Nitric oxide regulation of mitochondrial oxygen consumption I: cellular physiology

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Mitochondria are considered the main source of intracellular reactive oxygen species (ROS) under normal, physiological conditions via complex I and/or complex III (33). With the presence of NO, this situation had to be reviewed to include mitochondrial NO synthase (mtNOS). The latter molecule is produced by a constitutive nitric oxide synthase (NOS) in mitochondria (mtNOS; 4, 5, 29, 31, 33, 34, 44, 83) has provided the grounds to review several aspects of mitochondrial physiology.

The presence of a constitutive nitric oxide synthase (NOS) in mitochondria (mtNOS; 4, 5, 29, 31, 33, 34, 44, 83) has the change in therapeutic strategies of diseases related to mitochondrial oxidative/nitrative stress, such as amyotrophic lateral sclerosis and Alzheimer’s. These strategies may have to be modified when several new factors have to be considered: oxygen consumption and availability, ROS, mitochondrial metabolic state, and antioxidant defenses.

NO production in mitochondria is tightly regulated by temporal and spatial regulatory mechanisms. Calcium signal is central to temporal regulation of NO production in mitochondria. Upon stimulation of calcium release from endoplasmic reticulum (ER), calcium enters mitochondria via an electrogenic unipporter. Calcium activates mtNOS releasing NO. In turn, NO inhibits mitochondrial cellular respiration via binding to cytochrome c oxidase. This effect has been observed in a variety of biological settings (13, 19, 70; Giulivi and Cooper, unpublished observations). Spatial regulatory mechanisms of NO production allow a localized production of NO close to its targets. Controlled NO production sites have the advantage of minimizing secondary reactions (e.g., peroxynitrite production); however, at this point it is not clear how a highly diffusible (and hydrophobic) molecule as NO will be confined to a certain subcellular compartment. In the next section, we will discuss various aspects of the tight control of the temporal and spatial production of NO in mitochondria.

TEMPORAL REGULATION OF NO PRODUCTION

In vivo, two opposite processes regulate the steady-state concentration of NO: production and consumption of NO.

The main consumption pathways of NO include its reaction with oxyhemoglobin or oxyhemoglobin (yielding nitrate) (22, 52), with superoxide anion to produce the highly reactive species peroxynitrite (8, 62), and with oxygen (53). Although the third pathway, namely the reaction between NO and oxygen, is slow (53), it may represent a critical clearance mechanism in hydrophobic milieu (28). As a result, the concentration of NO can be adequately balanced by its production and consumption allowing NO to operate as a signaling molecule.

In mammals, NO is generated by nitric oxide synthase (NOS). In other organisms, alternative pathways had been found for the production of NO, e.g., nitrate reductase (59, 97–99). NO production can be regulated by expression of NOS and/or modulation of NOS activity by calcium. For instance, inducible NOS (iNOS) is overexpressed in response to infectious pathogens through transcription regulation (26, 27, 58, 90). Calcium signaling plays a crucial role in modulating constitutive NOS activity such as endothelial NOS (eNOS),

Giulivi, Cecilia, Kazunobu Kato, and Christopher Eric Cooper. Nitric oxide regulation of mitochondrial oxygen consumption I: cellular physiology. Am J Physiol Cell Physiol 291: C1225–C1231, 2006. First published August 2, 2006; doi:10.1152/ajpcell.00307.2006.—Mitochondrial biochemistry is complex, expanding from oxygen consumption, oxidative phosphorylation, lipid catabolism, heme biosynthesis, to apoptosis, calcium homeostasis, and production of reactive oxygen species, including nitric oxide (NO). The latter molecule is produced by a constitutive nitric oxide synthase (NOS) in mitochondria (mtNOS; 4, 5, 29, 31, 33, 44, 83) has provided the grounds to review several aspects of mitochondrial physiology.

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neuronal NOS (nNOS), and mtNOS (1, 31, 83). In addition, these enzymes are also regulated at the transcriptional or translational levels (10, 35, 42, 86).

The calcium-dependent mtNOS activity becomes relevant considering the critical role of mitochondria at maintaining calcium homeostasis. Calcium can regulate mitochondrial functions by activating key metabolic enzymes (pyruvate dehydrogenase, NAD+ -dependent isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase or α-ketoglutarate dehydrogenase) and mtNOS. The activation of calcium-dependent dehydrogenases leads to increased oxygen consumption, whereas that of mtNOS results in a decreased oxygen uptake (Fig. 1). The activation of the dehydrogenases or mtNOS is achieved at different calcium concentrations (31) being the former activated at concentrations as low as 0.2 μM, whereas the latter occurs at 1 μM (83, 84). Thus, ultimately the same signal, calcium, regulates opposing effects on mitochondrial oxygen consumption by means of different thresholds.

It is known that there is a heterogeneous distribution of calcium in the cell (56), and how this distribution may affect NO production is not well understood. Upon cell activation, mitochondria are exposed to different calcium concentrations; those close to endoplasmic reticulum (ER) calcium stores face microdomains of higher calcium concentrations through the opening of inositol trisphosphate-gated channel or ryanodine receptor (57, 65, 66). In addition, it has been indicated that ryanodine receptor isoform 1 is localized to the inner membrane of mitochondria, which may be one of the mechanisms for calcium influx into mitochondria (7). This, in addition to a calcium efflux antiporter different from the uniporter, evidences the complexity of calcium homeostasis in mitochondria, and difficulty in delineating the temporal regulatory mechanism of how calcium influences NO production and its inhibitory effect on cellular respiration.

One clue for better understanding about the correlation between calcium and NO-regulated mitochondrial respiration is the information about cytosolic and intramitochondrial calcium concentrations that affect mitochondrial motility and activity of the enzymes. Mitochondria have higher motility at resting level of cytosolic calcium; when calcium rises, mitochondria motility becomes arrested (90). If cytosolic calcium concentrations increase further, 1 μM [Ca2+]cyt activates NOS (86), thereby reducing oxygen consumption. Thus mitochondria are still able to produce ATP at a significant rate, preventing a local point of anoxia. These effects were calculated at 220 μM oxygen, concentrations considered hyperoxic for mitochondria; if hypoxic (or normoxic for mitochondria) conditions are considered (10–20 μM oxygen), these effects could be exacerbated (78). If cytosolic calcium concentrations increase further (e.g., 1 μM [Ca2+]cyt, equivalent to 2.3 μM [Ca2+]mt) as mitochondria are located closer to microdomains with higher calcium concentration, 71% of the mitochondria motility may become arrested. Simultaneously, the activity of dehydrogenases reaches almost maximal activation (92%), and mtNOS is 70% activated, resulting in a 35% decrease of the oxygen consumption. Therefore, activated mtNOS antagonizes the effects of dehydrogenases on mitochondrial respiration, implying that mtNOS functions as a negative regulator for mitochondrial respiration especially at higher calcium concentrations (84). Owing to this effect, mitochondria can still produce enough ATP while avoiding the occurrence of anoxic foci. This system allows oxygen to diffuse to mitochondria located away from calcium microdomains, and these mitochondria (exposed to lower calcium) have the potential to produce ATP via the activation of calcium-dependent dehydrogenases without activating mtNOS (Fig. 1). In this model, not all mitochondria operate in synchrony as an energy source, but rather they respond to the microenvironment changes. NO-induced inhibitory effect on mitochondrial respiration contributes to these mechanisms and acts as one of the important regulatory factors to control intracellular oxygen consumption.

Despite the clear evidences for the inhibitory role of NO on mitochondrial respiration and its susceptibility to calcium signaling, there is still an unresolved question about the correlation among calcium concentration, mitochondrial motility, and the temporal regulation for NO production. Provided higher calcium concentrations stop mitochondria motility (100), the density of mitochondria should become higher at ER sites.
However, no evidence for these settings has been provided (11, 100).

Factors other than calcium may regulate the recruitment (or derecruitment) of mitochondria to specific compartments in the cell; for example protein-protein interactions or docking/anchoring points. Obviously, calcium-regulated NO production in mitochondria will be affected by these mechanisms.

One of the key issues to resolve these questions is to consider the intracellular transport system for mitochondria. Docking of mitochondria to cytoskeletal structure, such as intermediate filaments or microtubes, is important for intracellular transport of mitochondria, which determines intracellular mitochondrial distribution (6, 54, 85). However, it is uncertain how docking of mitochondria may alter physiological processes, among them NO production and consumption.

Another significant issue that might affect mitochondrial NO production and its regulation is mitochondrial morphology. The typical representation of mitochondrion is a solitary, kidney-bean shaped organelle. A predominant percentage of mitochondria are identified as single, isolated particles (85). However, a growing amount of evidence has revealed that mitochondria can also have filamentous forms of long snake-like tube with branched reticulum, composing extended network throughout the cytosol (6, 91, 95). Mitochondrial fusion may contribute to the formation of this interconnected mitochondrial network in the cell (17, 37, 91, 95, 96). Importantly, the formation of an extensive mitochondrial network has functional importance (18, 77). For example, the network might be essential for intracellular delivery of energy to specific cell compartments of the cell (77). Using mammalian cells with mitochondria unable to fuse, Chen et al. (18) showed that morphological defects could lead to dysfunctional mitochondrial respiration. Mitochondrial fragmentation has been associated with increased ROS production during hyperglycemia (101). These data suggest that morphological changes (fusion and formation of the network) could be relevant to control mitochondrial respiration. Nonetheless, there have been few evidences on how calcium-regulated NO production is influenced by the dynamics of mitochondrial morphology. To this end, considering the calcium dependence of mtNOS and the three dehydrogenases, it will be of significance to elucidate how intracellular transportation and morphology of mitochondria affects the cross-talk between calcium and NO signaling in mitochondria.

In addition to calcium-mediated regulatory mechanisms of NO production in mitochondria, expression of mtNOS can be regulated at the transcriptional and translational levels (42). Considering the identification of mtNOS as an NOS isoform (24), it could be safely assumed that regulatory mechanisms for nNOS expression are operative for mtNOS. Several alternative spliced variants have been found for nNOS, and the expression of the variants is controlled in developmental stage-specific- or tissue-specific manners (51, 67, 76). These variants have different biological roles: for example distinct morphine tolerance (45) or unique function for myotube fusion in differentiated skeletal muscle (76). Some of nNOS variants lacking PDZ domain such as nNOS-β and -γ may have distinctive functions owed to defective protein-protein interactions (9). Moreover, a complex use of alternative promoters of nNOS (87) can affect transcription patterns and translational efficiencies (49). In this regard, a recent study indicated that hypoxia rapidly upregulated nNOS expression (89). These findings imply that living organisms have tightly regulated transcriptional/translational mechanisms for controlling nNOS expression in response to milieu changes. As an extension, it is possible that expression of mtNOS is tightly regulated at the transcription/translation steps.

**SPATIAL REGULATION OF NO**

NO is a small, hydrophobic species with a diffusion coefficient similar to that of oxygen (92). Applying Fick's law and assuming a half-life for NO of ~1 s (43), its diffusion distance would be ~140 μm. Bearing in mind that eukaryotic cells are 10 to 100 μm, then NO would have the potential to diffuse to neighboring cells. In support of this concept, NO produced from nNOS plays an important role as a diffusible neurotransmitter at synapse (50, 68, 69, 93). Analogously, it could be hypothesized that NO produced by NOs other than mtNOS could diffuse to other cells, affecting the cellular respiration at target cells. A few in vitro experiments endorse this hypothesis. NO produced from endothelial cells or macrophages can diffuse to co-cultured smooth muscle cells (48), and non-activated macrophages or fibroblasts (14), decreasing their oxygen consumption.

However, arguments based on biological observations may favor a localized NO effect through a variety of mechanisms. Some of these are the consumption of NO by mitochondria and the localization or targeting of NOs to specific subcellular compartments.

NO consumption by mitochondria can influence how NO exerts its biological effects. The consumption of NO is increased by 5 times in the presence of mitochondria (2 mg protein/ml; 75). With the use of an unique microsensor tip that can directly detect NO, withdrawing the tip (1-μm increment) from a single mitochondrion proved that NO generated by a mitochondrion could diffused only <10 μm (41). These experiments indicate that most of NO decays within the organelle and that extramitochondrial effects by NO endogenously produced from the organelles may be considered minimum. This entails NO production system (mtNOS) in the organelle, and the subcellular location of NOs would be critical for NO to have any biological effects on mitochondrial respiration.

Localization or targeting of NOs to specific subcellular compartments may assure a confined effect. For mtNOS, this could be achieved by means of posttranslational modifications and protein-protein interactions.

Co- or posttranslational modifications (myristoylation and palmitoylation) are essential for the proper localization of, for example, eNOS at caveolae (73, 79). Caveolae are specialized invaginations of the plasma membrane, found to be basic structures for NO-triggered signaling by eNOS (2, 20, 30). mtNOS has been found to be E-myristoylated (24), and based on the biological functions of acylation, E-myristoylation probably contributes to trafficking of mtNOS to mitochondria or to anchoring the enzyme to mitochondrial membranes.

Protein-protein interactions are another factor to determine NO localization at the subcellular level. nNOS-α has a PDZ domain important for protein scaffolding for signal transduction (9, 25, 47, 74). In this regard, nNOS can associate with postsynaptic density (PSD)-95, PSD-93 or skeletal muscle...
α₁-syntrophin via this domain (9, 46, 74), allowing a proper target to neuronal postsynaptic densities (9).

In terms of mtNOS, protein-protein interaction via PDZ motif has been recently observed (61; V. Haynes, N. N. Sinitsyna, and C. Giulivi, unpublished observations). A physical interaction via PDZ domain has been demonstrated between subunit Va of cytochrome c oxidase and mtNOS, using immunoelectron microscopy and immunoprecipitation experiments. Intriguingly, the interaction between cytochrome c oxidase and mtNOS depends on calcium (V. Haynes, N. N. Sinitsyna, and C. Giulivi, unpublished observations). This implies that the spatial control might be affected also by temporal control, specifically calcium signaling.

These temporospatial regulations of NO production in mitochondria may lead to important biological events. One of the downstream outcomes is intracellular oxygen redistribution, as previously discussed. Cells have intracellular gradients of oxygen (81, 82) from the plasma membrane to mitochondria, where oxygen is consumed at a high rate by coupling electron transport to oxidative phosphorylation. NO endogenously produced inhibits cytochrome c oxidase activity, decreasing oxygen consumption, thus allowing other enzymes with a higher \( K_m \) for oxygen to be active (32, 38).

An example of the effect of oxygen redistribution, resulting from the inhibition of the cellular respiration with NO, on the destabilization of the hypoxia-inducible transcription factor (HIF)-1α has been reported (36) (Fig. 2). The potential redistribution of oxygen allowed Pro hydroxylase to hydroxylate HIF-1α (destabilization), preventing the upregulation of genes related to glucose transport, glycolysis and erythropoiesis (21, 72). Intriguingly, it is not clear why such effect could be observed in cultured cells when oxygen diffusion is expected to be homogenous and not rate limiting. In addition, several studies have shown that HIF-1α expression/stabilization is not necessarily correlated with oxygen concentration in vivo (21). The issue of oxygen gradient redistribution mediated by NO becomes more puzzling when cells in culture are utilized. For most culture cells, the energy budget is unavailable, meaning whether main energy sources are from oxidative phosphorylation or from anaerobic glycolysis. Since Warburg’s earliest research (88), it has been known that cancer cells preferentially utilize anaerobic glycolysis over oxidative phosphorylation.

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**Fig. 2.** Effect of NO on the stabilization of hypoxia-inducible factor-1α (HIF-1α). When oxygen is available, it can be utilized as a substrate by the iron-dependent Pro hydroxylases and Asn hydroxylases. These enzymes hydroxylate the HIF-1α. Pro-hydroxylase introduces a hydroxyl group within HIF-1α oxygen-dependent degradation domain (ODDD; white domain) whereas Asn-hydroxylase hydroxylates the HIF-1α COOH-terminus end (gray domain). These modifications cause the recruitment of the von Hippel-Lindau tumor-suppressor protein (pVHL), which targets HIF-1α for proteosomal degradation. The proteolysis of HIF-1α prevents the transcriptional activity of this factor. Oxygen may become available to the hydroxylases when NO is inhibiting cytochrome c oxidase, promoting the diffusion of oxygen to other subcellular sites. Conversely, when oxygen is not available, i.e., hypoxia or no NO available (16), several pathways become activated (e.g., MAPK) that promotes the phosphorylation of HIF-1α (55, 64). Phosphorylation increases the transcriptional activity of HIF-1α via the dimerization with HIF-1β. The heterodimer activates the transcription of several factors like vascular endothelial growth factor (VEGF), erythropoietin (39), glucose transporters (GLUT1 and GLUT3), and several glycolytic genes. HO, heme oxygenase; PI-3K, phosphatidylinositol 3-kinase; UQ, ubiquinone.
For example, myxothiazol, a specific inhibitor of the respiratory chain, can lower ATP production by <25% in HeLa cells, and substantial suppression (>50%) on cellular respiration can be done with the combination of myxothiazol with a specific inhibitor for glycolysis like 2-deoxyglucose (40). Therefore, to ascertain effects of NO on cellular gradients, it is imperative to evaluate the contribution of anaerobic glycolysis and oxidative phosphorylation. In vitro experimental findings from cell culture system do not necessarily represent the actual biological events in vivo. This raises a concern about validity of cell culture experiments; it is a real challenge to recruit validated events in vivo. This raises a concern about validity of cell culture experiments; it is a real challenge to recruit validated experimental models to explore the in vivo events in mitochondria. Needless to say, this scenario applies for studies on isolated organelles performed under hypoxic conditions, and thus more studies are required to comprehend the mechanism for spatiotemporal regulation of NO production.

The implications of the modulation of mitochondrial respiration by NO via cytochrome c oxidase activity are a subject of controversial discussions and views, which raise challenging questions concerning the regulatory mechanisms and physiological relevance of in vivo modulation of mitochondrial oxygen consumption by NO.

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REFERENCES


