Biochemical and Biophysical Research Communications **272,** 129–133 (2000)
doi:10.1006/bbrc.2000.2748, available online at http://www.idealibrary.com on IDEAL®

Direct Evidence of Nitric Oxide Presence within Mitochondria

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Received April 26, 2000

Nitric oxide (NO) has been implicated in the modulation of mitochondrial respiration, membrane potential, and subsequently in apoptosis. Although the presence of a mitochondrial NO synthase (mtNOS) has been described, there is no direct evidence *in vivo* of the presence of NO within mitochondria. It was the aim of this study to demonstrate the *in vivo* production of NO within mitochondria. Using the novel fluorometric NO detection system, 4,5-diaminofluorescein diacetate (DAF-2/DA), we observed the presence of NO production in PC12 and COS-1 cells by conventional and confocal fluorescence microscopy. Part of the overall NO signal was colocalized within a subpopulation of mitochondria, labeled with the potential-dependent probe MitoTracker red. These findings demonstrate for the first time that the subcellular distribution of NO production is consistent with the presence of a mitochondrial NOS. Our results provide a new tool to directly study the modulatory role of NO in mitochondrial respiration and membrane potential, *in vivo*.

Key Words: **mitochondria; nitric oxide; diaminofluorescein; MitoTracker.**

Nitric oxide is a highly diffusible, highly reactive molecule that plays an important role in various physiological processes such as neurotransmission, immune response and vasodilatation. Since examination of this short-lived (0.5–5 s) species poses considerable technical problems, studies have focused on its synthesizing enzyme, nitric oxide synthase (NOS), or on selected NO derivatives. At least three subtypes of NOS, which are either free in the cytosol or bound directly or indirectly to membranes, have been characterized (for a review see (1–3)).

The presence of NOS within mitochondria has been previously demonstrated histochemically and immunohistochemically (4–7). More recently, a novel Ca^{2+} dependent NOS subtype, mtNOS, has been described in the inner membrane of isolated rat liver mitochondria (8–11). Moreover, production of NO-derivates by mtNOS has been indirectly characterized by spectroscopic techniques in Percoll-purified liver mitochondria (8, 11).

It has been suggested that the NO produced by mtNOS plays an important role in the modulation of the respiratory chain by binding to the heme group of cytochrome *c* oxidase (COX) or by controlling mitochondrial pH (12–19). In addition, a growing body of evidence suggests that NO, via formation of peroxynitrite ($ONOO^-$), is implicated in apoptosis and related neurological diseases, by altering the mitochondrial membrane potential (16, 20–23). Given the potential role of NO in all these mitochondrial processes, it is crucial to conclusively demonstrate the presence of NO within mitochondria of living cells. However, until recently there has been no reliable method for evaluating the NO *in vivo* production. Kojima and collaborators (24–27) have developed the DAF-2/DA system, which has been proven to be a simple direct method that allows the visualization of NO production at low concentrations (2–5 nM) in living cells, or in fresh, or frozen, brain tissue sections (25, 28–31). In a similar fashion, a novel mitochondrial-selective marker, MitoTracker, has been recently developed. MitoTracker has the ability of diffusing into living cells and labels only actively respiring mitochondria (32).

In sum, using the combination of a novel NO detection method and a selective marker for functional mitochondria, we conclusively demonstrated in the present paper the presence of NO within active respiring mitochondria of living cells by conventional and confocal microscopy. Furthermore, we demonstrated

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0006-291X/00 $35.00

the regulation of mitochondrial NO production in mitochondria by NO donors, scavengers and inhibitors.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's media (DMEM) was from Life Technologies (Rockville, MD). Fetal bovine serum (FBS) was from Hyclone Labs, Inc. (Logan, UT). 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3oxide (PTIO), *N*-[3-(aminomethyl)benzyl]acetamidine, dihydrochloride (1400W), N^{ω}-propyl-L-arginine (NPA) and 4,5-diaminofluorescein diacetate (DAF-2 DA) were all from Calbiochem (San Diego, CA). Sodium nitroprusside (SNP) was from Fisher Scientific (S-350, Fair Lawn, NJ). Mito-Tracker Red CM-H_2XRos was from Molecular Probes (Eugene, OR).

Cell culture. PC12 and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in phenol red-free DMEM supplemented with 10% FBS at 37°C with 5% CO_2. PC12 differentiation was induced in the presence of NGF (40 ng/ml).

NO detection system. NO production by cultured cells was bioassayed using the DAF-2/DA detection system as previously described (31). The cells were incubated in 10 μM DAF-2/DA for 30 min at 37°C in a humidified incubator under an atmosphere equilibrated with 5% CO_2. As a negative control, cells were incubated in medium lacking DAF-2/DA. The cells were then washed in PBS and cover-slipped using Aquamount as the mounting medium.

Control cultures were first washed and then incubated in DMEM for one hour in medium containing the NOS inhibitors 1400W and L-NPA, the NO scavenger, PTIO, and the NO donor, SNP (0.1–1 mM). Further controls consisted in fixation of the cells for 1 h in 4% paraformaldehyde. The cell cultures were then incubated in DAF-2/DA as previously described.

Mitochondrial colocalization. For mitochondrial colocalization, cells were incubated with DAF-2/DA and the marker of active respiring mitochondria, MitoTracker Red CM-H_2XRos (10–100 nM) (32), in the same solution for 20 min at 37°C. Then cells were washed in PBS and coverslipped.

Microscopy and image analysis. Processed cells were analyzed immediately under a Leica DHR epifluorescence microscope equipped with an excitation (450–490 nm) and emission (515–560 nm BP) green filter for fluorescein and an excitation (515–560 nm) and emission (590 nm LP) red filter. Images were digitized with 40× and 63× fluorescence objectives and a Sony DXC-970MD video camera and analyzed with the MCID image analysis system (Ontario, Canada). For confocal microscopy, both fluorescences were acquired in dual channel mode on a Nikon Diaphot 200 microscope equipped with a Noran confocal laser scanning imaging system and Silicon Graphics Indy workstation. The results are expressed as relative optical densities (ROD), which represent the mean optical density minus background, multiplied by the total target area. Background values were calculated as the mean of all pixels with lowest optical density outside a cell multiplied by 3.5 times its standard deviation. All values are expressed as a ratio of the control. Values are the result of the analysis of at least 8 images per experimental condition, with at least 20 cells per experimental condition and image. For each experiment, images were digitized under constant exposure time, gain and offset. Images were all taken within less than a minute, which was found to be within the linear range of the reaction speed. Images were deconvoluted using a No Neighbor deblurring algorithm from the MCID software to reduce the background and improve sharpening.

Statistical analysis. Data are expressed as mean ± SEM. The results were subjected to a one-way ANOVA followed by post hoc Dunnett's (vs corresponding control) multiple comparison test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Basal NO production. PC12 and COS-1 cells produce NO under basal conditions, as determined by the observation of green-fluorescent signal following incubation with the DAF-2/DA system in cells without fixation (Fig. 1). Autofluorescence was almost undetectable in control cells incubated without DAF-2/DA. Both types of cells exhibited an overall fluorescence signal with heterogeneous distribution within and between cells. Fiber-like structures were positive for DAF-2, especially when forming connections between cells. A very distinctive punctate pattern of intense fluorescence signal was observed within the cytoplasm of COS-1 cells. The subcellular pattern of fluorescence was patchy, with negative circular areas surrounded by positive areas with a pattern resembling the cytoskeleton. In contrast, and likely due to NO diffusion, cell nuclei were positively stained. Interestingly, paraformaldehyde fixation eliminated the NO-fluorescence signal.

Regulation of the NO production. To test the specificity of DAF-2/DA, the cell cultures were incubated in a solution containing DAF-2/DA together with NOS inhibitors, a NO scavenger and a NO donor. As seen in Fig. 2, incubation of cells with increasing concentrations (0.1 and 1 mM) of the NOS inhibitors 1400W and NPA, or the NO scavenger PTIO, resulted in a concentration dependent decrease in overall fluorescent intensity ($P < 0.01$ for 1 mM 1400, 1 mM NPA, 0.1 and 1 mM PTIO treatments). In contrast, incubation of cells with the NO donor SNP (0.1 and 1 mM), induced a significant increase in fluorescent intensity compared to the control ($P < 0.001$) (Fig. 2).

Localization of NO-fluorescence signal within mitochondria. Colocalization of the punctate NO-fluorescence signal within mitochondria was demonstrated by incubating the mitochondrial metabolic marker Mito Tracker (red) together with the DAF-2/DA (green), as described under Materials and Methods. We found by conventional and confocal microscopy (Fig. 1) that only a subpopulation of active mitochondria showed DAF-2 fluorescence. We also observed cases of mitochondrial-like structures positive for DAF-2 but not for mitochondrial marker fluorescence and vice versa.

DISCUSSION

Despite previous reports demonstrating the existence of mitochondrial NOS (5, 7, 8, 11), the direct determination of NO production has been hampered by the ultra short half-life of NO, and its highly reactive nature. In the present paper we demonstrate for the first time *in vivo* that a subpopulation of viable, coupled, mitochondria colocalized with NO production in living cells.

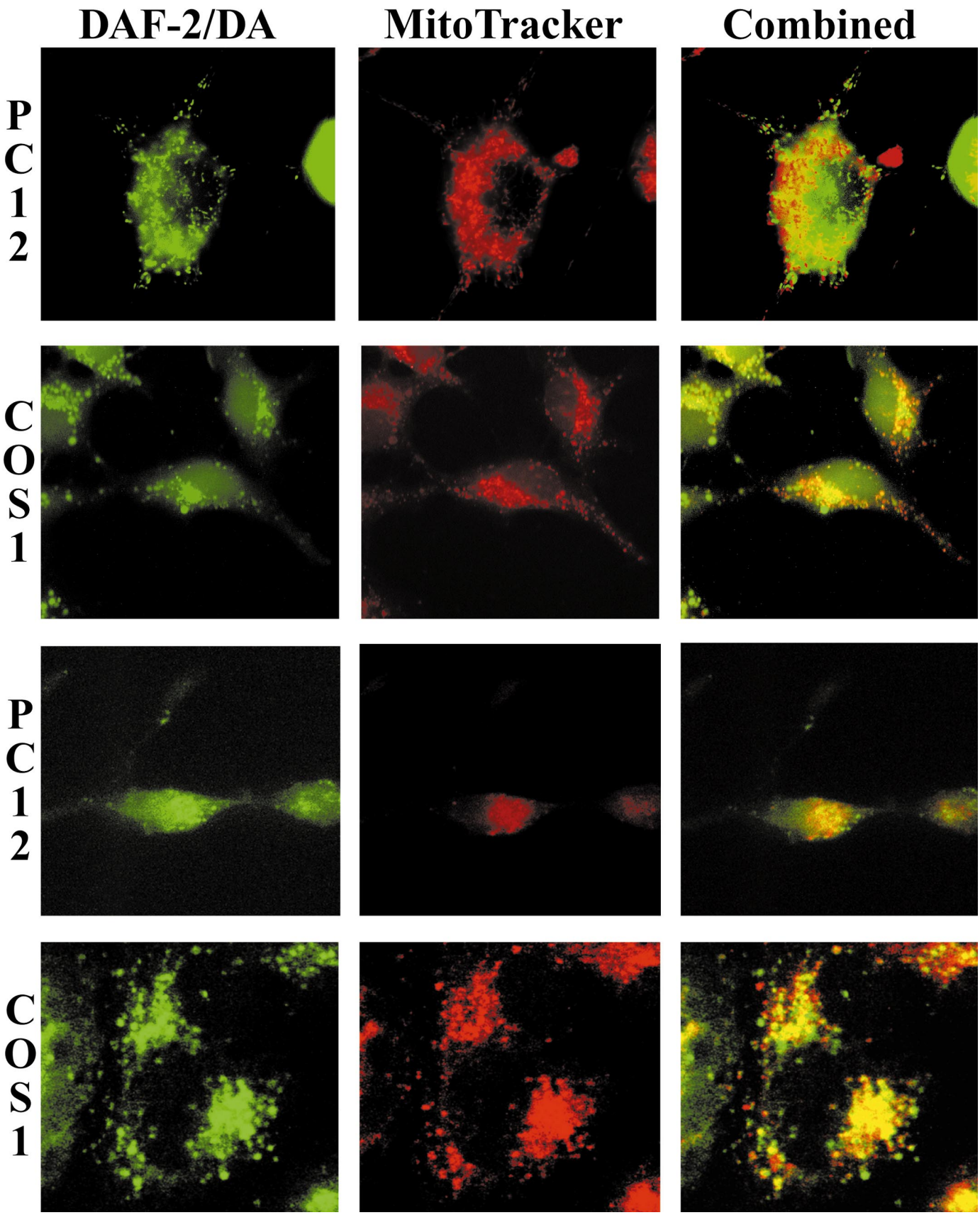

FIG. 1. Fluorescence micrographs of PC12 and COS1 cells loaded with DAF-2/DA (left column) and Mito tracker red (middle column). Yellow areas in superimposed images (right column) are indicative of colocalization. For the top two rows conventional microscopy (63×) was used. The lower two rows represent high-power confocal photomicrographs (63×) showing the localization of NO within mitochondria in the same cell lines. Some but not all of the Mito Tracker-stained mitochondria colocalize with the DAF 2/DA fluorescence signal and vice versa.

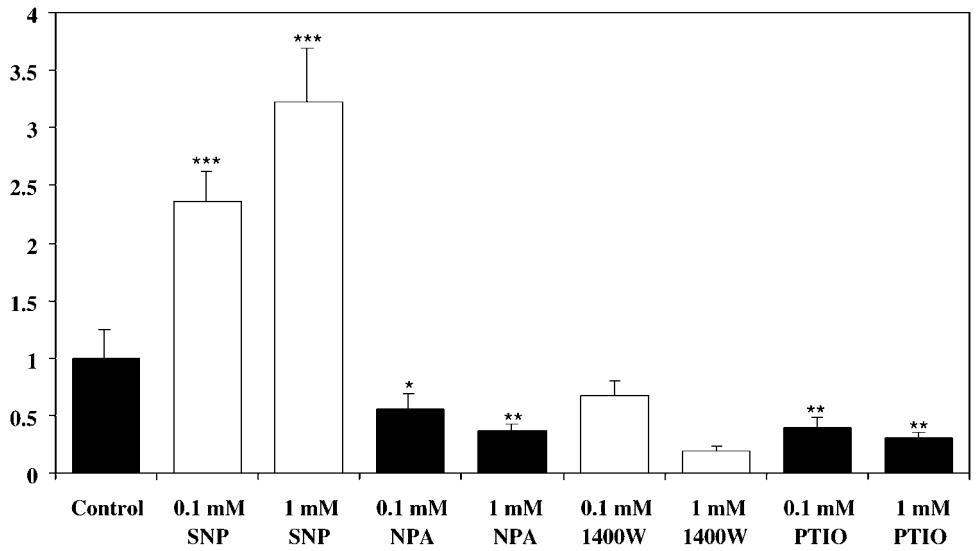

FIG. 2. Semiquantitative analysis of NO-fluorescence signal in cell cultures pretreated for 1 h with varying concentrations (0.1–1 mM) of the NOS inhibitors 1400W and L-NPA, the NO scavenger, PTIO, and the NO donor, SNP. Cells were then incubated in DAF-2/DA. Fluorescence intensities are represented in relative optical densities (ROD, see Methods). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

One of the main benefits of the DAF-2 system that we used is the visualization of the cellular and anatomical production of NO *in vivo*. In addition, the low detection limits of DAF-2 (2–5 nM) allows the detection of low levels of NO produced by the constitutive forms of NOS under basal conditions. Furthermore, the DAF-2 system provides sufficient resolution to locate NO sources to subcellular compartments, such as mitochondria. Finally, since DAF-2 is specific for NO, versus other reactive species such as O_2^-, H_2O_2, $ONOO^-$, NO_2^- and NO_3^- (27), DAF-2 fluorescence imaging appears to be a most useful method of detecting NO production in living cells.

The assumption that the DAF-2 method reflects the enzymatic production of NO was validated by the use of NOS modulators. Accordingly, in the presence of NOS inhibitors, DAF-2 fluorescence signal decreases in a dose-dependent manner. Moreover, the addition of a NO donor caused an increase in the fluorescence signal, whereas a NO scavenger blocked the fluorescence signal in a dose-dependent manner. Finally, we demonstrated that paraformaldehyde fixation negatively affects the detection of NO by the DAF-2 method, presumably by inactivation of the NOS activity. This last observation seems to be in agreement with a previous paper on the differential effect of fixation on NOS detection by immunohistochemistry and histochemistry (33).

In the present work we demonstrated that part of the cellular NO-fluorescence signal in COS-1 and PC12 cells is localized within active mitochondria under nonstimulated conditions, supporting the suggested role of NO in mitochondria respiration (8, 34, 35). However, we observed cases in which similar NO-fluorescence signal did not colocalize with the mitochondrial marker. Although one possibility is that the presence of NO is not limited to metabolically active mitochondria, another possibility is the observed loss of red-fluorescence signal of the employed mitochondrial marker, MitoTracker red, over time. Interestingly, the description of NOS in other subcellular compartments such as the Golgi apparatus, the endoplasmatic reticulum, or indirect binding to cellular membranes through PDZ-containing proteins, suggests that part of the DAF-2/DA signal might be localized to these structures (29, 36). Therefore, in view of the of the ability of NO to diffuse (37), we cannot discard the possibility that part of the signal observed in the mitochondria might have been the result of its diffusion from these other structures. Further studies are required to fully characterize the subcellular distribution of the NO-fluorescence signal in living cells. Determination of these issues might facilitate the understanding of the role of NO in neurophysiological disorders (12).

In conclusion, our data demonstrating the NO production in actively respiring mitochondria in cultured cells strongly suggest the involvement of NO in mitochondrial processes such respiration and apoptosis.

ACKNOWLEDGMENTS

Dr. L. C. Rønn was supported by grants from the Novo Norsdik, Carlsberg, and Weimann Foundations. We thanks Drs. Y. Hellsten and U. Frandsen for comments on the paper. The authors acknowledge technical support from Sharon Burke and Linda H. Gates. This project was supported by NIMH Program Project (MH42251) and The Pritzker Depression Network.

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