The nitric oxide-induced reduction in cardiac energy supply is not due to inhibition of creatine kinase

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Abstract

Objectives: While nitric oxide (NO) is a potent vasodilator already in the nM range, a cGMP-independent negative inotropic effect is observed at higher concentrations. Since inhibition of creatine kinase (CK) by NO-induced nitrosylation has been proposed as a possible mechanism of action, we measured the flux through CK in the intact heart. Methods: In saline perfused, paced guinea pig hearts \textsuperscript{31}P NMR spectroscopy was employed to directly assess the cardiac energy status, i.e. free energy of ATP hydrolysis ($\Delta G_{\text{ATP}}$) and flux through CK using magnetization transfer in absence and presence of NO. Results: NO (50 $\mu$M) doubled coronary flow and induced a rapid drop in left ventricular developed pressure (39±10 vs. 81±10 mmHg) and MVO\textsubscript{2} (1.3±0.8 vs. 3.7±0.5 $\mu$mol/min/g) (n=7). This effect was associated with an immediate decrease in phosphocreatine (PCr) ($269\%$) and $\Delta G_{\text{ATP}}$. During the subsequent 35 min of NO infusion cardiac function and MVO\textsubscript{2} remained depressed, while PCr partially recovered. NO had no effect on the unidirectional forward flux through CK (98±21 vs. 99±20 $\mu$mol/min/g, n=7) which was 5- to 10-fold greater than the rate of ATP turnover. Upon cessation of NO infusion both cardiac function and PCr rapidly returned to baseline values. The NO-induced fall in the myocardial energy status was associated with an increase in mitochondrial NADH (n=7) as assessed by surface fluorescence. The observed change in fluorescence was similar to that observed with short term ischemia. Conclusion: The NO-mediated depression of myocardial function, MVO\textsubscript{2} and energy status is not mediated by changes in CK flux. Most likely a partial blockade of mitochondrial oxidative phosphorylation at the level of cytochrome c oxidase is responsible for this effect. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In the heart, NO modulates contractile function and energy metabolism by a variety of mechanisms (for review see [1–3]). NO may influence contractility and thus energy demand by cGMP mediated modulation of L-type Ca\textsuperscript{2+}-channel activity [1,3]. cGMP mediated phosphorylation of phospholamban will enhance SR Ca\textsuperscript{2+}-ATPase activity, which may explain the relaxation-hastening effect of NO [2,4]. NO is also involved in the control of energy supply.

While NO, being a potent coronary vasodilator [5], enhances oxygen and substrate supply, it may also act as a tonic inhibitor of oxygen consumption (e.g. [6]), possibly due to the exquisite sensitivity of the mitochondrial respiratory chain to blockade by NO [7,8].

We have previously shown that application of authentic NO in the saline perfused guinea pig heart inhibits oxygen consumption and impairs the free energy of ATP hydrolysis. The negative inotropic effect observed was therefore attributed to a decreased energy supply [9]. The latter could be due to a direct inhibition of the respiratory chain by NO, and would result in a rise in mitochondrial NADH, as demonstrated by us recently in isolated cardiomyocytes [10]. While changes in mitochondrial NADH

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can be assessed also in the beating heart by surface fluorescence measurements \[11–13\], no data on the effects of NO on mitochondrial NADH have been reported to date in this model.

An alternative mechanism for the NO-induced decrease in energy supply could be inactivation of creatine kinase as first suggested by Gross et al. \[14\]. They reported a near-complete loss of the cardiac contractile reserve following 6 min exposure to the NO donor \(S\)-nitrosoacetylcyysteine (SNAC) associated with a decrease of cytosolic CK enzyme activity \(V_{\text{max}}\). Inhibition of CK in vitro had also been observed with other NO donors \[15,16\] and attributed to \(S\)-nitrosylation. Rapid inhibition of CK by irreversible thiol oxidation can be induced by peroxynitrite, formed in the reaction between superoxide and NO \[17\]. Mitochondrial CK was also highlighted as a prime target of peroxynitrite-induced inactivation \[18\]. Inhibition of either mitochondrial or cytosolic CK would interfere with ADP delivery to the mitochondrial ATP synthase and thus with energy supply, as suggested by the creatine kinase shuttle hypothesis (for review see \[19\]). However, because the NO-induced inactivation of CK isoforms was observed primarily in vitro it remained unclear whether CK inactivation and reduced creatine kinase flux can explain the in vivo cardiodepressant actions of NO.

It was therefore the aim of the present study to test whether creatine kinase flux decreases in the intact heart when exposed to authentic NO at concentrations that exert a pronounced negative inotropic effect. To this end, CK flux was measured by \(^{31}\)P NMR magnetization transfer techniques in saline perfused guinea pig hearts, both before and during application of authentic NO. In addition, the effects of NO on mitochondrial NADH were investigated using surface fluorescence.

2. Methods

2.1. General

Well oxygenated isolated guinea pig hearts according to Langendorff were studied as described previously \[9,20\]. Hearts were paced (310 bpm) and perfused at constant pressure (55 mmHg) with a bicarbonate buffer (95\% \(O_2\), 5\% \(CO_2\); pH 7.4; 37°C), containing in mM: 116 NaCl, 4.7 KCl, 1.1 MgSO\(_4\), 1.2 KHPO\(_4\), 24.9 NaHCO\(_3\), 2.5 CaCl\(_2\), 8.3 glucose, and 2 pyruvate. Coronary flow was measured by an ultrasonic flow meter (Transonics, Ithaka, USA) and left ventricular pressure using a saline-filled latex balloon within the left ventricle. The pulmonary artery was cannulated for \(O_2\) measurements by a Clark type micro-\(O_2\) electrode (Hugo Sachs, Marchstetten, Germany). The physiological parameters were recorded on a chart recorder (Gould, Erlensee, Germany) and also by an analogue to digital data acquisition system (Maclab 8e, ADI Instruments). Hearts were allowed to equilibrate for approximately 20 min before initiation of pacing, after which another 10 min were allowed for stable baseline starting conditions. The investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2.2. Infusion of authentic NO

NO was applied as described previously \[9\]. In brief, NO dissolved in deoxygenated saline and stored in an airtight glass syringe coupled to an airtight HPLC tube entered the perfusion line immediately above heart to avoid degradation of NO before infusion. The NO concentration of the stock solutions was appr. 1 mM and the arterial NO concentration required for a steady reduction of contractile function of about 50\% was determined to be 50 \(\mu\)M \((n=2)\), similar to previous data \[9\].

2.3. Protocols

The effects of NO on cardiac energy metabolism were investigated in two separate experimental series by either \(^{31}\)P NMR spectroscopy or surface fluorescence.

For the NMR experiments, the heart was placed in a 9.4 Tesla vertical bore magnet inside a 20 mm OD NMR tube and immersed in the coronary effluent. A continuous suction line was placed approximately 30 mm above the heart. A fully relaxed spectrum was acquired to determine the basal cardiac energy status, i.e. the high energy phosphate levels and the free energy of ATP hydrolysis \((\Delta G_{\text{ATP}})\). The flux mediated by CK was determined by \(^{31}\)P NMR magnetization transfer (MT) (see below) both under baseline conditions and during 50 \(\mu\)M NO application. To verify stable metabolic conditions during MT partially saturated spectra were acquired before and after the MT experiments, lasting 16 min each. Preliminary experiments \((n=2)\) had indicated that upon application of NO, a rapid decline in phosphocreatine (PCr) was followed by a partial recovery attaining a steady state within appr. 12 min. Therefore, the MT experiments were started thereafter. The NO application was stopped after 32 min and the recovery of cardiac function and energy status monitored for another 15 min.

When assessing mitochondrial NADH by surface fluorescence (SF), hearts were subjected to two brief (30s) ischemic episodes, each followed by a 10 min reperfusion phase. The SF signal was acquired before, during and after ischemia. Subsequently, NO (50 \(\mu\)M) was applied for 32 min and SF signals were monitored at frequent intervals before, during and until 1 min after the NO infusion. Zero flow ischemia of 10 min duration followed.

To exclude that the changes in SF at 480 nm induced by NO were simply due to altered tissue light absorbance, an additional series of hearts was loaded with succinimidyl \(N\)-methylanthranilate (100 \(\mu\)M for 35 min, 15 min wash-
out) as described by others before [21] and the effects of NO (50 μM) on SF spectra were tested.

2.4. NMR spectroscopy

$^{31}$P NMR spectroscopy was performed as described previously [22]. In brief, spectra were acquired on an AMX 400 WB spectrometer (Bruker, Rheinstetten, Germany) coupled to a 9.4 Tesla magnet (Oxford/Spectrospin) and equipped with a 20 mm probe (Fraunhofer Institute, St. Ingbert, Germany). After the heart was placed inside the magnet, the probe was tuned and the field homogeneity maximized. The resulting $^1$H H$_2$O resonance linewidth averaged 16 Hz.

$^{31}$P NMR spectra were acquired by sampling 6000 data points in the time domain using a sweep width of 6024 Hz. For fully relaxed spectra, 40 or 32 90° pulses (85 μs) at an interpulse delay of 15 s were applied (total acquisition time 10 and 8 min, respectively). Partially saturated spectra were obtained within 3 min, applying 64 70° pulses (60 μs) at 3 s intervals. Exponential multiplication (10 Hz) and Fourier transformation were followed by integration using the NMR1 software (Tripos).

To determine the forward flux through CK by saturation transfer, the $\gamma$-ATP resonance was selectively saturated by a 5 s low power, continuous wave irradiation prior to each 90° pulse and spectral acquisition, followed by a 10 s delay. 32 acquisitions were obtained per spectrum. Magnetization transfer from $\gamma$-ATP to PCr resulted in a decrease in the PCr signal; this was compared to a spectrum acquired in parallel in an interleaved mode with saturation downfield of PCr at a distance identical to the PCr-$\gamma$-ATP chemical shift difference. The forward flux through CK was calculated from the relative decrease of the PCr signal, assuming an intrinsic $T_1$ of phosphocreatine of 3.55 s [23].

Based on previous experimental data [22], basal ATP and total creatine of the saline perfused guinea pig heart were taken to be 6.98 and 22.2 mM, respectively. The PCr/ATP and P/ATP ratios in the initial, fully relaxed spectrum were used to determine PCr and P concentrations in each individual heart. In the following spectra, the relative changes in signal area were translated into changes in metabolite concentrations. Intracellular pH, free cytosolic ADP and the free energy change of ATP hydrolysis ($\Delta G_{\text{ATP}}$) were calculated as previously described [22].

The pseudo-first-order rate constant $k_{\text{for}}$ of the creatine kinase (CK) reaction was calculated according to the following equations [23]:

$$k_{\text{for}} = 1/\tau - 1/T_1$$

$$\tau = T_1 \times M_{\text{sat}} / M_0$$

($T_1$, intrinsic longitudinal relaxation time constant of PCr, $M_{\text{sat}}$ and $M_0$, magnetization of PCr in the presence and absence of an infinite $\gamma$-ATP saturation, respectively).

The forward CK flux was calculated as follows:

$$\text{CK Flux} = k_{\text{for}} \times [\text{PCR}]$$

and converted to μmol·min$^{-1}$·g$^{-1}$ wet wt by assuming a cytosolic water space of 60% of total wet weight. To compare the CK flux with the rate of ATP synthesis, MVO$_2$ was measured and a P:O ratio of 3 assumed.

2.5. Surface fluorescence

To assess changes in mitochondrial NADH, SF was measured as described by others before [12,13] using a custom built UV measurement system (T.I.L.L. Photonics, Martinsried, Germany). A Hg-Lamp (365 nm interference filter, bandwidth 10 nm) was coupled to a light guide for excitation of the tissue. The same light guide transmitted the emission signal which was coupled to two photomultipliers (410 and 480 nm interference filters). During measurements, the shutter was open only one second in every five to minimize bleaching.

In addition, in a limited number of experiments, a CCD detector system (1024×128 pixel) (Andor Technology, Belfast), permitted the acquisition of the emission spectrum in the range from 400 to 600 nm under basal conditions, during ischemia and upon NO application. All SF measurements were performed in the dark. After a background reference was obtained, the optic fiber was placed against and perpendicular to the left ventricular myocardium in a region with no visible epicardial vessels, minimizing motion artifacts.

2.6. Chemicals

Succinimidyl N-methylantranilate was obtained from Molecular Probes, Eugene, USA, all other chemicals from Merck, Darmstadt, Germany. NO gas was purchased from Air Liquide, Düsseldorf, Germany.

2.7. Statistical analysis

Results are presented as means±SD. ANOVA followed by Bonferroni’s correction for multiple measurements was employed where appropriate. Paired data were analyzed by Student’s t-test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Baseline function and energy status

Isolated guinea pig hearts ($n=7$), paced and perfused at a constant coronary perfusion pressure of 55 mmHg, developed a peak systolic pressure of $82±13$ mmHg. Basal
oxygen consumption was 3.6±0.5 μmol·min⁻¹·g⁻¹ and coronary flow 5.1±0.8 ml·min⁻¹·g⁻¹. Cardiac contractile function, oxygen consumption and coronary flow were stable prior to application of NO (Fig. 1). Under basal conditions, the PCr-to-ATP ratio as measured by ³¹P NMR spectroscopy was 1.96±0.26, corresponding to a PCr concentration of 13.7±1.8 mM at 6.98 mM ATP, similar to previously reported data [22]. Free cytosolic ADP was determined to be 37±13 μM, intracellular pH was 7.13±0.04, free cytosolic inorganic phosphate 2.1±0.7 mM and the free energy change of ATP hydrolysis (ΔG_{ATP}) was −60.3±1 kJ·mol⁻¹.

3.2. Functional and energetic consequences of NO infusion

Infusion of a saline solution saturated with NO gas at a final concentration of 50 μM rapidly induced contractile dysfunction (Fig. 1). Left ventricular developed pressure (LVDP) decreased by 50%. This was primarily due to an immediate fall in peak systolic pressure by 35 mmHg, while end-diastolic pressure (LVEDP) slowly but continuously increased from 0.1±2.1 to 8.9±4.3 mmHg during NO application. The decrease in developed pressure was associated with an almost proportional decline in oxygen consumption (Fig. 1). MVO₂ decreased from 3.7±0.5 to 1.3±0.8 μmol·min⁻¹·g⁻¹. During NO application, a small albeit significant recovery of oxygen consumption was observed, attaining 1.83±0.86 μmol·min⁻¹·g⁻¹ at the end of NO. As expected, the onset of 50 μM NO caused a massive coronary vasodilation (9.99±1.4 vs. 4.82±0.6 ml·min⁻¹·g⁻¹). After 32 min of NO, coronary flow was still 50% above baseline. The effect of NO on LVDP, LVEDP and MVO₂ was rapidly reversible upon cessation of NO infusion.

Cardiac energy status was stable under basal conditions (Fig. 2). The NO-induced contractile depression was associated with a rapid decline in PCr with only a marginal effect on ATP, resulting in a rise in inorganic phosphate up to 12.5±7.5 mM and a major decrease in ΔG_{ATP} down to −51.3±1.7 kJ·mol⁻¹ (from −60.3±1 kJ·mol⁻¹). This was associated with a drop in intracellular pH from 7.13±0.04 to 7.05±0.05. During the course of NO application, a partial recovery of PCr was observed, reaching a new steady state after about 12 min. Immediately following the end of the NO infusion, PCr and ΔG_{ATP} almost fully recovered.

3.3. Magnetization transfer experiments

To test the hypothesis that the NO-induced decrease in energy status and cardiac contractility was due to inhibition of creatine kinase (CK) [14], the flux mediated by CK was assessed by ³¹P NMR magnetization transfer (MT).
Since measurement of the unidirectional forward flux of CK requires metabolic steady state for a minimum period of 15 min, MT experiments were performed under baseline conditions preceding NO infusion and in the later course of NO application, when PCr, ATP and $\Delta G_{\text{ATP}}$ had attained a new steady state (min 14–30, Fig. 2). Due to the constant PCr during MT, forward and reverse fluxes can be assumed to be identical and were not determined separately.

In Fig. 3, the representative spectra obtained during the baseline period (control) and during application of authentic NO (50 μM) demonstrate MT from $\gamma$-ATP to PCr. To eliminate any time-dependent effects, interleaved spectra with $\gamma$-ATP and control irradiation were obtained. In the presence of selective irradiation of $\gamma$-ATP (5 s), the PCr signal decreased by approximately 40% owing to transfer of “saturated” $\gamma$-phosphate groups from ATP to creatine forming PCr. NO caused a major decrease in PCr and a smaller reduction in ATP, as already seen in Fig. 2. Notably, in the presence of NO, $\gamma$-ATP irradiation resulted in an even greater relative decrease in PCr, demonstrating on a qualitative level the sustained activity of creatine kinase. When calculating the pseudo first order rate constant $k_{\text{for}}$ in the absence and presence of NO, it was found for that NO actually significantly increased this measure of CK activity (Fig. 4). Taking the 50% decrease in PCr into account, the unidirectional forward flux of CK did not change at all in presence of NO.

The simultaneous measurement of oxygen consumption allowed the direct comparison of CK flux and aerobic ATP synthesis. Assuming a P:O ratio of 3 under control conditions, CK turnover exceeded that of ATP more than 4-fold, while during application of NO (50 μM) CK flux was even almost 10-fold greater than aerobic ATP synthesis (Fig. 4).

Fig. 3. Representative $^{31}$P NMR spectra of the saline perfused guinea pig heart in absence and presence of NO (50 μM). When comparing the control to saturation of $\gamma$-ATP, the major decrease in the PCr signal due to creatine kinase induced saturation transfer is evident. (32 scans each, 15 s pulse interval, 90° pulses (75 μs), 5 s frequency-selective continuous wave irradiation).

Fig. 4. The pseudo-first-order rate constant $k_{\text{for}}$ of the creatine kinase (CK) reaction, the PCr concentration, the CK flux as determined by $^{31}$P NMR saturation transfer and the ATP synthesis rate as calculated from measurements of oxygen consumption. Data were acquired before and during NO application. ($n=7$, **$P<0.01$ vs. control).
3.4. Surface fluorescence data

Since the magnetization transfer experiments virtually ruled out a functionally significant inhibition of creatine kinase, the NO (50 μM) induced simultaneous decrease of oxygen consumption and ΔGATP suggested a direct inhibition of mitochondrial oxidative phosphorylation. To test this hypothesis mitochondrial NADH was assessed by surface fluorescence (SF) in a separate experimental series. As shown in Fig. 5A, when excited at 365 nm basal SF spectra of the saline perfused guinea pig heart are almost identical to the spectrum of NADH in the range from 420 to 600 nm. Subjecting the heart to ischemia, known to increase mitochondrial NADH, elevates SF, the maximum being at 465 nm. Following application of NO (50 μM), a similar, albeit slightly smaller rise in SF was observed (Fig. 5A). This was clearly shifted to lower wavelengths, corresponding to a blue-shift.

In the range from 420 to 440 nm myoglobin contributes to tissue light absorbance [11]. Based on the formation of Met-myoglobin in presence of NO (50 μM) [24], we wondered whether NO alters tissue light absorption and thus shifts the spectrum in this range. Hearts were loaded with succinimidyl-N-methylanthranilate (SMA), a high-intensity fluorophore, and the effects of NO on the myocardial SF spectrum was assessed. As shown in Fig. 5B, NO induced a blue-shift of the SF spectrum also in the SMA-loaded hearts. Additionally, in vitro experiments indicated that NO has no direct effect on the fluorescence spectra of NADH and SMA in solution and does not induce a NAD signal. In consequence, the NO-induced rise in the myocardial SF signal at 460–480 nm (Fig. 5A) can be taken as measure of changes in mitochondrial NADH.

When studying the SF kinetics at 480 nm, short term ischemia of 30 s reversibly increased fluorescence intensity by approximately 60%. When starting NO application (50 μM), fluorescence rapidly increased (Fig. 5C), concomitant with the fall in left ventricular developed pressure (see Fig. 1). Maximum fluorescence was seen after 1 min of NO. Thereafter, a slow but continuous decline of SF was observed. Stopping NO induced a further drop in the fluorescence intensity by about 10–20%, that was consistently observed in all hearts.

To normalize the results of different experiments (n = 7), fluorescence was expressed in percent of the basal steady state fluorescence signal. As shown in Fig. 6, NO induced a significant rise in SF at 480 nm, consistent with a rise in mitochondrial NADH. This increase was 60% of that caused by 30 s of ischemia and about 1/3 of that seen after 10 min of ischemia.

![Fig. 5. Panel A: Fluorescence spectra (excitation wavelength 365 nm) obtained from 50 μM NADH in saline and from the cardiac surface under basal conditions, following the onset of NO (50 μM) and 30 s of ischemia. Panel B: Effect of NO on the surface fluorescence spectrum of succinimidyl-N-methylanthranilate (SMA) loaded guinea pig hearts. Spectra were acquired under basal conditions, after 30 s ischemia, following 35 min loading of 100 μM SMA and 15 min washout, and 1 min after start of 50 μM NO. Panel C. Representative time course of surface fluorescence (480 nm) intensity under basal conditions, during 20 s ischemia (I) and during 32 min NO (50 μM). The heart was illuminated for few seconds in regular intervals (see circles) only.](https://academic.oup.com/cardiovascres/article-abstract/51/2/313/293027)

![Fig. 6. Changes in mitochondrial NADH upon NO (50 μM) (n=7) as assessed by the 480 nm surface fluorescence emission signal (365 nm excitation) are compared to the effects of 30 s and 10 min of ischemia. Data are expressed relative to basal fluorescence intensity. P<0.05 vs. baseline.](https://academic.oup.com/cardiovascres/article-abstract/51/2/313/293027)
4. Discussion

In recent years, the NO-induced inactivation of the cytosolic or mitochondrial isoforms of creatine kinase attracted considerable attention [8,14–18]. Possible mechanisms of action include a direct nitrosylation [16] or thiol oxidation by the NO metabolite peroxynitrite [18]. Because most of these results were obtained using isolated CK isoforms, it remained unclear however, whether CK inactivation, and consequently a reduced CK flux, plays any relevant role in cardiac contractile dysfunction elicited by NO in vivo. Data presented in this study strongly suggest that NO-induced inactivation of CK can not explain the cardiodepressant actions of NO in the intact heart. The reasons are as follows:

- When measuring the unidirectional forward flux of CK in the intact heart by $^{31}$P NMR magnetization transfer, the CK flux was identical in absence and presence of NO concentrations (50 μM) which decreased contractile function by 50% (Fig. 4). Since these measurements were performed in the steady state, i.e. at constant PCR concentrations, forward and reverse fluxes must be the same.
- To test whether altered substrate concentrations (PCR, ADP, Cr, ATP) may have resulted in identical flux rates measured in spite of a partial CK inactivation, we have calculated the expected in vivo fluxes using an established kinetic model of the CK reaction [25]. Accounting for the kinetic constants reported for the MM isoform of muscle creatine kinase [26], and the relevant concentrations of PCR, Cr, ATP and ADP measured in the present study before and during NO application, the expected in vivo flux can be calculated to be 15% and 17% of $V_{max}$, respectively. This is in almost perfect agreement with the similarity of flux rates as determined by NMR.
- For CK to become rate-limiting, the flux mediated by the enzyme must be close to the rate of ATP synthesis. The CK flux, however, measured by $^{31}$P NMR magnetization transfer was several-fold higher than the rate of oxidative ATP formation (Fig. 4) under basal conditions, and almost 10-fold higher than ATP synthesis during NO application. This makes it highly unlikely that CK flux may have been limiting for energy supply or energy transduction.
- The rapid decrease in LVPD upon the onset of NO infusion was associated with an immediate decrease in PCr while ATP remained largely unchanged (Fig. 2). Thus CK activity was sufficient to catalyze the phosphoryl-transfer from PCr to ADP, contributing to the maintenance of ATP. These data are at odds with those of a previous study in which the NO donor SNAC (5 mM) only modestly depressed contractile function but caused a rapid drop in ATP with no effects on PCr under basal conditions [14]. Only when extracellular Ca$^{2+}$ was elevated a decline in PCr became visible. Possibly biochemical side-effects of the NO-donor SNAC may explain the discrepancy of findings.
- The decrease in left ventricular developed pressure and oxygen consumption on the onset of NO (50 μM) occurred rapidly (Fig. 1), in line with our previous observations [9]. In contrast, CK inhibition by NO donors requires between 10 and 30 min for full inactivation [14–16], even when applying NO donors in the millimolar range. Even after 32 min of NO exposure, cessation of NO resulted in an almost instantaneous recovery of left ventricular contractile function and cardiac energy status (Figs. 1 and 2). This rapid time course is incompatible with the proposed models of CK inactivation. Nitrosylation of the central cysteine of soluble CK, which has been shown to be essential for enzyme activity (Cys 283 in human B CK [27]), as well as thiol oxidation of the same cysteine residue would result in either slowly reversible [16] or irreversible inhibition of the enzyme [18].

Since in the intact heart, NO did not result in creatine kinase inactivation, the NO-induced decrease in oxygen consumption, impairment of energy status and cardiac function is most likely due to a direct inhibition of the respiratory chain, possibly at the ferrocytochrome a of the cytochrome c oxidase (for review see [28]). Consistent with this interpretation, immediately after the onset of NO (50 μM) a rise in mitochondrial NADH as assessed by surface fluorescence was observed, similar in magnitude to short term ischemia (Figs. 5 and 6). NO not only increased the intensity of the SF spectrum at 460–480 nm, but also shifted it to lower wavelengths. This was most pronounced in the range from 420 to 440 nm, where the absorbance of myoglobin [11] and its interaction with NO will have the greatest effect. Since we observed a similar shift following loading with succinimidyl-N-methylanthranilate (Fig. 5B) and have recently shown the NO-induced formation of Met-Myoglobin in the intact heart [24], we suggest that the blue shift of the SF spectrum in presence of NO is due to altered tissue light absorbance.

In contrast to ischemia, NO induced only a temporary rise in SF and thus in mitochondrial NADH: A rapid rise was followed by a slow decline. This was seen both at 480 nm (Fig. 5C) and at 460 nm (data not shown). The transient nature of the rise in NADH may be partly explained by a NADH-induced inhibition of pyruvate dehydrogenase, citrate synthase or α-ketoglutarate dehydrogenase, in presence of a slowed, but continuing NADH oxidation — in marked contrast to total global ischemia. An additional factor may be enhanced fatty acid oxidation induced by NO [29], reducing NADH and enhancing FADH$_{2}$ formation.

When considering the different sensitivities to NO determined in vitro for isolated mitochondria and purified CK enzymes, the results of the present study are not...
surprising. While NO donor and peroxynitrite concentrations of about 200 μM were necessary to partially inhibit purified CK enzymes [14–16], NO concentrations below 1 μM were sufficient to almost completely block mitochondrial respiration [30]. In fact, at physiologically relevant cytosolic PO2 values of about 5 mmHg [31] a Ki of less than 27 nM NO for blocking respiration was reported in isolated mitochondria [7]. This NO concentration is most likely in the range present in cardiomyocytes under basal conditions.

When applying NO in a previous study, we observed a dose-dependent negative inotropic effect only at concentrations well above those necessary for maximal vasodilatation and coronary venous cGMP release [9]. At high NO concentrations a substantial decrease in the free energy of ATP hydrolysis (ΔGATP) and major rise in inorganic phosphate appeared to govern cardiac contractility. In the present study, the NO concentration of 50 μM was purposely chosen to be within this range. It can thus be assumed that cGMP-mediated mechanisms did not contribute to any major extent to the reduction in energy turnover.

The main conclusion of the present study may be criticized for the following reasons.

- The unidirectional creatine kinase flux as determined by 31P NMR magnetization transfer may not represent a measure of total cellular CK activity. It may possibly assess cytosolic, but not mitochondrial and protein bound CK [32]. It should be kept in mind, however, that the postulated mechanisms of CK inactivation are identical for cytosolic [16] and mitochondrial CK [18]. This makes a selective inhibition of mitochondrial or bound CK unlikely. Moreover, provided that the unidirectional flux through mitochondrial CK plays an important role in ADP delivery to ATP synthase and energy transduction, inhibition of this enzyme could be expected to have consequences on the cytosolic CK flux, which however were not observed.

- The rate of aerobic ATP synthesis was determined from measurements of oxygen consumption, assuming a constant ratio of ATP formation to oxygen consumption (P/O-ratio) of 3:1. In fact, the P/O-ratio may be slightly lower, and an effect of NO on the mitochondrial proton leak reducing the P/O-ratio even further cannot be ruled out. In the present study, the flux mediated by CK was always much higher than the rate of aerobic ATP synthesis based on a P/O-ratio of 3:1. Any decrease in this ratio would even further lower the estimate for aerobic ATP synthesis but have no effect on the CK flux measured.

- In the present study, 50 μM NO was chosen to result in approximately a 50% decrease in contractility and oxygen consumption (Fig. 1). Due to the rapid decomposition of NO and various scavenging mechanisms along its route towards the cardiomyocyte’s cytosol, the precise concentration achieved intracellularly is not known but is probably much lower than the concentration applied intra-arterially. Our previous study [9] ruled out the involvement of stable metabolites such as nitrite and nitrate and the rapid recovery of function and energy status excludes long-term toxic effects.

### 4.1. Functional implications

The data of the present study clearly indicate that in the heart a sudden increase in cytosolic NO concentrations can reduce oxygen consumption and ATP synthesis, most likely by reversibly blocking the respiratory chain. This will be of major functional relevance when cardiac NO formation is enhanced, e.g. following the induced expression of the Ca2+-independent NOS2 in endotoxemia. It is only in the long term, that elevated NO levels may also result in S-nitrosylation of creatine kinase [14,16] or complexes of the mitochondrial respiratory chain [33], and thereby contribute to the contractile dysfunction.

In isolated mitochondria, NO has been shown to be a competitive inhibitor of O2 consumption at cytochrome c oxidase [7]. The low cytosolic PO2 in the heart [31] will increase the sensitivity of mitochondria to the effects of NO. It is thus well conceivable that in the presence of hypoxia, e.g. due to a coronary stenosis, the reduction of oxygen consumption and ATP generation is potentiated by the tonic presence of NO formed primarily in the endothelium but in cardiomyocytes as well. To what extent NO contributes to a control of oxygen consumption under physiological conditions by directly blocking mitochondrial respiration [6] remains to be seen. Also the functional relevance of mitochondrial NO synthase [34] in the context of regulation of myocardial oxidative phosphorylation needs to be firmly established.

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