Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells

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Mitochondrial calcium uptake stimulates nitric oxide production in mitochondria of bovine vascular endothelial cells. Am J Physiol Cell Physiol 286: C406–C415, 2004. First published September 24, 2003; 10.1152/ajpcell.00155.2003.—Although nitric oxide (NO) is a known modulator of cell respiration in vascular endothelium, the presence of a mitochondria-specific nitric oxide synthase (mtNOS) in these cells is still a controversial issue. We have used laser scanning confocal microscopy in combination with the NO-sensitive fluorescent dye DAF-2 to monitor changes in NO production by mitochondria of calf vascular endothelial (CPAE) cells. Cells were loaded with the membrane-permeant NO-sensitive dye 4,5-diaminofluorescein (DAF-2) diacetate and subsequently permeabilized with digitonin to remove cytosolic DAF-2 to allow measurements of NO production in mitochondria ([NO]mit). Stimulation of mitochondrial Ca2+ uptake by exposure to different cytoplasmic Ca2+ concentrations (1, 2, and 5 μM) resulted in a dose-dependent increase of NO production by mitochondria. This increase of [NO]mit was sensitive to the NOS antagonist L-NAME (1-miminoethyl)ornithine and the calmodulin antagonist calmidazolium (R-24571), demonstrating the endogenous origin of NO synthesis and its calmodulin dependence. Collapsing the mitochondrial membrane potential with the protonophore FCCP or blocking the mitochondrial Ca2+ uniporter with ruthenium red, as well as blocking the respiratory chain with antimycin A in combination with oligomycin, inhibited mitochondrial NO production. Addition of the NO donor spermine NONOate caused a profound increase in DAF-2 fluorescence that was not affected by either of these treatments. The mitochondrial origin of the DAF-2 signals was confirmed by colocalization with the mitochondrial marker MitoTracker Red and by the observation that disruption of caveolae (where cytoplasmic NOS is localized) formation with methyl-β-cyclodextrin did not prevent the increase of DAF-2 fluorescence. The activation of mitochondrial calcium uptake stimulates mtNOS phosphorylation (at Ser-1177) which was prevented by FCCP. The data demonstrate that stimulation of mitochondrial Ca2+ uptake activates NO production in mitochondria of CPAE cells. This indicates the presence of a mitochondria-specific NOS that can provide a fast local modulatory effect of NO on cell respiration, membrane potential, and apoptosis.

NITRIC OXIDE (NO) is known to have an inhibitory effect on cell respiration of different cell types, including vascular endothelial cells (4, 5, 12). Endogenous NO modulates cellular O2 consumption at the level of cytochrome oxidase, reducing the affinity of the enzyme for O2 (10, 11). The inhibitory effect of NO on the cytochrome oxidase depends on the levels of O2 and calcium and is more pronounced at low concentrations of O2 (3, 10, 12). Therefore, NO plays a physiological role in the regulation of the ability of cytochrome oxidase to use oxygen and to adjust to changes in O2 availability. This raises the question of whether mitochondria possess their own NO synthase (mtNOS) that would allow fast and local regulation of cell respiration or other NO-dependent mitochondrial functions.

Indeed, immunohistochemical techniques have shown the existence of mtNOS originally in mitochondria of nonsynaptic brain and liver tissues (1), smooth muscle (2, 25), heart and kidney (2) of adult rats, and human smooth muscle (16). The presence of functional constitutively expressed NOS in the inner membrane of rat liver mitochondria has been reported (20) and confirmed directly by purification and characterization of NOS from rat liver mitochondria (15, 23, 42). It has been shown that mtNOS became activated during mitochondrial Ca2+ uptake and that the NO produced exerted substantial control over mitochondrial respiration, membrane potential, and the transmembrane proton gradient (19, 20). Mitochondrial NO production was dependent on the presence of l-arginine and inhibited by N-methyl-l-arginine. Furthermore, NO production was linked to the respiratory state of mitochondria, suggesting that mitochondrial NADH plays a role in mitochondrial NO production (22). Various studies using biochemical, electrochemical, and fluorescent techniques consolidated the notion of the presence of mtNOS and mitochondrial NO production in different cell types (24, 26, 29, 43). However, little or no information is available in support of the existence of mtNOS in vascular endothelial cells (8) and the dynamic changes of NO production of individual mitochondria in single cells.

Agonist stimulation of endothelial cells causes an increase in cytosolic calcium concentration ([Ca2+]cytosol), which leads to subsequent accumulation of Ca2+ by mitochondria (14, 27). Ca2+ enters mitochondria via an electrogentic Ca2+ uniporter driven by the electrical potential difference across the inner mitochondrial membrane and can subsequently be extruded by either Na+/Ca2+ or H+/Ca2+ exchange (36). The increase of Ca2+ levels in the mitochondrial matrix ([Ca2+]mit) leads to the activation of key metabolic enzymes that are Ca2+-dependent (30). In the vascular endothelium, NO plays an important regulatory role. NO, which is synthesized in a Ca2+-dependent manner by the endothelial NOS (eNOS), acts on vascular smooth muscle cells in the vessel wall as an endothelium-derived relaxing factor (31). We have demonstrated previously that in vascular endothelial cells, NO synthesis is not only Ca2+-dependent but is also under autoregulatory control that involves NO-dependent [Ca2+] regulation (13). Considering the known effects of NO on cell respiration in endothelial cells, we raised the question of whether the autoregulatory function

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of NO in endothelial cells also entails mitochondrial NO production targeting local mitochondrial functions during the activation of mitochondrial Ca\textsuperscript{2+} uptake.

In the present study, we have used permeabilized calf pulmonary artery endothelial cells (CPAE) to examine the NO production from mitochondria during mitochondrial Ca\textsuperscript{2+} uptake via the ruthenium red-sensitive Ca\textsuperscript{2+} uniporter. The results of our experiments show that 1) the mitochondria of CPAE cells respond with a dose-dependent NO production to an increase in extramitochondrial Ca\textsuperscript{2+} concentration; 2) mitochondrial Ca\textsuperscript{2+} uptake stimulates the phosphorylation of mtNOS at Ser-1177; 3) the inhibition of mitochondrial Ca\textsuperscript{2+} uniporter by ruthenium red, collapsing the mitochondrial membrane potential with the protonophore FCCP as well as blocking the respiratory chain by antimycin A in combination with oligomycin, completely abolished mitochondrial NO production; and 4) the mitochondrial NO production was sensitive to inhibition of NOS and to the application of the calmodulin antagonist calmidazolium. The results indicate that mitochondria of CPAE cells possess a Ca\textsuperscript{2+}/calmodulin-dependent NOS that can produce NO in response to mitochondrial Ca\textsuperscript{2+} uptake.

METHODS

Cell Culture and Solutions

Experiments were performed on CPAE cells in nonconfluent cultures. The CPAE cell line was obtained as passage 15 from American Type Culture Collection (CCL-209; ATCC, 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 with 10% fetal bovine serum (GIBCO, Grand Island, NY) and 2 mM L-glutamine, and kept at 37°C in an atmosphere of 5% CO\textsubscript{2}-95% air. On confluence, the cells were washed with 135 NaCl, 10 NaCl, 20 HEPES, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH\textsubscript{2}PO\textsubscript{4}, 1 MgCl\textsubscript{2}, 5 EGTA, and 1.86 CaCl\textsubscript{2} to yield a free [Ca\textsuperscript{2+}] of about 100 nM. L-Arginine (100 μM) was added to all solutions, except in the study using National Institutes of Health ImageJ.

Fluorescence Measurements

[NO\textsubscript{int}] measurements. Laser scanning confocal microscopy (LSM 410, Carl Zeiss, Germany) was used to follow the changes in the measurements of NO production in mitochondria ([NO\textsubscript{int}] during activation of mitochondrial Ca\textsuperscript{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). Direct measurements of NO production from mitochondria were performed on cells loaded with the fluorescent NO-sensitive dye 4,5-diaminofluorescein (DAF-2; Ref. 32) and MitoTracker Red CMXRos (34). Cells were exposed to 5 μM of the membrane-permeant form of the indicator DAF-2 diacetate (DAF-2 DA; Calbiochem, San Diego, CA) for 40 min at 37°C in 1 ml of standard Tyrode solution containing (in mM) 135 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES (pH 7.3). During the final 10 min of dye loading, 200 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) was added. Cells were subsequently washed for 10 min. The cells were then placed in Ca\textsuperscript{2+}-free Tyrode solution and permeabilized by exposure to 10 μM digitonin added to the “intracellular” solution consisting of (in mM) 135 KCl, 10 NaCl, 20 HEPES, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH\textsubscript{2}PO\textsubscript{4}, 1 MgCl\textsubscript{2}, 5 EGTA, and 1.86 CaCl\textsubscript{2} to yield a free [Ca\textsuperscript{2+}] of ~100 nM. L-Arginine (100 μM) was added to all solutions, except in experiments in which NO was inhibited with L-N\textsuperscript{6}-l-arginine-ethyl-) ornithine (l-NIO). Both indicators were excited with the 488-nm line of the argon laser, and the emitted fluorescent signals were measured simultaneously at 510–525 nm (DAF-2) and 590 nm (MitoTracker Red CMXRos). Changes in mitochondrial DAF-2 fluorescence intensities (F) in each experiment were normalized to the level of fluorescence recorded before stimulation (F\textsubscript{0}) but after cell permeabilization (see Fig. 1C). Changes in [NO\textsubscript{int}] are expressed as ΔF/F\textsubscript{0} and thus represent %increases above basal levels. Activation of DAF-2 by NO is irreversible, and, therefore, fluorescence intensity remains constant even if NO levels decrease. The MitoTracker Red signal was used to identify individual mitochondria and to position regions of interest of ≤9 μm\textsuperscript{2} for the [NO\textsubscript{int}] measurements. Thus the [NO] measurements shown in this study represent NO signals from a small (<10) number of mitochondria.

Western Blot Analysis

Immunoblot of lysates from intact, permeabilized CPAE cells and mitochondrial fractions were carried out to analyze phosphorylation of mtNOS at Ser-1177 using the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ). Briefly, control cells and cells exposed to different treatments were scraped from the flask surface and centrifuged at 100 g for 10 min at 4°C. Cells were then lysed in ice-cold buffer of the following components: 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 1.0 mM EGTA, 1.0 mM Na\textsubscript{3}VO\textsubscript{4}, 10 mM Na-pyrophosphate, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4. Samples were centrifuged at 10,000 g at 4°C for 10 min to remove insoluble material, and protein contents were determined by using BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of proteins (25–50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, which was subsequently immunoblotted with anti-phospho-eNOS antibody (Ser-1177) (Transduction Laboratories, San Diego, CA) at room temperature for 1 h. Protein bands were detected by using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences) and analyzed quantitatively by using National Institutes of Health ImageJ.

Chemicals

The protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), ruthenium red (RR), methyl-β-cyclodextrin (cyclo-dextrin), digitin, antimycin A, oligomycin, Na\textsubscript{3}VO\textsubscript{4}, sodium pyrophosphate, NaF, sodium deoxycholate, SDS, Tris-base, leupeptin, aprotinin, and PMSF were obtained from Sigma (St Louis, MO); spermine NONOate (Sper/NO), l-NIO, and manganese (III) tetrakis (4-benzoic acid)porphyrin (MnTBAP) were from Calbiochem (San Diego, CA); calmidazolium chloride (R-24571) was purchased from Amersham Biosciences. The NO-sensitive dye Sper/NO was purchased from RBI (Natick, MA); glycerol and Triton X-100 were from Lab Chem (Pittsburgh, PA).

Statistical Analysis

Statistical differences of the data were determined with the Student’s t-test for unpaired data and 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

Direct Measurements of NO in Mitochondria of Single Permeabilized CPAE Cells

For direct measurement of NO production by mitochondria of vascular endothelial cells, we used the NO-sensitive fluorescent indicator DAF-2. This indicator has been employed successfully to estimate NO levels in endothelial cells produced by the constitutive eNOS (6, 13, 32). Cells were co-
localed with DAF-2 DA for 40 min at 37°C (i.e., in conditions that allow the dye to compartmentalize into mitochondria) and with the mitochondrial marker MitoTracker Red (see METHODS for details). Figure 1A (DAF-2, cntrl) shows that DAF-2 was distributed homogeneously throughout the cell, including mitochondria. Plasma membrane permeabilization with digitonin removed the cytosolic and nuclear DAF-2 (Fig. 1A, DAF-2, digitonin), revealing the particulate and punctate pattern of fluorescence typical for mitochondria (Fig. 1A, MitoTracker Red, digitonin). The colocalization of DAF-2 fluorescence remaining after cell permeabilization with MitoTracker Red fluorescence proved the mitochondrial origin of the DAF-2 signal (Fig. 1A, overlay, digitonin). In the following experiments, the MitoTracker Red signal served to identify mitochondrial regions and to position the regions of interest (≤9 μm²) for the measurements of [NO]ₘ. Figure 1B shows that under basal conditions (0.1 μM Ca²⁺), mitochondria of CPAE cells reveal relatively low basal levels of NO. However, stimulation of mitochondrial Ca²⁺ uptake by increasing extramitochondrial calcium ([Ca²⁺]ₑ) to 2 μM significantly enhanced the intensity of DAF-2 fluorescence, suggesting endogenous NO production by mitochondria (Fig. 1B, 2 μM Ca²⁺). Over a period of 4 min, ΔF/F₀ increased by 0.62 ± 0.04 (n = 11, P < 0.001) over basal level. To prove the specificity of DAF-2 for NO, the NO donor Sper/NO was applied to the permeabilized cells at the end of the experiment, which led to a rapid and robust increase in DAF-2 fluorescence (Fig. 1B, 300 μM Sper/NO). The average ΔF/F₀ after 2 min of Sper/NO exposure was 3.19 ± 0.29 (n = 11, P < 0.001). Figure 1C presents the typical time course of DAF-2 fluorescence intensity changes from mitochondria of a single CPAE cell before and after cell permeabilization with digitonin and after activation of mitochondrial Ca²⁺ uptake by increasing extramitochondrial Ca²⁺ to 2 μM, followed by application of Sper/NO. The Sper/NO-induced rate of increase of ΔF/F₀ was approximately eightfold faster than observed upon stimulation of mitochondrial Ca²⁺ uptake.

**Disruption of Caveolae Does Not Affect Mitochondrial NO Production**

eNOS is primarily associated with plasma membrane caveolae. Caveolae are 50–100 nm diameter invaginations of the plasma membrane that are rich in cholesterol, sphingolipids,
and lipid-anchored membrane proteins and are resistant to solubilization by detergents (37). Experimentally lowering the cholesterol level of caveolar fractions disrupts the molecular assembly and ultrastructure of the caveolar domain (40). To exclude a possible contribution from eNOS located at caveolae in our measurement of [NO]mt, cells were pretreated with 1 mM methyl-
H9252/cyclodextrin for 1 h at 37°C. Ultrastructure studies indicate that this treatment disrupts the caveolar organization without any other obvious structural changes within cells (28, 40). Figure 2A (cntrl) presents the image of a CPAE cell loaded with DAF-2 after cyclodextrin treatment. The initial level of DAF-2 fluorescence in the cyclodextrin-treated cell was significantly decreased compared with control (cf. Fig. 1A, cntrl), especially in the area near the plasma membrane. Cell permeabilization with digitonin removed the cytosolic and nuclear staining of DAF-2, revealing the same punctate mitochondrial fluorescence pattern as the untreated cell (compare Figs. 1A and 2A, digitonin). The integrity of mitochondria was unchanged after cyclodextrin treatment as revealed by MitoTracker Red staining (Fig. 2A, digitonin). Furthermore, the overlay of both signals indicated that the remaining DAF-2 fluorescence after cell permeabilization originated from mitochondria. Figure 2B compares the intensity of DAF-2 fluorescence as an indicator of NO production during stimulation of mitochondrial Ca2+/H11001 uptake (2 μM Ca2+) and Sper/NO application (300 μM Sper/NO) with the unstimulated condition (0.1 μM Ca2+). A typical example of DAF-2 fluorescence changes from a cyclodextrin-treated CPAE cell is shown in Fig. 2C. The quantitative analysis of DAF-2 fluorescence changes revealed that cyclodextrin treatment affected neither the mitochondrial NO production during Ca2+/H11001 uptake (ΔF/F0 = 0.59 ± 0.07; n = 7) nor the Sper/NO-induced increase in [NO]mt (ΔF/F0 = 2.88 ± 0.18; n = 7) compared with control cells (the means were not significantly different). These data confirmed that the DAF-2 fluorescence measurements in permeabilized CPAE cells reflect noncytoplasmic (i.e., mitochondrial) NO production.

**Block of Mitochondrial NO Production by Inhibition of Constitutive NOS**

To verify that the observed NO production was indeed due to endogenous NOS activity, CPAE cells were preincubated for >10 min with 10 μM l-NIO, a specific inhibitor of constitutive NOS. When the activity of NOS was blocked by l-NIO, exposure to 2 μM Ca2+ failed to induce NO production.
Fig. 3. Effect of l-N^3-(1-iminoethy])-ornithine (l-NIO) on mitochondrial [NO]_mt. A: representative traces of changes in DAF-2 fluorescence from mitochondria of permeabilized CPAE cells in response to an increase of \([\text{Ca}^{2+}]_{\text{em}}\) from 0.1 to 2 \(\mu\text{M}\), followed by exposure to 300 \(\mu\text{M}\) Sper/NO in control conditions (cntrl) and after cells were pretreated with the NOS inhibitor l-NIO (+l-NIO; 10 \(\mu\text{M}\)). B: summary of changes in mitochondrial DAF-2 fluorescence (\(\Delta F/F_0\)) obtained after an increase of \([\text{Ca}^{2+}]_{\text{em}}\) from 0.1 to 2 \(\mu\text{M}\) and exposure to Sper/NO, respectively, in the presence and absence of l-NIO. Numbers in parentheses indicate the number of cells.

by mitochondria, whereas the NO donor Sper/NO was still able to increase DAF-2 fluorescence (Fig. 3A). Thus inhibition of NOS blocked the ability of mitochondria to produce NO, confirming that the source of NO was indeed intramitochondrial. These data are summarized in Fig. 3B. Under control conditions, mitochondrial \(\text{Ca}^{2+}\) uptake increased NO production (\(\Delta F/F_0\) of 0.62 ± 0.04; \(n = 11\)), and this effect was completely blocked by l-NIO (\(\Delta F/F_0 = 0.02 \pm 0.01\) measured after 4 min; \(n = 5\), \(P < 0.001\)). The Sper/NO-induced rise of DAF-2 fluorescence was not affected by l-NIO as would be expected from an exogenous source of NO. The average \(\Delta F/F_0\) induced by Sper/NO was 3.02 ± 0.24 (\(n = 5\)) in the presence of l-NIO and 3.19 ± 0.29 (\(n = 11\)) under control conditions (difference not statistically significant). In summary, the data indicate that NO is synthesized by NOS located within mitochondria in response to mitochondrial \(\text{Ca}^{2+}\) uptake.

**Dependence of Mitochondrial NO Production on \([\text{Ca}^{2+}]_{\text{em}}\)**

To examine the \([\text{Ca}^{2+}]_{\text{em}}\) dependence of mitochondrial NO production, permeabilized cells were exposed to various concentrations of extramitochondrial \(\text{Ca}^{2+}\) (Fig. 4). Figure 4A shows an overlay of normalized traces of mitochondrial NO production obtained with \([\text{Ca}^{2+}]_{\text{em}}\) of 1, 2, and 5 \(\mu\text{M}\), indicating that the increasing \([\text{Ca}^{2+}]_{\text{em}}\) resulted in a concentration-dependent (threshold for uptake 0.8 \(\mu\text{M}\), \(k_{0.5} = 3 \mu\text{M}\)) increase in the rate and magnitude of mitochondrial NO production. We used the normalized NO level (\(\Delta F/F_0\)) reached after 4 min of \(\text{Ca}^{2+}\) exposure for the quantitative characterization of the dose dependence of mitochondrial NO production. The averaged data of normalized NO production from mitochondria in response to application of 1, 2, and 5 \(\mu\text{M}\) of \([\text{Ca}^{2+}]_{\text{em}}\) are presented in Fig. 4B. Because we have shown previously (36) that an increase of \([\text{Ca}^{2+}]_{\text{em}}\) results in a dose-dependent increase in mitochondrial \(\text{Ca}^{2+}\) uptake in CPAE cells, the results here indicate that mitochondrial NO production is also \(\text{Ca}^{2+}\) concentration dependent.

**Mitochondrial Inhibitors Prevent NO Production in Mitochondria**

Figure 5A shows the effect of dissipating the mitochondrial membrane potential (\(\Delta \psi\)) with the uncoupler of oxidative phosphorylation FCCP on mitochondrial NO production. Mitochondrial \(\text{Ca}^{2+}\) uptake was initiated by elevating \([\text{Ca}^{2+}]_{\text{em}}\) from 0.1 to 5 \(\mu\text{M}\) and resulted in an increase of mitochondrial DAF-2 fluorescence (Fig. 5Aa, cntrl). In contrast, when cells were pretreated with 1 \(\mu\text{M}\) FCCP, elevation of \([\text{Ca}^{2+}]_{\text{em}}\) did not produce any changes in DAF-2 fluorescence (Fig. 5A, FCCP). As summarized in Fig. 5Ab, in the presence of 1 \(\mu\text{M}\) FCCP, the amplitude of \(\Delta F/F_0\) measured 4 min after \([\text{Ca}^{2+}]_{\text{em}}\) elevation reached 0.054 ± 0.01 (\(n = 4\)) compared with 0.83 ± 0.09 (\(n = 6\)) in control (\(P < 0.001\)). Sper/NO-induced elevation in DAF-2 fluorescence was not affected by this treatment \(\Delta F/F_0\) was on average 3.23 ± 0.74 (\(n = 4\)) in the presence of FCCP vs. 3.20 ± 0.52 (\(n = 6\)) in control; data not shown.

Fig. 4. \([\text{Ca}^{2+}]_{\text{em}}\) dependence of mitochondrial NO production. A: measurements of NO production in permeabilized CPAE cells exposed to various concentrations of \([\text{Ca}^{2+}]_{\text{em}}\). \([\text{Ca}^{2+}]_{\text{em}}\) was elevated from 0.1 to 1, 2, and 5 \(\mu\text{M}\). B: average changes of \([\text{NO}]_{\text{mt}}\) (expressed as \(\Delta F/F_0\) of DAF-2 fluorescence) are presented as a function of \([\text{Ca}^{2+}]_{\text{em}}\). Values for \(\Delta F/F_0\) were determined 4 min after increasing \([\text{Ca}^{2+}]_{\text{em}}\) from 0.1 \(\mu\text{M}\). Data represent means ± SE. Numbers in parentheses indicate the number of cells.
Figure 5Ba represents the typical trace of DAF-2 fluorescence upon stimulation of mitochondrial Ca\(^{2+}\) uptake in control conditions and when the respiratory chain was blocked by 5 \(\mu\)g/ml antimycin A in combination with 1 \(\mu\)g/ml oligomycin. Antimycin A inhibits electron transfer from cytochrome b to cytochrome c\(_{1}\) (complex III), whereas oligomycin stops ATP synthesis by blocking mitochondrial H-ATP synthase (complex V). Because the processes of electron transfer and ATP synthesis are coupled tightly, the use of these inhibitors results in complete inhibition of cell respiration and oxidative phosphorylation. The inhibitors were applied 2 min before stimulation of Ca\(^{2+}\) uptake by addition of 2 \(\mu\)M Ca\(^{2+}\) to the extramitochondrial solution. As shown in Fig. 5Bb, blocking the respiratory chain resulted in a significant \((P < 0.001)\) inhibition of mitochondrial NO production \([\Delta F/F_0]\) was on average 0.013 \pm 0.007 \((n = 6)\) vs. 0.70 \pm 0.09 \((n = 4)\) in control. Thus these results indicate that the activity of the respiratory chain might play an important role in the regulation of mtNOS.

In the next set of experiments (Fig. 6A), we blocked the mitochondrial Ca\(^{2+}\) uniporter with 10 \(\mu\)M RR. After the cell was permeabilized with digitonin (Fig. 6A), 10 \(\mu\)M RR was added to the intracellular solution for 2 min before \([\text{Ca}^{2+}]_{\text{em}}\) was increased. In the presence of RR, there were no significant changes in \([\text{NO}]_{\text{em}}\) in response to increasing \([\text{Ca}^{2+}]_{\text{em}}\) from 0.1 \(\mu\)M to 2 \(\mu\)M (Fig. 6A, +RR) compared with control (Fig. 6A, cnt). However, the subsequent application of the NO donor stimulated an increase in DAF-2 fluorescence (data not shown), suggesting that in the presence of RR, the mitochondrial DAF-2 was still capable of sensing changes in NO. In summary, in cells pretreated with RR, the level of fluorescence \((\Delta F/F_0)\) was 0.03 \pm 0.006 \((n = 7)\) after \([\text{Ca}^{2+}]_{\text{em}}\) elevation vs. 0.61 \pm 0.04 \((n = 11, P < 0.001)\) in control (Fig. 6C) and 3.06 \pm 0.16 \((n = 7)\) after Sper/NO application vs. 3.19 \pm 0.29 \((n = 11)\) in control (Fig. 3B).

These data indicate that the increase in mitochondrial NO production after elevation of \([\text{Ca}^{2+}]_{\text{em}}\) was due to a Ca\(^{2+}\)-uniporter-mediated, \(\Delta\varphi_{\text{em}}\)-dependent Ca\(^{2+}\) uptake into the mitochondria leading to the activation of Ca\(^{2+}\)-dependent mitochondrial NOS.

**Effect of Calmidazolium on Mitochondrial NO Production**

To explore the calmodulin dependence of mitochondrial NOS and to determine the effect of calmodulin inhibition on NO production by the mitochondria, cells were preincubated with 10 \(\mu\)M calmidazolium (R-24571) for >10 min before the \([\text{NO}]_{\text{em}}\) measurements. As shown in Fig. 6B, treatment with 10 \(\mu\)M calmidazolium completely inhibited the increase in \([\text{NO}]_{\text{em}}\), normally induced by mitochondrial Ca\(^{2+}\) uptake, suggesting the constitutive nature of mtNOS in CPAE cells. In a total of 5 cells, the average change of DAF-2 fluorescence \((\Delta F/F_0)\) was 0.03 \pm 0.02 compared with 0.61 \pm 0.04 in control \((n = 11, P < 0.001)\) (Fig. 6C). The Sper/NO-induced increase in mitochondrial NO level was not affected by this treatment \((\Delta F/F_0) = 3.20 \pm 0.26\) vs. control of 3.19 \pm 0.29\((n = 11)\); data not shown). Thus these data suggest the presence of a constitutive calcium/calmodulin-dependent NOS in mitochondria of CPAE cells.

**Effect of Superoxide Scavenger MnTBAP on Mitochondrial NO Production**

It has been reported that DAF-2 can sense free radicals and reactive species aside from NO (41). Considering that mitochondria are very well known to produce such compounds, we evaluated the potential 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 in our conditions, in separate sets of experiments we pretreated cells with a cell-permeable superoxide dismutase (SOD) mimic and peroxynitrite scavenger, Mn-TBAP (50 \(\mu\)M), and examined changes in mitochondrial DAF-2 signal upon stimulation of mitochondrial Ca\(^{2+}\) uptake. As shown in Fig. 7A, MnTBAP not only failed to prevent a Ca\(^{2+}\)-induced rise of the DAF-2 signal, it even increased mitochondrial NO production [from \(\Delta F/F_0 = 0.58 \pm 0.06\) in control (\(n = 5\)) to \(1.01 \pm 0.04\) in Mn-TBAP-treated cells (\(n = 9\), \(P < 0.05\)]. This \(\sim 75\%\) increase in mitochondrial DAF-2 fluorescence upon SOD mimetic treatment suggests that either formation of superoxide radicals suppresses the NO production and/or part of the formed NO is transformed quickly to peroxynitrite. Nevertheless, the data provide strong evidence that the observed changes in DAF-2 fluorescence were due to mitochondrial NO production upon stimulation of mitochondrial Ca\(^{2+}\) uptake.

**Ca\(^{2+}\) Uptake Stimulates the Phosphorylation of eNOS at Ser-1177**

Activation of eNOS and NO production can be induced by phosphorylation of eNOS at Ser-1177 by activated serine/threonine protein kinase B (Akt/PKB) (17). In an attempt to demonstrate that a mitochondrial form of NOS exists in CPAE cells and that the enzyme becomes activated during mitochondrial Ca\(^{2+}\) uptake, we performed Western blot analysis of eNOS phosphorylation at Ser-1177. In the first set of experiments, protein levels of phosphorylated eNOS were determined in intact cells pretreated with 5 \(\mu\)g/ml of the calcium ionophore A-23187 for 5 min at 37\(^\circ\)C. The results are shown in Fig. 8A and are expressed as a percent of control. The amount of eNOS protein phosphorylated at Ser-1177 was significantly (\(P < 0.01\)) increased in the cells pretreated with A-23187 compared with controls (157 \(\pm\) 14\%, \(n = 3\)). Furthermore, when cells were pretreated with FCCP to dissipate the mitochondrial membrane potential and to remove the driving force
for mitochondrial Ca\textsuperscript{2+} uptake, stimulation of cells with A-23187 resulted in a smaller amount of phosphorylated NOS compared with treatment without FCCP (110 ± 12%, n = 3, P < 0.01). It is worth noting that FCCP alone slightly decreased the phosphorylation of eNOS in intact cells, indicating involvement of mitochondria in basal NOS activity. In addition, we isolated mitochondrial fractions from intact cells with and without A-23187 treatment and showed that the activity of mtNOS was significantly higher in A-23187-treated cells (data not shown). Taken together, these findings indicate that a significant amount of NOS activity measured from the intact cells during stimulation with A-23187 originated from mitochondria. In the second set of experiments, we directly compared the level of phospho-eNOS from lysates obtained from intact cells, permeabilized cells with and without stimulation of the mitochondrial Ca\textsuperscript{2+} uptake. The amount of phospho-eNOS in digitonin-treated cells was significantly lower compared with the nonpermeabilized cell (~40% of intact cell preparation; Fig. 8B). This level of phosphorylation was defined as control (100%) to normalize the data obtained with different treatments in permeabilized cells (see Fig. 8B). When mitochondrial Ca\textsuperscript{2+} uptake was stimulated by increasing [Ca\textsuperscript{2+}]\textsubscript{em} from 0.1 to 2 μM, the level of phospho-eNOS was significantly enhanced (197 ± 21%, n = 4, P < 0.01). Moreover, the phosphorylation of eNOS during mitochondrial Ca\textsuperscript{2+} uptake in permeabilized cells was completely blocked when permeabilized cells were pretreated with FCCP before stimulation of Ca\textsuperscript{2+} uptake (106 ± 6%, n = 4, P < 0.01). These findings directly correlate with NO measurements from mitochondria (see above). Thus these data indicate that mitochondrial Ca\textsuperscript{2+} uptake increases serine phosphorylation of eNOS, a marker of increased eNOS activity (17).

**DISCUSSION**

Using the NO-sensitive dye DAF-2 and permeabilized CPAE cells, we performed real-time measurements of NO production by mitochondria which was induced by calcium uptake through the electrogenic mitochondrial Ca\textsuperscript{2+} uniporter. The NO-sensitive fluorescent dye DAF-2 DA in combination with MitoTracker red was successfully used previously to stain mitochondria and reveal NO production from these organelles (8, 29, 43). However, these studies were performed in intact cells, and doubts remain regarding the accuracy of these measurements because the mitochondrial DAF-2 signal could be contaminated to a significant degree by cytosolic DAF-2 fluorescence. We improved this technique by using cell membrane permeabilization to remove cytosolic DAF-2 fluorescence. The technique of plasma membrane permeabilization was chosen to maintain the relative integrity of the cell and mitochondrial metabolism. Using the same approach of cell permeabilization, we have shown previously that an elevation of [Ca\textsuperscript{2+}]\textsubscript{em} resulted in a dose-dependent increase in the rate of Ca\textsuperscript{2+} accumulation into mitochondria via the mitochondrial Ca\textsuperscript{2+} uniporter (36). The present study now presents evidence for NO production within mitochondria of permeabilized CPAE cells during activation of mitochondrial Ca\textsuperscript{2+} uptake.

Several lines of evidence indicate the mitochondrial origin of NO production in our study. First, [NO]\textsubscript{mt} increased under conditions that result in mitochondrial Ca\textsuperscript{2+} uptake (Fig. 1). Preincubation of cells with the eNOS inhibitor L-NIO resulted in a block of Ca\textsuperscript{2+}-induced NO production, whereas the Sper/NO-induced DAF-2 signal was not affected by this treatment. Moreover, the experiments with the SOD mimetic Mn-TBAP ruled out the possibility of DAF-2 signal contamination by reactive oxygen species (ROS). If DAF-2 sensed ROS as well as NO, the fluorescence signal would be expected to decrease during application of Mn-TBAP and not to increase, as we observed (Fig. 7). This confirms the endogenous source of NO and specificity of the DAF-2 measurements. The potential influence of eNOS located in caveolae, the typical location of cytoplasmic NOS, was dismissed because no effect of cycloedrin, a caveolea disrupting agent, on [NO]\textsubscript{mt} was found (Fig. 2). Abolition of NO production by collapsing the mitochondrial membrane potential with the uncoupler of oxidative phosphorylation FCCP (Fig. 5A), by de-energizing mitochondria by antimycin A in combination with oligomycin (Fig. 5B), and by mitochondrial Ca\textsuperscript{2+}-uniporter blocker ruthenium red (Fig. 6, A and C) presents strong evidence for the mitochondrial origin of NO production. Dependence of mitochondrial NO production on Ca\textsuperscript{2+} (Fig. 4) and calmodulin...
(Fig. 6, B and C) further supports the idea that mitochondria of CPAE cells contain eNOS. Furthermore, using the Western blot technique, we showed the reactivity of mtNOS with eNOS antibody. Besides, we demonstrated that the level of mtNOS phosphorylation (Ser-1177) was significantly increased during stimulation of mitochondrial Ca$^{2+}$ uptake. The phosphorylation was blocked when cells were pretreated with FCCP to collapse mitochondrial membrane potential and to block Ca$^{2+}$ uptake. Activation of eNOS and NO production can be induced by phosphorylation of eNOS at Ser-1177 by activated Akt/ PKB. Serine phosphorylation of eNOS at Ser-1177 has been shown to correlate directly with increased NO production (17). The calmodulin dependence of NOS and the crossreactivity of mtNOS with eNOS but not with inducible (iNOS) or brain type of NOS (bNOS) has been used to demonstrate the existence of a constitutive endothelial isoform of NOS in liver mitochondria (1, 2, 26), although other studies showed similarity of mtNOS with iNOS in rat liver mitochondria (42). Recently, mtNOS isolated from rat liver was identified as bNOS$\alpha$ with two posttranslational modifications consisting of acylation with myristic acid and phosphorylation at the COOH terminus (15). In addition, the bNOS isoform has been identified in mitochondria of human skeletal muscle (16) and in mouse cardiomyocytes (24), suggesting that the nature of mitochondrial NOS might be species dependent.

Even though evidence is accumulating that various cell types contain a mitochondrial NOS isoform and are capable of mitochondrial NO synthesis, the physiological (or pathophysiological) role of mitochondrial NO has remained elusive. The production of NO by mitochondria might have important physiological relevance because NO can modulate the level of mitochondrial Ca$^{2+}$ and regulate the availability of O$_2$ for other cellular functions. For example, it has been shown that pretreatment with an NO donor induces sustained mitochondrial depolarization and protects cardiomyocytes from simulated ischemia and reoxygenation injury by limiting mitochondrial Ca$^{2+}$ accumulation, providing a mechanism to account for NO-induced cardioprotection (39). Furthermore, the activity of NOS in isolated mitochondria from mouse liver was functionally upregulated after hypoxia (26). Together with the known effects of NO on mitochondrial function during hypoxia (7, 9), one might speculate that the ability of mitochondria to regulate their own NO levels constitutes an important mechanism for cell protection and survival.

In addition, mtNOS may also play an important role in cell apoptosis. Recent reports have suggest that eNOS is regulated by heat shock protein Hsp90 (18, 35). It was shown that NO generation was greatly increased when Hsp90 associated with eNOS in endothelial cell cultures (18). Inhibition of Hsp90 function uncouples eNOS activity to increase eNOS-dependent O$_2$ generation that can lead to cell apoptosis (35). Furthermore, mitochondrial Ca$^{2+}$ uptake is an essential step in Ca$^{2+}$-induced apoptosis (33, 38). Our results show that mitochondrial Ca$^{2+}$ release stimulates NO production in the mitochondria of CPAE cells. Close proximity of an NO source to O$_2$ release from mitochondria in parallel with an increase of lipid peroxidation, both typical signs of apoptosis. The cytochrome $c$ release was not mediated by mitochondrial permeability transition pore (MTP) because this release was not prevented by cyclosporin A treatment. Our data with SOD-mimetic Mn-TBAP, which can also scavenge peroxynitrite, suggest that part of the formed NO is quickly transformed into peroxynitrite (Fig. 7), which can further induce apoptosis. Even though mitochondrial Ca$^{2+}$ uptake induces a profound drop in $\Delta\psi_m$, we did not find (data not shown) any evidence of MTP formation during 2 $\mu$M Ca$^{2+}$ application to permeabilized CPAE cells. Although we do not have any direct evidence for induction of apoptosis in our experiments, our data indirectly support the finding by Ghafoorifar et al. (21) that the activation of mtNOS could induce apoptosis in a MTP-independent manner.

In conclusion, our results provide strong evidence that mitochondrial Ca$^{2+}$ uptake stimulates Ca$^{2+}$-dependent NO production in the mitochondria of CPAE cells. Thus locally produced NO by mtNOS provides a mechanism to regulate $\Delta\psi_m$ and mitochondrial Ca$^{2+}$ homeostasis and may play a significant role in cell apoptosis.

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