Modulation of mitochondrial Ca\(^{2+}\) by nitric oxide in cultured bovine vascular endothelial cells

Elena N. Dedkova and Lothar A. Blatter
Department of Physiology, Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois

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Dedkova, Elena N., and Lothar A. Blatter. Modulation of mitochondrial Ca\(^{2+}\) by nitric oxide in cultured bovine vascular endothelial cells. Am J Physiol Cell Physiol 289: C836–C845, 2005. Published May 18, 2005; doi:10.1152/ajpcell.00011.2005.—In the present study, we used laser scanning confocal microscopy in combination with fluorescent indicator dyes to investigate the effects of nitric oxide (NO) produced endogenously by stimulation of the mitochondria-specific NO synthase (mtNOS) or applied exogenously through a NO donor, on mitochondrial Ca\(^{2+}\) uptake, membrane potential, and gating of mitochondrial permeability transition pore (PTP) in permeabilized cultured calf pulmonary artery endothelial (CPAE) cells. Higher concentrations (100–500 \(\mu\)M) of the NO donor spermine NONOate (Sper/NO) significantly reduced mitochondrial Ca\(^{2+}\) uptake and Ca\(^{2+}\) extrusion rates, whereas low concentrations of Sper/NO (<100 \(\mu\)M) had no effect on mitochondrial Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_{\text{m}}\)). Stimulation of mitochondrial NO production by incubating cells with 1 \(\text{mM}\) \(L\)-arginine also decreased mitochondrial Ca\(^{2+}\) uptake, whereas inhibition of mtNOS with 10 \(\mu\)M \(L\)-NAME-(1-iminoethyl)ornithine resulted in a significant increase of [Ca\(^{2+}\)]\(_{\text{m}}\). Sper/NO application caused a dose-dependent sustained mitochondrial depolarization as revealed with the voltage-sensitive dye tetramethylrhodamine ethyl ester (TMRE). Blocking mtNOS hyperpolarized basal mitochondrial membrane potential and partially prevented Ca\(^{2+}\)-induced decrease in TMRE fluorescence. Higher concentrations of Sper/NO (100–500 \(\mu\)M) induced PTP opening, whereas lower concentrations (<100 \(\mu\)M) had no effect. The data demonstrate that in calf pulmonary artery endothelial cells, stimulation of mitochondrial Ca\(^{2+}\) uptake can activate NO production in mitochondria that in turn can modulate mitochondrial Ca\(^{2+}\) uptake and efflux, demonstrating a negative feedback regulation. This mechanism may be particularly important to protect against mitochondrial Ca\(^{2+}\) overload under pathological conditions where cellular NO can reach very high levels. 

Although mtNOS has been discovered in a variety of cell types (3, 4, 20, 21, 25, 26, 35, 36, 38, 49, 52), the functional implications of NO produced locally by mitochondria for mitochondrial energy metabolism and Ca\(^{2+}\) homeostasis are much less explored and not well understood. NO is known to be a mediator of Ca\(^{2+}\) homeostasis in a highly complex and cell-specific manner (17), which can affect mitochondrial Ca\(^{2+}\) homeostasis as well. Several studies have addressed the effect of NO on mitochondrial Ca\(^{2+}\) homeostasis; however, the results are inconsistent and appear to depend on the cell types and experimental approaches (intact cells or isolated mitochondria) as well as the sources and the concentrations of NO used. For example, local suppression of mitochondrial Ca\(^{2+}\) handling by NO was proposed to be a key mechanism in the regulation of capacitative Ca\(^{2+}\) entry in human embryonic kidney cells (50), whereas no role for mitochondria in the regulation of capacitative Ca\(^{2+}\) entry by NO was found in platelets (51) and vascular endothelial cells (19). The experiments performed on isolated mitochondria have shown that application of NO donors inhibited Ca\(^{2+}\) uptake by mitochondria (9) or induced Ca\(^{2+}\) efflux from Ca\(^{2+}\)-loaded mitochondria (2, 45), whereas blocking of mtNOS increased Ca\(^{2+}\) buffering capacity of mitochondria (27). These effects of NO were explained either by the ability of NO to decrease mitochondrial membrane potential (\(\Delta\Psi_{\text{m}}\)) (2, 9, 27) or by the ability of NO to open the mitochondrial permeability transition pore (PTP) (9). It has also been suggested that the effects of NO on the PTP were not mediated by NO itself but rather by other reactive nitrogen species such as peroxynitrite (ONOO\(^-\)) (27, 48). It is noteworthy that in experiments performed on isolated mitochondria, higher concentrations of NO donors were used compared with experiments in intact cells, which could explain the difference in the results.

Bearing in mind the inconsistencies of previous reports on the effect of NO on mitochondrial Ca\(^{2+}\) handling, the present study was designed to examine the effect of a wide range of NO concentrations on mitochondrial Ca\(^{2+}\) uptake, \(\Delta\Psi_{\text{m}}\), and activity of the PTP in permeabilized calf pulmonary artery endothelial (CPAE) cells. The results of our study show that NO provided by the exogenous NO donor Sper/NO induced a dose-dependent inhibition of mitochondrial Ca\(^{2+}\) uptake and reduction of Ca\(^{2+}\) extrusion rates, but only when higher concentrations (100–500 \(\mu\)M) were used. Lower concentrations of Sper/NO (<100 \(\mu\)M) did not affect mitochondrial Ca\(^{2+}\) uptake; stimulation of mitochondrial NO production by supplying extra amounts of the mtNOS substrate \(L\)-arginine also resulted in an impairment of mitochondrial Ca\(^{2+}\) uptake, whereas...
abolishing mitochondrial NO production by blocking mtNOS with 1-NIO resulted in an increase of mitochondrial Ca²⁺ uptake. NO induced a dose-dependent depolarization of the mitochondrial membrane, whereas 1-NIO partially prevented the drop in membrane potential during mitochondrial Ca²⁺ uptake. Finally, higher concentrations of Sper/NO (100–500 μM) induced the opening of the mitochondrial PTP, which serves as additional mechanism for Ca²⁺ efflux from mitochondria. The results indicate that in CPAE cells, mitochondrially produced NO plays a protective role against mitochondrial Ca²⁺ overload through a negative feedback regulation of its own synthesis.

METHODS

Cell Culture and Solutions

Experiments were performed on CPAE cells in nonconfluent cultures. The CPAE cell line was obtained at passage 15 from American Type Culture Collection (ATCC CCL-209, Manassas, VA). The cells were cultured in Eagle’s minimum essential medium, supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and 2 mM L-glutamine, and kept at 37°C in an atmosphere of 5% CO₂ and 95% air. Once per week, the cells were dispersed with the use of a Ca²⁺/Ca²⁺-free (0.1% EDTA) 0.25% trypsin solution and subcultured onto glass coverslips for later experimentation. Cells were passaged up to 6 times after they were obtained from ATCC. All experiments were carried out at room temperature (20–22°C) on single cells in nonconfluent cultures within 1 wk after being plated.

Fluorescence Measurements

Mitochondrial Ca²⁺ measurements. Laser scanning confocal microscopy (model LSM 410, Zeiss) was used to follow the changes in the mitochondrial Ca²⁺ level ([Ca²⁺]ₘt) during activation of mitochondrial Ca²⁺ 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, 1.3 numerical aperture, Zeiss). Measurements of [Ca²⁺]ₘt were performed on cells loaded with the fluorescent Ca²⁺-sensitive dye fluo-3. Cells were exposed to 25 μM of the membrane-permeant form of the indicator fluo-3 AM (Molecular Probes, Eugene, OR) for 40 min at 37°C in 1 ml of standard Tyrode solution containing (in mM) 135 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH 7.3). Cells were subsequently washed for 10 min. The cells were then placed in Ca²⁺/Ca²⁺-free Tyrode solution and permeabilized by exposure to 10 μM digitonin for 60 s. Digitonin was added to the “intracellular” solution consisting of (in mM) 135 KCl, 10 NaCl, 20 HEPES, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH₂PO₄, 1 MgCl₂, 5 EGTA, and 1.86 CaCl₂ to yield a free [Ca²⁺] of ~100 nM. Fluo-3 fluorescence was excited with the 488-nm line of an argon ion laser, and the emitted fluorescence signals were measured at 510–525 nm. Changes in mitochondrial fluo-3 fluorescence intensities (F) in each experiment were normalized to the level of fluorescence recorded before stimulation (F₀) but after cell permeabilization (see Fig. 1D). Changes in [Ca²⁺]ₘt are expressed as ΔF/F₀. [Ca²⁺]ₘt measurements were performed from small mitochondria-rich regions of interest of ~5 μm², representing a small (~10) number of mitochondria.

Changes in ΔΨₘ were followed using the potential-sensitive dye tetramethylrhodamine ethyl ester (TMRE). CPAE cells were exposed to 0.2 μM TMRE for 15 min at 37°C before experiments and then permeabilized with digitonin. All solutions contained 0.2 μM TMRE during recordings. TMRE fluorescence was excited at 514 nm and recorded at 590 nm. For measurements of the time-dependent TMRE fluorescence changes, data were acquired every 2 s. Because the relationship between TMRE fluorescence and ΔΨₘ is governed by the

Nernst equation, TMRE fluorescence recordings are shown on a logarithmic scale.

Activity of mitochondrial permeability transition pore was monitored using the fluorescent dye calcein in permeabilized CPAE cells. Opening of PTP resulted in the loss of mitochondria-trapped calcein (620 kDa) and a decrease of fluorescence (34). CPAE cells were loaded with 5 μM of the membrane-permeant form of the fluorescent probe calcein AM (Molecular Probes) for 40 min at 37°C. After dye loading, the cells were placed in dye-free Tyrode solution for 10 min to wash off excess dye. The calcein fluorescence was excited at 488 nm, and the emitted fluorescence signal was measured at ~510 nm.

Chemicals

The protonophore carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), ruthenium red (RutRed), decylubiquinone (DQ), and digitonin were obtained from Sigma (St. Louis, MO). Spermine NONOate (Sper/NO), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3 oxide (PTIO), and 1-N²-(1-iminoethyl)ornithine (1-NIO) were purchased from Calbiochem (San Diego, CA). Sper/NO was dissolved as a 15 mM stock in water before the experiments and used within 4 h.

Statistical Analysis

Statistical differences of the data were determined using Student’s t-test for unpaired or paired data and were considered significant at P < 0.05. Results are reported as means ± SE for the indicated number (n) of cells. Each experiment was conducted on a separate cell culture.

RESULTS

Effect of NO on Mitochondrial Ca²⁺ Uptake

For the direct measurement of Ca²⁺ uptake by mitochondria of vascular endothelial cells, we used a method based on the ability of the Ca²⁺-sensitive fluorescent indicator flou-3 AM to compartmentalize into mitochondria, followed by subsequent removal of cytoplasmic flou-3 by surface membrane permeabilization with digitonin. We have developed this method for CPAE cells and employed it successfully to estimate Ca²⁺ levels in mitochondria of endothelial cells (46).

Figure 1A (before digitonin) shows that flou-3 fluorescence was distributed relatively homogeneously throughout the cell with higher intensities of fluorescence around the nuclei. Plasma membrane permeabilization with digitonin removed cytosolic and nuclear flou-3 (Fig. 1B, after digitonin), revealing the particular and punctate fluorescence pattern typical for mitochondria (20). The mitochondrial origin of the flou-3 signal was confirmed by colocalization with the potentiometric dye TMRE used to localize mitochondria (46). Elevating extramitochondrial [Ca²⁺] ([Ca²⁺]ₑm) from 0.1 to 2 μM resulted in an increase of the mitochondria-trapped flou-3 signal due to mitochondrial Ca²⁺ uptake (Fig. 1C).

Figure 1D presents the typical time course of flou-3 fluorescence intensity changes from mitochondria of CPAE cells before and after cell permeabilization with digitonin and after activation of mitochondrial Ca²⁺ uptake by increasing [Ca²⁺]ₑm transiently from 0.1 to 2 μM (control, red trace). The level of fluorescence after digitonin treatment (F₀) was on average only 21 ± 1% (n = 50) of the initial mitochondrial fluorescence. This level represents the contribution of mitochondria and was used to normalize the fluorescence signal (F₀/F₀). When [Ca²⁺]ₑm was raised from 0.1 to 2 μM, F₀/F₀ increased to 5.81 ± 0.19 (measured at the peak of the response; n = 50). Figure 1D (black trace) shows the effect of dissipation of the ΔΨₘ with the uncoupler of oxidative phos-
phorylation FCCP on mitochondrial Ca\(^{2+}\) accumulation. When cells were pretreated with 1 \(\mu\)M FCCP, elevation of [Ca\(^{2+}\)]\(_{\text{mt}}\) was virtually abolished (Fig. 1D, FCCP). Next we blocked the mitochondrial Ca\(^{2+}\) uniporter with 10 \(\mu\)M RutRed (Fig. 1D). After the cell was permeabilized with digitonin, 10 \(\mu\)M RutRed was added to the intracellular solution for 2 min before [Ca\(^{2+}\)]\(_{\text{em}}\) (blue trace) was increased. In the presence of 1 \(\mu\)M RutRed, F/F\(_{0}\) increased to only 1.09 \(\pm\) 0.02 \((n = 8)\) in response to increasing [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 \(\mu\)M up to 2 \(\mu\)M, while in the presence of 1 \(\mu\)M FCCP, the amplitude of F/F\(_{0}\) increased to 1.51 \(\pm\) 0.08 \((n = 9)\). In both instances the increases of F/F\(_{0}\) were significantly smaller \((P < 0.001)\) than under control conditions. The rise in [Ca\(^{2+}\)]\(_{\text{mt}}\) amounted on average to only 2 and 11\% of control in the presence RutRed and FCCP, respectively. Thus the increase of the fluo-3 signal after elevation of [Ca\(^{2+}\)]\(_{\text{em}}\) represents a Ca\(^{2+}\) uniporter-mediated, \(\Delta\Psi_m\)-dependent Ca\(^{2+}\) uptake into the mitochondria.

After establishing the basic experimental protocol, we studied the effect of NO on mitochondrial Ca\(^{2+}\) uptake. Figure 2A shows a typical control recording of [Ca\(^{2+}\)]\(_{\text{mt}}\) from permeabilized CPAE cells in response to stimulation of mitochondrial Ca\(^{2+}\) uptake induced by increasing [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 to 2 \(\mu\)M. After [Ca\(^{2+}\)]\(_{\text{em}}\) was switched back to the initial level (0.1 \(\mu\)M), mitochondrial Ca\(^{2+}\) also decreased quickly due to Ca\(^{2+}\) extrusion via mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchange (46). The subsequent second increase of [Ca\(^{2+}\)]\(_{\text{em}}\) resulted in an identical [Ca\(^{2+}\)]\(_{\text{mt}}\) response (Fig. 2A, gray trace). This dual-application protocol was used in the following experiments to normalize the changes in [Ca\(^{2+}\)]\(_{\text{em}}\) caused by Sper/NO (or other interventions) in individual cells. We used the NO donor Sper/NO in the range of 50–500 \(\mu\)M, which produces NO levels of 0.5–3 \(\mu\)M as estimated by us and reported by others (10, 13, 47). Therefore, the low concentrations of Sper/NO (50–100 \(\mu\)M) imitate NO production observed under physiological conditions \((~0.5–1 \mu\text{M NO})\), whereas higher concentrations of Sper/NO (>100 \(\mu\)M) produce NO in the range typical for pathological conditions (>1 \(\mu\text{M NO}) (24).

The application of the NO donor Sper/NO (300 \(\mu\)M) 2 min before elevation of [Ca\(^{2+}\)]\(_{\text{em}}\) significantly decreased mitochondrial Ca\(^{2+}\) uptake (53 \(\pm\) 3\% of control, \(n = 15\), \(P < 0.001\); Fig. 2B, gray trace) and slowed Ca\(^{2+}\) extrusion after removal of extramitochondrial Ca\(^{2+}\) (Fig. 2B, gray trace). The rate of Ca\(^{2+}\) extrusion was calculated from a monoexponential fit to the decline of [Ca\(^{2+}\)]\(_{\text{mt}}\) during Ca\(^{2+}\) removal and normalized to control values (% of control). In the presence of 300 \(\mu\)M Sper/NO the rate of Ca\(^{2+}\) extrusion was reduced to 47 \(\pm\) 6\% of control \((n = 15\), \(P < 0.001)\). Low concentration of Sper/NO (50 \(\mu\)M) had no significant effect on mitochondrial.
Ca\textsuperscript{2+} uptake (94 ± 8% of control, n = 15; Fig. 2C) as well as on the rate of Ca\textsuperscript{2+} extrusion (102 ± 9% of control, n = 15; Fig. 2C). All data are summarized in Fig. 2C and indicate that the ability of mitochondria to sequester and extrude Ca\textsuperscript{2+} was significantly impaired when cells were treated with 100–500 μM Sper/NO. Lower concentrations of Sper/NO (<100 μM) had no effect. The close correlation between the degree of inhibition of mitochondrial Ca\textsuperscript{2+} uptake and the degree of inhibition of Ca\textsuperscript{2+} extrusion suggests that Ca\textsuperscript{2+} extrusion was slowed down as a result of the Sper/NO effect on mitochondrial Ca\textsuperscript{2+} uptake rather than a direct effect on Ca\textsuperscript{2+} extrusion mechanisms.

Effect of Stimulation and Inhibition of Mitochondrial NO Production by Constitutive mtNOS on Mitochondrial Ca\textsuperscript{2+} Uptake

In the above experiments, we used an exogenous NO source (NO donor Sper/NO) to evaluate the effect of NO on mitochondrial Ca\textsuperscript{2+} uptake. However, we have shown previously that stimulation of mitochondrial Ca\textsuperscript{2+} uptake activates mtNOS to produce NO inside of mitochondria of vascular endothelial cells, and with the experimental approach used here (permeabilized cells) the observed changes of [Ca\textsuperscript{2+}]\textsubscript{mt} were exclusively due to mtNOS activity (20). For this reason, we set out to manipulate (increase or decrease) mitochondrial NO production to evaluate the effect of endogenously produced NO on [Ca\textsuperscript{2+}]\textsubscript{mt}. To enhance mitochondrial NO production, we preincubated CPAE cells with the mtNOS substrate l-arginine. Despite the fact that endothelial cells can synthesize basal amounts of l-arginine, NO production is strongly dependent on the availability of exogenous l-arginine (30, 32). Therefore, we enhanced mitochondrial NO production by supplying an extra amount of l-arginine and evaluated how this affected mitochondrial Ca\textsuperscript{2+} uptake. Figure 3A presents a typical trace recorded from a cell pretreated with 1 mM l-arginine for 4 min before the second Ca\textsuperscript{2+} application. The experiment shows that stimulation of mitochondrial NO production resulted in an attenuation of mitochondrial Ca\textsuperscript{2+} uptake. The average increase of [Ca\textsuperscript{2+}]\textsubscript{mt} during l-arginine treatment amounted to only 61 ± 3% of control (n = 19, P < 0.001; Fig. 3C).

To decrease mitochondrial NO production, we blocked the activity of mtNOS with l-NIO, an inhibitor of the constitutive eNOS. Figure 3B shows that application of 10 μM l-NIO just 4 min before the second exposure to 2 μM Ca\textsuperscript{2+} enhanced mitochondrial Ca\textsuperscript{2+} uptake. Mitochondrial Ca\textsuperscript{2+} uptake in the presence of l-NIO amounted to an increase of F/F\textsubscript{0} to 128 ± 3% of control (n = 12, P < 0.001; Fig. 3C).

In summary, these data strongly suggest that NO can serve as a negative modulator of mitochondrial Ca\textsuperscript{2+} uptake and its own synthesis.

Effect of Sper/NO on \Delta \Psi\textsubscript{m}

To investigate mitochondrial function in permeabilized CPAE cells further, we used the potentiometric dye TMRE. Sequestration of TMRE into mitochondria is governed by the highly negative \Delta \Psi\textsubscript{m}, which is maintained by proton translocation by the electron transport system. When electron transport is inhibited pharmacologically or when the proton gradient is abolished by a protonophore, the membrane depolarizes, and TMRE is released into the cytoplasm, which leads to a decrease in TMRE fluorescence (Fig. 4A). We applied the protonophore FCCP (1 μM) at the end of each experiment. FCCP induced a complete depolarization of the mitochondrial membrane and a maximal decrease of TMRE fluorescence. We used this signal to normalize the changes in fluorescence produced by Sper/NO and other agents. TMRE exhibits a Nernstian distribution across the inner mitochondrial membrane; therefore, TMRE fluorescence signals are presented on a logarithmic scale. Sper/NO application caused a dose-dependent de-
polarization of $\Delta \Psi_m$. Figure 4A shows a representative example of measurements of $\Delta \Psi_m$-dependent TMRE fluorescence in response to application and withdrawal of 300 $\mu$M Sper/NO and subsequent application of 1 $\mu$M FCCP. Sper/NO produced a rapid drop in TMRE fluorescence that was stable during 2 min of Sper/NO exposure. The withdrawal of Sper/NO resulted in recovery of TMRE fluorescence to the initial level. While application of 300 $\mu$M Sper/NO had a profound effect on $\Delta \Psi_m$, lower concentrations of Sper/NO had no or little effect (Fig. 4, B and F). Figure 4F summarizes the effect of different Sper/NO concentrations on the $\Delta \Psi_m$. When cells were pretreated with 50 $\mu$M of the NO scavenger PTIO (1), Sper/NO-induced changes in TMRE fluorescence were abolished (11 ± 2% of the effect achieved with 300 $\mu$M Sper/NO alone; $n = 9$; $P < 0.001$; Fig. 4, C and F). Pretreatment with Mn-TBAP (50 $\mu$M), a cell-permeable superoxide dismutase (SOD) mimetic and ONOO$^-$ scavenger, did not significantly change the effect of 300 $\mu$M Sper/NO on mitochondrial TMRE fluorescence (Fig. 4, D and F). In a total of 5 cells, Sper/NO-induced changes in TMRE fluorescence in the presence of Mn-TBAP were 116 ± 5% of the effect achieved with 300 $\mu$M Sper/NO alone (not significantly different; Fig. 4, D and F). The decrease in $\Delta \Psi_m$ induced by Sper/NO could be the result of the opening of the PTP, which renders the inner mitochondrial membrane permeable to molecules up to 1,500 Da (28, 53). The decrease in TMRE fluorescence induced by 300 $\mu$M Sper/NO was partially prevented by cell treatment with 100 $\mu$M of the PTP inhibitor DQ (46 ± 2% of the effect achieved with 300 $\mu$M Sper/NO alone; $n = 14$, $P < 0.001$), a PTP inhibitor with a reported efficiency to block PTP comparable to that exerted by cyclosporin A (CsA) (23). We chose DQ over CsA because DQ has no effect on cell respiration (23) and CsA has been shown to be an unreliable blocker of PTP in CPAE and other cells (15, 33, 53).

In summary, the experiments shown in Fig. 4 provide evidence that the observed changes in TMRE fluorescence were due to a specific action of NO on $\Delta \Psi_m$ and indicate the involvement of PTP in this regulation.

Effect of mtNOS Inhibition on $\Delta \Psi_m$

Mitochondrial Ca$^{2+}$ uptake by respiring mitochondria induced a large membrane depolarization amounting to 82 ± 1%
of the maximum decrease in TMRE fluorescence evoked by FCCP (Fig. 5A; n = 13). Cell treatment with the NOS inhibitor L-NIO (10 \mu M) for 4 min after cell permeabilization resulted in a partial prevention of the Ca\(^{2+}\)-induced depolarization (Fig. 5B). In the presence of L-NIO, the magnitude of Ca\(^{2+}\)-induced depolarization was only 66 \pm 2\% (P < 0.001; n = 15; Fig. 5C) compared with Ca\(^{2+}\)-dependent depolarization in the absence of L-NIO. In addition, the initial level of fluorescence was higher in L-NIO-treated cells compared with control. In a total of 17 cells, the initial TMRE fluorescence level was 129 \pm 6\% of control (P < 0.01). These data suggest that mitochondrial NO production can modulate the \Delta \Psi_m and therefore can increase or decrease the driving force for mitochondrial Ca\(^{2+}\) uptake.

Effect of NO on Mitochondrial Permeability Transition Pore

The data in Fig. 4E suggest that NO can modulate the activity of the PTP in mitochondria of CPAE cells. This suggests that the PTP can serve as an additional mechanism for Ca\(^{2+}\) release during mitochondrial Ca\(^{2+}\) overload. To explore the effect of NO on the PTP directly, we used a method based on the observation that relatively large mitochondria-trapped molecules (such as the fluorescent probe calcein with a molecular weight of \sim 620 \text{Da}) can be released from isolated mitochondria (34) and from mitochondria in intact or permeabilized cells (42) after opening of the PTP. The release of calcein is associated with a decrease in fluorescence in permeabilized cells and can be blocked with cyclosporin A (42), directly suggesting that the pathway of calcein release involves the PTP. We loaded intact CPAE cells with the ester form of calcein in the presence of 100 \mu M Sper/NO and subsequent application and withdrawal of 1 \mu M FCCP (Fig. 6A). Application of 100 \mu M Sper/NO and subsequent application of 1 \mu M FCCP in control conditions. C: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the NO scavenger PTIO (50 \mu M). D: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the superoxide dismutase (SOD) mimetic and peroxynitrite scavenger Mn-TBAP (50 \mu M). E: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the PTP inhibitor decylubiquinone (DQ; 100 \mu M). F: summary of the normalized \Delta \Psi_m-dependent TMRE fluorescence changes upon application of 1 \mu M FCCP (100\%) and different Sper/NO concentrations ((Sper/NO) in \mu M indicated by the numbers on the bottom of bars), 300 \mu M Sper/NO in the presence of 50 \mu M PTIO, 300 \mu M Sper/NO in the presence of 50 \mu M Mn-TBAP, 300 \mu M Sper/NO in the presence of 100 \mu M DQ. The fluorescence level after FCCP addition was used to normalize the Sper/NO-induced changes in TMRE signal. All data are presented as \% of \Delta \Psi_m observed after FCCP application. The numbers in parentheses indicate the number of cells tested.

Fig. 4. Effect of NO on mitochondrial membrane potential (\Delta \Psi_m). A: tetramethylrhodamine ethyl ester (TMRE) fluorescence changes during application and withdrawal of 300 \mu M Sper/NO and subsequent application and withdrawal of 1 \mu M FCCP. B: application 100 \mu M Sper/NO and 1 \mu M FCCP in control conditions. C: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the NO scavenger PTIO (50 \mu M). D: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the superoxide dismutase (SOD) mimetic and peroxynitrite scavenger Mn-TBAP (50 \mu M). E: application of 300 \mu M Sper/NO and 1 \mu M FCCP in the presence of the PTP inhibitor decylubiquinone (DQ; 100 \mu M). F: summary of the normalized \Delta \Psi_m-dependent TMRE fluorescence changes upon application of 1 \mu M FCCP (100\%) and different Sper/NO concentrations ((Sper/NO) in \mu M indicated by the numbers on the bottom of bars), 300 \mu M Sper/NO in the presence of 50 \mu M PTIO, 300 \mu M Sper/NO in the presence of 50 \mu M Mn-TBAP, 300 \mu M Sper/NO in the presence of 100 \mu M DQ. The fluorescence level after FCCP addition was used to normalize the Sper/NO-induced changes in TMRE signal. All data are presented as \% of \Delta \Psi_m observed after FCCP application. The numbers in parentheses indicate the number of cells tested.
vascular endothelial cells (6, 7, 18). It has been shown that endogenous NO modulates cellular O₂ consumption. NO acts at the level of cytochrome oxidase, reducing the affinity of the enzyme for O₂ (12, 16). Disruption of mitochondrial respiration by NO has been proposed to be partially responsible for the energy depletion in neurons (10) that led to the disruption of Ca²⁺ homeostasis (11). We have demonstrated previously that in vascular endothelial cells, NO inhibits capacitative Ca²⁺ entry and enhances endoplasmic reticulum Ca²⁺ uptake; however, the effect of NO on mitochondrial Ca²⁺ homeostasis has not been evaluated in details (19). Surprisingly, although numerous data exist on the effect of NO on cell respiration, relatively little is known about the effect of NO on mitochondrial Ca²⁺ homeostasis.

Mitochondria are capable of Ca²⁺ accumulation resulting from agonist-induced cytoplasmic [Ca²⁺] elevations. Ca²⁺ enters mitochondria via an electrogenic Ca²⁺ uniporter driven by the electrical potential difference across the inner mitochondrial membrane and is extruded by either Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchange (41). Using fluo-3 trapped inside mitochondria, we evaluated the effect of NO on mitochondrial Ca²⁺ uptake through the electrogenic mitochondrial Ca²⁺ uniporter in digitorin-permeabilized CPAE cells. The method of cell membrane permeabilization has proved to be extremely valuable to measure the activities of mitochondria and other cellular organelles in situ without the need for isolation procedures that can adversely affect mitochondrial functions. The method has the unique advantage that it allows control of the environment surrounding mitochondria (i.e., the cytoplasm environment) and to monitor the interactions between different organelles in their native structural arrangement (22). In the present study, we demonstrated that the application of high concentrations of NO (supplied by 100–500 μM Sper/NO) resulted in mitochondrial membrane depolarization and subsequent decrease of mitochondrial Ca²⁺ uptake. Mitochondrial Ca²⁺ extrusion was also decreased in these experiments; however, we believe that this was a consequence of the inhibitory effect of Sper/NO on Ca²⁺ uptake and decreased [Ca²⁺]mt levels required for stimulation of Ca²⁺ extrusion mechanisms such as Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchangers. Because Na⁺/Ca²⁺ or H⁺/Ca²⁺ exchangers are Ca²⁺ dependent, decreasing of [Ca²⁺]mt will result in a lowering of the rate of Ca²⁺ extrusion.

Low concentrations of Sper/NO (<100 μM), which generate physiological [NO] levels, had no effect on [Ca²⁺]mt. These results are in agreement with our previous observations in intact CPAE cells (19) that low concentrations of NO had no effect on mitochondrial Ca²⁺ content releasable by ionomycin. Our new data with L-arginine and l-NIO from permeabilized CPAE cells favor the possibility that mitochondrially produced NO can help regulate mitochondrial Ca²⁺ fluxes. In intact cells, caveolae-located NOS is the main source of NO, thus [Ca²⁺]mt is unlikely to be controlled solely by mtNOS. Under pathological conditions, however, caveolar NOS can be compromised (see, e.g., Ref. 35) and mtNOS may serve as a compensatory mechanism to control mitochondrial Ca²⁺ transport. Altogether, our data indicate that the modulation of mitochondrial Ca²⁺ uptake by NO might play an important role in the prevention of mitochondrial Ca²⁺ overload. For example, it was shown that NO provided protection during simulated ischemia and reoxygenation in isolated guinea pig (31) and neonatal rat ventricular cardiomyocytes (44). The cardiopro-
mitochondrial Ca$^{2+}$ and NO in endothelium

It is well established that an essential link between mitochondrial electron transport and ATP synthesis is the maintenance of $\Delta \Psi_m$ with negative intramitochondrial polarity. In isolated mitochondria, values of $\Delta \Psi_m$ on the order of $-180$ mV or more have been obtained during state 4 respiration (for review, see Ref. 28). In our experiments, we have investigated the effect of different NO concentrations on $\Delta \Psi_m$. We found that application of Sper/NO induced a dose-dependent and reversible depolarization of the membrane potential. Moreover, inhibition of mtNOS with L-NIO led to an increased initial depolarization by NO donors and SOD did not affect the depolarization induced by NO (10). We also did not find any significant effect of the SOD mimetic and ONOO$^-$ scavenger Mn-TBAP on Sper/NO-induced changes in $\Delta \Psi_m$ (Fig. 4, D and F). Although the effect was small and not statistically significant, we found a slightly more pronounced depolarization by 300 $\mu$M Sper/NO in the presence of Mn-TBAP, consistent with an enhanced NO production in mitochondria and reduced NO conversion to ONOO$^-$ (20). Cell treatment with the NO scavenger PTIO, however, resulted in complete prevention of Sper/NO-induced membrane depolarization (Fig. 4, C and F). Furthermore, the effects of ONOO$^-$ have been reported to be irreversible compared with those of NO, further indicating that the observed changes in $\Delta \Psi_m$ were due to rapid and reversible effects of NO on the respiratory chain. The effect was mediated by NO interaction with cytochrome oxidase because mitochondria treatment with sodium cyanide (blocks respiratory chain at the level of cytochrome oxidase) prevented the NO-induced depolarization of mitochondrial membrane, whereas other inhibitors of respiratory chain did not affect it (data not shown).

An alternative explanation for the decrease of [Ca$^{2+}$]$_{\text{mit}}$ by NO is the opening of the mitochondrial permeability transition pore. In isolated mitochondria, conditions have been described that cause a Ca$^{2+}$-dependent increase in mitochondrial permeability to ions and solutes with molecular weights up to 1,500 Da, matrix swelling and uncoupling of oxidative phosphorylation. The pore is protected from opening by low pH, a high sodium cyanide (blocks respiratory chain at the level of cytochrome oxidase) prevented the NO-induced depolarization of mitochondrial membrane, whereas other inhibitors of respiratory chain did not affect it (data not shown).

Fig. 6. Effect of NO on the activity of the mitochondrial permeability transition pore. Representative traces of mitochondria-trapped calcein fluorescence changes from CPAE cells after permeabilization with 10 $\mu$M digitonin. A: application of 300 $\mu$M Sper/NO induced a decrease in calcein fluorescence while 50 $\mu$M Sper/NO had no effect. The subsequent application of the pore-forming antibiotic alamethicin (40 $\mu$g/ml) resulted in a rapid loss of calcein fluorescence, providing a positive control for maximal calcein release from the mitochondrial matrix. B: treatment with 100 $\mu$M of the PTP inhibitor decylubiquinone (DQ), significantly decreased the Sper/NO-induced loss of calcein from mitochondria. C: representative trace of calcein fluorescence changes after subsequent application of 100 and 200 $\mu$M Sper/NO. D: summary of the effects of different Sper/NO concentrations on the rate of calcein release from mitochondria. The data were normalized in each individual cell for the initial rate of fluorescence decline (100%). The numbers in parentheses indicate the number of cells tested.

$+P < 0.05$, $\#P < 0.01$, $*P < 0.001$, statistical significance. The effects of Sper/NO alone were compared with control. The effect of 300 $\mu$M Sper/NO+DQ was compared with 300 $\mu$M Sper/NO alone.
NO donors, has also been reported to have a dual effect on PTP, depending on NO concentration. NO reversibly inhibited PTP opening with IC_{50} of 11 nM NO/s, whereas at supraphysiological release rates (>2 μM/s), NO accelerated PTP opening (9). Similar results were found in rat liver mitochondria, where the application of lower NO donor concentrations [1 to 20 μM of GEA 3162 (1,2,3,4-oxatriazolium,5-amino-3-5-dichloro-phenyl)-chloride], 3-morpholinosydnonimine, and SNAP] had no effect or delayed PTP opening, whereas doses from 20 to 100 μM accelerated Ca^{2+} overload-induced PTP (43). We used a fluorescence assay (42) to evaluate the effect of NO on the PTP. PTP opening causes matrix-trapped calcein to be released from mitochondria, which results in a decrease of mitochondrial calcein fluorescence. Because application of high concentrations of Sper/NO induced a rapid and sustained drop in membrane potential, which was partially prevented by the PTP inhibitor DQ (Fig. 4, E and F), we tested the hypothesis that the PTP is involved. Indeed, we found that the concentrations of Sper/NO (100–500 μM) that were able to induce mitochondrial membrane depolarization also initiated opening of the PTP. The decrease in calcein fluorescence induced by Sper/NO application was partially prevented by cell treatment with 100 μM DQ, a PTP inhibitor (23), which confirmed that the observed effect was due to opening of the PTP. The correlation between a sustained drop in ΔΨ_m and PTP opening was observed previously in CPAE cells (33). Moreover, it was shown that individual mitochondria displayed repetitive opening and closing of the PTP (“flickering”) spontaneously (41) as well as during exposure to oxidative stress and/or Ca^{2+} overload (33, 54). These short openings of PTP might serve as an emergency mechanism allowing the dissipation of the driving force for mitochondrial Ca^{2+} uptake, leading to fast release of accumulated Ca^{2+} ions and the decreased generation of endogenous oxygen radicals.

In conclusion, herein we have presented evidence that the higher levels of NO, usually reached during pathological conditions, produced either by plasma membrane-associated eNOS or locally by mitochondrially located NOS, would provide protection against mitochondrial Ca^{2+} overload. This protection is mediated by decreasing ΔΨ_m, which leads to the decreased driving force for mitochondrial Ca^{2+} uptake. The reversible opening of PTP might provide an additional emergency mechanism preventing mitochondrial Ca^{2+} overload.

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