

## Mitochondrial nitric oxide synthase

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## 1. ABSTRACT

Nitric oxide (NO) regulates several cellular functions via reversible regulation of mitochondrial respiration. Nitric oxide also reacts with mitochondrial superoxide anion to produce the potent oxidative species peroxynitrite that irreversibly hinders mitochondrial activities. Recent findings demonstrating that mitochondria produce NO via mitochondrial NO synthase (mtNOS) has intrigued several laboratories revealing crucial roles for mtNOS-derived NO and peroxynitrite in regulating the functions of mitochondria, cells and organs. The present article reviews the current understanding of the interactions between mitochondria, and NO and peroxynitrite.

## 2. INTRODUCTION

Mitochondria are organelles present in the cytoplasm of eukaryotic cells that produce most of cellular ATP in expense of consuming  $O_2$ . Therefore, inhibition of mitochondrial  $O_2$  consumption affects numerous cellular activities. Nitric oxide (NO) is a molecule with physicochemical properties similar to  $O_2$  that behaves as physiological reversible inhibitor of mitochondrial  $O_2$  consumption. By competing with  $O_2$  for the  $O_2$ -binding site of cytochrome C oxidase (COX), NO reversibly inhibits

mitochondrial respiration and regulates cellular functions. However, NO congeners such as peroxynitrite (ONOO<sup>-</sup>), formed by the reaction of NO with superoxide anion ( $O_2^-$ ), irreversibly hinder mitochondrial functions, cause cellular malfunctioning, and cell death. Several factors are involved in the switch from the physiologically relevant regulation of cellular respiration by NO, to the pathologic inhibition of respiration by peroxynitrite.

Although mitochondrial respiratory chain complexes are tightly arranged in a redox potential hierarchy, 2 to 5% of the electrons flowing down the chain leak out and generate  $O_2^-$  (1). Thus, the mitochondrial respiratory chain is one of the prime cellular producers of  $O_2^-$ . Superoxide reacts with NO at a diffusion-controlled rate and produces peroxynitrite. While mitochondria maintain multiple lines of defense barriers allowing effective biotransformation of  $O_2^-$ , they possess limited protective mechanisms against peroxynitrite. Thus, peroxynitrite can potently interact with multiple mitochondrial components including proteins, lipids, and nucleic acids, and irreversibly damage those targets. Peroxynitrite is extremely reactive and its short biological life time does not allow peroxynitrite formed in the

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cytoplasm to diffuse to reach the mitochondria (2); however, peroxynitrite-modified proteins, lipids and nucleic acids have been shown to exist in mitochondria.

Mitochondria produce NO via mitochondrial NO synthase (mtNOS). Association of mtNOS with the mitochondrial inner membrane provides a unique condition allowing mtNOS-derived NO to reversibly inhibit the activity of COX, also located at the inner mitochondrial membrane, or react with  $O_2^-$ , produced by the mitochondrial respiratory complexes located at the inner membrane, to generate peroxynitrite. The current knowledge on how reversible regulation of respiration by mtNOS-derived NO vs. irreversible modification of mitochondrial targets by mtNOS-derived peroxynitrite is harmonized is rapidly developing. It seems that reduced glutathione (GSH), that is one of the few anti-peroxynitrite defense mechanisms available in mitochondria, play a decisive role.

### 3. NITRIC OXIDE AND ITS METABOLIC FATES IN BIOLOGY

Nitric oxide is a colorless gas at temperature above  $-151.8\text{ }^\circ\text{C}$  that readily dissolves in aqueous solutions. In deoxygenated aqueous solutions, concentration of dissolved NO reaches up to 2 mM (3). Nitric oxide predominantly reacts with molecules containing metalloids atoms, such as oxygen or sulfur, transition metals, such as iron, or with free radicals, particularly with  $O_2^-$ . One of the most abundant oxygen-atom containing molecules is  $O_2$ . The reaction of NO with  $O_2$  is second order in NO and first order in  $O_2$  concentration. Therefore, at physiological concentrations of NO and  $O_2$ , simple autoxidation of NO is not a major metabolic fate for NO (3). Most biological environments are enriched in sulfur- or iron-containing molecules, including thiols and hemoproteins. Nitric oxide reacts with a reduced thiol, *i.e.* -SH, to produce S-nitrosothiol, *i.e.* S-N=O, and with ferrous-heme, *i.e.* R-Fe-R, to produce nitrosyl heme-iron, *i.e.* R-Fe-N=O. Under most biological environments both reactions occur at the rate of  $10^5$  to  $10^6\text{ M}\cdot\text{s}^{-1}$  (4) that is fast enough for most NO produced within the cells to react with abundant cellular thiols and hemoproteins. However, both reactions are reversible, *i.e.* reducing thiol or oxidizing the heme-iron readily liberates the NO. Thus, S-nitrosation and heme-nitrosylation are not terminal metabolic fates for NO in biology. Nitric oxide reacts with  $O_2^-$  with the rate constant of  $1.9 \times 10^{10}\text{ M}^{-1}\text{ s}^{-1}$  (5) that is 4 to 5 orders of magnitude faster than S-nitrosation or heme-nitrosylation. The reaction produces peroxynitrite, an extremely reactive NO-derived species that irreversibly reacts with several biological targets including amino acids, peptides, proteins, lipids, and nucleic acids. In contrast to S-nitrosated and iron-nitrosylated products that release NO back to the biological environment, the products of the reactions of peroxynitrite with its targets do not release - peroxynitrite. Thus, the reaction of NO with  $O_2^-$  to produce peroxynitrite is the preferred metabolic fate for NO in biology.

### 4. NITRIC OXIDE SYNTHASES

The discovery that endothelium-derived relaxation factor (6) is NO (7,8) opened new windows in

biomedical researches and changed our view of NO from being considered a noxious gas to that of a beneficial molecule of principal importance in biology (9).

Synthesis of NO in biology is catalyzed by the members of the NO synthase (NOS) family. The synthesis of NO is a two-step five-electron oxidation of the terminal guanidino nitrogen of L-arginine, produces N-hydroxy-L-arginine as the intermediate, stoichiometrically consumes  $O_2$ , requires 5 electrons donated from NADPH, and produces L-citrulline as the final co-product. To date, three distinct NOS isozymes have been well characterized in mammalian tissues. Although there is no tissue-specific pattern of expression for these isozymes, they are generally known as the endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS). All characterized mammalian NOS isozymes are heme-containing proteins that are dimeric in native conditions with monomer molecular mass of about 126-160 kDa. nNOS and eNOS are constitutively expressed, whereas iNOS is expressed once cells challenged with immunological or inflammatory stimuli. The constitutive NOS isozymes are activated upon elevation of cytosolic  $Ca^{2+}$  and exert a typical reversible interaction with calmodulin. However, iNOS forms a tight complex with  $Ca^{2+}$ -calmodulin at very low  $Ca^{2+}$  concentrations and remains active as long as substrates are available.

Recent reports have demonstrated a novel NOS isozyme in plant *Arabidopsis thaliana* (AtNOS) (10). The AtNOS utilizes L-arginine, produces NO and L-citrulline in a  $Ca^{2+}$ -sensitive manner, and regulates plant growth and response to light. The mammalian ortholog of AtNOS, the mAtNOS, has been also characterized (11), although the functions of mAtNOS need to be further studied.

### 5. NITRIC OXIDE AND MITOCHONDRIA

Before it was known that activated macrophages produce NO, it was found that cytotoxic macrophages inhibit the respiration of the neighboring cells by inhibiting the activity of mitochondrial respiratory complexes I and IV(12,13). About a decade later and after the discovery of NO in biology (7), several laboratories reported that physiologically-relevant concentrations of NO inhibit mitochondrial respiration by reversible inhibition of COX (complex IV) in mitochondria or submitochondrial particles of brain (14,15), heart (16,17), skeletal muscle (18) and liver (15).

Cytochrome C oxidase is a heme-copper complex protein found in the bacterial membrane and the inner mitochondrial membrane of eukaryote cells. Mammalian COX consists of 13 subunits and pumps protons from the matrix into the mitochondrial intermembrane space. The oxygen-binding site of COX is consists of two copper,  $Cu_A$  and  $Cu_B$ , and two hemes, cytochrome *a* and cytochrome *a<sub>3</sub>*, centers, located at the mitochondrial inner membrane facing the mitochondrial matrix. The oxidized form of COX becomes reduced by receiving electrons from reduced cytochrome *c*, as its substrate. Reduction of COX increases the affinity of its  $O_2$ -binding site to bind to  $O_2$  and to reduce it  $H_2O$ .

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Nitric oxide has been used since long time ago as a probe to study O<sub>2</sub> binding to COX (19) because of the similar diatomic structure and physico-chemical properties of NO and O<sub>2</sub>. Nitric oxide binds to the O<sub>2</sub>-binding site of the reduced COX and inhibits mitochondrial respiration. At nanomolar concentrations, NO inhibits COX by binding to the cytochrome a<sub>3</sub>-Cu<sub>B</sub> center of reduced COX, and dissociation of NO from COX allows COX reactivation (20). At physiologically relevant concentrations of NO and O<sub>2</sub>, the inhibition by NO of COX is reversible and competitive, in a manner representing a pharmacological competitive antagonism between NO and O<sub>2</sub> (1). For example, at 145 microM O<sub>2</sub> that is about the arterial O<sub>2</sub> concentration, inhibition by NO of mitochondrial respiration occurs with an IC<sub>50</sub> of 270 nM, whereas at 30 microM O<sub>2</sub> that is about the tissue level of O<sub>2</sub>, the IC<sub>50</sub> of NO is 60 nM (14). Because COX consumes more than 95% of the O<sub>2</sub> taken up by mammalian cells and mitochondrial ATP generation is coupled to O<sub>2</sub> consumption, the regulation of COX activity by NO at physiological concentrations of NO and O<sub>2</sub> has a vast biological relevance.

NO also reacts with the oxidized form of COX; however at micromolar concentrations. The reaction of NO with oxidized COX is irreversible and not in competition with O<sub>2</sub> (21) and, therefore, physiologically irrelevant.

## 6. PEROXYNITRITE AND MITOCHONDRIA

As mentioned above, the reaction of NO with O<sub>2</sub><sup>-</sup> to form peroxynitrite occurs at the diffusion-controlled rate of 4.3 x 10<sup>9</sup> to 1.9 x 10<sup>10</sup> M<sup>-1</sup> s<sup>-1</sup> (5,22). Peroxynitrite formed *in vivo* plays a significant role in pathologic conditions including acute endotoxemia, inflammatory bowel disease, acute lung injury, neurological disorders, and atherosclerosis (23-25). Interaction of peroxynitrite with mitochondria causes irreversible modification of mitochondrial respiratory chain at multiple sites including mitochondrial complex I (2,17,26), mitochondrial complex II (2,17,26,27), mitochondrial complex III (2,27) and mitochondrial complex IV (27,28). Since peroxynitrite is extremely reactive and cells contain high concentrations of the peroxynitrite scavenger GSH, under physiologic conditions it is unlikely that peroxynitrite formed in the cytoplasm reaches mitochondria to inhibit mitochondrial respiratory complexes, unless peroxynitrite is formed within the mitochondria (2). However, under pathologic conditions such as hypoxia/reperfusion (H/R), cellular GSH is depleted and O<sub>2</sub><sup>-</sup> formation is elevated (29). Loss of cellular GSH and/or elevation of O<sub>2</sub><sup>-</sup> switches the reversible regulatory effects of NO on mitochondrial respiration to the irreversible peroxynitrite-induced mitochondrial damage (30-32).

## 7. MITOCHONDRIAL NITRIC OXIDE SYNTHASE

Between 1995 and 1996 studies suggesting the presence of a NOS in mitochondria were published. Immunohistochemical studies (33-36) with co-localization of NOS antibodies with mitochondrial markers (35) and cross-reaction of mitochondria with NOS antibodies

(33,34,36) suggested that mitochondria might contain a NOS-like protein. In 1997, Ghafourifar and Richter reported the presence of a constitutively expressed and continuously active NOS in mitochondria (mtNOS), the Ca<sup>2+</sup>-dependence of mtNOS and its association with the mitochondrial inner membrane (37). In 1998, Giulivi *et al.* (38) reported on the mitochondrial production of NO, and definitively confirmed the observation. Soon thereafter, several laboratories also confirmed the formation of NO by the mitochondria of various organs and revealed multiple novel functions for mtNOS (39-45). However, there are few published studies on controversial results concerning the isozyme specificity of mtNOS and its Ca<sup>2+</sup> dependence (reviewed in 1).

Similar to the NO produced by cytoplasmic NOS isozymes, mtNOS-derived NO decreases mitochondrial O<sub>2</sub> consumption (37,46,47), mitochondrial inner membrane potential ( $\Delta\psi$ ) (37, 46,47) and mitochondrial ATP formation (48). Inhibition of the endogenous mtNOS activity increases mitochondrial O<sub>2</sub> consumption and  $\Delta\psi$  (37,46,47), and increases transmembrane  $\Delta$ pH and mitochondrial Ca<sup>2+</sup> retention capacity (46,47) indicating a continuous regulation by mtNOS-derived NO of mitochondrial respiration and respiration-dependent functions.

## 8. MITOCHONDRIAL Ca<sup>2+</sup> HOMEOSTASIS

Mitochondria remain one of the main components of cellular Ca<sup>2+</sup> homeostasis and actively participate in physiological cellular Ca<sup>2+</sup> turnover (49-52). Although  $\Delta\psi$  enables mitochondria to take up relatively large amounts of Ca<sup>2+</sup> very rapidly, the intramitochondrial ionized calcium concentration ([Ca<sup>2+</sup>]<sub>m</sub>) is maintained very low by multiple mechanisms, primarily by precipitating the Ca<sup>2+</sup> to electron-dense granules (53-56). These granules may have various compositions at different physiological and pathological conditions (57,58). However, they consist mainly of tricalcium phosphate and hydroxyapatite. Earlier reports have suggested the [Ca<sup>2+</sup>]<sub>m</sub> at the range of 1-2 nmol Ca<sup>2+</sup> per mg rat liver and heart mitochondrial protein. Considering each mg mitochondrial protein contains 7.2 x 10<sup>9</sup> mitochondria, and the volume of each mitochondrion is 7.1 micro-m<sup>3</sup> (59), the [Ca<sup>2+</sup>]<sub>m</sub> can be estimated about 2-4 microM. More recent studies have reported even lower values, e.g. less than 100 nM [Ca<sup>2+</sup>]<sub>m</sub> in heart mitochondria (55,60).

## 9. Ca<sup>2+</sup> DEPENDENCE OF HEART mtNOS

Several groups have reported on heart mtNOS and its functions (43-45,61). Most studies have shown that heart mitochondria produce NO in a typical Ca<sup>2+</sup>-sensitive manner (43-45). However, one study (61) did not observe Ca<sup>2+</sup> dependence of heart mtNOS. It is important to notice that some buffers used to investigate mitochondrial functions, including those used in that study (61), contain high concentrations (1-5 mM) of Mg<sup>2+</sup> that is a known mitochondrial Ca<sup>2+</sup> uptake blocker (62-64). mtNOS is Ca<sup>2+</sup>-sensitive and prevention of mitochondrial Ca<sup>2+</sup> uptake decreases mtNOS activity (3,43). A dose-dependent inhibition by Mg<sup>2+</sup> of mtNOS activity has been shown (45).

## 10. MITOCHONDRIA, mtNOS, AND APOPTOSIS

Apoptosis is an evolutionarily conserved mechanism that regulates normal cell and tissue homeostasis (65). Unwanted apoptosis is the primary mechanism underlying numerous pathological conditions including H/R-induced cardiac cell injury (66). Mitochondria play a significant and central role in apoptosis (67,68). Endogenously formed NO induces apoptosis (3,68-77) through formation of peroxynitrite (69,75,78). This form of apoptosis occurs with mitochondrial dysfunction (69,79) and perturbed mitochondrial redox status (69). Prolonged elevated cytoplasmic  $Ca^{2+}$  also induces apoptosis (80) through mechanisms that involve elevation of  $[Ca^{2+}]_m$  (70,79,81) and increased NOS activity (70,79). Elevation of  $[Ca^{2+}]_m$  increase the activity of mtNOS and intramitochondrial formation of NO, that potently reacts with mitochondrial  $O_2^-$  to produce peroxynitrite. Peroxynitrite induces apoptosis by releasing the apoptogenic protein cytochrome *c* from mitochondria (47,68,77,82). Thus, it is plausible that mtNOS mediates the elevated  $[Ca^{2+}]_m$ -induced apoptosis.

### 11. DOES mtNOS RELATE H/R, PEROXYNITRITE, AND APOPTOSIS?

Hypoxia of the cardiomyocytes causes necrotic cell death, whereas hypoxia followed by reoxygenation (H/R) induces apoptosis (66) predominantly through mitochondria (83,84) and release of cytochrome *c* (85). Inhibition of cytochrome *c* release inhibits reoxygenation-induced apoptosis (83). Although the exact mechanism for H/R-induced cytochrome *c* release remains elusive, it has been shown that H/R elevates myocardial  $Ca^{2+}$  (85), increases  $[Ca^{2+}]_m$  (84,86), and causes mitochondrial malfunctioning (87). Additionally, NO and peroxynitrite are increased during H/R-induced apoptosis (31,88-90). Attenuation of increased  $Ca^{2+}$  (91), NO or peroxynitrite (92), or augmentation of the natural cellular peroxynitrite scavenger GSH (90) prevents the apoptosis of cardiomyocytes. Likewise, lessening mitochondrial  $O_2^-$  (31,93) or scavenging peroxynitrite (31) protects myocardial cells against H/R-induced injury. Mitochondria possess a  $Ca^{2+}$ -sensitive mtNOS and elevation of  $[Ca^{2+}]_m$  stimulates mtNOS activity followed by generation of peroxynitrite, release of mitochondrial cytochrome *c* and mitochondria malfunction. Thus, mtNOS may play a hitherto unrevealed crucial role in the pathology of H/R, and provide the link between H/R,  $[Ca^{2+}]_m$  elevation, peroxynitrite formation, release of cytochrome *c*, and apoptosis.

## 12. CONCLUSIONS AND PERSPECTIVES

The reversible inhibition of COX by physiologically relevant concentrations of NO is a fundamental physiologic mechanism by which NO regulates mitochondrial respiration. The reaction of NO with  $O_2^-$  produces the powerful oxidant peroxynitrite that causes mitochondrial and cellular injury. Formation of NO within mitochondria provides a unique possibility for NO to react with  $O_2^-$ , and makes mitochondria one of the

primary cellular peroxynitrite producers. How mitochondria harmonize the reversible regulation by NO of mitochondrial functions, vs. the irreversible modification of mitochondrial targets by peroxynitrite is the subject of ongoing investigations.

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