Characteristics and function of cardiac mitochondrial nitric oxide synthase

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We used laser scanning confocal microscopy in combination with the nitric oxide (NO)-sensitive fluorescent dye DAF-2 and the reactive oxygen species (ROS)-sensitive dyes CM-H2DCF and MitoSOX Red to characterize NO and ROS production by mitochondrial NO synthase (mtNOS) in permeabilized cat ventricular myocytes. Stimulation of mitochondrial Ca\(^{2+}\) uptake by exposure to different cytoplasmic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\) = 1, 2 and 5 \(\mu\)M) resulted in a dose-dependent increase of NO production by mitochondria when l-arginine, a substrate for mtNOS, was present. Collapsing the mitochondrial membrane potential with the protonophore FCCP or blocking the mitochondrial Ca\(^{2+}\) uniporter with Ru360 as well as blocking the respiratory chain with rotenone or antimycin A in combination with oligomycin inhibited mitochondrial NO production. In the absence of l-arginine, mitochondrial NO production during stimulation of Ca\(^{2+}\) uptake was significantly decreased, but accompanied by increase in mitochondrial ROS production. Inhibition of mitochondrial arginase to limit l-arginine availability resulted in 50% inhibition of Ca\(^{2+}\)-induced ROS production. Both mitochondrial NO and ROS production were blocked by the nNOS inhibitor (4S)-N-(4-amino-5-[aminoethyl]aminopentyl)-N'-(4-amino-5[methylaminopentyl])-N'-nitroguanidine and the calmodulin antagonist W-7, while the eNOS inhibitor l-N\(^5\)-(1-iminoethyl)ornithine (l-NIO) or iNOS inhibitor N-(3-aminomethyl)benzylacetamidine, 2HCl (1400W) had no effect. The superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP abolished Ca\(^{2+}\)-induced ROS generation and increased NO production threefold, suggesting that in the absence of MnTBAP either formation of superoxide radicals suppressed NO production or part of the formed NO was transformed quickly to peroxynitrite. In the absence of l-arginine, mitochondrial Ca\(^{2+}\) uptake induced opening of the mitochondrial permeability transition pore (PTP), which was blocked by the PTP inhibitor cyclosporin A and MnTBAP, and reversed by l-arginine supplementation. In the presence of the mtNOS cofactor (6R)-5,6,7,8,-tetrahydrobiopterin (BH\(_4\); 100 \(\mu\)M) mitochondrial ROS generation and PTP opening decreased while mitochondrial NO generation slightly increased. These data demonstrate that mitochondrial Ca\(^{2+}\) uptake activates mtNOS and leads to NO-mediated protection against opening of the mitochondrial PTP, provided sufficient availability of l-arginine and BH\(_4\). In conclusion, our data show the importance of l-arginine and BH\(_4\) for cardioprotection via regulation of mitochondrial oxidative stress and modulation of PTP opening by mtNOS.

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In the cardiovascular system, nitric oxide (NO) plays an integral role in the regulation of ion channels, myocyte contraction, oxygen consumption, substrate utilization, apoptosis and hypertrophy (Massion & Balligand, 2003; Massion et al. 2003; Belge et al. 2005; Davidson & Duchen, 2006). It is now well established that NO is constitutively generated in the heart, not only by endothelial cells but also by cardiac myocytes themselves (Kaye et al. 1996; Kelly et al. 1996; Kanai et al. 1997; Dedkova et al. 2002, 2003, 2007; Wang et al. 2002, 2005b; Dedkova & Blatter, 2008). NO is formed during enzymatic conversion of l-arginine to l-citrulline by nitric oxide synthase (NOS) in the presence of O\(_2\) and NADPH. Several reducing cofactors such as flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), (6R)-5,6,7,8,-tetrahydrobiopterin (BH\(_4\)), ferroprotoporphyrin IX (haeme) and the regulatory protein calmodulin are required for NO
generation (Moncada et al. 1991). The catalytic mechanism of NOS involves flavin-mediated electron transport from NADPH in the reductase domain to haeme in the amino-terminal oxidase domain, where oxygen binds and is incorporated into NO and L-citrulline (Griffith & Stuehr, 1995). However, if the electron flow from NADPH to haeme is interrupted, NOS can generate superoxide (O$_2^-$) instead of NO by causing dissociation of the ferrous-dioxygen complex (Vasquez-Vivar et al. 1998, 1999, 2003; Schulz et al. 2008). This process is called NOS uncoupling, i.e. referring to the uncoupling of NADPH oxidation and NO synthesis (Pou et al. 1992; Berka et al. 2004; Yokoyama & Hirata, 2007; Berka et al. 2008).

Although the molecular mechanisms underlying NOS uncoupling still remain to be determined fully, a deficiency of NOS substrate L-arginine and the cofactor NADPH may be a major contributing factor (Heinzel et al. 1992; Wever et al. 1997; Vasquez-Vivar et al. 1998).

Three distinct isoforms of NOS derived from separate genes are well characterized: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Until recently, eNOS (localized to caveolae) was considered to be the only isoform constitutively expressed in ventricular myocytes and thus the source of NO involved in the autocrine regulation of myocardial contraction and Ca$^{2+}$ homeostasis. However, NOS targeted to the cardiac sarcoplasmic reticulum (SR) (Xu et al. 1999) and NOS localized to inner mitochondrial membrane of cardiac mitochondria (mtNOS) (Kanai et al. 2001; Elfering et al. 2002) have been identified. Despite evidence for the existence of cardiac mtNOS from immunohistochemical (Bates et al. 1996), biochemical (Elfering et al. 2002; Boveris et al. 2003; Zanella et al. 2004; Gonzales et al. 2005), spectrophotometrical (Costa et al. 2002; Boveris et al. 2003; Gonzales et al. 2005; Zaobornyj et al. 2005, 2007; La Padula et al. 2008), and electrochemical (Kanai et al. 2001, 2004) studies, skepticism regarding its existence, origin, as well as functional role has remained (Brookes, 2004; Tay et al. 2004; Lacza et al. 2006a,b; Csordas et al. 2007). The skepticism is mainly attributed to the impurity of mitochondrial preparations and the fact that the detected NOS activity in mitochondrial fractions might be related to NOS contamination from other organelles and membrane fractions. All three mtNOS isoforms were proposed as candidates for mtNOS; however, in the heart, the most convincing data implicate nNOS (Kanai et al. 2001, 2004; Elfering et al. 2002; Dedkova & Blatter, 2006; La Padula et al. 2008) as a primary candidate for mtNOS. At the same time, however, also evidence in favour of eNOS (Bates et al. 1996; Hotta et al. 1999; Zanella et al. 2004) or iNOS (French et al. 2001; Valdez et al. 2004; Zanella et al. 2004; Gonzales et al. 2005; La Padula et al. 2008) was forwarded. In addition, Lacza et al. (2003) were unable to relate mitochondrial NO production to any of the three known NOS isoforms, and therefore speculated that a bacterial NOS-like oxygenase enzyme (Lacza et al. 2006b) or even the respiratory chain itself (Lacza et al. 2006a) could be a source of mitochondrial NO. Indeed, the proteins with NOS-like activities were discovered in plants and lower organisms, such as bacteria and Arabidopsis thaliana (Adak et al. 2002a,b; Guo & Crawford, 2005; Gupta et al. 2005). mAtNOS1, the mammalian homologue of the Arabidopsis thaliana NOS-like gene (AtNOS1) was reported in mouse (Zemojtel et al. 2006) and human (Parihar et al. 2008ab,d), demonstrating the possible existence of a novel NOS enzyme different from the well-characterized eNOS, nNOS and iNOS isoforms.

The methods of mitochondrial NO detection have also been criticized extensively for possible sources of artifacts (Brookes, 2004; Tay et al. 2004). For example, it has been cautioned (Tay et al. 2004) that the Griess assay employed for NO detection can yield false positive results not related to mitochondrial NO production. Furthermore, confocal microscopy studies using NO-sensitive dyes to identify mitochondrial NO production have been criticized (Brookes, 2004) on the basis that NO can freely diffuse within the cell, rendering it difficult to pinpoint mitochondria themselves as the NO source. While this argument may hold for intact cells, the utilization of cell permeabilization techniques or using isolated mitochondria should overcome this problem. Despite these critiques, the functional studies performed on isolated mouse (Kanai et al. 2001, 2004) and rat (Manzo-Avalos et al. 2002; Saavedra-Molina et al. 2003; Zanella et al. 2004; Zaobornyj et al. 2005; Valdez et al. 2006; Nazarewicz et al. 2007; Valdez & Boveris, 2007; Zenebe et al. 2007) cardiac mitochondria clearly demonstrated mitochondrial NO production upon activation of mitochondrial Ca$^{2+}$ uptake.

In summary, all methodological approaches used so far have failed to reach an unequivocal conclusion whether mitochondria of cardiomyocytes contain NO within the organelle and whether this enzyme is able to produce physiologically relevant amounts of NO or O$_2^-$ (Brookes, 2004; Davidson & Duchen, 2006; Dedkova & Blatter, 2008). In the present study, we directly measured NO production in permeabilized cat ventricular myocytes, and determined that mtNOS plays an important role in the regulation of the mitochondrial permeability transition pore (PTP). A previous account of this work was presented in abstract form (Dedkova & Blatter, 2006).

Methods

Cell isolation and solutions

Left ventricular myocytes were isolated from cat hearts (Rubenstein & Lipsius, 1995). Cats ($n = 40$) were obtained...
from R & R Research (Howard City, MI, USA). The vendor and the procedure for cell isolation were fully approved by the Institutional Animal Care and Use Committee. Adult cats of either sex were anaesthetized with sodium pentobarbital (50 mg kg\(^{-1}\), i.p.). Following thoracotomy hearts were quickly excised, mounted on a Langendorff apparatus, and retrogradely perfused with a bicarbonate-buffered Tyrode solution for approximately 5 min, followed by perfusion with a nominally Ca\(^{2+}\)-free Tyrode solution. After 5 min the perfusion was switched to a low Ca\(^{2+}\) (36 μM) Tyrode solution containing 0.06% collagenase (type II, Worthington Biochemical Corp., Lakewood, NJ, USA) for 25–30 min. Following collagenase digestion, small pieces of ventricular muscle were scraped from the free wall of the left ventricular endocardial surface and agitated in fresh 0.06% collagenase, and 0.01% protease (Sigma, Type XIV) for 5 min. Cells were placed in Hepes-buffered modified Tyrode solution containing (in mM): NaCl 145, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 2, Hepes 5, glucose 11, titrated with NaOH to a pH 7.4, and used within 6 h after isolation. All experiments were performed at room temperature (20–22°C).

Permeabilized ventricular myocytes

The sarcolemma of cardiomyocytes was permeabilized by brief (60 s) exposure to 10 μM digitonin in an ‘intracellular’ solution containing (in mM): 135 KCl, 10 NaCl, 20 Hepes, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH\(_2\)PO\(_4\), 0.5 MgCl\(_2\), 15 2,3-butanedione monoxime (BDM), 5 EGTA, and 1.86 CaCl\(_2\) to yield a free [Ca\(^{2+}\)] of ∼100 nM.

Fluorescence measurements

Confocal microscopy. Laser scanning confocal microscopy (LSM 410, Carl Zeiss, Germany) was used to monitor changes in the [NO]_{mt}, ROS production, and PTP opening during activation of mitochondrial Ca\(^{2+}\) uptake. For fluorescence measurements the coverslip with attached cells was mounted on the stage of an inverted microscope equipped with a 40× oil immersion objective (Plan-Neofluar, NA = 1.3, Carl Zeiss, Germany).

[NO]_{mt} measurements. Direct measurements of NO production from mitochondria were performed on permeabilized ventricular myocytes loaded with the fluorescent NO-sensitive dye 4,5-diaminofluorescein diacetate (5 μM DAF-2 DA; Calbiochem/EMD Biosciences, San Diego, CA, USA) (Kojima et al. 1998) for 40 min at 37°C. DAF-2 was excited with the 488 nm line of the argon laser, and emitted fluorescence was measured at 510–525 nm. Mitochondrial DAF-2 fluorescence intensity (F) in each experiment was normalized to the level of fluorescence recorded prior to stimulation (F\(_0\)) but after cell permeablization. Changes in [NO]_{mt} are expressed as ΔF/F\(_0\), thus representing the total percentage increase above basal level. In a subset of experiments 200 nM MitoTracker Red CMXRos (Invitrogen/Molecular Probes, Carlsbad, CA, USA) was added during the final 10 min of DAF-2 loading to stain mitochondria (Poot et al. 1996). Both indicators were excited at 488 nm, and the emitted fluorescence signals were measured simultaneously at 510–525 nm (DAF-2) and 590 nm (MitoTracker Red CMXRos). Regions of interest (ROIs; ≤ 40 μm\(^2\)) were positioned over mitochondria, thus representing [NO]_{mt} measurements from a small number of mitochondria (≤ 40). L-Arginine (100 μM) was added to all solutions, unless specifically indicated. The NO donor spermine NONOate (Sper/NO; 300 μM) was added at the end of each experiment to produce a maximal DAF-2 signal and demonstrate that pharmacological interventions had no effect on DAF-2 fluorescence.

ROS production. ROS production was measured in permeabilized ventricular myocytes loaded with either 10 μM 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H\(_2\)DCF DA, Invitrogen/Molecular Probes) or 1 μM MitoSOX Red (Invitrogen/Molecular Probes) for 30 min at 37°C. CM-H\(_2\)DCF (DCF) was excited at 488 nm and emitted fluorescence was recorded at > 510 nm, while MitoSOX Red was excited at 514 nm with fluorescence measured at 590 nm. Changes in mitochondrial dye fluorescence intensity (F) were normalized to the level of fluorescence recorded prior to stimulation (F\(_0\)), and expressed as ΔF/F\(_0\). The rate of ROS production (d(ΔF/F\(_0\))/dt) was estimated from the initial linear phase of the DCF or MitoSOX fluorescence increase in order to minimize potential problems arising from mitochondrial dye saturation and leakage.

PTP activity. PTP activity was monitored using the fluorescent dye calcein in permeabilized ventricular myocytes. Opening of PTP resulted in the loss of mitochondria-trapped calcein (620 Da) and a decrease of fluorescence (Huser et al. 1998; Dedkova & Blatter, 2005). Cells were loaded with 5 μM of the membrane-permeant form of the fluorescent probe calcein/AM (Invitrogen/Molecular Probes) for 40 min at 37°C. After dye loading cells were placed in dye-free Tyrode solution for 10 min to wash off the excess dye. The calcein fluorescence was excited at 488 nm, and the emitted fluorescence signal was measured at > 510 nm. At the end of each recording 10 μg ml\(^{-1}\) of the pore-forming antibiotic alamethicin (Marsh, 1996) was applied to provide a control measure for maximum calcein release from mitochondria. Loss of mitochondrial calcein induced by elevating extramitochondrial [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{em}\)) was
quantified as the rate of decline of fluorescence calculated from the linear fit to the initial decrease of calcein fluorescence. The rate of decline was normalized to the basal decline of calcein fluorescence in control conditions ([Ca2+]_{em} = 0.1 μM; 100%).

Chemicals

The protonophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), methyl-β-cyclodextrin (MCD) (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH$_4^-$), digitonin, antimycin A (AntA), oligomycin, rotenone, and alamethicin were obtained from Sigma (St Louis, MO, USA). Spermine NONOate (Sper/NO), Ru360, L-N$^2$-(1-iminoethyl)ornithine (L-NIO), (4S)-N-(4-amino-5[aminoethyl]aminopentyl)-N$'$-nitroguanidine (nNOS blocker I), N-(3-aminomethyl)benzylacetamidine, 2HCl (1400W), N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W-7), (S)-(2-boronoetyl)-l-cysteine (BEC), and Mn(III)tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP) were from Calbiochem/EMD Bioscience.

Statistical analysis

Statistical differences of the data were determined with Student’s $t$ test for unpaired data and considered significant at $P < 0.05$. Results are reported as means ± standard error of the mean (S.E.M.) for the indicated number ($n$) of cells.

Results

Direct measurements of [NO] in mitochondria of single permeabilized cat ventricular myocytes

For the direct measurement of NO production by mitochondria of cat ventricular myocytes we used the NO-sensitive fluorescent indicator DAF-2. This dye has been employed successfully to estimate NO levels in endothelial cells as well as in cardiomyocytes produced by the constitutive NOS (Dedkova & Blatter, 2002; Wang et al. 2002, 2005b; Dedkova et al. 2003, 2004, 2007). After cell loading with DAF-2 DA under conditions favouring mitochondrial dye compartmentalization (see Methods for details), intact cells showed relatively homogeneous dye distribution throughout the cell, with the exception of the intense fluorescence around the nuclei (Fig. 1A, DAF-2, cntrl). Plasma membrane permeabilization with digitonin removed the cytosolic and nuclear DAF-2 (Fig. 1A, DAF-2, digitonin), revealing the particulate and punctate fluorescence pattern characteristic of mitochondria (Fig. 1A, Mitotracker Red, digitonin). The colocalization of DAF-2 fluorescence remaining after cell permeabilization with Mitotracker Red fluorescence indicated the mitochondrial origin of the DAF-2 signal (Fig. 1A, overlay, digitonin). Figure 1B shows that under basal conditions (0.1 μM Ca$^{2+}$) mitochondria revealed relatively low levels of NO. However, stimulation of mitochondrial Ca$^{2+}$ uptake by increasing [Ca$^{2+}$]$_{em}$ to 2 μM significantly enhanced the intensity of DAF-2 fluorescence suggesting NO production by mitochondria (Fig. 1B; 2 μM Ca$^{2+}$). On average the increase of ΔF/F$_0$ above basal level after 10 min exposure to 2 μM Ca$^{2+}$ was 1.21 ± 0.07 ($n$ = 25; $P < 0.001$). Figure 1C presents the typical time course of DAF-2 fluorescence intensity changes from mitochondria of ventricular myocytes before and after cell permeabilization with digitonin, and after activation of mitochondrial Ca$^{2+}$ uptake by increasing extramitochondrial Ca$^{2+}$ from 0.1 to 2 μM, followed by application of Sper/NO (cntrl). The Sper/NO-induced rate of increase of ΔF/F$_0$ was 5.3-fold faster than observed upon stimulation of mitochondrial Ca$^{2+}$ uptake. We have previously estimated that 300 μM Sper/NO releases ~3.2 μM NO in our experimental conditions (Dedkova & Blatter, 2005). This gives a rough estimate of NO produced during stimulation of mitochondrial Ca$^{2+}$ uptake of ~600 nM per 40 mitochondria or ~15 nM NO for a single mitochondrion. In separate sets of experiments (Fig. 1C), permeabilized cardiomyocytes were treated with 10 μM haemoglobin, a well known NO scavenger. Haemoglobin abolished both the Ca$^{2+}$- and Sper/NO-induced increase in DAF-2 fluorescence (the average ΔF/F$_0$ increase after 10 min of Ca$^{2+}$ exposure was 0.19 ± 0.03 ($n$ = 5; $P < 0.001$ compared to 10 min control Ca$^{2+}$ exposure) and after 2 min of Sper/NO exposure was 0.29 ± 0.06 ($n$ = 5; $P < 0.001$)), again confirming the specificity of DAF-2 for NO.

Dependence of mitochondrial NO production on [Ca$^{2+}$]$_{em}$

To examine the [Ca$^{2+}$]$_{em}$ dependence of mitochondrial NO production, permeabilized cells were exposed to various concentrations of extramitochondrial Ca$^{2+}$ (Fig. 2). Figure 2A shows an overlay of normalized traces of mitochondrial NO production obtained with different concentration of [Ca$^{2+}$]$_{em}$. First, we monitored changes in DAF-2 fluorescence from permeabilized myocytes maintained in 0.1 μM Ca$^{2+}$. As demonstrated in Fig. 2A (lower trace and representative images), no
substantial increase in DAF-2 fluorescence was observed during 10 min of recording ($\Delta F/F_0 = 0.11 \pm 0.04; n = 9$). However, an elevation of $[Ca^{2+}]_{em}$ from 0.1 to 1, 2 and 5 $\mu$M evoked a dose-dependent increase in DAF-2 fluorescence, indicating that the increasing $[Ca^{2+}]_{em}$ resulted in an increase in the rate and magnitude of mitochondrial NO production. The averaged data of normalized NO production ($\Delta F/F_0$) from mitochondria reached after 10 min of $Ca^{2+}$ exposure to 1, 2 or 5 $\mu$M of $[Ca^{2+}]_{em}$ were 0.56 ± 0.04 ($n = 4$), 1.21 ± 0.07 ($n = 25$) and 1.17 ± 0.08 ($n = 5$), respectively (Fig. 2B). We have shown previously (Sedova et al. 2006) that an increase of $[Ca^{2+}]_{em}$ results in a dose-dependent increase in mitochondrial $Ca^{2+}$ uptake in cat ventricular myocytes.

Figure 1. Measurements of NO production by mitochondria of permeabilized ventricular myocytes with compartmentalized dye DAF-2

A, from left to right are confocal images of a ventricular myocyte loaded with DAF-2 (green, emitted fluorescence recorded at 510–525 nm) and MitoTracker Red (red, > 590 nm) before (cntrl) and after cell permeabilization with digitonin (digitonin). The right panel shows the overlay of the two individual images. Colocalization of DAF-2 and MitoTracker Red is represented by shades of yellow. B, confocal images of a permeabilized DAF-2-loaded cell under unstimulated conditions ($[Ca^{2+}]_{em}$ = 0.1 $\mu$M), after an increase in $[Ca^{2+}]_{em}$ to 2 $\mu$M $Ca^{2+}$, and after subsequent addition of 300 $\mu$M Sper/NO (lower panel). Upper panel represents images at higher magnification from the area marked in the lower panel. The shades of grey were used to reflect the difference between the bright mitochondria, showing the longitudinal arrangement of individual mitochondria. C, time course of DAF-2 fluorescence recorded from a region of interest ($\leq 40 \mu m^2$) under conditions shown in A (cntrl) and B. When a different cell was treated with the NO scavenger haemoglobin (10 $\mu$M), the $Ca^{2+}$-induced increase in DAF-2 fluorescence was abolished (grey trace, Hg).

Disruption of caveolae and depletion of the sarcoplasmic reticulum do not affect the $Ca^{2+}$-induced NO production

The molecular identity or isoform of mtNOS has remained controversial. To date no unique gene has been identified with a half-maximal activation at $[Ca^{2+}]_{em} = 4.4 \mu$M. The data presented here indicate that mitochondrial NO production is also $Ca^{2+}$-concentration dependent; however, a maximum activation of mtNOS appeared to occur already at $[Ca^{2+}]_{em} = 2 \mu$M. Therefore, 2 $\mu$M $Ca^{2+}$ was used for all subsequent experiments.
for mtNOS, and all three types of NOS have been suggested to mediate mtNOS production (Brookes, 2004; Kato & Giulivi, 2006). In cardiomyocytes, the well established localization of the endothelial isoform of NOS (eNOS) are the caveolae of the plasma membrane (Feron & Balligand, 2006). Caveolae are 50–100 nm invaginations of plasma membrane that are rich in cholesterol, sphingolipids and lipid-anchored membrane proteins. Caveolae are rather resistant to solubilization by detergents (Shaul, 2002). Experimentally lowering the cholesterol level of caveolar fractions with methyl-β-cyclodextrin (MCD) has been shown to disrupt the molecular assembly and ultrastructure of the caveolar domain (Rodal et al. 1999).

To exclude a possible contribution from eNOS located at caveolae in our measurements of [NO]mt, cells were pretreated with 1 mM MCD for 1 h at 37°C. Under these conditions MCD disrupts caveole structure (Lohn et al. 2000; Calaghan et al. 2008; Horikawa et al. 2008) and blocks eNOS-related NO production (Dedkova et al. 2003; Wang et al. 2005b) without causing other structural changes within cardiomyocytes (Lohn et al. 2000; Horikawa et al. 2008). As shown in Fig. 3A, stimulation of mitochondrial Ca2+ uptake in methyl-β-cyclodextrin pretreated cells (+ MCD) induced mitochondrial NO production similar to control, indicating that eNOS located in caveolae was not a source of the observed Ca2+-induced NO generation.

To rule out a potential contribution from NOS located in the SR, we repeated our experiments in cardiomyocytes pretreated with the SR-ATPase inhibitor thapsigargin (TG; 1 μM) for 10 min before cell permeabilization at a concentration that has been shown to block SR-ATPase in intact cells within 90 s (Bassani et al. 1993). In permeabilized cells, however, even nanomolar

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**Figure 2.** [Ca2+]em dependence of mitochondrial NO production

A, measurements of NO production in permeabilized ventricular myocytes exposed to various [Ca2+]em. [Ca2+]em was elevated from 0.1 to 1, 2, and 5 μM or maintained at 0.1 μM for 10 min. Inserts are representative images of different cells maintained for 10 min in 0.1 (lower panel) or exposed to 2 μM Ca2+ (upper panel). B, average changes of [NO]mt as a function of [Ca2+]em measured 10 min after increasing [Ca2+]em.

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**Figure 3.** Disruption of caveolae and depletion of the sarcoplasmic reticulum do not affect the Ca2+-induced NO production

A, representative traces of changes in DAF-2 fluorescence from mitochondria of permeabilized myocytes in response to an increase of [Ca2+]em from 0.1 to 2 μM under control conditions and after cells were pretreated with 1 mM methyl-β-cyclodextrin (MCD, 60 min, 37°C) and 1 μM thapsigargin (TG, 10 min, room temperature). B, summary of changes of mitochondrial DAF-2 fluorescence (∆F/F0) obtained after an increase of [Ca2+]em from 0.1 to 2 μM Ca2+ in the presence and absence of MCD or TG. Numbers in parentheses indicate the number of cells tested. ∆F/F0 was measured 10 min after increasing [Ca2+]em.
concentrations of TG were sufficient to block the enzyme activity completely within 5 s (Hove-Madsen & Bers, 1993). Treatment with TG prevents Ca\textsuperscript{2+} uptake into the SR (Thastrup et al. 1990), and therefore the activation of NOS located in the SR. In the event of SR NOS activity, we would expect Ca\textsuperscript{2+} activity to be decreased or completely abolished. However, we did not observe any difference in Ca\textsuperscript{2+}-induced mitochondrial NO production to the intracellular solution (i.e. in the absence of L-arginine) and therefore the role of SR-localized NOS is likely to be minimal. The quantitative analysis of DAF-2 fluorescence changes revealed that neither MCD (ΔF/F₀ = 1.23 ± 0.27; n = 5) nor TG treatment (ΔF/F₀ = 1.23 ± 0.21; n = 8) affected mitochondrial NO production during Ca\textsuperscript{2+} uptake compared with control cells (ΔF/F₀ = 1.21 ± 0.07; n = 25; the means were not significantly different). These data demonstrate that the observed DAF-2 fluorescence increase was not due to activation of eNOS located in the plasma membrane caveolae or nNOS located in the SR.

Ca\textsuperscript{2+}-induced NO production critically depends on the availability of intracellular L-arginine and is modulated by tetrahydrobiopterin

All our experiments presented so far were performed in the presence of 100 μM L-arginine in the 'intracellular' solution, where L-arginine is the substrate for NO formation. The reported intracellular levels of L-arginine varied from ~21 μM in arterioles from soleus muscle (estimated from Delp et al. 2008), to ~30 μM in rat heart (estimated from Desrois et al. 2003), ~70 μM in human embryonic kidney 293 cells (Xia et al. 1996) and ~100–800 μM in cultured endothelial cells (Closs et al. 2000; Vukosavljevic et al. 2006). Thus supplementation with 100 μM L-arginine imitates an intact extra-mitochondrial environment. To determine the importance of L-arginine as substrate for mtNOS, we studied mitochondrial NO production in the absence and presence of 100 μM L-arginine. As shown in Fig. 4A, omitting L-arginine from the ‘intracellular’ solution significantly decreased mitochondrial NO production (ΔF/F₀ = 0.17 ± 0.01; n = 20, P < 0.001) compared to conditions where L-arginine was present (control: ΔF/F₀ = 1.21 ± 0.07; n = 25). Addition of 10 μM of tetrahydrobiopterin (BH\textsubscript{4}), an essential mtNOS cofactor, to the intracellular solution in the absence of L-arginine did not evoke an increase in DAF-2 fluorescence (ΔF/F₀ = 0.23 ± 0.07; n = 8, P < 0.001 compared to control). This concentration of BH\textsubscript{4} (10 μM) is frequently used in studies on isolated mitochondria (Giulivi et al. 1998; French et al. 2001) and in vitro NOS studies (Gao et al. 2007a,b), and it is similar to the reported intracellular BH\textsubscript{4} levels in adult cardiomyocytes (1.4 pmol (mg protein)\textsuperscript{-1} which corresponds to ~0.21 μM as estimated from Kalivendi et al. (2005)). Simultaneous application of 100 μM L-arginine and 10 μM BH\textsubscript{4} (Fig. 4C, L-arg + 10 μM BH\textsubscript{4}) did not increase DAF-2 fluorescence above control (e.g. L-arginine alone). However, when BH\textsubscript{4} concentration was elevated from 10 to 100 μM, the combined application of BH\textsubscript{4} and L-arginine increased mitochondrial NO production to ~26% above control (ΔF/F₀ = 1.65 ± 0.36; n = 5, P < 0.05; Fig. 4A and C, L-arg + 100 μM BH\textsubscript{4}). Addition of 100 μM BH\textsubscript{4} alone to the intracellular solution (i.e. in the absence of L-arginine) induced an increase in mitochondrial NO production; however, this increase (ΔF/F₀ = 0.34 ± 0.08; n = 13, P < 0.001) was significantly smaller than the increase induced by 2 μM Ca\textsuperscript{2+} in the presence of L-arginine (the trace is not shown, summary in Fig. 4C). These data are summarized in Fig. 4C. The results indicate that mitochondrial NO production was critically dependent on the availability of intracellular L-arginine, and was modulated by BH\textsubscript{4}.

Mitochondrial NO production is mediated by Ca\textsuperscript{2+}/calmodulin-dependent nNOS

To verify whether the observed NO production was indeed due to the activation of the mitochondrial NOS and to determine the isoform of NOS responsible for NO production by mitochondria, cardiomyocytes were preincubated with three different inhibitors of NOS, which have different selectivity toward nNOS, eNOS and iNOS. First, we used the well known inhibitor of eNOS, N\textsuperscript{3}-(1-iminoethyl)ornithine, dihydrochloride (L-NIO), which at a concentration of 1 μM has an 8-fold selectivity for eNOS over iNOS and nNOS (Moore et al. 1994). Figure 4B shows that cell pretreatment with 1 μM L-NIO for 5 min after cell permeabilization did not affect mitochondrial NO production during stimulation of mitochondrial Ca\textsuperscript{2+} uptake. To block nNOS activity, we treated cells with 240 nM (4S)-N\textsuperscript{3}-(3-aminomethyl)benzylacetamidine, (referred to here as nNOS blocker I), a novel inhibitor that at this concentration displays > 2500-fold and 320-fold selectivity over eNOS and iNOS, respectively (Hah et al. 2001). When the activity of nNOS was blocked by nNOS blocker I, exposure to 2 μM Ca\textsuperscript{2+} failed to induce NO production by mitochondria, while the NO donor Sper/NO was still able to increase DAF-2 fluorescence (Fig. 4B and C). Furthermore, the Ca\textsuperscript{2+} dependence of mitochondrial NO production by itself would rule out the contribution of iNOS in this process since iNOS is a Ca\textsuperscript{2+}-independent enzyme. To exclude the contribution of iNOS to mitochondrial NO production, we pretreated cells with 15 nM of the highly selective and irreversible iNOS inhibitor N\textsuperscript{3}-(3-aminomethyl)benzylacetamidine, 2HCl (1400W, K\textsubscript{d} = 7 nM) which exhibits greater
than 5000- and 200-fold inhibitory potency against human iNOS relative to eNOS and nNOS, respectively, and greater than 1000-fold potency against rat iNOS relative to eNOS (Garvey et al. 1997). As expected, cell treatment with iNOS blocker 1400W did not affect Ca^{2+}-induced mitochondrial NO production (Fig. 4B and C). Mitochondrial Ca^{2+} uptake-induced NO production was completely blocked by nNOS blocker I treatment ($\Delta F/F_0 = 0.04 \pm 0.01; n = 7, P < 0.001$) and was not affected by l-NIO ($\Delta F/F_0 = 1.16 \pm 0.13; n = 6, P = 0.64$) or 1400W ($\Delta F/F_0 = 1.02 \pm 0.08; n = 6, P = 0.21$) (Fig. 4B and C). In summary, these data suggest that the observed increase in DAF-2 fluorescence during activation of mitochondrial Ca^{2+} uptake was due to NO production by nNOS.

To explore the calmodulin dependence of mitochondrial NO production, cells were preincubated with the calmodulin inhibitor W-7 (50 μM; 10 min) prior to [NO]_{mt} measurements. As shown in Fig. 4B, W-7 significantly inhibited the increase in [NO]_{mt} normally induced by mitochondrial Ca^{2+} uptake, suggesting the constitutive nature of mtNOS in ventricular myocytes.

Figure 4. Effects of NOS inhibitors, calmodulin antagonist, l-arginine, and tetrahydrobiopterin (BH4) on mitochondrial NO production
A, representative traces of changes in DAF-2 fluorescence from mitochondria of permeabilized myocytes in response to an increase of $[Ca^{2+}]_{em}$ from 0.1 to $2 \mu M$ Ca^{2+}, followed by exposure to $300 \mu M$ Sper/NO under control conditions (i.e. in the presence of 100 μM l-arginine), in the absence of l-arginine (no l-arg), in the presence of 100 μM BH4 alone (no l-arg + BH4), and during simultaneous application of 100 μM l-arginine and 100 μM BH4 (l-arg + 100 μM BH4). B, representative traces of changes in DAF-2 fluorescence from mitochondria of permeabilized myocytes in response to an increase of $[Ca^{2+}]_{em}$ from 0.1 to $2 \mu M$ Ca^{2+}, followed by exposure to $300 \mu M$ Sper/NO after cell pretreatment with 240 nM nNOS inhibitor (4S)-N-(4-amino-5[aminomethyl]aminopentyl)-N′-nitroguanidine (nNOS blocker I), 1 μM eNOS inhibitor (L-NIO), 15 nM iNOS inhibitor N-(3-aminomethyl)benzylacetamidine (1400W) or 50 μM of the calmodulin antagonist W-7 (W-7). C, summary of changes in mitochondrial DAF-2 fluorescence ($\Delta F/F_0$) obtained after an increase of $[Ca^{2+}]_{em}$ from 0.1 to $2 \mu M$ Ca^{2+} in the presence and absence of l-arginine, BH4, nNOS blocker I, l-NIO, 1400W or W-7. Statistical significance: $^{*}P < 0.001$, $^{\circ}P < 0.05$ versus control.

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In a total of seven cells the average change of DAF-2 fluorescence ($\Delta F/F_0$) was 0.02 ± 0.01 compared with 1.21 ± 0.07 in control ($n = 25$, $P < 0.001$; Fig. 4B and C). Thus, these results suggest that mitochondrial Ca$^{2+}$ uptake leads to the activation of a constitutive Ca$^{2+}$/calmodulin-dependent nNOS possibly located in mitochondria.

**Mitochondrial dependence of Ca$^{2+}$-induced NO production**

Figure 5 shows the effect of inhibition of the mitochondrial Ca$^{2+}$ uniporter with Ru360 (5 $\mu$M; added for 2 min before increasing [$Ca^{2+}]_{em}$) on mitochondrial NO production. In the presence of Ru360 there were no significant changes in [$NO]_m$ in response to increasing [$Ca^{2+}]_{em}$ from 0.1 $\mu$M to 2 $\mu$M (Fig. 5A, Ru360) compared with control (Fig. 5A, cntrl). In summary, in cells pretreated with Ru360 the level of fluorescence ($\Delta F/F_0$) was 0.12 ± 0.04 ($n = 6$) after [$Ca^{2+}]_{em}$ elevation versus 1.21 ± 0.07 ($n = 25$, $P < 0.001$) in control (Fig. 5B).

In the next set of experiments we determined the effect of dissipating the mitochondrial membrane potential ($\Delta \Psi$) with the oxidative phosphorylation uncoupler FCCP. When cells were pretreated with 1 $\mu$M FCCP, elevation of [$Ca^{2+}]_{em}$ did not produce any changes in DAF-2 fluorescence (Fig. 5, FCCP). As summarized in Fig. 5B, in the presence of 1 $\mu$M FCCP $\Delta F/F_0$ (measured 10 min after [$Ca^{2+}]_{em}$ elevation) reached 0.08 ± 0.05 ($n = 7$) in comparison to 1.21 ± 0.07 ($n = 25$) in control ($P < 0.001$).

Next, we blocked the respiratory chain using rotenone (1 $\mu$M) or antimycin A (5 $\mu$g ml$^{-1}$) in combination with oligomycin (1 $\mu$g ml$^{-1}$) (Fig. 5A). Rotenone inhibits mitochondrial complex I of the respiratory chain, antimycin A inhibits the complex III of the respiratory chain, whereas oligomycin stops ATP synthesis by blocking mitochondrial H$^+$/ATP synthase. Since the processes of electron transfer and ATP synthesis are tightly coupled, the use of these inhibitors results in complete inhibition of cell respiration and oxidative phosphorylation. As shown in Fig. 5B blocking the respiratory chain resulted in nearly complete inhibition of mitochondrial NO production ($\Delta F/F_0$) was 0.15 ± 0.02 ($n = 8$) in the presence of rotenone and 0.11 ± 0.03 ($n = 5$) in the presence of antimycin A/oligomycin compared to 1.21 ± 0.07 ($n = 25$) in control; $P < 0.001$). Thus, these results indicate that the activity of the respiratory chain plays an important role in the regulation of mtNOS.

The Sper/NO-induced increase in DAF-2 fluorescence was not affected by these inhibitors indicating that mitochondrial-trapped DAF-2 was still capable of sensing changes in [$NO]_m$ (Fig. 5A). Taken together, these data suggest that the increase in mitochondrial NO production following elevation of [$Ca^{2+}]_{em}$ was due to Ca$^{2+}$ uniporter-mediated, $\Delta \Psi$-dependent Ca$^{2+}$ uptake into the mitochondria leading to the activation of the Ca$^{2+}$-dependent mitochondrial NOS.

**Effect of superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP on mitochondrial NO production**

It has been cautioned that DAF-2 may not be an ideal NO detector because it might sense other free radicals and reactive species such as peroxynitrite (Roychowdhury et al. 2002). Considering that mitochondria produce such species, we evaluated the contribution of superoxide anions to the Ca$^{2+}$-induced increases in DAF-2 fluorescence from mitochondria. To estimate whether
DAF-2 senses free oxygen radicals under our conditions we pretreated cells with the cell-permeant superoxide dismutase (SOD) mimic and peroxynitrite scavenger MnTBAP (50 μM), and examined changes in mitochondrial DAF-2 signal upon stimulation of mitochondrial Ca\(^{2+}\) uptake. As shown in Fig. 6, instead of attenuating the Ca\(^{2+}\)-induced rise of the DAF-2 signal, MnTBAP dramatically increased mitochondrial NO production (from ΔF/F₀ = 1.21 ± 0.07 in control (n = 25) to 3.08 ± 0.26 in MnTBAP-treated cells (n = 6, P < 0.001). This ~300% increase in mitochondrial DAF-2 fluorescence upon SOD mimetic treatment unmasked an even more pronounced NO production, and suggests that in the absence of Mn-TBAP either formation of superoxide radicals suppresses NO production or part of the formed NO is transformed quickly to peroxynitrite. Thus, the observed changes of DAF-2 fluorescence upon stimulation of mitochondrial Ca\(^{2+}\) uptake are consistent with underestimated levels of NO generation rather than peroxynitrite formation.

**Mitochondrial Ca\(^{2+}\) uptake stimulates ROS production in the absence of l-arginine**

Because the Ca\(^{2+}\)-induced NO production was significantly (3-fold) increased during cell treatment with MnTBAP, we tested whether mitochondrial Ca\(^{2+}\) uptake stimulates ROS production. For this purpose, we used the ROS-sensitive fluorescent dye CM-H\(_2\)DCF (DCF), which is oxidized to a fluorescent product by ROS. Therefore, the rate of DCF fluorescence increase serves as a measure of ROS generation. As indicated in Fig. 7A, stimulation of mitochondrial Ca\(^{2+}\) uptake by elevating [Ca\(^{2+}\)]\(_{em}\) from 0.1 to 2 μM resulted in the generation of ROS. Ca\(^{2+}\) uptake caused a rapid increase in the rate of rise of the DCF signal (d(ΔF/F₀)/dt = 0.0046 ± 0.0007 s\(^{-1}\); n = 30; Fig. 7A and C). This ROS production was abolished by cell treatment with MnTBAP (50 μM), which indicates that DCF senses both H\(_2\)O\(_2\) and peroxynitrite (ONOO\(^-\)) (d(ΔF/F₀)/dt = 0.00046 ± 0.00007 s\(^{-1}\); n = 12; Fig. 7A and C). Cell treatment with FeTPPS (10 μM), a selective ONOO\(^-\) scavenger with minimal SOD mimetic activity, also decreased the rate of DCF fluorescence increase (d(ΔF/F₀)/dt = 0.00096 ± 0.00002 s\(^{-1}\); n = 5; Fig. 7A and C), but was unable to block it completely. These data indicate that the major part (~80%) of the DCF signal was due to peroxynitrite formation under conditions of L-arginine depletion, and ~20% of the signal represents H\(_2\)O\(_2\) formation from superoxide. Moreover, cell treatment with the mitochondrial uncoupler FCCP prevented the Ca\(^{2+}\)-induced increase in ROS generation (d(ΔF/F₀)/dt was reduced to 0.00024 ± 0.00004 s\(^{-1}\); n = 11; Fig. 7A and C) demonstrating the mitochondrial dependence of ROS generation.

Interestingly, this Ca\(^{2+}\)-induced ROS production was also abolished by preincubation with nNOS blocker I (240 nM), but was unaffected by eNOS inhibitor L-NIO (1 μM) or iNOS blocker 1400W (15 nM). The rate of rise of DCF fluorescence was 0.00031 ± 0.00007 s\(^{-1}\) (n = 10) after inhibition of nNOS, 0.0048 ± 0.0006 s\(^{-1}\) (n = 6) after eNOS inhibition, and 0.0044 ± 0.0002 s\(^{-1}\) (n = 5) after iNOS inhibition (Fig. 7B and C). These data indicate that uncoupled nNOS was the source of ROS generation in these experiments. Because nNOS is a Ca\(^{2+}\)/calmodulin-dependent enzyme (Fig. 4), we tested whether calmodulin inhibition with W-7 (50 μM)
affects ROS generation in mitochondria. As shown in Fig. 7B, the increase of DCF fluorescence observed during stimulation of mitochondrial Ca\(^{2+}\) uptake was attenuated by W-7 (\(d(\Delta F/F_0)/dt = 0.00039 \pm 0.00033\) s\(^{-1}\); \(n = 6\); Fig. 7B and C), suggesting that Ca\(^{2+}\) uptake-induced ROS production by nNOS localized to mitochondria was also calmodulin dependent.

All ROS measurements presented so far were performed in the absence of added external L-arginine. Despite the presence of the sufficient L-arginine levels required to activate and couple mtNOS in intact cardiomyocytes (Desrois et al. 2003), cell permeabilization will lead to depletion of this NOS substrate. The negligible capacity of cardiac myocytes for de novo synthesis of L-arginine makes these cells dependent on L-arginine uptake (Simmons et al. 1996a,b). Therefore, we determined whether availability of L-arginine altered ROS generation during stimulation of mitochondrial Ca\(^{2+}\) uptake. The Ca\(^{2+}\) uptake-stimulated ROS production in cardiomyocytes was inhibited almost completely in the presence of 1 mM L-arginine (\(d(\Delta F/F_0)/dt\) decreased from 0.0046 \(\pm 0.0004\) s\(^{-1}\) (\(n = 30\)) to 0.0034 \(\pm 0.00016\) s\(^{-1}\) (\(n = 11\)); \(P < 0.001\); Fig. 8A and C), strongly suggesting that the observed ROS generation was mediated by nNOS deprived of the substrate L-arginine.

L-Arginine is also a substrate for arginase, an enzyme which catalyses the conversion of L-arginine to L-ornithine and urea within mitochondria (Jung et al. 2006; Steppan et al. 2006). Therefore, arginase has a potential role in limiting L-arginine availability for mtNOS. We found that pharmacological inhibition of arginase with 10 \(\mu\)M (S)-(2-boronoethyl)-L-cysteine (BEC) resulted in 50\% inhibition (\(d(\Delta F/F_0)/dt\) decreased from 0.0046 \(\pm 0.0007\) s\(^{-1}\) (\(n = 30\)) to 0.0022 \(\pm 0.0003\) s\(^{-1}\) (\(n = 11\)) by 50\% inhibition (\(d(\Delta F/F_0)/dt\) decreased from 0.0046 \(\pm 0.0007\) s\(^{-1}\) (\(n = 30\)) to 0.0022 \(\pm 0.0003\) s\(^{-1}\) (\(n = 11\)).

**Figure 7.** Mitochondrial Ca\(^{2+}\) uptake stimulates ROS production

A, mitochondrial ROS production during elevation of [Ca\(^{2+}\)]\(_{em}\) from 0.1 to 2 \(\mu\)M in the absence and presence of 50 \(\mu\)M MnTBAP, 10 \(\mu\)M FeTPPS or 1 \(\mu\)M FCCP. B, mitochondrial ROS production during elevation of [Ca\(^{2+}\)]\(_{em}\) from 0.1 to 2 \(\mu\)M in the presence of l-NIO (1 \(\mu\)M), 1400W (15 \(nM\)), the nNOS blocker I (240 \(nM\)) or the calmodulin antagonist W-7 (50 \(\mu\)M). C, summary of the effects of MnTBAP, FeTPPS, FCCP, nNOS blocker I, l-NIO, 1400W and W-7 on Ca\(^{2+}\)-induced changes in CM-H\(_2\)DCF (DCF) fluorescence (expressed as the rate of rise of DCF fluorescence, \(d(\Delta F/F_0)/dt\)). Statistical significance: *\(P < 0.001\) versus control.
Tetrahydrobiopterin (BH₄) is another factor known to regulate NOS uncoupling from NO production to ROS generation (Wever et al. 1997; Vasquez-Vivar et al. 1999; Bevers et al. 2006). BH₄ is known to stabilize the dimeric structure of NOS (Klatt et al. 1995) and increases substrate affinity and NADPH consumption (Thony et al. 2000; Rosen et al. 2002). Therefore, one would expect that the addition of exogenous BH₄ could potentially reduce mtNOS-dependent ROS production. We studied the Ca²⁺-induced ROS production in the absence and presence of 10 μM BH₄ (Fig. 8A). As shown in Fig. 8A and C, addition of 10 μM BH₄ slightly reduced the rate of Ca²⁺-induced ROS production. However, the effect was not statistically significant (d(ΔF/F₀)/dt = 0.0042 ± 0.0002 s⁻¹; n = 11; P = 0.25 versus control). Simultaneous exposure to L-arginine and BH₄ (10 μM) resulted in complete inhibition of Ca²⁺-induced ROS production (97 ± 1% inhibition compared to control; d(ΔF/F₀)/dt = 0.00014 ± 0.00004 s⁻¹; n = 17; P < 0.001; Fig. 8A and C). However, a higher concentration of BH₄ (100 μM) was able to decrease DCF fluorescence even in the absence of L-arginine (d(ΔF/F₀)/dt = 0.00015 ± 0.00002 s⁻¹; n = 15; P < 0.001; Fig. 8A and C). Addition of 100 μM BH₄ in the presence of L-arginine resulted in complete inhibition of Ca²⁺-induced ROS production (d(ΔF/F₀)/dt = 0.000046 ± 0.00007 s⁻¹; n = 4; P < 0.001; Fig. 8C). These data demonstrate that
ROS production by mtNOS primarily occurs during L-arginine depletion which can be prevented by L-arginine or decreased by BH4 supplementation.

We repeated our experiments using MitoSOX Red (MSR) as a detector of mitochondrial ROS generation. MSR is selectively targeted to the mitochondrial matrix, and reports only mitochondrial superoxide production (i.e. it does not react with peroxynitrite or hydrogen peroxide). Figure 8B demonstrates that stimulation of mitochondrial Ca2+ uptake in the absence of L-arginine induced mitochondrial superoxide production (d(\Delta F/\Delta F_0)/dt = 0.00156 ± 0.00006 s⁻¹; n = 6; Fig. 8B and D), which was prevented by 100 μM L-arginine (d(\Delta F/\Delta F_0)/dt = 0.00005 ± 0.000008 s⁻¹; n = 4; Fig. 8B and D) and decreased by 100 μM BH4 supplementation (d(\Delta F/\Delta F_0)/dt = 0.00021 ± 0.00005 s⁻¹; n = 7; Fig. 8B and D). Altogether, these data demonstrate that in the absence of L-arginine and BH4, mtNOS becomes 'uncoupled', i.e. it generates small amounts of NO, but elevated superoxide amounts, leading to peroxynitrite formation.

L-Arginine and BH4 inhibit Ca2+-induced opening of mitochondrial permeability transition pore (PTP)

To assess whether mtNOS-mediated ROS generation caused cellular injury, we monitored the activity of PTP induced by increased mitochondrial Ca2+ in the absence and presence of L-arginine. We evaluated PTP activity using a method based on the observation that relatively large mitochondria-trapped molecules (such as the fluorescent probe calcein with a molecular weight of ~620 Da) can be released from isolated mitochondria (Huser et al. 1998) and from mitochondria in intact or permeabilized cells (Petronilli et al. 1999; Dedkova & Blatter, 2005) after opening of the PTP. The release of calcein is associated with a decrease in fluorescence in permeabilized cells and can be blocked with the PTP inhibitor cyclosporin A (Petronilli et al. 1999), indicating that calcein is released through the PTP. Figure 9A shows that application of 2 μM [Ca2+]em in the absence of L-arginine-induced calcein release from mitochondria (rate of fluorescence decline was 266 ± 39% of control; n = 13; control refers to the fluorescence decline in [Ca2+]em = 0.1 μM and is defined as 100%). This decrease in calcein fluorescence was completely prevented by 5 μM cyclosporin A (108 ± 13%; n = 7, P < 0.01 compared to 2 μM Ca2+), confirming that the observed effect was due to opening of the PTP. Cell treatment with 50 μM MnTBAP (94 ± 19%; n = 5, P < 0.01) abolished the opening of the PTP (Fig. 9A and C) indicating that mitochondrial generation of ROS was responsible for PTP opening during mitochondrial Ca2+ uptake. Furthermore, when these experiments were performed in the presence of the mtNOS substrate L-arginine, we never observed an opening of PTP during elevation of the [Ca2+]em to 2 μM (96 ± 7%; n = 8; P < 0.001; Fig. 9B and C). When cells were pretreated with 240 nM nNOS blocker I for 15 min before cell permeabilization, the application of L-arginine was not able to prevent Ca2+-induced opening of the mitochondrial PTP (215 ± 39%; n = 8; P = 0.19; Fig. 9B and C), confirming that the protective effect of L-arginine was mediated by 're-coupling' of mtNOS from ROS generation towards NO production rather than direct radical scavenging by L-arginine. Addition of 10 μM BH4 in the absence of L-arginine had no effect on Ca2+-induced opening of PTP (231 ± 31%; n = 7; P = 0.58), however, simultaneous application of 10 μM BH4 with L-arginine prevented Ca2+-induced PTP opening (103 ± 9%; n = 4, P < 0.01, Fig. 9B and C). When the BH4 concentration was elevated from 10 to 100 μM, BH4 by itself decreased (but did not block completely) Ca2+-induced PTP opening (167 ± 18%; n = 11, P < 0.05, Fig. 9B and C). These data demonstrate that both intracellular L-arginine and BH4 may modulate the activity of the PTP, with L-arginine being more effective at inhibiting the PTP. These data are in agreement with the ability of L-arginine and BH4 to modulate mitochondrial ROS production through uncoupling of mtNOS, and indicate that stimulation of mtNOS can be cardioprotective or detrimental to cells depending upon composition of the cellular environment.

Discussion

The present study provides new insight into the interplay between mitochondrial generation of NO and ROS by nNOS localized to mitochondria upon stimulation of Ca2+. Using the NO-sensitive fluorescent dye DAF-2 trapped in mitochondria and subsequent permeabilization of the plasma membrane to eliminate cytosolic DAF-2, we were able to show that mitochondrial Ca2+ uptake stimulates dose-dependent NO production (Figs 1 and 2). Ca2+-stimulated NO production was sensitive to a specific nNOS blocker and the calmodulin antagonist W-7, while cell treatment with the eNOS blocker l-NIO and iNOS inhibitor 1400W had no effect (Fig. 4). These data indicate that the observed NO production was due to activation of a constitutive nNOS located in mitochondria. The potential contribution of the eNOS located in caveolae was dismissed because the caveolae disruption with cyclodextrin had no effect on the observed NO production (Fig. 3). Moreover, cell treatment with thapsigargin, an inhibitor of the SR Ca2+-ATPase, ruled out activation of nNOS localized in the SR (Xu et al. 1999). Abolishing NO production by collapsing the mitochondrial membrane potential with the oxidative phosphorylation uncoupler FCCP, by
the mitochondrial Ca$^{2+}$-uniporter blocker Ru360, or by de-energizing mitochondria with rotenone or antimycin A plus oligomycin (Fig. 5) is strong additional evidence for the mitochondrial origin of NO. The Ca$^{2+}$/calmodulin dependence of NO production by itself points towards a constitutive isoform of mtNOS (i.e. nNOS or eNOS). The sensitivity of NO production to FCCP and respiratory chain inhibitors (which also affect ΔΨ) is in agreement with reports that mtNOS is a voltage-dependent enzyme, with mtNOS activity being very sensitive to small changes in ΔΨ in the physiological range (∼150–180 mV) (Valdez et al. 2006; Valdez & Boveris, 2007). The peak level of NO produced by a single mitochondrion upon stimulation of mitochondrial Ca$^{2+}$ uptake was estimated to be ∼15 nM in our study, which is very close to data reported earlier for mouse heart (∼28 nM) (Kanai et al. 2001).

Even though evidence is accumulating that various cell types contain a mitochondrial NOS isoform and that mitochondria are capable of synthesizing NO, the physiological (or pathophysiological) role of

![Figure 9](https://example.com/image.png)

**Figure 9.** L-Arginine and tetrahydrobiopterin (BH$_4$) supplementation decrease Ca$^{2+}$-induced opening of the mitochondrial permeability transition pore

A, representative traces of mitochondrial-trapped calcine fluorescence (arbitrary fluorescence units, a.u.) from permeabilized ventricular myocytes during stimulation of mitochondrial Ca$^{2+}$ uptake by increasing [Ca$^{2+}$]$_{em}$ from 0.1 to 2 μM in the absence of L-arginine. Effects of 5 μM cyclosporin A (CsA) and 50 μM superoxide and peroxynitrite scavenger MnTBAP (MnTBAP) are compared with 2 μM Ca$^{2+}$ (ctrl). Application of the pore-forming antibiotic alamethicin (10 μg ml$^{-1}$) at the end of the experiment resulted in a rapid loss of calcine fluorescence, providing a positive control for maximal calcine release from the mitochondrial matrix. B, representative traces of mitochondrial-trapped calcine fluorescence after increasing [Ca$^{2+}$]$_{em}$ in the presence of 1 mM L-arginine (L-arg), 10 μM BH$_4$ (10 μM BH$_4$) or 100 μM BH$_4$ (100 μM BH$_4$) alone, or the combination of both (L-arg + BH$_4$; [BH$_4$] = 10 μM). When cells were pretreated with 240 nM nNOS inhibitor for 15 min, application of 1 mM L-arginine did not prevent the opening of the PTP (nNOS blocker I + L-arg). C, summary data of the rate of decline of calcine fluorescence upon elevation of [Ca$^{2+}$]$_{em}$. The data were normalized in each individual cell for the initial rate of fluorescence decline (100%) in [Ca$^{2+}$]$_{em}$ = 0.1 μM (control). Statistical significance: *P < 0.001, #P < 0.01, †P < 0.05 versus [Ca$^{2+}$]$_{em}$ = 2 μM.
mitochondrial-derived NO in heart has remained elusive. While some reports revealed that up to 55% of total cytosolic NO may be provided by NO diffusion from mitochondria (Valdez et al. 2004; Zaoborniy et al. 2005; Valdez & Boveris, 2007) indicating that mitochondrial NO may have a significant impact on cardiac excitation–contraction coupling, others claimed no physiological importance for mitochondrially produced NO (French et al. 2001; Tay et al. 2004; Csordas et al. 2007). These differences can be explained by the composition of experimental buffers, which in the latter studies contained 1–5 mM MgCl2, a known mitochondrial Ca2+ uptake blocker. It has been shown that an elevation of extramitochondrial Mg2+ to more than 1 mM significantly decreased (Manzo-Avalos et al. 2002) or completely prevented (Zenebe et al. 2007) mitochondrial NO production. We found that stimulating mitochondrial Ca2+ uptake in the absence of the mtNOS substrate L-arginine led to diminished NO production, enhanced superoxide production, and activation of the PTP (Figs 4, 7, 8 and 9). These effects could all be prevented by L-arginine or decreased by BH4 supplementation. The fact that an nNOS blocker inhibited not only mitochondrial NO production but also the generation of ROS as well as the Ca2+-induced PTP opening indicates that uncoupled mitochondrially localized nNOS was the source of ROS production under conditions of L-arginine depletion. In addition, mitochondrial ROS production was also Ca2+/calmodulin-dependent and sensitive to the mitochondrial uncoupler FCCP, demonstrating a regulation similar to NO production by mtNOS. There is substantial evidence that in endothelial cells NO can function as a NO- or a O2−-generating enzyme, the latter being the result of uncoupling oxygen reduction and L-arginine oxidation when substrates or cofactors are depleted (Vasquez-Vivar et al. 1998, 1999). Uncoupled eNOS is thought to be a prominent source of endothelial ROS during hypertension (Landmesser et al. 2003), neurohormonal stimulation, and hyperglycaemia (Mollnau et al. 2002), as well as following exposure to ONOO− (Zou et al. 2002). However, the evidence for ROS production by uncoupled NO (eNOS, nNOS or mtNOS) in cardiomyocytes is sparse. Data obtained from murine heart indicate that uncoupling of eNOS plays a major role in pressure overload-induced myocardial ROS production and subsequent chamber remodelling and hypertrophy (Takimoto et al. 2005). Moreover, eNOS uncoupling was accompanied by reduced eNOS dimer and BH4 levels, while cotreatment with BH4 prevented eNOS uncoupling and inhibited ROS formation (Takimoto et al. 2005). Furthermore, a follow up study (Moens et al. 2008) not only confirmed that reduced BH4 levels were responsible for the enhanced ROS production by uncoupled eNOS, but also demonstrated that exogenous BH4 supplementation re-coupled eNOS and reversed advanced hypertrophy/dilatation more effectively than a less specific treatment with the antioxidant Tempol. These data demonstrate that the efficient re-coupling of NO can be used as a therapeutic strategy and emphasize the importance of the balance between NO and ROS generation. Opposing effects of coupled and uncoupled NOS activity on the Na+−K+ pump were reported in rat cardiac myocytes (White et al. 2008). Here, the authors demonstrated that NO, produced by coupled NOS, stimulated Na+−K+ pump activity while uncoupling of NOS caused O2−-mediated pump inhibition. Supplementation with 10 μM L-arginine re-coupled NOS, resulting in increased NO production, and Na+−K+ pump activity. At the same time perfusion with 10 μM BH4 had no effect on pump activity. Cell incubation in L-arginine-free medium decreased intracellular L-arginine concentration from 70 μM to 16 μM and led to the diminished NO production while O2−-generation was significantly increased in nNOS-transfected human embryonic kidney cells (Xia et al. 1996). While only small amounts of NO were detected (reminiscent to our data presented in Fig. 4), NO was able to react with O2− leading to the peroxynitrite formation and cellular injury by uncoupled nNOS.

We found that L-arginine availability determines the extent of mtNOS uncoupling and ROS production, which in turn regulates PTP opening (Figs 7–9). Studies performed with liver mitochondria suggested that L-arginine availability should not limit mitochondrial NO production based on the estimated mitochondrial L-arginine levels of 150–300 μM and Km of the mtNOS for L-arginine equal 5 μM (Giulivi et al. 1998; Valdez et al. 2006). However, rather lower intracellular levels of L-arginine were reported in cardiomyocytes (Desrois et al. 2003) compared to other cell types (Closs et al. 2000; Vukosavljevic et al. 2006), which suggests that the availability of L-arginine could become critical for mtNOS activation and coupling in these cells, particularly under pathological conditions. L-Arginine can be formed endogenously from intracellular L-ornithine or L-citrulline pools in the Krebs–Henseleit urea cycle, mainly in the liver and kidney (Reyes et al. 1994) but also by macrophages (Wu & Brosnan, 1992) and endothelial cells (Hecker et al. 1990; Sessa et al. 1990; Wu & Meininger, 1993). However most tissues, including cardiomyocytes, cannot synthesize L-arginine de novo and depend on L-arginine uptake (Simmons et al. 1996a,b). L-Arginine is transported into cardiomyocytes by Na+−independent cationic amino acid transport (CAT) proteins consisting of CAT-1, CAT-2B (Simmons et al. 1996a; Remillard & Yuan, 2007) and CAT-2A (Peluffo, 2007). Despite the fact that the liver is the major site of L-arginine synthesis, it does not contribute significantly to the maintenance of plasma levels of L-arginine since L-arginine synthesized in this organ is routed toward local utilization (Reyes et al. 1994).
Thus maintenance of normal plasma levels of \( \text{L-arginine} \) is mainly dependent on dietary intake and synthesis by the kidney (Reyes et al. 1994). The plasma levels of \( \text{L-arginine} \) in healthy humans are around 100–200 \( \mu \text{M} \) (van Haefen & Konings, 1989; Mendes Ribeiro et al. 2001), and were significantly decreased to \(~59 \mu \text{M} \) during congestive heart failure (CHF) (Mendes Ribeiro et al. 2001). Moreover, \( \text{L-arginine} \) uptake in ventricular myocytes from patients with CHF was significantly decreased (\(~30\%)\) in parallel with a 38% reduction in the expression of CAT-1 while the expression of NOS proteins (\(\text{eNOS and iNOS}\) was unchanged (Kaye et al. 2002). Oral (Rector et al. 1996; Doutreleau et al. 2006) or intravenous (Koifman et al. 1995) \( \text{L-arginine} \) supplementation significantly improved peripheral blood flow at rest, during exercise, and in response to the endothelium-dependent vasodilators in patients with CHF. Our data suggest that mtNOS could be a key contributor to the oxidative stress observed in conditions of heart failure (Ide et al. 1999, 2000) which was mediated by the deficiency of the mitochondrial respiratory complex I (Ide et al. 1999). The tight association and functional coupling (Parihar et al. 2008c,e) of mtNOS with complex I allows for conditions where reduced activity of the respiratory chain may lead to enhanced ROS production by mtNOS. Another limiting factor for the \( \text{L-arginine} \) availability is the consumption of \( \text{L-arginine} \) by the mitochondrial urea-cycle arginase (Post & Pieske, 2006). Arginase exists in two isoforms, arginase I (\( \text{liver type} \)) and arginase II (\( \text{non-liver type} \)), and catalyses the conversion of \( \text{L-arginine} \) to \( \text{L-ornithine} \) and urea. The expression of arginases is well documented in mitochondria of kidney (Moradi et al. 2006), liver (Patterson et al. 2000) and endothelial cells (Topal et al. 2006); however, only recently the expression of arginase-II in mitochondria of mouse cardiac myocytes (Steppan et al. 2006) and arginase I in feline cardiac myocytes (Jung et al. 2006) was demonstrated. While the study of Jung et al. (2006) did not specify the subcellular localization of arginase, both studies demonstrated that arginase had substantial impact on cardiomyocyte contractility, which was probably mediated by changes in NOS activity and NO production (Jung et al. 2006; Steppan et al. 2006). Our data show that inhibition of arginase with BEC resulted in a \(~50\%)\) reduction of \( \text{Ca}^{2+} \)-induced ROS production in mitochondria (Fig. 8). This indicates that modulation of intracellular \( \text{L-arginine} \) levels by either increased supplementation or decreased consumption resulted in diminished ROS production and prevention of mtNOS uncoupling.

The important regulator of NOS uncoupling, BH4 at a concentration of 10 \( \mu \text{M} \), neither provided protection against opening of the mitochondrial PTP, nor decreased \( \text{Ca}^{2+} \)-induced mitochondrial ROS production (Figs 8 and 9). However, supplementation with 100 \( \mu \text{M} \) BH4 decreased both \( \text{Ca}^{2+} \)-induced mitochondrial ROS generation and PTP opening (Figs 8 and 9) while it had only a small effect on \( \text{Ca}^{2+} \)-induced NO production (Fig. 4). Thus it becomes critical whether cardiac mitochondria have high enough BH4 to maintain redox reactions and mtNOS coupling, since intracellular BH4 levels in cardiomyocytes are \(~30\) times lower compared to endothelial cells (1.4 \( \pm \) 0.6 \( \text{versus} \) 42.1 \( \pm \) 6.8 pmol (mg protein)\(^{-1}\), or 0.21 \( \text{versus} \) 13 \( \mu \text{M} \)) where fluctuations in intracellular BH4 levels have been shown to be responsible for eNOS uncoupling (Kalivendi et al. 2005). Moreover, it has been suggested that BH4 is not essential for \( \text{L-arginine} \) oxidation by nNOS, but rather enables nNOS to generate NO instead of nitroxyl (NO\(^\cdot\)) (Adak et al. 2000). This is particularly important as new evidence (Parihar et al. 2008b) suggests that peroxynitrite is formed from nitroxyl anion (NO\(^\cdot\)) rather than NO, as the nitroxyl scavenger l-cysteine potently prevented the irreversible inhibition of cytochrome oxidase whereas superoxide dismutase did not provide the same protective effect.

Under physiological conditions (i.e., with \( \text{L-arginine} \) and cofactors present) no opening of the PTP was observed during mitochondrial \( \text{Ca}^{2+} \) uptake (Fig. 9B). These data are in agreement with results obtained from isolated rat liver mitochondria (Brookes et al. 2000), where physiological concentrations of NO (with IC\(_{50}\) \( 0.7 \mu \text{M} \) and NO release rates of \( \sim 11 \text{nm s}^{-1} \)) prevented \( \text{Ca}^{2+} \)-induced opening of the PTP, while supraphysiological NO levels (release rates \( > 2 \mu \text{M s}^{-1} \)) accelerated PTP opening. It is likely that at these concentrations, NO (or nitroxyl anion, see above) may react with superoxide to form peroxynitrite (ONOO\(^\cdot\)), which has been shown to induce PTP opening (Packer & Murphy, 1994; Brown & Borutaite, 2007), cytochrome c release (Ghaforifar et al. 1999) and apoptosis (Levrand et al. 2006; Obrosova, 2006). We demonstrated that scavenging ROS with the SOD mimetic and peroxynitrite scavenger MnTBAP in the presence of intracellular \( \text{L-arginine} \) led to a dramatical increase (\(~3\)-fold) in the amount of NO generated by mitochondria (Fig. 6) and prevented \( \text{Ca}^{2+} \)-induced PTP opening (Fig. 9). Similar results were previously obtained in vascular endothelial cells (Brodsky et al. 2002; Dedkova et al. 2004), where a 75% increase in NO production was observed following application of MnTBAP (Dedkova et al. 2004). It is unclear whether superoxide radicals produced by the respiratory chain suppress mtNOS activity and NO production, or whether a portion of NO is simply transformed to ONOO\(^\cdot\). Evidence for ONOO\(^\cdot\) formation by mtNOS was originally presented in rat liver mitochondria (Ghaforifar et al. 1999; Bringold et al. 2000). It was demonstrated that mtNOS-mediated ONOO\(^\cdot\) formation promoted \( \text{Ca}^{2+} \) (Bringold et al. 2000) and cytochrome c (Ghaforifar et al. 1999) release from mitochondria leading to Bcl-2-dependent apoptosis. More recent data from the Ghaforifar group
(Zenebe et al. 2007) indicate that mtNOS is a key source for peroxynitrite formation in oxidative injury (i.e. cytochrome c release, lipid peroxidation and protein carboxylation) caused by ischaemia–reperfusion in the heart. The injury was prevented when mtNOS was inhibited or mitochondria were supplemented with antioxidant peroxynitrite scavengers (Zenebe et al. 2007). These data are in agreement with our data presented in Fig. 9, where the inhibition of mtNOS uncoupling or superfusion with the ONOO− scavenger MnTBAP prevented Ca2+-induced opening of the mitochondrial PTP. The importance of functional nNOS activity and NO production towards balancing ATP supply and demand during simulated ischaemia–reperfusion was demonstrated in cultured rat myocytes (Kawahara et al. 2006). Either inhibition of nNOS or the scavenging NO during simulated ischaemia–reperfusion resulted in a significant decrease in cellular ATP levels and survival rate of myocytes (Kawahara et al. 2006). Moreover, it was previously reported that NO donors protect murine myocardium against ischaemia–reperfusion injury via inhibition of the mitochondrial PTP (Wang et al. 2005a). Inhibition of the mitochondrial PTP at the onset of reoxygenation protected human myocardium against lethal hypoxia–reoxygenation injury (Shanmuganathan et al. 2005), and therefore it was concluded that the mitochondrial PTP could be a target for cardioprotection in human heart.

In summary, our data indicate that mtNOS can generate ROS instead of NO under conditions of L-arginine depletion, leading to opening of the mitochondrial PTP. Addition of L-arginine and BH4 suppressed both ROS generation and PTP opening. It has been shown that addition of L-arginine to a cardioplegic solution protects the heart from reperfusion injury and preserves the intracellular amino-acid pools during heart transplantation (Desrois et al. 2000, 2003). Moreover, addition of L-arginine to the diet of the patients with stable angina, when used as an adjunct to traditional therapy, improved vascular function, exercise capacity and aspects of quality of life (Maxwell et al. 2002). Our data show the importance of L-arginine and BH4 in the regulation of mitochondrial oxidative stress and modulation of PTP opening by mtNOS. Thus, the maintenance of physiological L-arginine and BH4 levels is cardioprotective, which seems to be of critical importance under pathological conditions such as CHF or when the heart is particularly vulnerable (e.g. during heart transplantation).

References


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