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Mitochondrial nitric oxide synthase

Pedram Ghafourifar[1] and Enrique Cadenas[2]

[1]Department of Pharmacology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25704, USA
[2]Department of Molecular Pharmacology & Toxicology, School of Pharmacy, University of Southern California, Los Angeles, CA 90089, USA

Mitochondria produce nitric oxide (NO) through a Ca^{2+}-sensitive mitochondrial NO synthase (mtNOS). The NO produced by mtNOS regulates mitochondrial oxygen consumption and transmembrane potential via a reversible reaction with cytochrome *c* oxidase. The reaction of this NO with superoxide anion yields peroxynitrite, which irreversibly modifies susceptible targets within mitochondria and induces oxidative and/or nitrative stress. In this article, we review the current understanding of the roles of mtNOS as a crucial biochemical regulator of mitochondrial functions and attempt to reconcile apparent discrepancies in the literature on mtNOS.

Discovery of mitochondrial nitric oxide synthase

The discovery that the endothelium-derived relaxing factor is nitric oxide (NO) [1] opened new horizons in biomedical research. The cellular synthesis of NO is catalyzed by NO synthase (NOS) isozymes, three of which are well characterized. Although expression of these enzymes is not tissue specific, they are referred to as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Each isozyme consumes L-arginine, produces equal amounts of NO and L-citrulline, and requires Ca^{2+}–calmodulin for activity. The activity of eNOS and nNOS are regulated tightly by alterations in Ca^{2+} status but, because iNOS forms a complex with calmodulin at very low concentrations of Ca^{2+}, its activity is not regulated by Ca^{2+} alterations.

NO exerts a broad spectrum of functions in several system, including the cardiovascular system, PNS, CNS and immune system. These functions are mediated through the reactions of NO with targets that include hemoproteins, thiols and superoxide anions. Mitochondria possess several hemoproteins (e.g. cytochrome *c* oxidase), thiols (e.g. glutathione) and cysteine-containing proteins, and they are major cellular sources of superoxide anion. Consequently, mitochondria are important targets of NO and contribute to several of the biological functions of NO [2].

Several laboratories have addressed the possibility that NOS is present in mitochondria. The cross-reaction of mitochondria with antibodies to Ca^{2+}-sensitive eNOS was reported almost simultaneously by two laboratories. In rats, mitochondria from skeletal muscle fibers from the diaphragm [3], non-synaptosomal brain [4], and heart, skeletal muscle and kidney [5] cross-react with eNOS antibodies. Other laboratories also report an association between eNOS and mitochondria in rat heart [6], brain and liver [7]. The association of eNOS with mitochondria has been examined systematically by Gao *et al.* [8] to reveal that eNOS associates only with the cytoplasmic face of the outer mitochondrial membrane. This study shows that eNOS associates with the outer membrane of mitochondria, even when denatured with urea. However, the denatured enzyme does not associate with the cytoplasmic membrane. Removal by proteinase K of proteins that bind nonspecifically the mitochondrial outer membrane abolishes the association between eNOS and mitochondria. Deletion of a segment comprising five basic residues (628–632) abolishes the association between eNOS and the mitochondrial outer membrane but not the cytoplasmic membrane [8]. These findings indicate that eNOS is not the *bona fide* mitochondrial NOS.

Immunolabeling of cytochrome *c* oxidase from the inner mitochondrial membrane of human vastus lateralis skeletal muscle has been shown to be similar to that of nNOS, the other Ca^{2+}-sensitive NOS [9]. The similarity between mitochondrial NOS (mtNOS) and nNOS has been observed in several laboratories [10–14].

The presence of a constitutively active mtNOS and the determination of mtNOS activity were reported first in mitochondria from rat liver [15]. This study used the oxyhemoglobin assay and reported that NO was not observed with either intact mitochondria or mitoplasts (mitochondria devoid of the outer membrane and intermembrane space). This ruled out contamination of mitochondria with non-mitochondrial NOS and association of NOS with the cytoplasmic face of the inner membrane. However, adding exogenous nNOS to these mitochondrial preparations resulted in the production of detectable amounts of NO, which validates the assay. Using the same assay, this study also showed that sub-mitochondrial particles (the mitochondrial inner membrane flipped inside-out), but not the mitochondrial-matrix fraction, produce NO and are sensitive to conventional NOS inhibitors, thus indicating that mtNOS is associated with the matrix face of the mitochondrial inner membrane and produces NO enzymatically. Intact mitochondria and mitoplasts produce L-citrulline in a manner that is sensitive to inhibitors of NOS but not to the arginase inhibitor L-lysine [15]. The activity of mtNOS depends, typically, on Ca^{2+}. Thus, increasing the intramitochondrial Ca^{2+}concentration $\{[Ca^{2+}]_m\}$ increases the activity of mtNOS, which decreases both the mitochondrial consumption of O_2 and the mitochondrial transmembrane potential ($\Delta\psi$). Conversely, inhibiting the basal activity of mtNOS increases

Corresponding author: Ghafourifar, P. (ghafourifar@marshall.edu).
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mitochondrial consumption of O_2 and $\Delta\psi$. These findings indicate that mtNOS continuously downregulates O_2 consumption and $\Delta\psi$ in intact rat liver mitochondria [15].

Although the occurrence and functions of mtNOS have been confirmed in many tissues, organs and cells, the isozyme of NOS that accounts for mtNOS is a matter of debate. In the following paragraphs we outline a consensus on mtNOS research and describe a role for mtNOS as a crucial regulator of mitochondrial function.

mtNOS and the regulation of mitochondrial functions

Electrons flow down the mitochondrial respiratory chain following the redox-potential hierarchy of the respiratory complexes and reduce O_2 to H_2O at the terminal member of the chain, cytochrome *c* oxidase. Coupled to the electron flow, protons are pumped from the mitochondrial matrix into the intermembrane space. The chemiosmotic principle, which was established by the pioneering work of Mitchell in the 1950s [16], postulates two immediate consequences of this proton extrusion. These are an electrochemical gradient ($\Delta\psi$) and a proton gradient (ΔpH) across the inner membrane of the mitochondria. The former renders the matrix face of the inner membrane negative and the latter maintains an alkaline matrix. Inhibiting the mitochondrial electron-transport chain decreases $\Delta\psi$ and ΔpH. Although the O_2-binding site of cytochrome *c* oxidase is specialized, similarity between the physicochemical properties of NO and O_2 enables NO to bind to cytochrome *c* oxidase, thus inhibiting the electron flow (Figure 1). Inhibition of O_2 consumption by NO occurs at physiologically relevant concentrations of NO, and is competitive, reversible and dose dependent. Thus, NO is considered to be a pharmacological competitive antagonist of O_2 [17]. NO decreases mitochondrial O_2 consumption, $\Delta\psi$ [18] and ΔpH [15,19], and, therefore, the formation of ATP [20].

However, some studies have suggested that mtNOS has a minimal role in regulating mitochondrial bioenergetics [21–24]. This apparent discrepancy might be reconciled by considering the crucial effect of Ca^{2+} in regulating mtNOS activity. First, Mg^{2+} is known to block Ca^{2+} uptake by mitochondria [25–27] and inhibition of mitochondrial Ca^{2+} uptake by, for example, either ruthenium red or collapsing $\Delta\psi$, prevents stimulation of mtNOS activity [11,28,29]. Moreover, Mg^{2+} is a potent, dose-dependent inhibitor of mtNOS activity [30]. Second, although $\Delta\psi$, which is the driving force for mitochondrial Ca^{2+} uptake, enables mitochondria to take up and accommodate relatively large quantities of Ca^{2+}, $[Ca^{2+}]_m$ is maintained at a low level by several mechanisms, including calcium precipitation to form matrix electron-dense granules (Figure 1) [31–33]. Although the content of these granules varies in different physiological and pathological conditions [34,35], they consist mainly of tricalcium phosphate and hydroxyapatite. Earlier reports have suggested that rat liver and heart mitochondria contain 1–2 nmol ionized Ca^{2+} per mg mitochondrial protein [32]. Considering a volume of 7.1 μm^3 for each mitochondrion and 7.2×10^9 mitochondria in each mg of mitochondrial protein [36], the concentration of $[Ca^{2+}]_m$ can be estimated as 2–4 μM. Recent studies detect lower $[Ca^{2+}]_m$ (100–500 nM in mitochondria from rat heart) [37,38] and lower levels of ionized Ca^{2+} in the matrix of mitochondria than that in the endoplasmic reticulum [39]. Because $[Ca^{2+}]_m$ must be elevated for maximal mtNOS activity in rat liver [15,19,28], brain [12], heart [11,14,30] and endothelial cells [29], the presence of Mg^{2+} ($\geq$1 mM) in buffers used to investigate mtNOS functions [21–23], combined with lack of efforts to elevate $[Ca^{2+}]_m$, account for the low activity and modest contribution of mtNOS to the regulation of mitochondrial bioenergetics (reviewed in [40]).

NO decreases $\Delta\psi$ and, consequently, decreases mitochondrial uptake of Ca^{2+} and $[Ca^{2+}]_m$. Stimulation the synthesis of NO by mtNOS decreases the rate and extent of mitochondrial Ca^{2+} uptake and $[Ca^{2+}]_m$, and inhibition of constitutive endogenous mtNOS activity increases the rate and extent of mitochondrial uptake of Ca^{2+} [19]. This finding indicates that mtNOS is involved in mitochondrial Ca^{2+} homeostasis via a feedback mechanism that protects the organelles against overload by Ca^{2+}. Thus, elevation of $[Ca^{2+}]_m$ increases NO formation by mtNOS, which decreases $\Delta\psi$. In turn, this releases Ca^{2+} from the organelles, which is followed by inactivation of mtNOS.

Which NOS isozyme is mtNOS?

The only effort to purify and characterize the amino acid sequence of mtNOS used mitochondria from rat liver. A protein from the mitochondrial matrix of rat liver that generates L-citrulline from L-arginine in a Ca^{2+}-independent manner has been purified using ADP-affinity chromatography [21]. Because the purified protein cross-reacts with an antibody to iNOS (which also generates L-citrulline from L-arginine in a Ca^{2+}-insensitive manner) it was concluded that mtNOS was iNOS. However, it must be noted that liver mitochondria form L-citrulline via routes other than NOS. Terrestrial ureotelic organisms deposit ammonia in liver mitochondria. This is converted to urea in the urea cycle, which encompasses enzymes of cytoplasm and mitochondria. The mitochondrial component is the matrix enzyme carbamoyl phosphate synthetase 1 (CPS-1; EC 2.7.2.5). This catalyzes the conversion of ammonia to carbamoyl phosphate, which is further condensed with L-ornithine to yield L-citrulline. CPS-1 is an abundant matrix protein ($\sim$20% of the total protein in the mitochondrial matrix of rat liver), the activity of which is regulated by the concentrations of Mg^{2+} and ATP in mitochondria [41,42]. Thus, it is likely that the mitochondrial matrix protein reported in this study [21] is CPS-1. A subsequent study used two-dimensional gel electrophoresis to characterize the mitochondrial protein purified by ADP-affinity purification [13]. The spots that cross-reacted with the iNOS antibody were excised, the amino acid sequence determined using matrix-assisted laser desorption ionized time-of-flight (MALDI-TOF) and quadruple mass spectrometry with time of flight (Q-TOF), and a protein with 100% homology to nNOS and 21% homology to iNOS identified. However, NOS activity associated only with the inner membrane of mitochondria and there was a correlation between a protein that cross-reacted with the nNOS antibody and the mitochondrial inner-membrane marker cytochrome *c* oxidase [13]. These discrepancies have been addressed subsequently. A study of mtNOS in

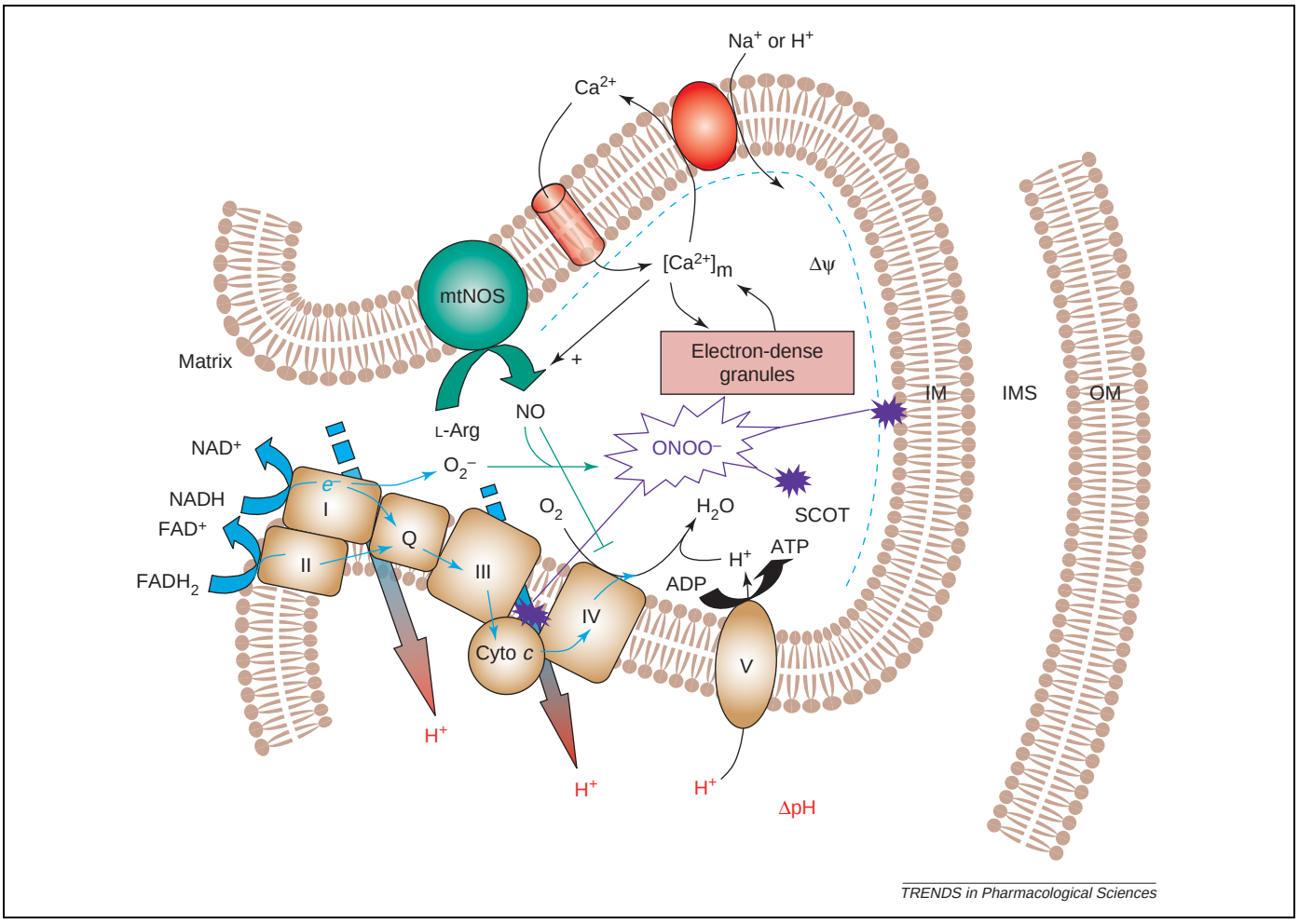

Figure 1. Mitochondria and mtNOS. Highly compartmentalized mitochondria consist of an outer membrane (OM), an intermembrane space (IMS), an inner membrane (IM) and the matrix. The mitochondrial respiratory chain is embedded in the IM and consists of complexes I–IV, coenzyme Q [ubiquinone (Q)] and ATP synthase (which is also referred to as complex V). Cytochrome *c* oxidase (cyto *c*) is the only member of the chain that is present in the IMS. These respiratory chain complexes are arranged functionally in an electrochemical hierarchy based on their redox potentials. The chain provides a unique broad spectrum of redox potentials that varies from −280 mV for complex I to +250 mV for complex IV. Electrons (blue arrows) enter the chain through oxidation of either NADH at complex I or $FADH_2$ at complex II and flow down the chain to complex IV to reduce O_2 to H_2O. Although most of the O_2 consumed in mitochondria is reduced fully to water at complex IV, some O_2 is reduced incompletely to superoxide anion (O_2^-) by other respiratory chain complexes. Coupled to the electron flow, protons are extruded from the matrix into the IMS (red arrows). Because the IM is impermeable to protons, and protons can re-enter the matrix only through the ATP synthase machinery, proton extrusion establishes a transmembrane potential ($\Delta\psi$; negative inside) and an electrochemical gradient (ΔpH; alkaline inside) across the coupling membrane. $\Delta\psi$ is the driving force for mitochondria to take up relatively large quantities of Ca^{2+}. However, $[Ca^{2+}]_m$ is kept low by several mechanisms. Mitochondria precipitate the $[Ca^{2+}]_m$ to form pools of non-ionized calcium, the matrix electron-dense granules, that consist mainly of calcium phosphate and hydroxyapatite. Ca^{2+} also leaves mitochondria when $\Delta\psi$ decreases and in exchange with other cations such as H^+ and Na^+. The latter mechanism is non-electrogenic and occurs with preserved $\Delta\psi$. Mitochondria possess a NOS (termed mtNOS), which is associated with the IM and generates NO in a Ca^{2+}-sensitive fashion. NO formed in mitochondria competes with O_2 for binding to complex IV and regulates mitochondrial respiration and its consequences such as $\Delta\psi$, ΔpH and $[Ca^{2+}]_m$ retention. NO produced by mtNOS reacts readily with superoxide anion to produce the powerful oxidative species peroxynitrite ($ONOO^-$). Peroxynitrite produced inside mitochondria causes the release of cyto *c*, increases the peroxidation of mitochondrial membrane lipids and oxidatively damages susceptible targets within mitochondria such as succinyl-CoA:3-oxoacid CoA-transferase (SCOT) (purple).

intact mitochondria isolated from mouse heart [11] reveals that mtNOS generates NO in response to elevation of $[Ca^{2+}]_m$ in a manner that is sensitive to the Ca^{2+}-uptake blocker ruthenium red and depletion of $[Ca^{2+}]_m$ following collapse of $\Delta\psi$. A knockout approach demonstrates the absence of mtNOS activity in $nNOS^{-/-}$ mice but not in $eNOS^{-/-}$ and $iNOS^{-/-}$ mice, which indicates that the mtNOS in mouse heart is related to nNOS [11]. Poderoso's group has demonstrated that mtNOS from rat brain is a 144-kDa nNOS that is distinct from 157-kDa nNOS in cytoplasm; the mitochondrial enzyme associates with the mitochondrial inner membrane and produces NO in a Ca^{2+}-sensitive manner [12]. Debates about the isozyme specificity of mtNOS continue following a recent study [24] that used a fluorescent probe, 4,5-diamino-fluorescein diacetate, and reported the lack of formation of NO by isolated intact mitochondria from mouse brain. However, the presence of 5 mM Mg^{2+} in the NOS assay medium (as discussed previously), and experimental conditions that involved depleting the medium of O_2 and low temperature might account for the lack of NO formation. The same study also used western blot analysis and was unable to identify proteins that cross-reacted with either iNOS or eNOS in mitochondria from mouse brain. However, a protein with a molecular size of ~70 kDa that cross-reacts with nNOS was observed [24]. Whether the protein that cross-reacts with the nNOS antibody is mtNOS from mouse brain or whether it is a fragmented

enzyme caused by sonicating and boiling mitochondrial samples before gel electrophoresis has not been investigated. Boveris' group report that mouse brain mtNOS is a 147-kDa protein that cross-reacts with an nNOS antibody and is associated with the mitochondrial inner membrane [43]. NO produced by mtNOS from mouse brain decreases mitochondrial respiration by competing with O_2 for the O_2-binding site of cytochrome *c* oxidase, and inhibiting endogenous activity of mtNOS in the brain using conventional NOS inhibitors increases mitochondrial respiration, which supports the regulatory role of mtNOS on mitochondrial respiration. Moreover, the calmodulin antagonist chlorpromazine inhibits mtNOS activity in the brain, which indicates that mtNOS in mouse brain is Ca^{2+} sensitive [43].

Taken together, most reports agree that mtNOS is associated with the mitochondrial inner membrane and generates NO in a Ca^{2+}-sensitive manner, and that NO produced by mtNOS regulates mitochondrial respiration. However, the amino acid composition of mtNOS remains to be characterized.

NO in mitochondria

Of the electrons that flow through the respiratory chain, ~2–5% leak out [44,45]. These electrons account for the fraction of the total oxygen that is consumed by mitochondria to generate superoxide anion and hydrogen peroxide. Although the chemical reactivity of superoxide anion is modest, its reaction with NO with the rate constant of 1.9×10^{10} M s^{-1} [46] is nearly diffusion-controlled and results in the formation of peroxynitrite, a highly reactive NO-derived species. Mitochondria provide the two reactants for peroxynitrite formation. These are superoxide anion, which is produced during electron transfer through the inner membrane respiratory chain, and NO, which is produced by the inner membrane mtNOS. Thus, it is likely that NO produced by mtNOS reacts with superoxide to form peroxynitrite [47]. In fact, utilization of NO by superoxide anions at the mitochondrial inner membrane to yield peroxynitrite occurs at the rate of 9.5×10^{-8} M s^{-1}, which exceeds the utilization of NO by cytochrome *c* oxidase (0.8×10^{-8} M s^{-1}) [48]. This amount of peroxynitrite accounts for 15% of the superoxide generated by the mitochondrial inner membrane, with the remaining 85% yielding H_2O_2 as final product [48,49]. Several groups report that mtNOS generates peroxynitrite [14,28,29,48–53]. The first report on the generation of peroxynitrite by mtNOS showed that mtNOS-derived peroxynitrite induces oxidative stress and promotes the release of cytochrome *c* from mitochondria [28]. These effects of mtNOS are prevented by the anti-apoptotic protein Bcl-2, which indicates a role for mtNOS-derived peroxynitrite in apoptosis. Peroxynitrite derived from mtNOS induces mitochondrial dysfunction and contractile failure in rat and human skeletal muscle [51], and diminishes the oxidative-phosphorylation capacity in mouse cardiomyocytes [14]. A role for mtNOS in the apoptosis of SH-SY5Y neural cells has also been reported [54]. Several studies provide evidence to support the notion that a substantial amount of NO produced in mitochondria converts to peroxynitrite [7,14,29], and mtNOS has been called peroxynitrite synthase [55].

How mitochondria harmonize the formation of NO and peroxynitrite has been questioned since the early reports of mtNOS. The different electrochemical properties, redox state, pH, and enzyme and ionic content in the mitochondrial matrix, intermembrane space and membranes provide many possible reactions for NO within highly compartmentalized mitochondria. The vectorial release of superoxide from the inner membrane into the matrix [56] and the higher pH of the matrix, which stabilizes the reaction of NO with superoxide [57], provide a suitable environment for the generation of peroxynitrite and its and reactions within the mitochondrial matrix. Supporting this view, several proteins that contain nitrated tyrosine residues and can account as peroxynitrite markers [58] have been identified in this compartment [59–61].

Nitrosation of SH moieties of proteins by NO is another important reaction in mitochondria [62]. Although the higher pH and high concentration of inorganic phosphate in the mitochondrial matrix do not favor *S*-nitrosation of proteins [63], and the matrix enzymes glutathione peroxidase [64] and thioredoxin reductase [65] accelerate the decomposition of *S*-nitrosothiols within the matrix, the inner mitochondrial membrane and intermembrane space appear to be preferred sites of protein *S*-nitrosation because *S*-nitrosation is favored by lower pH [2] and lipophilic membranous environments [66]. The *S*-nitrosation of a mitochondrial intermembrane protein, caspase-3 [62], and inner membrane embedded proteins, the complex I [67], might indicate a crucial role for mitochondria in the regulation of cell signaling by NO.

Concluding remarks

The first report on mtNOS activity in 1997 stimulated several laboratories to study this enzyme. By competing for the O_2-binding site of cytochrome *c* oxidase, NO produced by mtNOS modulates mitochondrial respiration, $\Delta\psi$ and ΔpH, and, thus, regulates mitochondrial bioenergetics. NO produced by mtNOS can generate peroxynitrite, which induces oxidative and/or nitrative stress and the release of cytochrome *c* from mitochondria in addition to inactivation of susceptible mitochondrial enzymes. These functions indicate a pro-apoptotic role for mtNOS. Conversely, *S*-nitrosation of caspase-3, which renders the protein inactive apoptotically, indicates an anti-apoptotic role for mtNOS that protects cells from unwanted apoptosis and organelles from proteolytic activity of the caspase. Distinct sub-organelle environments that stem from the tight compartmentalization of mitochondria coordinate the generation, reactions and functions of mtNOS-derived NO and peroxynitrite.

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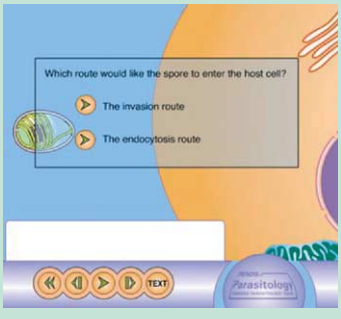

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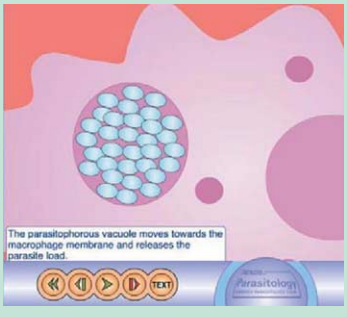

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