Attenuation of myocardial ischemia/reperfusion injury in mice with myocyte-specific overexpression of endothelial nitric oxide synthase

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Abstract

Objective: The role of nitric oxide (NO) in myocardial ischemia/reperfusion injury remains controversial as both NO donors and NO synthase (NOS) inhibitors have shown to be protective. We generated transgenic (TG) mice that overexpress endothelial NOS (eNOS) exclusively in cardiac myocytes to determine the effects of high cardiac NO levels on ischemia/reperfusion injury and cellular Ca\textsuperscript{2+} homeostasis. Wild-type (WT) mice served as controls.

Methods: Hearts were perfused in vitro and subjected to 20 min of total no-flow ischemia and 30 min of reperfusion (n=5 per group). Left ventricular function, cGMP levels and intracellular Ca transients (Ca\textsuperscript{i}) were determined.

Results: Left ventricular pressure was reduced (maximum, −33%) and basal cardiac cGMP was increased (twofold) in TG hearts, and the changes were reversed by NOS blockade with N\textsuperscript{-}nitro-L-arginine methyl ester (L-NAME). Relative to baseline, recovery of reperfusion contractile function was significantly better in hearts from TG (98%) than WT (51%) mice, and L-NAME abolished this effect. Heart rate and coronary perfusion pressure were not different between groups. Systolic and diastolic Ca\textsuperscript{i} concentrations were similar in WT and TG hearts, but Ca\textsuperscript{2+} overload during early reperfusion tended to be less in TG hearts. Kineti\textsuperscript{c} analysis of pressure curves and Ca transients revealed a faster left ventricular diastolic relaxation and abbreviated aequorin light signals in TG hearts at baseline and during reperfusion.

Conclusions: High levels of NO/cGMP strongly protect against ischemia/reperfusion injury, the protection is largely independent of changes in Ca\textsuperscript{2+} modulation, but relates to reduced preischemic performance. Myocyte-specific NO augmentation may aid in studies of the (patho)physiological roles of cardiac-derived NO.

Keywords: Calcium (cellular); Contractile function; Ischemia; Nitric oxide; Reperfusion; Ventricular function

1. Introduction

Nitric oxide (NO) is formed from L-arginine and oxygen by the enzyme nitric oxide synthase (NOS). There are three known isoforms of the enzyme, all of which are expressed in the heart: neuronal NOS (nNOS, NOS 1), endothelial NOS (eNOS, NOS 3), and inducible NOS (iNOS, NOS 2). The main source of cardiac NO is generated through eNOS expressed by coronary endothelial cells and cardiac myocytes [1], whereas iNOS expression is low or absent in uninfected hearts. Under normal physiological conditions in the heart, NO regulates coronary vasodilator tone and inhibits platelet aggregation and cell adhesion to the vascular endothelium [2]. In addition, NO is known to modulate cardiac contractile function in vitro and in vivo, but these effects are variable and their physiological relevance remains partly equivocal [3].

Myocardial ischemia and reperfusion are associated with marked impairment of endothelium-dependent coronary relaxation, reduced contractility and cardiac arrhythmias. The role of NO in reperfusion injury has been studied intensively over the last decade and yielded largely, but not...
entirely consistent results [4]. In many studies, reperfusion injury has been attributed to a reduced formation or activity of NO because the NO precursor l-arginine [5,6]. NO donors of different chemical structures [7,8] and interventions resulting in stimulated NO production involving endothelial receptors [9] have all been found to protect the heart. Likewise, approaches to remove NO by pharmacological inhibition of NOS [8,10] and eNOS gene deletion [11,12] have also been shown to aggravate ischemic and reperfusion injury. On the other hand, in some studies NOS blockade improved myocardial reperfusion function [13]. With respect to the phenomenon of ischemic preconditioning, enhanced biosynthesis of NO by eNOS is essential to trigger ischemia-induced late preconditioning, and enhanced NO production by iNOS mediates the anti-stunning and infarct-limiting actions of late preconditioning [4].

Clearly, comparisons between these studies are difficult because of differences in study design and experimental protocols, frequent lack of dose–response studies, and the use of different pharmacological agents that themselves may alter the cardiovascular response to various pathophysiological events. The approaches using knockout animals are hampered because they assumed a reduction in NO synthesis which may not always have been the case [18]. Recently, we have generated mice overexpressing eNOS exclusively in cardiac myocytes to determine the role of NO in the neurohormonal control of myocardial contractility and heart rate [14]. We showed that NO is positively inotropic in spontaneously beating hearts from wild-type (WT) mice, whereas transgenic (TG) hearts had reduced basal contractility. Acetylcholine was positively inotropic in unstimulated hearts and negatively inotropic after β-adrenergic stimulation, and these responses were identical in WT and TG hearts. Finally, the beat-to-beat responsiveness to Ca²⁺ was reduced in TG hearts. We concluded that high levels of NO blunt myofilament tissue levels of cGMP in TG hearts (see Fig. 2D) indicate activities cannot be equated with NO levels, the higher sensitivity and have a minimal effect on autonomic modulation of contractility.

In the present study, we used this model to study the role of eNOS-derived NO in myocardial ischemia/reperfusion injury. Specifically, we tested the hypothesis that (i) eNOS-derived NO overproduction protects against myocardial ischemia/reperfusion injury in crystalloid-perfused hearts and that, (ii) changes in intracellular Ca²⁺ homeostasis ([Ca²⁺]), may be at the origin of protection [15].

2. Methods

2.1. Mice

All mice used in the experiments were male, ~3 months of age, housed in small groups and provided food and water ad libitum. Wild-type (WT) C57BL/6 mice were obtained from Charles River Laboratory (Sulzfeld, Germany). Transgenic (TG) mice with overexpression of eNOS exclusively in cardiac myocytes were prepared as follows [14]: the full length cDNA for human eNOS (kindly provided by J. Liao, Boston, MA) and the PCR-cloned 3’UTR were subcloned into a vector (clone 26) containing the murine cardiac α-myosin heavy chain (MHC) gene promoter. All cloning junctions were subsequently sequenced. After digestion with NotI and extensive purification, the 10.6 kb minigene (Fig. 1), containing the 5.5 kb α-MHC gene promoter, the first 3 exons and 2 introns of the α-MHC gene, the human eNOS cDNA from bp −19 to the end of the 3’UTR and an additional 579 bp of the 3’ flanking region, was microinjected into oocytes of pseudopregnant mice (F₁ of CBA×C57BL/6). Transgene-positive mice were backcrossed with C57BL/6 mice for at least three generations.

In the present report, hearts from WT and two TG lines were studied, i.e. TG line 11 with 38-fold overexpression and TG line 23 with 88-fold overexpression of eNOS. The corresponding NOS activities, determined as [³H]-citrulline formation, were 26.5±5.7 and 61.8±11.8 pmol mg⁻¹ min⁻¹, respectively. NOS activity in hearts from WT mice was 0.7±0.3 pmol mg⁻¹ min⁻¹ [14]. Although NOS activities cannot be equated with NO levels, the higher tissue levels of cGMP in TG hearts (see Fig. 2D) indicate that NO production was higher in TG than WT hearts. Substrate availability was not limiting. Body weight, arterial blood pressure and heart rate were not different between groups (means of all three groups, 29.6±0.6 g, 112±5 mmHg and 462±26 min⁻¹, respectively; n=15). Mice from two groups (WT and TG line 23) were treated with the NOS inhibitor, N⁵-nitro-l-arginine methyl ester (l-NAME), which was added to the drinking water for 2.

![Fig. 1. Schematic representation of the eNOS minigene used for cardiac eNOS overexpression. The construct contains the 5.5 kb α-myosin heavy chain (α-MHC) gene promoter, 3 exons and 2 introns of the α-MHC gene, the complete eNOS cDNA including the 3’-untranslated region (UTR) and the polyadenylation signal, and 579 bp of the 3’ flanking region of the eNOS gene. Arrow, transcription start site.](image-url)
2.2. Isolated mouse heart preparation and measurement of contractile function

Animals received care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (revised 1996). Animals were anesthetized with urethane (1 g kg\(^{-1}\)), the hearts excised, the aorta was cannulated and the heart retrogradely perfused at 2.0 ml min\(^{-1}\) (pH 7.4, 37 °C) [14]. A fluid-filled balloon (~50 µl) made of polyethylene film was inserted into the left ventricle and connected to a pressure transducer. The following cardiac parameters were monitored: left ventricular developed pressure (LVDevP), left ventricular end-diastolic pressure (LVEDP), maximal rate of rise and fall of left ventricular pressure (+dP/dt\(_{\text{max}}\) and −dP/dt\(_{\text{max}}\)), heart rate and coronary perfusion pressure (via a second pressure transducer attached to the aortic cannula). We used recovery of LVDevP during reperfusion as a measure of functional performance, rather than infarct size, because functional recovery ‘encompasses’ infarct size which makes it a similarly meaningful parameter.

2.3. Experimental protocol

Hearts were equilibrated for 30 min (baseline), subjected to 20 min of no-flow ischemia at 37 °C followed by 30 min of reperfusion at 2 ml min\(^{-1}\) (total duration of experiment: 80 min). Some hearts were perfused at normoxic conditions for 80 min to verify the stability of the preparation (time-matched controls).

2.4. cGMP measurements

Hearts were perfused in the presence of isobutyl methyl xanthine (IBMX; 1 mmol l\(^{-1}\)) to inhibit degradation of cGMP and coronary venous effluent was collected prior to ischemia and during reperfusion. At the end of reperfusion, the heart was freeze-clamped and processed as described [8]. Effluents and homogenates were analyzed for cGMP by radioimmunoassay. Standard curves were established in perfusion buffer containing 1 mmol l\(^{-1}\) IBMX and results expressed as amount cGMP per litre effluent or per mg tissue (wet weight).

2.5. Ca\(^{2+}\) measurements

Intracellular Ca\(^{2+}\) transients were measured on a beat-to-beat basis as previously described [14,17]. Hearts were equilibrated (10 min) and arrested by reducing the perfusate Ca\(^{2+}\) and Mg\(^{2+}\) concentration to 0.5 and 0.6 mmol l\(^{-1}\), respectively. Aequorin (1 µg ml\(^{-1}\); Friday Harbor Photoproteins, Friday Harbor, WA) was slowly macroinjected into the epicardium at the apex of the heart, perfusate Ca\(^{2+}\) and Mg\(^{2+}\) were gradually increased to 1.2
mmol l⁻¹, the temperature was slowly raised to 37 °C, and pacing was initiated at 6.7 Hz (400 min⁻¹). Hearts were subjected to isothermic ischemia and reperfusion as described above, and contractile parameters and aequorin signals were continuously recorded. Pacing was discontinued 30 s after initiation of ischemia and was reinitiated ~3 min after start of reperfusion, when the first spontaneous contractions appeared.

2.6. Data analysis and statistics

\[ [\text{Ca}^{2+}]_i \] was determined using the method of fractional luminescence (L/L_{max(t)}) which relates the recorded light signals (L) to the amount of light emitted after lysis of cells with Triton X-100 (TTX, 5%), plus the amount consumed throughout the experiment [18]. L/L_{max(t)} was converted to \( \mu \text{mol l}^{-1} \text{Ca}^{2+} \) using an in vitro calibration curve (37 °C) as described previously [18]. The averaged light and pressure signals were analyzed to obtain the parameters time to peak light (TPL), time to 90% decline from peak light (T_{90}L), time to peak pressure (TPP) and time to 90% decline from peak pressure (T_{90P}). To analyze \( \text{Ca}^{2+} \) overload on reperfusion, the time integrals of the first 2 min were calculated in intervals of 30 s and normalized by L_{max(t)} [19].

Group data are presented as arithmetic mean ± S.E.M. Differences were considered significant at \( P<0.05 \) (two-way ANOVA).

3. Results

3.1. Stability of preparation

The stability of the preparation was tested by perfusing hearts for 80 min with normoxic solution. Functional parameters were stable over time (\( P=NS \) for 30 vs. 80 min of perfusion; \( n=3 \), data not shown). LVEDP was set at 5 mmHg at the beginning of the experiment and was unchanged in both groups. However, basal left ventricular contractility was lower in TG hearts (see Section 3.2).

3.2. Ischemia/reperfusion contractile function

Basal LVDevP was 93±4 mmHg in WT and significantly lower in TG hearts (line 11, 69±1 mmHg (−26%); line 23, 62±2 mmHg (−33%)) (\( P<0.05 \), \( n=5 \); see baseline in Fig. 2A). No-flow ischemia resulted in cessation of left ventricular contractile function within 2 min and an increase in LVEDP. After 30 min of reperfusion LVDevP remained depressed to 51% of preischemic value in WT hearts, while TG hearts approached preischemic values (TG line 11, 83%; TG line 23, 98% recovery; Fig. 2A) (the data for TG line 11 are not shown in the figure for the sake of clarity). Treatment with L-NAME reduced basal LVDevP in hearts from WT animals (−20%; \( P<0.05 \)) and abolished the cardioprotective effect of eNOS overexpression in TG hearts (\( P=NS \) vs. WT, squares in Fig. 2A).

Data for \( +dP/dt_{\text{max}} \) are shown in Fig. 2B. Basal \( +dP/dt_{\text{max}} \) was 4322±258 mmHg s⁻¹ in WT and significantly lower in TG hearts (line 11, −26%; line 23, −36%; \( P<0.05 \), \( n=5 \); see baseline in Fig. 2B). Contractility remained depressed after 30 min of reperfusion in WT hearts (48% of preischemic value), but was significantly improved in TG hearts (line 11, 81%; line 23, 95% recovery). The improved percent recoveries in TG hearts were partly due to reduced preischemic inotropy (i.e. the lower reference LVDevP), as well as an absolute increase in contractility; however, the latter was smaller than the former. Again, the NOS blocker abolished the improvement (\( P=NS \) vs. WT, squares in Fig. 2B). Similar results were obtained for the diastolic parameter \( -dP/dt_{\text{max}} \) (\( n=5 \); data not shown).

Fig. 2C shows corresponding cGMP levels. Both at baseline and during reperfusion, cGMP efflux was significantly higher in TG (line 23) than WT hearts (basal, 2.3-fold; reperfusion, 1.8-fold; \( P<0.05 \), \( n=4 \). L-NAME treatment of TG mice for 2 days abolished the differences in cGMP efflux (squares in Fig. 3C; \( P=NS \) vs. WT). Similar results were obtained in tissue homogenates (Fig. 2D).

3.3. Diastolic function

During ischemia, LVEDP increased eightfold in WT, but less than sixfold in the two TG lines; after 30 min of reperfusion, LVEDP was elevated 3.6-fold above basal in WT and 1.7-fold in TG hearts (\( n=5 \), \( P<0.05 \) vs. WT) (Fig. 3). After NOS blockade the amelioration of diastolic

![Fig. 3. Diastolic function. Left ventricular end-diastolic pressure (LVEDP) development during ischemia and reperfusion in wild-type (WT, open circles) hearts and hearts from transgenic animals (TG line 23, solid circles). Data are mean±S.E.M., \( n=5 \). Squares, animals treated with L-NAME. *\( P<0.05 \) TG vs. WT; L-NAME abolished the differences (\( P=NS \) for TG vs. WT).](image-url)
function in TG hearts was abolished, so that LVEDP was no longer different from WT (for TG line 23, see squares in Fig. 3; data for line 11 not shown for the sake of clarity).

3.4. Coronary function and heart rate

NO overexpression had no effect on coronary perfusion pressure or heart rate at baseline or during reperfusion. Coronary perfusion pressure rose post-ischemia, but the increase was similar in WT and TG hearts (baseline, mean of all three groups 69±1 mmHg; at end of reperfusion, 102±2 mmHg; \( P = \text{NS WT vs. TG} \)). Preischemic heart rate was 405±12 min\(^{-1}\) (mean of all groups; \( P = \text{NS WT vs. TG} \)) and recovered to ~90% of preischemic value at the end of reperfusion (\( P = \text{NS vs. pre-ischemia; } n = 5 \) in each group). Accordingly, the product of heart rate and LVDevP (rate–pressure product) was consistently lower at baseline (~29%) and higher at end of reperfusion (+24%) in TG compared to WT hearts (\( P < 0.05 \)).

Fig. 4. Original recordings of LV pressure (LVP) and aequorin light signals (L) in a WT (A, B) and TG line 23 (C, D) heart during ischemia (Isch) and reperfusion (Rep). The light signal was normalized to \( L_{\text{max}} \). [18]

Fig. 5. Summary of \([\text{Ca}^{2+}]\) levels in ischemia and reperfusion. Systolic (circles) and diastolic (diamonds) levels of intracellular \([\text{Ca}^{2+}]\) (\([\text{Ca}^{2+}]\) ) are given for hearts from WT (open symbols) and TG (line 23, solid symbols) animals. Data are mean±S.E.M., \( n = 5 \) in each group. \([\text{Ca}^{2+}]\) rose significantly during ischemia and reperfusion compared to preischemia (basal) (\( P < 0.05 \); not shown), but there were no differences between experimental groups.

3.5. \([\text{Ca}^{2+}]\) in ischemia and reperfusion

Fig. 4 illustrates continuous simultaneous recordings of aequorin light signals and isovolumic LV pressure during 20 min ischemia followed by reperfusion in a WT and TG heart (line 23). After interruption of coronary flow, there was an abrupt fall in LV pressure and an increase in diastolic and peak light. Upon resumption of perfusion, peak \([\text{Ca}^{2+}]\) transients increased in both groups. A detailed analysis of the light signals and conversion to \([\text{Ca}^{2+}]\) values is presented in Fig. 5. Peak systolic \([\text{Ca}^{2+}]\) was similar in both groups at baseline and during reperfusion (circles in Fig. 5). (In our previous report [14], the mean...
difference in peak systolic [Ca\(^{2+}\)], was 0.04 \(\mu\)mol l\(^{-1}\) which was statistically significant.) Of note, [Ca\(^{2+}\)], remained similarly elevated above baseline in both groups at the end of reperfusion (\(P<0.05\)) despite a substantial difference in recovery of LV pressure (63 and 95%; see Fig. 2). Similarly, levels of resting (diastolic) [Ca\(^{2+}\)], were not different between groups (diamonds in Fig. 5; \(n=5\)).

[Ca\(^{2+}\)], during early reperfusion was analyzed in more detail (Fig. 6). The time integral of the light signal, an index of Ca\(^{2+}\) overload, appearing in the first 2 min of reperfusion, tended to be lower in TG hearts. This tendency reached statistical significance at 60–90 s after initiation of reperfusion (\(P<0.05\)). However, there was no significant difference when integrating the entire first 120 s after start of reperfusion (\(P=NS\)). Finally, we also analyzed the kinetics of Ca\(^{2+}\) transients during reperfusion. As expected after an ischemic episode, time to peak pressure (TPP) and time to peak light (TPL) were prolonged during reperfusion compared to baseline (\(P<0.05\)), and both parameters were significantly less in TG hearts (Fig. 7A and C). Likewise, time to 90% relaxation from peak pressure (\(T_{90}\)P) and peak light (\(T_{90}\)L) was prolonged throughout reperfusion (\(P<0.05\)) and both parameters were significantly smaller in TG hearts (\(P<0.05\)), indicating a faster diastolic relaxation (Fig. 7B and D).

4. Discussion

The results of this study documented that TG mouse hearts that overexpress eNOS within their myocytes are less susceptible to ischemia/reperfusion injury compared with WT mouse hearts, suggesting that the availability of additional NO/cGMP is beneficial, not deleterious, to the hearts. The rise in systolic and diastolic [Ca\(^{2+}\)], during ischemia and reperfusion was similar in WT and NO overproducing hearts, indicating that protection was not primarily due to the avoidance of Ca\(^{2+}\) overload. Rather, the improved postischemic function was related to the reduced basal contractility in TG hearts prior to ischemia.

A series of studies from Lefer’s group [11,20] and others using NO donors and eNOS knockout mice found
NO to be cardioprotective, whereas in several other reports competitive inhibitors of the NOS enzyme ameliorated reperfusion function (see discussion in Ref. [21]). The latter investigations imply a deleterious role for NO which may result from the reaction of NO with superoxide giving peroxynitrite which is generally considered to be cytotoxic [13]. In the present novel experimental approach we generated high levels of endogenous eNOS-derived NO exclusively within cardiac myocytes and found that it consistently ameliorated reperfusion contractile function, both as percent recovery relative to the reduced preischemic function as well as in absolute terms (mmHg of LVEDP). The improvement correlated with higher cGMP levels in cardiac homogenates and coronary effluents throughout reperfusion, suggesting that NOS overexpression stimulated the NO/cGMP pathway and causally contributed to the functional improvement. Likewise, the rate–pressure product was consistently higher in TG than in WT hearts, indicating that not only the contractile state, but performance was improved during reperfusion, probably as a result of the lower preischemic energy demand which entails less ATP breakdown during ischemia [22]. The genetic manipulation had no effect on coronary perfusion pressure in our isolated perfused hearts, either at baseline or during reperfusion, indicating that cardioprotection in TG hearts was a local myocardial effect.

Basal contractile function was depressed in both TG lines, and the depression was antagonized after NOS blockade, suggesting that the negative inotropy was mediated by NO and was not due to nonspecific effects of transgenesis. This interpretation is not contradicted by the lack of a significant effect of l-NAME in line 11, because in this line contractility is as high as in WT hearts treated with the blocker, i.e. at the maximal possible level. The mechanism of the negative inotropic effect of NO was not specifically investigated but may relate to cGMP-dependent phosphorylation of troponin I which leads to an sion mechanical function relates to an additional role of specific investigated but may relate to cGMP-dependent phosphorylation of troponin I which leads to an enhanced mechanical function.
during ischemia or reperfusion but rather involved blunted myofilament sensitivity to $\text{Ca}^{2+}$ prior to and/or during ischemia which subsequently translated into improved mechanical function that was accompanied by shortened diastolic $\text{Ca}^{2+}$-transients. These data strongly support the view that NO may be cardioprotective in different clinical situations associated with reduced formation or heightened inactivation of NO.

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References


