

Protein Expression in Isolated Mitochondria (nNOS, L-type Ca2+ Channel, SERCA2a)

Enriched mitochondrial fraction from mouse heart was prepared according to a modified protocol developed by R&D Systems (Minneapolis, Minn). Mitochondria were further enriched on a gradient of Percoll (See the online-only Data Supplement for more details). For Western blot analysis, mitochondria samples were probed with purified mouse monoclonal anti-nNOS antibody 1:200 (BD Transduction Laboratories), polyclonal anti–L-type Ca2+ channel antibody 1:500 (Alomone Laboratories), or polyclonal anti-SERCA2a antibody 1:5000 (Badrilla). As a loading control, antiprohibitin antibody 1:200 (abcam) was used.

Coimmunoprecipitation Experiments

Frozen hearts were homogenized in radioimmunoprecipitation assay buffer. The coimmunoprecipitation experiments were done with the Immunoprecipitation Starter Pack (GE Healthcare). Protein 300 to 600 μg was used. Antigens were coupled to 2.5 μg purified antibody (anti-nNOS, anti–inducible NOS [iNOS], or anti–endothelial NOS [eNOS]; all Transduction Laboratories). Western blot analysis was performed with monoclonal anti–heat shock protein 90 (HS90) antibody 1:5.000 (abcam).

Transfection of Neonatal Rat Cardiomyocytes and Treatment With Geldanamycin

Isolated neonatal cardiomyocytes were plated in MEM/5 on 6-well plates at a density of 1 million cells per well. Forty-eight hours after preparation, cells were transfected with lipofectamine (Invitrogen, Carlsbad, Calif). The transfection was performed with the nNOS plasmid described earlier. Cells were treated with 2 μmol/L geldanamycin (a specific inhibitor of HS90 activity). After 24 hours, mitochondria were isolated and Western blot analysis was performed. Mitochondria samples were probed with purified mouse monoclonal anti-nNOS antibody 1:200 (BD Transduction Laboratories). Anti–cytochrome c antibody was used as a loading control.

Nitrite Level in Isolated Mitochondria

This measurement was performed with a modified version of the Nitric Oxide Colorimetric Assay Kit from Biomol. See the online-only Data Supplement for more details.

Cytochrome c Oxidase Activity

Cytochrome c oxidase activity was measured with a modified version of the Cytochrome C Oxidase Assay Kit from Sigma. Instead of DTT, 10 mmol/L ascorbate was used to reduce cytochrome c, followed by removal of the reductant by passage through a Sephadex G-25 column. The colorimetric assay in this kit is based on the observation of a decrease in absorbance at 550 nm ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. Whole mouse hearts were homogenized in radioimmunoprecipitation assay buffer, and mitochondria were isolated as described above. Protein concentration was measured, and a final concentration of 0.4 μg/μL was used for the assay. Activity was measured with a kinetic program: 5-second delay, 10-second interval, 6 readings, and room temperature. To investigate whether cytochrome c oxidase activity was inhibited by NO binding to caveolin or to the heme, we added either DTT or hemoglobin. DTT (50 μmol/L) or hemoglobin (4 μmol/L) was added, and measurement of cytochrome c oxidase activity was started immediately.

Myocardial O2− Consumption

O2− consumption measurements were performed as described previously. For more details, see the online-only Data Supplement.

O2− Production in Isolated Mitochondria

O2− production was determined by the oxidation of mito-hydroethidine (Mito-HE/MitoSOX Red; Invitrogen) to 2-hydroxy-mito-ethidium with high-performance liquid chromatography–electrochemical analysis as described previously with some modifications. Mitochondria were isolated from the left ventricle with the Qproteome Mitochondria Isolation Kit (QIAGEN). Isolated mitochondria were incubated with Mito-HE (10 μmol/L), and the conversion to 2-hydroxy-mito-ethidium was monitored by high-performance liquid chromatography–electrochemical analysis.
NADPH Oxidase Activity and Protein Expression of NADPH Oxidase Subunits

NADPH oxidase activity was measured in homogenized hearts (radioimmunoprecipitation assay buffer) by lucigenin-enhanced chemiluminescence. Then, 100 µg protein lysate was incubated with 1 mmol/L lucigenin and 300 µmol/L NADPH, and luminescence was recorded for 20 minutes in 30-second intervals.

Western blot analysis was performed for different subunits of the NADPH oxidase (p47\textsuperscript{PHOX}, p67\textsuperscript{PHOX}, and rac 1). Protein samples were probed with purified mouse monoclonal anti-rac 1 antibody 1:500, purified mouse monoclonal anti-p47\textsuperscript{PHOX} antibody 1:500, or purified mouse monoclonal anti-p67\textsuperscript{PHOX} antibody (all BD Transduction Laboratories). As a loading control, anti-GAPDH antibody 1:8000 (chemicon) was used.

XOR Activity and Protein Expression

The measurement of XOR activity was carried out with the Amplex Red Xanthine Oxidase Assay Kit (Molecular Probes, Carlsbad, Calif; see the online-only Data Supplement). For Western blot analysis of XOR protein expression, samples were probed with purified rabbit polyclonal anti-XOR antibody 1:1000 (abcam). As a loading control, anti-GAPDH antibody 1:8000 (chemicon) was used.

Statistical Analysis

Statistical analysis was performed by use of the statistical software packages SPSS 18.0 and R 2.10.1. For evaluation of statistical significance between 2 groups, the nonparametric Wilcoxon rank-sum test for unpaired samples was used. For comparison between 3 groups, the nonparametric Kruskal Wallis test was performed first, followed by posthoc pairwise comparisons with Bonferroni adjustment. For box plots in figures, the lower and upper bounds of the boxes indicate the 25th and 75th percentile (Q.25/Q.75) values, and the horizontal lines indicate the median. Longitudinal data were analyzed by generalized estimated equation techniques with the gee pack package.

Results

nNOS Reduced Infarct Size In Vivo After Ischemia/Reperfusion Injury

The infarct size within the area at risk was significantly decreased in nNOS-overexpressing nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{+} mice compared with noninduced nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{−} animals (median, 37.5%; Q.25/Q.75, 31.25/40.90%; versus median, 59.65%; Q.25/Q.75, 54.60/63.57%; n = 12; P < 0.0001). Application of SMTC again weakens the positive effects of the transgene nNOS overexpression (estimated average LVDP increase, 0.832 mm Hg/min; n = 12). Recovery of ventricular function was improved over the whole reperfusion period in nNOS-overexpressing animals. Heart rate did not differ significantly between groups (noninduced, 558±33 bpm; nNOS overexpression, 581±27 bpm; nNOS overexpressing plus SMTC, 567±21 bpm; n = 12; P = NS). Rate-pressure product was changed in parallel to LVDP (Figure I in the online-only Data Supplement). Both in vivo and in vitro ischemia/reperfusion experiments showed a cardioprotective effect of conditional myocardial nNOS overexpression.

nNOS Protein Expression in the Mitochondria

To investigate the subcellular distribution of nNOS, we performed immunogold labeling, immunofluorescence staining, and Western blot analysis. Electron microscopy of mouse myocardium from noninduced and nNOS-overexpressing nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{+} animals showed that nNOS is additionall lokalized in mitochondria after the induction of nNOS expression (Figure 2A). Immunogold labeling was positive for the 6xHN tag that indicated conditional nNOS overexpression. Immunogold labeling also showed a close proximity of nNOS and cytochrome c oxidase in the mitochondria (Figure 2B and 2C). We also performed immunofluorescence staining of isolated adult cardiac myocytes (Figure III in the online-only Data Supplement). These images indicated a colocalization of nNOS and cytochrome c oxidase (as mitochondrial marker) in nNOS-overexpressing nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{+} mice.

To confirm these results, we isolated mitochondria from hearts of noninduced and nNOS-overexpressing nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{−} mice. Western blot analysis showed a significantly increased nNOS protein expression in isolated mitochondria of nNOS-overexpressing nNOS\textsuperscript{−/−}/αMHC-tTA\textsuperscript{−} mice (median, 219.70 relative percent, Q.25/Q.75, 200.03/243.98 relative percent versus median, 104.04 relative percent, Q.25/Q.75, 85.58/118.20 relative percent; P < 0.0001; Figure 2D). To test for contamination of the isolated mitochondria, we performed different Western blot analyses of isolated mitochondria. Figure 2E shows no
detectable protein expression of L-type Ca$^{2+}$ channel and SERCA2a in either noninduced nNOS$^+/\alpha$MHC-tTA$^+$ animals or nNOS-overexpressing nNOS$^+/\alpha$MHC-tTA$^+$ mice. For control experiments on contamination, see also Figure IV in the online-only Data Supplement.

Of note, endogenous nNOS is also located at the mitochondria in noninduced nNOS$^+/\alpha$MHC-tTA$^+$ animals, but endogenous nNOS was increased in isolated mitochondria of wild-type mouse hearts after ischemia/reperfusion (median, 306.70 relative percent, Q.25/Q.75, 303.20/312.40 relative percent versus median, 100.70 relative percent, Q .25/Q.75, 95.20/103.20 relative percent; n=7; P=0.002; Figure 2F).

**Translocation of nNOS to Mitochondria**
To analyze the transport mechanism of nNOS into mitochondria, we performed coimmunoprecipitation experiments. Coimmunoprecipitation analysis showed an interaction between nNOS and HSP90 in both noninduced and nNOS-overexpressing nNOS$^+/\alpha$MHC-tTA$^+$ mice. Notably, immunoreactivity in the nNOS-overexpressing mice was stronger than in the noninduced animals. There was no interaction between iNOS and HSP90, whereas the interaction between eNOS and HSP90 again was detectable in both nNOS-overexpressing nNOS$^+/\alpha$MHC-tTA$^+$ and noninduced nNOS$^+/\alpha$MHC-tTA$^+$ mice (Figure 3A). To further assess the mechanism of nNOS translocation, neonatal rat cardiomyocytes were transfected with nNOS and treated with geldanamycin (a specific inhibitor of HSP90 activity). Western blot analyses of isolated mitochondria showed a significant suppression of nNOS protein expression in geldanamycin-treated cardiomyocytes (median, 17.19 relative percent, Q.25/Q.75, 6.25/31.25 relative percent versus median, 100.00 relative percent, Q.25/Q.75, 90.63/109.38 relative percent; n=11; P<0.0001; Figure 3B). Obviously, nNOS is shuttled into the
mitochondria via interaction with HSP90, which was sufficiently inhibited by geldanamycin.

**Nitrite Level of Isolated Mitochondria and Impairment of Cytochrome c Oxidase Activity**

It was previously shown that endogenous NO reversibly inhibits oxygen consumption and ATP synthesis by competitive inhibition of cytochrome c oxidase. Here, we demonstrated that nitrite levels were significantly increased in isolated mitochondria of nNOS-overexpressing nNOS+/αMHC-tTA− mice compared with noninduced littermates (median, 18.65 μmol/L, Q25/Q75, 14.78/19.72 μmol/L versus median, 11.15 μmol/L, Q25/Q75, 10.25/12.10 μmol/L; n=10; P<0.0001; Figure 4A).

Figure 2. A, Electron microscopy of mouse myocardium from nNOS-overexpressing nNOS+/αMHC-tTA− mice and noninduced nNOS+/αMHC-tTA− animals. Immunogold labeling indicates the 6xHN tag of overexpressed nNOS (arrows) and demonstrates nNOS localization. Overexpressed (transgenic) nNOS is additionally localized in mitochondria in hearts of nNOS-overexpressing nNOS+/αMHC-tTA− mice. In contrast, there is no transgene nNOS localization in mitochondria (M) of noninduced animals. B, Immunogold labeling of mouse myocardium from noninduced nNOS+/αMHC-tTA− and nNOS-overexpressing nNOS+/αMHC-tTA− animals. The polyclonal anti-nNOS antibody was used, followed by a second antibody that was coupled to 12-nm gold particles. Endogenous nNOS is located at the mitochondria in both noninduced and nNOS-overexpressing nNOS+/αMHC-tTA− animals.
Simultaneously, nNOS overexpression significantly suppressed cytochrome c oxidase activity (median, 71.50 U/mL, Q₂₅/Q₇₅, 63.93/81.23 U/mL versus median, 111.75 U/mL, Q₂₅/Q₇₅, 108.83/115.48 U/mL; n₁₁₀₀₅; P < 0.0001; Figure 4B) and first-order rate constant (0.0089 first-order rate constant of nNOS-overexpressing mice versus 0.0178 first-order rate constant of nNOS-overexpressing animals; n₇; P < 2e⁻¹⁶; Figure 4C and Table II in the online-only Data Supplement). To investigate whether cytochrome c oxidase activity was inhibited by NO binding to cysteine or to the heme, we added either DTT (which destroyed S-nitrosothiols) or hemoglobin (which scavenged NO of the heme in cytochrome c oxidase).

Application of DTT completely reversed the inhibiting effect of nNOS overexpression (~0.0089 first-order rate constant of nNOS-overexpressing mice versus ~0.0178 first-order rate constant of nNOS-overexpressing animals plus DTT; n₇; P < 2e⁻¹⁶). In contrast, application of hemoglobin further suppressed cytochrome c oxidase activity significantly (~0.00321 versus ~0.0089 first-order rate constant; n₇; P < 2e⁻¹⁶; Figure 4D and Table III in the online-only Data Supplement). Application of DTT had no significant
effect on cytochrome c oxidase activity in noninduced nNOS⁺/αMHC-tTA⁺ mice (−0.01779 first-order rate constant of noninduced mice versus −0.0155 first-order rate constant of noninduced animals plus DTT; n=7; P=0.21). Again, application of hemoglobin suppressed cytochrome c oxidase activity significantly (−0.0057 versus −0.01767 first-order rate constant; n=7; P<2e−16; Figure 4E and Table IV in the online-only Data Supplement). From these experiments, we concluded that inhibition of cytochrome c oxidase activity is inhibited by NO binding to a cysteine thiol of cytochrome c oxidase (rather than binding to the heme).

**Myocardial Oxygen Consumption**

To investigate whether NO derived from nNOS directly affects myocardial oxygen consumption (MV$_\text{O}_2$), we examined the effects of conditional nNOS overexpression on MV$_\text{O}_2$ in isolated muscle strips. Already under resting conditions, we observed a significant inhibition of MV$_\text{O}_2$ in isolated muscle strips from
nNOS-overexpressing nNOS\(^+/\alpha\)MHC-tTA\(^+\) animals compared with noninduced nNOS\(^+/\alpha\)MHC-tTA\(^+\) mice (median, 0.016 mL O\(_2\)·mm\(^{-3}\)·min\(^{-1}\), Q.25/Q.75, 0.015/0.019 mL O\(_2\)·mm\(^{-3}\)·min\(^{-1}\) versus median, 0.024 mL O\(_2\)·mm\(^{-3}\)·min\(^{-1}\), Q.25/Q.75, 0.022/0.027 mL O\(_2\)·mm\(^{-3}\)·min\(^{-1}\); n=13; \(P<0.0001\); Figure 5A). This effect was sustained during work (stimulation at 5 Hz for 30 minutes). O\(_2\) consumption was given as ratio of MVO\(_2\) to force-time integral (median, 69.00 relative percent, Q.25/Q.75, 65.50/74.00 relative percent versus median, 100.00 relative percent, Q.25/Q.75, 95.75/105.25 relative percent; n=14; \(P<0.0001\); Figure 5B). The ratio of MVO\(_2\) to force-time integral was taken as 100% in the noninduced animals. nNOS-mediated alterations of MVO\(_2\) under rest indicate a direct inhibitory effect of nNOS on mitochondrial function because the effect of cross-bridge cycling kinetics under rest is negligible.

### O\(_2\)\(^{-}\) Production and Generation of ROS

Measurement of O\(_2\)\(^{-}\) production in isolated mitochondria showed a significant decrease in nNOS-overexpressing nNOS\(^+/\alpha\)MHC-tTA\(^+\) mice compared with noninduced animals (median, 0.35 U, Q.25/Q.75, 0.31/0.35 U versus median, 0.57 U, Q.25/Q.75, 0.47/0.59 U; n=5; \(P=0.009\); Figure 6A). ROS have been implicated in cardiac dysfunction. XOR and NADPH oxidase are known to be the major enzymes generating ROS in cardiac myocytes. In this study, we investigated ROS generation during nNOS overexpression and in noninduced nNOS\(^+/\alpha\)MHC-tTA\(^+\) mice.

The ROS concentration was significantly decreased in hearts of nNOS-overexpressing nNOS\(^+/\alpha\)MHC-tTA\(^+\) mice compared with noninduced animals (median, 6.08 \(\mu\)mol/L, Q.25/Q.75, 5.83/6.75 \(\mu\)mol/L versus median, 14.54 \(\mu\)mol/L, Q.25/Q.75, 13.91/14.72 \(\mu\)mol/L; n=8; \(P=0.001\); Figure 6B). O\(_2\)\(^{-}\) is produced via several mechanisms, including XOR and NADPH oxidase. Thus, we investigated the effects of allopurinol, an inhibitor of XOR, and apocynin, an inhibitor of NADPH oxidase, in both nNOS-overexpressing and noninduced nNOS\(^+/\alpha\)MHC-tTA\(^+\) mice.
In noninduced nNOS\(^+/−\)/αMHC-tTA\(^+/−\) animals, both application of apocynin (median, 14.54 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 13.91/14.72 \(\mu\)mol/L versus median, 7.17 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 6.92/7.85 \(\mu\)mol/L; \(n=8\); \(P<0.0001\)) and application of allopurinol (0.1 mmol/L for 1 minute at room temperature) caused a significant decline in ROS concentration (median, 14.54 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 13.91/14.72 \(\mu\)mol/L versus median, 7.17 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 6.92/7.85 \(\mu\)mol/L; \(n=8\); \(P<0.0001\); Figure 6C). In nNOS-overexpressing nNOS\(^+/−\)/αMHC-tTA\(^+/−\) animals, application of apocynin also significantly inhibited ROS generation via inhibition of NADPH oxidase activity (median, 6.08 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 5.83/6.75 \(\mu\)mol/L versus median, 3.22 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 2.97/3.43 \(\mu\)mol/L; \(n=8\); \(P<0.0001\)). However, in contrast to the noninduced animals, application of allopurinol had no significant inhibitory effect on ROS generation (median, 6.08 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 5.83/6.75 \(\mu\)mol/L versus median, 5.36 \(\mu\)mol/L, Q\(_{25}/Q_{75}\), 4.99/5.69 \(\mu\)mol/L; \(n=8\); \(P<0.0001\)) in nNOS-overexpressing nNOS\(^+/−\)/αMHC-tTA\(^+/−\) animals, whereas XOR activity was already suppressed by nNOS (Figure 6D). These data demonstrate that conditional nNOS overexpression in cardiac myocytes decreased ROS generation selectively by inhibition of XOR but had no effect on NADPH oxidase activity.

**NADPH Oxidase Activity and Protein Expression**

NADPH oxidase is one major enzyme generating ROS. It catalyzes the production of superoxide from oxygen and NADPH. It is a complex enzyme consisting of 2 membrane-bound elements (gp91\(_{PHOX}\) and p22\(_{PHOX}\)), 3 cytosolic components (p67\(_{PHOX}\), p47\(_{PHOX}\), and p40\(_{PHOX}\)), and a low-molecular-weight protein (either rac 1 or rac 2).

Measurement of NADPH oxidase activity in hearts of nNOS-overexpressing and noninduced animals showed no significant difference (median, 98.30 relative percent, Q\(_{25}/Q_{75}\), 96.70/104.00 relative percent versus median, 100.00 relative percent, Q\(_{25}/Q_{75}\), 98.00/103.00 relative percent; \(n=7\); \(P=0.522\); Figure 7A).

Protein expression of p47\(_{PHOX}\), p67\(_{PHOX}\), and rac 1 was investigated by Western blot analyses. Only protein expression of rac 1 (important for the activation of the NADPH oxidase) was significantly reduced in nNOS-overexpressing mice (median, 108.41 relative percent, Q\(_{25}/Q_{75}\), 85.96/
116.83 relative percent versus median, 162.62 relative percent, $Q_{25}/Q_{75}$, 143.92/194.39 relative percent; $n=10$; $P<0.0001$; Figure 7B), whereas protein expression of p47$^{PHOX}$ (median, 99.50 relative percent, $Q_{25}/Q_{75}$, 95.75/110.50 relative percent versus median, 128.00 relative percent, $Q_{25}/Q_{75}$, 120.75/138.25 relative percent; $n=10$; $P=0.06$) and p67$^{PHOX}$ (median, 97.17 relative percent, $Q_{25}/Q_{75}$, 91.35/101.00 relative percent versus median, 131.78

Figure 5. Specific MVO$_2$ of isolated muscle strips. In independent experiments, oxygen consumption was investigated during rest or during stimulation with 5 Hz. A, Specific oxygen consumption was significantly reduced in nNOS-overexpressing nNOS$^{-/}\alpha$MHC-tTA$^+$ animals already during rest. B, During stimulation with 5 Hz, nNOS-overexpressing nNOS$^{-/}\alpha$MHC-tTA$^+$ animals also displayed decreased O$_2$ consumption. In this case, O$_2$ consumption is measured as the ratio of MVO$_2$ to force-time integral and given in relative percent.
relative percent, Q_{25}/Q_{75} 124.96/142.43 relative percent; n = 10; \( P = 0.06 \) was not significantly changed (Figure 7C).

**XOR Activity and Protein Expression**

XOR activity was significantly decreased in nNOS-overexpressing mice (median, 0.091 nU/mL, Q_{25}/Q_{75}, 0.084/0.095 nU/mL versus median, 0.397 nU/mL, Q_{25}/Q_{75}, 0.381/0.418 nU/mL; n = 8; \( P = 0.001 \); Figure 8A).

Protein expression of XOR was investigated by Western blot analysis. XOR protein expression of nNOS-overexpressing mice showed no significant difference compared with noninduced animals (median, 94.44 relative percent, Q_{25}/Q_{75}, 87.04/111.11 relative percent versus median, 120.37 relative percent, Q_{25}/Q_{75}, 103.70/124.07 relative percent; n = 7; \( P = 0.073 \); Figure 8B). These results were consistent with the results of ROS generation.

**Discussion**

Recent studies have consistently demonstrated that nNOS is cardioprotective in different disease states. Specifically, nNOS has been shown to delay the transition to heart failure in response to pressure overload, to protect the myocardium from functional deterioration after myocardial infarction, and to decrease mortality after myocardial infarction. In contrast, the precise molecular mechanisms of nNOS action in the myocardium during rest and after myocardial damage are still being debated.

In a model with nNOS overexpression restricted to cardiac myocytes, we recently demonstrated that nNOS decreased myocardial contractility via inhibition of the \([Ca^{2+}]_i\) amplitude and \([Ca^{2+}]_i\) transients. We now additionally identified mitochondria and XOR as further targets for nNOS in this model. Because suppression of XOR activity by nNOS has been linked to improved outcome after myocardial infarction, we now hypothesized that nNOS also is cardioprotective after ischemia/reperfusion injury. Indeed, we found preserved LVDP and a significant decrease in infarct size after ischemia/reperfusion injury. In further studies on subcellular localization of nNOS, we found a strong enrichment of overexpressed nNOS and low levels of endogenous nNOS in mitochondria. After ischemia/reperfusion injury, endogenous nNOS was also clearly accumulated in the mitochondria. This was interesting in the context that mitochondria play an important role in cell death and cardioprotection. It has been demonstrated that endogenous NO interacts with mitochondrial respiration at several steps of the electron transfer, although some groups doubt the existence of nNOS and the production of significant NO levels via NOS in mitochondria. Recent findings support the notion that the PDZ-binding domain of the nNOS isoforms that are expressed in striated muscle (nNOSα and

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Figure 6. A, \( \text{O}_2^- \) production was measured in isolated mitochondria of nNOS-overexpressing nNOS\(^{-/-}\)αMHC-tTA\(^{+}\) and noninduced nNOS\(^{-/-}\)/αMHC-tTA\(^{+}\) mice. \( \text{O}_2^- \) production was significantly reduced in nNOS-overexpressing nNOS\(^{-/-}\)/αMHC-tTA\(^{+}\) mice. B, The ROS concentration was significantly decreased in hearts of nNOS-overexpressing mice. C and D, ROS production is caused by XOR. In noninduced nNOS\(^{-/-}\)/αMHC-tTA\(^{+}\) animals, application of apocynin (NADPH oxidase inhibitor) and allopurinol (XOR inhibitor) caused a significant decline in ROS concentration. In nNOS-overexpressing nNOS\(^{-/-}\)/αMHC-tTA\(^{+}\) animals, application of apocynin also inhibited ROS generation significantly. However, in contrast to the noninduced animals, application of allopurinol had no significant inhibitory effect on ROS generation in nNOS-overexpressing nNOS\(^{-/-}\)/αMHC-tTA\(^{+}\) animals.
nNOS is responsible for interaction with specific binding partners and for targeted subcellular localization. The nNOS adaptor protein CAPON (also known as NOS1AP) was demonstrated to be important for routing nNOS from the sarcoplasmic reticulum to the plasma membrane after myocardial infarction. nNOS-CAPON complexes were also found within mitochondria. Here, we demonstrated that nNOS overexpression potently inhibited myocardial oxygen consumption mainly as a result of inhibition of cytochrome c oxidase. This is in line with earlier findings but raises 2 questions: How is nNOS translocated into the mitochondria, and how can nNOS produce NO in mitochondria where it is in competition for O₂ with other enzymes of the respiratory chain?

We now identified HSP90 as a carrier protein to translocate nNOS across the outer mitochondrial membrane to cytochrome c oxidase via the TOM complex. This could be specifically inhibited by the HSP90 inhibitor geldanamycin. Similarly, a recent study described HSP90 as the carrier responsible for translocation of connexin 43 into the mito-

**Figure 7.** A, There was no significant difference between noninduced and nNOS-overexpressing animals in terms of NADPH oxidase activity. B, Western blot analysis of rac 1 (a low-molecular-weight G protein important for the activation of NADPH oxidase) protein expression showed a significant decrease in hearts of nNOS-overexpressing animals compared with noninduced mice. C, Western blot analysis showed no significant difference in the protein expression of the cytosolic components p47<sup>PHOX</sup> and p67<sup>PHOX</sup> of NADPH oxidase. GAPDH was used as a loading control.
chondria, providing protection from ischemia/reperfusion injury. Damy et al. also reported an increased HSP90-nNOS interaction in failing human hearts. However, the \( K_M \) values for nNOS are rather high, so nNOS might be substrate limited by oxygen in an area close to the cytochrome c oxidase. However, besides NO generation via NOS enzymes, NO generation independent of the NOS enzymes was described earlier. Hemoglobin and myoglobin can reduce nitrate and nitrite to NO. In our nNOS-overexpressing mouse model, the abundant nNOS generated elevated levels of NO as published earlier. This NO then is likely to be oxidized to nitrite by ceruloplasmin. Nitrite from this source might serve as an NO store once ischemia occurs. Accordingly, we found significantly increased nitrite levels in isolated mitochondria from nNOS-overexpressing mice.

In a scenario with low oxygen tension (like ischemia), it has been demonstrated previously that hemoglobin and myoglobin can form NO from nitrite at low oxygen apart from NOS enzymes. Clearly, nitrite had to be formed by abundant NO previously. NO from these nitrite sources might then inhibit cytochrome c oxidase during acute ischemia. After translocation of nNOS to the mitochondria, as occurs in ischemia/reperfusion injury and during subsequent reperfusion with gradually increasing \( O_2 \) levels, NO that is generated from nNOS at the mitochondria might then take over and continue to inhibit cytochrome c oxidase (which might be easier at this point because cytochrome c oxidase was inhibited earlier and could therefore not compete for \( O_2 \)). Accordingly, we found increased nitrite levels in isolated mitochondria from nNOS-overexpressing mice that are in favor of this mechanism.

**Figure 8.** A, Measurement of XOR activity showed a significant decrease in nNOS-overexpressing animals. B, Western blot analysis of XOR protein expression showed no significant difference in hearts of nNOS-overexpressing animals compared with noninduced mice. GAPDH was used as a loading control.
Recent work already identified NO derived from the other NOS enzymes (eNOS, iNOS) as factors responsible for cardioprotection in ischemia and reperfusion. It was demonstrated that iNOS inhibited mitochondrial permeability transition, and the authors suggested that iNOS acts upstream of mitochondrial permeability transition. In addition, in the case of eNOS overexpression, the protective effect has been consistently demonstrated by several studies. An explanation for this effect might come from a study in eNOS-overexpressing animals in which Massion et al showed that eNOS, targeted to caveolae in cardiomyocytes, attenuated the effect of high concentrations of catecholamines. Therefore, all 3 NOS isoforms as a net effect decreased ischemic damage. However, it appears that the molecular and subcellular processes behind this uniform protection are different for each case.

From further experiments with isolated muscle strips, we found that increased nNOS expression decreased myocardial contractility and simultaneously decreased myocardial oxygen consumption both at work and at rest. Especially from the decrease in MVO$_2$ at rest, we concluded that an increase in nNOS expression might act as preconditioning measure before ischemia/reperfusion.

During ischemia, ATP depletion leads to a rise in Ca$^{2+}$, which further accelerates ATP depletion. The rise in Ca$^{2+}$ during ischemia and reperfusion leads to mitochondrial Ca$^{2+}$ accumulation, particularly during reperfusion when oxygen is reintroduced. This can result in opening of the mitochondrial permeability transition pore, which further compromises cellular energetics. If downregulation of mitochondrial function occurs before ischemia, this may downregulate cellular energetics and prevent cell death.

To this end, however, it is not fully clear whether this direct effect of nNOS on mitochondrial function is cardioprotective or detrimental because one would expect that inhibition of mitochondrial function cuts off energy supply. In contrast and of particular interest, it was observed that S-nitrosoglutathione treatment resulted in increased S-nitrosylation of the mitochondrial F1-ATPase, which resulted in decreased activity. It has been reported that approximately half of the ATP generated during ischemia by glycolysis is consumed by the reverse mode of the mitochondrial F1-ATPase. Therefore, inhibition of the F1-ATPase during ischemia would conserve ATP. Inhibition of the F1-ATPase could thus be beneficial by conserving cytosolic ATP and by reducing Ca$^{2+}$ uptake into the mitochondria.

Additional effectors of ischemia/reperfusion injury in cardiomyocytes are ROS. Reducing ROS has already been reported to reduce ischemic injury. In this study, we have shown that the overexpression of nNOS in cardiac tissue balances the production between the production of ROS and NO. Notably, in a similar study, XOR activity was elevated at baseline in NOS$^{-/-}$ relative to wild-type mice and remained persistently elevated, whereas wild-type mice exhibited a transient elevation that was restored to normal by 4 weeks after myocardial infarction. This phenomenon is in accordance with our findings demonstrating decreased XOR activity in nNOS-overexpressing mice, resulting in lower ROS levels. NADPH oxidase activity was not altered by nNOS overexpression. Mitochondrial ROS generation was also decreased in our nNOS-overexpressing animals. It has been shown that S-nitrosylation of the complex I of the electron transport chain inhibited the activity of this complex and decreased mitochondrial-derived ROS formation.

The mechanisms underlying nNOS- and eNOS-mediated cardioprotection after myocardial infarction include the maintenance of an equilibrium between ROS and reactive nitrogen species in different cellular compartments. In infarcted nNOS$^{-/-}$ mice, there is a clear mismatch between the increase in ROS, especially in that resulting from XOR activity and NO production. XOR activity is persistently upregulated after myocardial infarction in nNOS$^{-/-}$ mice, whereas it increases transiently in control mice after myocardial infarction. The increased XOR activity was attributed to diminished XOR inhibition in the absence of nNOS-derived NO. Therefore, the absence of nNOS within the myocardium creates a nitroso-redox imbalance that was shown to be sustained in both acute and chronic myocardial infarction. We can now demonstrate that reducing ROS, possibly by overexpression of nNOS, reduced ischemic injury.

Recently, an additional mechanism for the protective effects of nNOS translocation has been suggested in a study by Sun and colleagues, who examined male and female mice after ischemia/reperfusion. A major membrane target for nNOS after translocation appeared to be the L-type Ca$^{2+}$ channel. In female hearts, there was increased S-nitrosylation of the L-type Ca$^{2+}$ channel. Functionally, this led to decreased L-type Ca$^{2+}$ current [\(I_{Ca(L)}\) with reduced Ca$^{2+}$ entry into the cell, which in turn protected the cell from Ca$^{2+}$ overload injury. This is in line with our previous studies and that of Sears et al that demonstrated inhibition of \(I_{Ca(L)}\) by nNOS. In contrast, others have observed unchanged L-type  Ca$^{2+}$ currents in nNOS$^{-/-}$ mice after ischemia/reperfusion. The latter studies, however, did not focus on ischemia/reperfusion injury.

**Limitations**

We used isoflurane in the ischemia/reperfusion experiments. In general, volatile anesthetics seem to be cardioprotective in ischemia and therefore may bias the observed cardioprotective effects. Nevertheless, we observed a protective effect of nNOS on top of this preexisting protection by isoflurane, which we believe is in favor for our hypothesis of cardioprotection by nNOS.

**Conclusions**

Conditional transgenic overexpression of nNOS localizes to the mitochondria and inhibits XOR, resulting in a decrease in ROS formation. Mitochondrial respiration is also downregulated in response to nNOS accumulation at the mitochondria. Together with nNOS-mediated inhibition of L-type Ca$^{2+}$ currents, these multiple actions of nNOS potently protect cardiomyocytes from ischemia/reperfusion injury. The versatile actions of nNOS at multiple subcellular sites have been demonstrated in an
array of recent studies. It appears that the different kinds of cardiac disease and the different disease states are mainly responsible for the prevailing function of nNOS, depending on the respective subcellular localization.

Sources of Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG Ro 1083/4–1), Interdisziplinaries Zentrum fuer klinische Forschung Wuerzburg (IZKF E-33), and the Deutsche Stiftung fuer Herzforschung (F24/04) to Dr Ritter. Dr Frantz was supported by the DFG (Sonderforschungsbereich 688, TPA10). Dr Hoffmann also received a grant from the IZKF Wuerzburg. Dr Widder was supported by the Deutsche Forschungsgemeinschaft (DFG Wi 2110/2–1) and a grant from the IZKF Wuerzburg.

Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Myocardial ischemia is the major threat to cardiac myocytes in acute myocardial infarction. Yet, despite infarct-sparing interventions like early reperfusion therapy by percutaneous coronary intervention, myocardial scars develop, even if call-to-balloon time is <90 minutes. Therefore, there is a need for additional options to enhance myocardial resistance to ischemia. Ischemic preconditioning or drugs that stimulate cardioprotective signaling (pharmacological preconditioning) might prove to be beneficial in this setting. Endothelial nitric oxide synthase (NOS) and inducible NOS have already been proven to be cardioprotective in ischemia/reperfusion experiments. We now add data on the remaining NOS isoform, neuronal NOS (or NOS1). Neuronal NOS overexpression in an animal model restored myocardial function compared with wild-type mice after reperfusion caused by inhibition of xanthine oxidoreductase (which decreased reactive oxygen series formation) and by inhibition of cytochrome c oxidase (which decreased myocardial oxygen consumption).

It appears that transgenic overexpression in the myocardium of each of the 3 NOS isoforms acts as a net and prevents reperfusion damage, at least in part. However, the precise molecular mechanism behind each isoform is probably different, depending on the subcellular localization of each isoform. It has also been demonstrated that neuronal NOS may inhibit arrhythmogenesis by maintaining intracellular Ca\(^{2+}\) homeostasis in cardiomyocytes via S-nitrosylation of Ca\(^{2+}\)-handling proteins, leading to reductions in ventricular arrhythmias and death after myocardial infarction. Hence, adding NO as a drug to the therapeutic regimen in acute myocardial infarction is likely to be less successful than increasing neuronal NOS expression in the myocardium.
Conditional Overexpression of Neuronal Nitric Oxide Synthase Is Cardioprotective in Ischemia/Reperfusion

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_Circulation_. 2010;122:1588-1603; originally published online October 4, 2010; doi: 10.1161/CIRCULATIONAHA.109.933630

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/122/16/1588

Data Supplement (unedited) at:
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Supplementary data to manuscript “Conditional overexpression of neuronal nitric oxide synthase is cardioprotective in ischemia-reperfusion” by Burkard et al.

CIRCULATIONAHA/2009/933630/R2

Supplementary methods:

Animal model
In brief, transgenic mice with the Tet-Off system (BD Biosciences, Heidelberg, Germany) were used. For this system two different mouse strains were crossbred. The first strain encodes for the regulatory protein tTA (tetracycline-controlled transactivator) under control of the αMHC promoter (FVB.Cg-Tg(Myh6-tTA)6Smbf/J, 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 nNOSα cDNA was cloned into the pTRE-6xHN vector (BD Bioscience). pTRE-6xHN-nNOS mice were crossbred with αMHC-tTA mice, and the expression of the tTA-responsive transgene (nNOS) was examined. All maternal mice as well as offsprings were treated with DOX to suppress transgenic nNOS expression during fetal development. This suppression was released by DOX removal in the postnatal period to study effects of cardiac nNOS overexpression in the adult myocardium. The induction of nNOS overexpression by removal of DOX was started minimum 10 days before any experiment. Animals of both gender were used. Age of mice for experiments was < 3 months.
Infarct size measurement after ischemia-reperfusion

After anesthesia (1.5 % isoflurane) and intubation, mice were ventilated with a volume-cycled rodent respirator. Three different groups of animals were investigated (nNOS overexpressing, non-induced and nNOS overexpressing+SM TC, 12 mice each group). For ischemia-reperfusion studies, ligation of the anterior descending branch of the left coronary artery (LAD) was achieved by tying 8-0 silk suture around the artery. After occlusion of the anterior descending branch of the LAD for 30 min, blood flow was restored by removing the ligature and polyethylene tubing. For infarct size measurement, after 24 h of reperfusion, the mouse chest was opened and 5 % Evansblue was injected into the apex of the heart. The heart was removed, washed with NaCl and frozen in Tissue Tek at -20°C. The frozen heart was cut into five parallel transverse slices, which were stained with 2 % triphenyl-tetrazolium-chloride (TTC) for 10 min at 37°C. After TTC staining, viable myocardium stains red and the infarcted areas appear pale. After fixation in formalin, slices were weighed, imaged and the area of infarction for each section was determined by computerized planimetry using an image analysis software program. The size of infarction was determined by the following equations.

Weight of infarction = (A1 x Wt1) + (A2 x Wt2) + (A3 x Wt3) + (A4 x Wt4) + (A5 x Wt5),
where A is percent area of infarction by planimetry and Wt is the weight of each section.
Percentage of infarcted left ventricle = (weight of infarction/weight of left ventricle) x 100.
Area at risk as a percentage of left ventricle = (weight of left ventricle - weight of left ventricle stained blue)/weight of left ventricle.

Electron microscopy with immunogold labeling

The excised hearts were fixed in 4 % Paraformaldehyde (PFA) for 20 min, cut in small pieces (< 1 mm) and incubated in 4 % PFA for additionally one hour at 4°C. After fixation, specimen were washed with PBS at 4°C, incubated with 50 mM NH₄Cl for 15 min and again washed several times in dH₂O. After dehydration with an ascending alcohol bank, heart
pieces were embedded in LR-White, transferred into capsules out of gelatine and polymerized at 40°C. For detection of transgene nNOS a polyclonal anti-6xHN antibody 1:50 (BD Biosciences) was used followed by a secondary antibody coupled to 12 nm gold particles. For detection of endogenous nNOS the polyclonal anti-nNOS antibody 1:50 (Zymed) was used followed by a second antibody that was coupled to 12 nm gold particles. This anti-nNOS antibody was reactive for both endogenous and overexpressed nNOS.

When nNOS and cytochrome c oxidase expression was investigated simultaneously, the monoclonal anti-cytochrome c oxidase antibody 1:200 (MitoSciences) was used followed by a second antibody that was coupled to 12 nm gold particles and the polyclonal anti-nNOS antibody was used followed by a second antibody that was coupled to 6 nm gold particles.

**Protein expression in isolated mitochondria**

Enriched mitochondrial fraction from mouse heart was prepared according to a modified protocol developed by R&D Systems. Mouse hearts were homogenized in RIPA buffer (containing 150 mM NaCl, 50 mM Tris, 1 % Igepal, 0,5 % sodium deoxycholate, dH2O) centrifuged for 30min at 15,000g and the resulting pellet was homogenized in buffer A (5ml/0.5g tissue) (containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 1 mg/ml fatty acid free BSA, 10 mM HEPES, pH 7.4). The resulting homogenate was centrifuged for 10 min at 15,000g (4°C). The pellet was homogenized in 500 µl buffer B (containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4) and centrifuged for 10 min at 15,000 g (4°C). The pellet was resuspended in 600 µl buffer C (containing 395 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4). Mitochondria were further enriched on a gradient of Percoll by centrifugation in polyallomer tubes in an ultracentrifuge. All solutions, the centrifuge, and tubes were prechilled to 4°C. 500 µl of 60 % Percoll (Percoll diluted with buffer D) (buffer D containing 1.28 M sucrose, 0.4 mM EGTA, 40 mM HEPES, pH 7.4) added to the bottom of the centrifuge tube was carefully overlaid with 900 µl 26 % Percoll.
(Percoll diluted with buffer D). 300 µl of the pellet resuspended in buffer C was overlaid on the 26 % Percoll.

The material was centrifuged for 30 min at 40,000 g (4°C). After centrifugation, the mitochondria were collected from the interface formed between the 26 % and 60 % Percoll. For Western Blot analysis of different protein expression, isolated mitochondria were electrophoretically transferred onto nitrocellulose membrane, while immersed in transfer buffer. Mitochondria samples were probed with purified mouse monoclonal anti-nNOS antibody 1:200 (BD Transduction Laboratories), polyclonal anti-L-type Ca²⁺-channel antibody 1:500 (Alomone Laboratories) or polyclonal anti-SERCA2a antibody 1:5,000 (Badrilla) for 1h followed by horseradish peroxidase-conjugated anti-mouse IgG 1:5,000 (GE Healthcare) for 1h. Bands were visualized by enhanced chemi-luminescence (GE Healthcare).

As a loading control, mitochondria samples were probed with purified rabbit polyclonal anti-prohibitin antibody 1:200 (abcam) for 1h followed by horseradish peroxidase-conjugated anti-rabbit IgG 1:5,000 (GE Healthcare) for 1h.

To test for quality of mitochondria preparation and mitochondria integrity (shown in supplementary figure 3), Western Blot analysis was performed with either cytochrome c antibody (MitoSciences, 1:1,000) or porin antibody (MitoSciences, 1:1,000).

To test for contamination of mitochondria preparation (shown in supplementary figure 3), Western Blot analysis was performed with either L-type Ca²⁺-channel antibody (Alomone Laboratories, 1:500) or SERCA2a antibody (Badrilla, 1:5.000). As a loading control, ponceau staining was performed.

**Nitrite level in isolated mitochondria**

Nitrite level was determined in isolated mitochondria of nNOS overexpressing nNOS⁺/αMHC-tTA⁺ and non-induced nNOS⁺/αMHC-tTA⁺ mice. The measurement was performed with a modified version of the Nitric Oxide Colorimetric Assay Kit from Biomol.
The kit involves the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm.

**Myocardial O₂ consumption**

Muscle stripes were excised from the right ventricle (4 x 0.5 x 0.5mm). The muscle preparations were transferred to a chamber containing oxygenated KHS solution at 37°C. Muscle stripes were fixed between steel clamps and a force transducer (Scientific instruments; Heidelberg, Germany). Before measuring O₂-consumption, the protective solution containing BDM was washed out. The oxygen measurement setup consists of a metal cylinder with heating unit and a closed plexiglas block containing the muscle chamber. The muscle stripe is suspended between two steel clamps, providing means for fixation and electrical stimulation. One of these clamps is connected with a force transducer. An inlet and outlet are used for perfusion. The Clark-oxygen electrode is located perpendicular to the long axis of the muscle strip, providing direct access to the perfusate. For the measurement, perfusion with oxygenated solution was stopped and the decrease of oxygen partial pressure at a defined distance from the muscle surface was recorded for 15-25 sec. Muscle strips were stimulated with 1-5 Hz (60-300 beats/min). After a steady state was reached, MVO₂ was recorded. Muscle fibers that showed a significant loss in force development compared with the initial 1-Hz value were excluded. Analysis of oxygen data (ml O₂ x mm⁻³ x min⁻¹) was performed using Muscle Research System software from Scientific Instruments. Recording of isometrically developed force, force–time-integral (FTI) was performed by “twitch” software (Scientific Instruments). FTI (in N × s/min/mm²) is defined as the area between peak systolic force and diastolic force during the stimulation interval. It represents an equivalent of work in isometrically contracting myocardium and is a major determinant of MVO₂. For better display ratio of MVO₂ / FTI was given in rel.% where MVO₂ / FTI at rest was set as 100 %.
Reactive oxygen free radicals (ROS)-concentration

For measuring the concentration of reactive oxygen free radicals in homogenized hearts of non-induced and nNOS overexpressing mice, the TAC-Peroxyl assay kit (Applied Bioanalytical Labs) was used. This assay is based on inhibition of luminescence caused by peroxyl radicals that are involved in lipid peroxidation in vivo. Biological relevant peroxyl free radicals are generated by thermal decomposition of 2,2'-azobis(2-amidinopropane) (ABAP). The ABAP decomposition products are a pair of C-centered free radicals R• and a nitrogen molecule. The R• free radicals further react with oxygen molecules to form peroxyl radicals ROO•, which are similar to those found in vivo during lipid peroxidation. These peroxyl radicals react with an indicator molecule, luminol (LH2), to generate a luminol radical (LH•) which result in emission of blue lights centered at ~425 nm. When antioxidants are present, such a light production is inhibited until the antioxidants are exhausted. The time of inhibition or the induction time to light production is proportional to the total concentration of antioxidants. The antioxidants concentration is determined by comparing induction time to that of a water-soluble Vitamin E (tocopherol) analog, Trolox.

To determine if the ROS are produced by the NADPH oxidase or the XOR, heart samples were incubated with different inhibitors. Apocynin (300 µM, 30 min incubation at 37°C) as an inhibitor for the NADPH oxidase and allopurinol (0.1 mM, 1 min incubation at room temperature) for the XOR.

Xanthine oxidoreductase activity

The measurement of the XOR activity in hearts of non-induced and nNOS overexpressing mice, was carried out by using the Amplex Red Xanthine Oxidase Assay Kit (Molecular probes). In this assay, XOR catalyzes the oxidation of xanthine to uric acid and superoxide. In the reaction mixture, the superoxide spontaneously degrades to hydrogen peroxide (H2O2) and the H2O2, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with
Amplex Red reagent to generate the red-fluorescent oxidation product resorufin. Resorufin has an absorbance and fluorescence emission maxima of approximately 571nm and 585nm. As a first step, a XOR standard curve with concentrations of 0 to 10mU/ml was assed. In the assay, the homogenized hearts (RIPA buffer) of the non-induced and the nNOS overexpressing mice were used in a final concentration of 5µg/µl. The samples were diluted in a working solution containing Amplex Red reagent solution, HRP solution, xanthine and reaction buffer. This mixture was filled into a 96-microplate, incubated for 30min at 37°C and measured at 560nm.
Supplementary figures:

Supplementary figure 1)

Supplementary figure 1 shows rate pressure product of non-induced, nNOS overexpressing and nNOS overexpressing + SMTC nNOS+/αMHC-tTA+ animals. Rate pressure product was changed in parallel to LVDP (figure 1C). In hearts of nNOS overexpressing nNOS+/αMHC-tTA+ mice, HRxLVDP increased at a higher rate than in hearts of the non-induced group (during 30 min post-ischemia: estimated average HRxLVDP increase equals 503 bpm/mmHg in non-induced animals vs. 866 bpm/mmHg). Application of SMTC again weakens the positive effects of the transgene nNOS overexpression (during 30 min post-ischemia, estimated average HRxLVDP increase is 557 bpm/mmHg, n = 12)
The evolution of rate pressure product from time 20 to 50 can be captured by the model

\[ LVDP = a \times (t - 20)^{0.771} \]

The parameter \( a \) was estimated by generalized estimating equations (GEE) assuming an unstructured working correlation matrix. Numerical optimization of the mean squared error yielded the exponent 0.771. The table below reports the results, showing different, highly significant increases of rate pressure product for the three groups. Standard errors are calculated by the fully iterated jackknife technique.

|                     | Estimate \( \hat{a} \) | Std. err | Wald   | Pr(>|W|)  |
|---------------------|------------------------|----------|--------|-----------|
| nNOS overexpressing | 1848.27                | 23.52    | 6176   | <2e-16 ***|
| NOS overexpressing + SMTC | 1188.17             | 20.71    | 3293   | <2e-16 ***|
| non-induced         | 1073.22                | 8.47     | 16037  | <2e-16 ***|
Supplementary figure 2)

Representative heart section of nNOS overexpressing nNOS⁺/αMHC-tTA⁺ mice, non-induced littermates and nNOS overexpressing nNOS⁺/αMHC-tTA⁺ mice treated with the specific nNOS inhibitor SMTC. Red encircled = area at risk; black encircled = infarcted area.
Supplementary figure 3)

Isolated adult cardiac myocytes of nNOS overexpressing mice

Isolated adult cardiac myocytes of non-induced mice

Immunofluorescence staining of isolated adult cardiac myocytes showed a colocalization of nNOS and cytochrome c oxidase in nNOS overexpressing nNOS$^{+/-}$/$\alpha$MHC-tTA$^+$ mice. Yellow in the overlay panels indicates colocalization of nNOS and cytochrome c oxidase. Red = cytochrome c oxidase, green = nNOS.
Supplementary figure 4)

Western Blot analysis of isolated mitochondria:

Mitochondria were isolated as described in supplementary methods. The supernatant fraction was saved after spin one and two. 40µg of isolated mitochondria and 40µg of supernatant were loaded onto each lane. To test for quality of mitochondria preparation and mitochondria integrity, Western Blot analysis was performed with either cytochrome c antibody (MitoSciences, 1:1,000) or porin antibody (MitoSciences, 1:1,000). Western Blot showed that minimal loss of cytochrome c (attached to the inner mitochondrial membrane) and porin (localized in the outer membrane of mitochondria) occurs during mitochondria isolation.

To test for contamination of mitochondria preparation, Western Blot analysis was performed with either L-type Ca\(^{2+}\)-channel antibody (Alomone Laboratories, 1:500) or SERCA2a (Badrilla, 1:5,000). Western Blot showed that the level of contamination was reduced during mitochondria isolation.
Supplementary tables:

Supplementary table 1): estimated results of figure 1C

The evolution of LVDP from time 20 to 50 can be captured by the model \( LVDP = a \times (t - 20)^{0.721} \). The parameter \( a \) was estimated by generalized estimating equations (GEE) assuming an unstructured working correlation matrix. Numerical optimization of the mean squared error yielded the exponent 0.721. The table below reports the results, showing different, highly significant increases of LVDP for the three groups. Standard errors are calculated by the fully iterated jackknife technique.

|                  | Estimate | Std. err | Wald   | Pr(>|W|)  |
|------------------|----------|----------|--------|-----------|
| nNOS overexpressing | 4.1265   | 0.0675   | 3735   | <2e-16 ***|
| NOS overexpressing + SMTC | 2.1484   | 0.0712   | 911    | <2e-16 ***|
| non-induced      | 1.5587   | 0.0215   | 5267   | <2e-16 ***|

Supplementary table 2): estimated results of figure 4C

|                | Estimate  | Std. err | Wald   | Pr(>|W|)   |
|----------------|-----------|----------|--------|-----------|
| (Intercept)    | -2.19363  | 0.01376  | 25433.30 | < 2e-16 ***|
| time           | -0.01779  | 0.00042  | 1838.10 | 1.0e-10 ***|
| NOS overexpressing | 0.12337  | 0.01909  | 41.80   | 2e-16 ***   |
| NOS overexpressing:time | 0.00889  | 0.00073  | 147.60  | < 2e-16 ***|
Supplementary table 3): estimated results of figure 4D

|                        | Estimate | Std. err | Wald     | Pr(>|W|)  |
|------------------------|----------|----------|----------|-----------|
| (Intercept)            | -2.19552 | 0.01415  | 24082.10 | < 2e-16 ***|
| Time                   | -0.00886 | 0.00061  | 211.90   | < 2e-16 ***|
| nNOS overexpressing + DTT | -0.12292 | 0.01932  | 40.50    | 2.0e-10 ***|
| nNOS overexpressing + Hb | 0.00044  | 0.02230  | 0.00     | 0.98      |
| nNOS overexpressing + DTT:time | -0.00895 | 0.00076  | 140.30   | <2e-16 ***|
| nNOS overexpressing + Hb:time | 0.00565  | 0.00089  | 40.50    | 2e-10 *** |

Supplementary table 4): estimated results of figure 4E

|                        | Estimate | Std. err | Wald     | Pr(>|W|)  |
|------------------------|----------|----------|----------|-----------|
| (Intercept)            | -2.30844 | 0.01782  | 16779.37 | <2e-16 ***|
| time                   | -0.01767 | 0.00043  | 1683.07  | <2e-16 ***|
| non-induced + DTT      | -0.00610 | 0.02603  | 0.05     | 0.815     |
| non-induced + Hb       | 0.05097  | 0.02508  | 4.13     | 0.042 *   |
| non-induced + DTT:time | 0.00219  | 0.00176  | 1.55     | 0.213     |
| non-induced + Hb:time  | 0.01198  | 0.00077  | 240.75   | <2e-16 ***|