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Mitochondrial nitric oxide synthase, a voltage-dependent enzyme, is responsible for nitric oxide diffusion to cytosol

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1. ABSTRACT

The mitochondrial metabolic state regulates the rate of mitochondrial NO production and release to the cytosol. Nitric oxide release of rat heart mitochondria decreased markedly from 2.2 to 1.2 nmol NO/min. mg protein in the state 4 to state 3 transition. The activity of mtNOS, responsible for NO release, is driven by the membrane potential and not by intramitochondrial pH changes. The release of NO by rat liver mitochondria showed an exponential dependence on membrane potential. A similar behavior was reported for heart mitochondrial H_2O_2 production. The fraction of heart cytosolic NO provided by diffusion from mitochondria is 90 %. The intramitochondrial concentrations of L-arginine and NADPH are higher than their K_M values, and the changes in their concentrations in the state 4-state 3 transition are not enough to explain the changes in NO release. These data indicate that the redox state of the respiratory chain components regulates H_2O_2 production and mitochondrial membrane potential modulates NO release, and support the speculation that NO and H_2O_2 are a biological signal that reports a high mitochondrial energy charge to the cytosol. The marked regulation of mtNOS activity, as a voltage-dependent enzyme and at the physiological range of membrane potentials, makes mtNOS a highly sensitive enzyme that in turn regulates mitochondrial O_2 uptake and H_2O_2 production.

2. INTRODUCTION

There is a generalized concept that in physiological conditions, mitochondria provide to the rest of the cell both energy and signals to command the genetic expression and metabolic regulation (1). Hydrogen peroxide (H_2O_2) and nitric oxide (NO) are uncharged molecules, highly diffusible through biological membranes and suitable for cellular and subcellular signaling. However, the mechanisms by which NO and H_2O_2 may regulate genetic expression and metabolism (2-5) as well as the molecular signals involved in the cross-communication of mitochondrial and cytosolic functions are processes that are starting to be understood. At the present time, the recognition of a mitochondrial nitric oxide synthase (mtNOS) activity in a series of organs with similar specific activities, the use of spectroscopic (6,7), electrochemical (8) and fluorescent (9) assays to detect NO production, the sequencing of liver mtNOS (10), and the dependence of mtNOS activity on metabolic state and membrane potential (11,12), strongly support both the recognition of mtNOS as a constitutive integral protein of the inner mitochondrial membrane and the concept that mitochondrial NO release is a physiological regulator of cytosolic and cellular functions. There is current point of view that NO and H_2O_2 are diffusible molecules that jointly with ATP report a high mitochondrial energy charge to the cytosol (12). The diffusion of both NO and H_2O_2 has been considered as a

Table 1. Oxygen consumption, H_2O_2 and NO release from rat heart mitochondria

Substrate	O_2 consumption (ng-at O/min. mg protein)		H_2O_2 release (nmol/min. mg protein)		NO release (nmol/min. mg protein)	
	State 4	State 3	State 4	State 3	State 4	State 3
Malate-glutamate	45 ± 4	248 ± 11	0.80 ± 0.04	0.05 ± 0.01	2.20 ± 0.07	1.25 ± 0.04
Succinate	92 ± 8	394 ± 35	0.60 ± 0.03	0.04 ± 0.01	2.23 ± 0.06	1.22 ± 0.05
Physiological situation[1]	122[2]		0.42 ± 0.02		1.75 ± 0.03	

Data reproduced with permission from (11), [1] The physiological situation for tissue mitochondria was considered to be the simultaneous oxidation of 3 NADH (malate-glutamate) and 1 succinate with 53 % of heart mitochondria in state 4 and 47 % of mitochondria in state 3 (20). [2] Calculated from reference 21, taking into account 50 mg heart mitochondrial protein/g organ; isolated beating heart, 240 beats/min

pleitropic signal involved in the regulation of cell metabolism and proliferation (2,4,5).

3. MITOCHONDRIAL METABOLIC STATES AND H_2O_2 PRODUCTION

Chance & Williams (13) described the redox states of the components of the mitochondrial respiratory chain and defined the concepts of mitochondrial metabolic states and of respiratory control (RC). The latter concept describes the state 4 to state 3 transition in which ADP regulates the rate of mitochondrial electron transfer and respiration. High rates of O_2 uptake and ATP production are observed in mitochondria supplemented with substrates and ADP in metabolic state 3 or active respiration; whereas without ADP, the resting respiration of metabolic state 4 is established with low rates of O_2 uptake. The mitochondrial transition from state 4 to state 3 establishes a fast flow of H^+ through the F_0 position of the F_1-ATPase complex, a change that increases the rates of electron transfer, decreases the reduction level of the components of the mitochondrial respiratory chain, decreases the membrane potential and increases the sensitivity of cytochrome oxidase to NO (14).

3.1. Mitochondrial O_2^- and H_2O_2 production

Mitochondria were recognized by Boveris and Chance in 1972-1973 (15,16) as an active source of H_2O_2 in a process that depends on the mitochondrial metabolic state. The rate of H_2O_2 generation in state 4 is about 4-16 times higher than in state 3 (17,18). The rates of H_2O_2 production of mitochondria isolated from mammalian organs are in the range of 0.4-0.9 nmol H_2O_2/min. mg protein in state 4 and 0.05-0.15 nmol H_2O_2/min. mg protein in state 3 (18,19). In the case of heart mitochondria, H_2O_2 production is about 0.6-0.8 nmol H_2O_2/min. mg protein in state 4, and 0.04-0.05 in state 3, using malate glutamate or succinate as substrates (Table 1). These rates of H_2O_2 production accounts for about 2 % of the O_2 consumption in state 4 (17 and Table 1) and for about 1 % of the O_2 consumption in physiological conditions. Hydrogen peroxide release of heart mitochondria under physiological conditions has been calculated as about 0.42 nmol H_2O_2/min. mg protein, considering the simultaneous oxidation of 3 NADH (malate-glutamate) and 1 succinate, in the tricarboxylic acid cycle and taking into account that heart mitochondria oscillate between a NO-inhibited state 3 (47 %) and state 4 (53 %) (20). Boveris and Chance showed that when malate-glutamate were used as a source of reducing equivalents, H_2O_2 production is inhibited by rotenone, and when the electron flow is blocked by antimycin, heart mitochondria exhibit high rates of H_2O_2 production (15-17). The fact that H_2O_2 production is maximal in the highly reduced states, such as in state 4 or in the presence of antimycin, and minimal in the oxidized states, as in state 3 or 3u, indicates that a component of the respiratory chain changing its redox state in the state 4-state 3 transition is the H_2O_2 generator.

Superoxide anion (O_2^-) is the stoichiometric precursor of H_2O_2 (18,19). Two main generating O_2^- reactions have been described for the mitochondrial respiratory chain (16,17,19). In both cases the intermediate semiquinone form of redox pairs components of the respiratory chain, ubiquinol (UQH_2)/ubiquinone (UQ), and the $FMNH_2$/FMN component of NADH dehydrogenase, *i.e.* $UQH^\bullet$ and $FMNH^\bullet$, respectively, are collisionally and non-enzymatically auto-oxidized by molecular O_2 to yield O_2^-. The main part of O_2^- (about 70 %) is vectorially released to the mitochondrial matrix where Mn-SOD catalyzes the O_2^- disproportionation reaction to yield H_2O_2 and the remaining 30 % is released to the mitochondrial intermembrane space (21).

3.2. H_2O_2 release and redox state

It was first observed that the rate of mitochondrial O_2^- production was approximately linear with the degree of reduction of the components of the respiratory chain which are more reduced in state 4 than in state 3 (15-18). However, it was later recognized that ionophores and protophores (22) and membrane potentials themselves (23-25) markedly regulate the rates of H_2O_2 production. Korshunov *et al.* (23) reported that the effects of malonate, ADP and of an uncoupler on the production of H_2O_2 by rat heart mitochondria are proportional to their effects on $\Delta\Psi$. The observed regulation showed an exponential dependence of H_2O_2 generation on $\Delta\Psi$ with a threshold at the state 3 $\Delta\Psi$ value above which a strong, up to 10 times, increase in H_2O_2 production takes place. In addition, Hansford *et al.* (24) showed that H_2O_2 production by heart mitochondria requires both a high fractional reduction of Complex I and a high membrane potential.

4. MITOCHONDRIAL NO PRODUCTION

4.1. Mitochondrial nitric oxide synthase (mtNOS)

Mitochondrial NO production is carried out by the mitochondrial nitric oxide synthase (mtNOS), an isoenzyme of the NOS family located in mitochondrial

inner membrane that requires NADPH, L-arginine, O_2 and Ca^{2+} for enzymatic activity (26-28). The production of NO has been observed in mitochondria and in mitochondrial membranes isolated from a series of mammalian organs: liver (26,27,29), heart (8,30,31), kidney (32), brain (33,34), diaphragm (35), and thymus (36). In 2002, the sequence of rat liver mtNOS was reported as the splice variant α of the nNOS isoform, with post translational modifications: acylation with myristic acid at the N-terminal and phosphorylation at the C-terminal region (10).

Nitric oxide production by mitochondrial membranes and NO release by intact mitochondria are conveniently measured by following the oxidation of oxyhemoglobin (HbO_2) to methemoglobin at 37ºC, in an air-saturated reaction medium (185 μM O_2). The reaction is considered one of the important processes for *in vivo* NO catabolism due to both the high rate constant between HbO_2 and NO ($k = 3 \times 10^7$ M^{-1} s^{-1}, 37) and the blood HbO_2 concentration (2.5 mM as tetrameric form). The NO assay is performed using a diode array spectrophotometer in which the active wavelength is set at 577 nm and the reference wavelength at the isosbestic point at 591 nm (ε = 11.2 mM^{-1} cm^{-1}) (6,38). The method is based on the original assay developed by Murphy and Noack (6) for perfused organs in which the HbO_2 gamma-band is used to follow NO production and has been adapted to the hemoprotein alpha-band for its use with neutrophils and lymphocytes (39,40). The alpha-band is more suitable for the high light scattering conditions of cellular and mitochondrial suspensions due to the close vicinity of the active (577 nm) and the reference (591 nm) wavelengths (38).

The rates of NO production of mitochondrial membranes from mammalian organs using the HbO_2 method are in the range of 0.5-1.4 nmol NO/min. mg protein (31,32,34,35,38). The similar rates of NO production by mitochondria isolated from different organs strongly argues against the measured activity being caused by non-mitochondrial contaminants (41).

4.2. Heart mtNOS: activity and expression

Nitric oxide production by heart mtNOS was determined in a series of studies (8, 30,31,42-44). Kanai and co-workers (8) showed the presence of a NOS activity in mouse cardiac mitochondria by measuring, in an individual mitochondrion, the NO production that followed to Ca^{2+} addition to the reaction medium, by using a porphyrinic microsensor. Moreover, Boveris group (30,31,43,44) reported a heart mtNOS activity of 0.7-1.2 nmol NO/min. mg protein using the HbO_2 technique.

Heart mtNOS activity was found susceptible of pharmacological and physiological regulations. Treatment with enalapril markedly increased heart mtNOS activity (30,38,43) and rats exposed to hypobaric hypoxia (53 kPa O_2) from 2 to 18 months of age showed a 20 to 60 % increased mtNOS activity; this latter an effect marked enough to counteract the age-dependent decline in mtNOS activity (44). This up-regulation of mtNOS activity by hypoxia was associated with a retardation of the decline in the mechanical activity of papillary muscle upon aging and with an improved recovery after anoxia-reoxygenation (44-46).

The immunological nature of the heart mtNOS isoform remains controversial, a situation that extends to all reported mtNOS activities (47). Elfering and co-workers (10) used an anti-iNOS antibody in two-dimensional electrophoresis to identify a mtNOS spot of the liver mitochondrial proteome and found that the sequence that best matched the digested protein fragments was nNOS-α. Furthermore, Kanai *et al.* (8) showed the absence of NO production in the mitochondria of knockout mice for the neuronal, but not the endothelial or inducible NOS (8). However, other groups identified heart mtNOS as eNOS or iNOS isoforms (48-50). The 51-57 % homology reported for nNOS, iNOS and eNOS could contribute to explain these conflicting results (51). In our laboratory, a protein of about 130 kDa of heart mitochondrial membranes, identified as mtNOS, reacted specifically with the anti-iNOS antibodies (38,52), and did not react with anti-nNOS or anti-eNOS antibodies. The heart post-mitochondrial supernatant showed the reactions of a 130 kDa protein with the anti-iNOS antibodies and of a 135 kDa protein with the anti-eNOS antibodies. The 130 kDa protein was identified as the mtNOS of the mitochondrial fragments of the post-mitochondrial supernatant, whereas the 135 kDa protein was identified as the eNOS of the caveolae and plasma membrane (53-54). This finding is in agreement with the results reported by Buchwalow *et al.* (55), who showed that an iNOS was targeted predominantly to the particulate component of cardiomyocytes, a fraction that includes mitochondria, T-tubules and the Golgi complex.

4.3. NO release and mitochondrial membrane potential

Mitochondrial metabolic state regulates the rate of mitochondrial NO production (11,12). Nitric oxide release from heart mitochondria supplemented with either malate-glutamate or succinate as substrates decreased markedly from 2.2 to 1.2 nmol NO/min. mg protein in the state 4–state 3 transition (Table 1). Similarly, in liver, kidney and brain mitochondria the rates of NO release were 40-45 % lower in state 3 than in state 4 (11,12). Table 1 shows that the slower rates of O_2 consumption of the resting respiration (state 4) were associated with faster rates of NO release, and the faster rates of O_2 consumption of the active respiration (state 3) were associated with slower rates of NO release. This fact is consistent with the findings published by Brookes *et al.* (56) who showed that mitochondrial respiration is more sensitive to exogenous NO in state 3 than in state 4, at physiological O_2 levels.

Nitric oxide release by isolated heart mitochondria accounts for about 5-10 % of the corresponding O_2 consumption in state 4 (Table 1) and 0.6-1 % of the O_2 uptake in state 3. Heart NO release in physiological conditions, calculated as described before for mitochondrial H_2O_2 production and release, resulted 1.75 nmol NO/min. mg protein, a value that accounts for about 3 % of heart O_2 consumption in physiological conditions.

The addition of SOD (to avoid interference by O_2^-) and catalase (to avoid HbO_2 oxidation by H_2O_2) to the reaction

Table 2. Nitric oxide release by heart mitochondria

Experimental condition	**NO release (M x 10^{-6}/min)**	
	State 4	State 3
Malate-glutamate	1.05 ± 0.03	0.63 ± 0.02
+ SOD + catalase	1.10 ± 0.04	0.65 ± 0.03
Succinate	1.07 ± 0.02	0.62 ± 0.02
+ SOD + catalase	1.03 ± 0.03	0.64 ± 0.03
+ GSNO + DTT[1]	1.28 ± 0.01	1.19 ± 0.02

Additions: 6 mM malate + 6 mM glutamate, 8 mM succinate, 0.5 mM ADP, 4 μM Cu,Zn-SOD, 0.5 μM catalase, 5 μM GSNO, 50 μM DTT, 0.3 mg/ml heart mitochondrial protein, [1] The addition of GSNO-DTT system to the reaction medium produced a sustained NO release of 1.79 ± 0.09 μM/min.

medium did not modify the rate of NO release by heart mitochondria both in state 4 and state 3, or using succinate or malate-glutamate as substrates (Table 2). Recovery experiments using GSNO and DTT as a NO-generating system (Table 2) showed an active NO onsumption by mitochondria, being this NO utilization slightly higher in state 4 than in state 3, in accordance with the known higher production of O_2^- in state 4 (16-18) and being this latter species capable of consuming NO in a diffusion-controlled reaction to yield $ONOO^-$ (57,58).

The decrease in NO release in the state 4 to state 3 transition is opposite to what could be expected from the matrix acidification that follows to the considered transition and the pH dependence of mtNOS activity (11,12). In intact tightly coupled succinate-energized mitochondria, the matrix pH is close to 7.8 with a $\Delta\psi$ of 180 mV and a proton motive force of 240 mV (59,60). Taking into account matrix pH values of about 7.5 in state 3 and of about 7.8 in state 4, heart mtNOS show a 15 % higher activity in state 3 (pH 7.5) than in state 4 (pH 7.8) (35). The higher NO release in state 4 is not due to pH changes (11).

Moreover, the difference observed in NO release in states 4 and 3 can not be explained either by the variations in mitochondrial NADPH content that follow the transition from state 4 to state 3, or by the matrix L-arginine concentration (11). Considering NADPH concentration values in state 4 mitochondria (1.78 x 10^{-3} M) and in state 3 (1.04 x 10^{-3} M) and the NOS K_M value for NADPH (0.1-1 x 10^{-6} M) (61) the intramitochondrial NADPH concentration is not rate limiting and can not explain the difference observed in the NO release rate in state 4 and 3. Similarly, mitochondrial L-arginine availability is neither rate limiting NO release by mitochondria, due to the intramitochondrial concentrations of L-arginine, that is about 3.1 x 10^{-4} M (11,62), 60 times higher than the reported K_M of liver mtNOS (5 x 10^{-6} M) (27).

Nitric oxide release from liver mitochondria shows a very strong and exponential dependence on mitochondrial membrane potential ($\Delta\psi$) (11,12). This dependence appears more important in the physiological range of $\Delta\psi$ (150-180 mV) where small changes of mitochondrial membrane potential produce marked modifications of mitochondrial NO release. Results of our laboratory showed that agents that decrease or abolishm membrane potential are able to minimize NO release (to the minimal, non-energized rate of 0.48 nmol NO/min. mg protein), while the addition of oligomycin (the inhibitor of ATP synthase), that produces mitochondrial hyperpolarization due to the blockade of H^+ flow through F_0-ATPase, generates the maximal measured NO release, 1.4 nmol NO/min. mg protein (11,12). A marked decrease of NO release was observed when mitochondrial membrane potential was collapsed by the uncoupler of oxidative phosphorylation m-CCCP. This concept is in agreement with the report by Dedkova *et al.* (63) who showed that collapsing the mitochondrial membrane potential with FCCP or blocking mitochondrial Ca^{2+}-uniporter with ruthenium red, inhibited NO production.

Considering that the permeability of mitochondrial membranes to NO is constant, the changes in NO release appear to reflect the effect of membrane potential in the rate of NO production by mtNOS. A similar exponential dependence on mitochondrial membrane potential was reported for mitochondrial H_2O_2 as mentioned above. It is then clear that a high H^+ electrochemical potential across the inner membrane is linked to high production rates of NO and H_2O_2. The data seems to support the speculation that NO and H_2O_2 constitute a pair of diffusible molecules that signal a high mitochondrial energy charge to the cytosol. In summary, the evidence reported here sustains the notions that mitochondria-specific NOS (mtNOS) is associated to the inner mitochondrial membrane and that its activity is regulated by the mitochondrial membrane potential, as a voltage-dependent enzyme activity.

4.4. Heart mtNOS contribution to total NO production

In order to assess the relevance of mitochondrial NO release for heart cellular function, we calculated the relative contribution of heart mtNOS to total cardiomyocyte NO production. The rate of NO production by the heart post-mitochondrial supernatant was 0.63 nmol NO/min. mg protein (Table 3). Taking cytochrome oxidase activity as an indicator of the amount of mitochondrial fragments present in the post-mitochondrial fraction, we calculated the mitochondrial protein content and the mtNOS activity of the post-mitochondrial supernatant. For this, the cytochrome oxidase activity of heart mitochondria and of the post-mitochondrial fractions were determined and the values are shown in Table 3. The activity of heart cytosolic NOS was calculated subtracting the NOS activity

Table 3. Heart mitochondrial contribution to cellular NO generation under physiological conditions

	Heart NOS activity		COX activity
	(nmol/min. mg protein)	(nmol/min. g tissue)	(1/min. mg protein)
Mitochondria	1.75 ± 0.05	87.5 ± 2.5	18.5 ± 1.0
Postmitoch. supernatant	0.63 ± 0.05	-	5.73 ± 0.21
Cytosol	0.09	9.0	-

The activity of heart cytosol NOS (eNOS) was calculated from the measured activity of postmitochondrial supernatant by subtraction of the activity of the fraction of mitochondrial fragments in the 7000*g* supernatant fraction determined by cytochrome oxidase (COX) activity. The rates of NO production in mitochondria and cytosol per g of tissue were calculated taking into account 50 mg mitochondrial protein/g heart and of 100 mg cytosolic protein/g heart.

due to mitochondrial fragments present in the post-mitochondrial supernatant from the total post-mitochondrial supernatant NOS activity. Considering the determined protein contents of 50 mg mitochondrial protein/g heart (52,64) and of 100 mg cytosolic protein/g heart (52), the NO production by mtNOS accounts for about 90 % of total cellular NO production. The data clearly indicate that mtNOS and mitochondria are the main source of cellular NO in cardiomyocytes.

4.5. Heart NO production and mitochondrial NO steady state concentrations

The rate of NO production by heart mtNOS can be calculated taking into account the NO metabolism in the mitochondrial matrix (utilization term) and the NO release (diffusional term) as shows Eq. 1:

$$[+d[NO]/dt]_{mtNOS} = [-d[NO]/dt]_{utilization} + [-d[NO]/dt]_{release} \quad (1)$$

The diffusional term was measured as mitochondrial NO release (Table 1) and the NO matrix utilization term (Eq. 2) was calculated considering the two main NO utilization reactions: the termination reaction with O_2^- to yield peroxynitrite ($ONOO^-$) and the reduction by UQH_2 to produce nitroxyl anion (NO^-):

$$[-d[NO]/dt]_{utilization} = k_a [O_2^-] [NO] + k_b [UQH_2] [NO] \quad (2)$$
$$[-d[NO]/dt]_{release} = k_c [NO] \quad (3)$$

Equations 2 and 3, written for states 4 and 3, and expressed as a ratio (Eq. 4), give an useful form that relates ratios of mtNOS activity, NO steady state levels, NO utilization rates and NO diffusion rates (12).

$$[d[NO]/dt]_{mtNOS\text{-}st\ 4} / [d[NO]/dt]_{mt\ NOS\text{-}st\ 3} = \{[NO]_{st\ 4} (k_a [O_2^-]_{st\ 4} + k_b [UQH_2]_{st\ 4} + k_c)\} / \{[NO]_{st\ 3} (k_a [O_2^-]_{st\ 3} + k_b [UQH_2]_{st\ 3} + k_c)\} \quad (4)$$

Heart mitochondrial NO steady state concentration in state 3 was estimated as 200 nM NO, considering the mathematical model that describes cytochrome oxidase inhibition by NO (14) and the fraction of state 3 O_2 uptake (25 %) that is affected by arginine and NOS inhibitors (Table 4) (65). Consequently, the NO steady state level in state 4 is estimated as 366 nM NO, considering the 1.83 times higher NO release in state 4 (Table 1) than in state 3. Equation 4 can be solved by placing the NO steady states levels and available values for heart mitochondria, as follows: $[UQH_2]_{st\ 4} = 5.6 \times 10^{-4}$ M and $[UQH_2]_{st\ 3} = 0.85 \times 10^{-4}$ M (66); $[O_2^-]_{st\ 4} = 1.5 \times 10^{-10}$ M and $[O_2^-]_{st\ 3} = 0.32 \times 10^{-10}$ M (19); $k_a = 1.9 \times 10^{10}$ M^{-1} s^{-1} (67); $k_b = 2.1 \times 10^3$ M^{-1} s^{-1} (57); and $k_c = 0.5$ s^{-1} (12). Solved equation 4 indicates a rate of NO production of 0.26×10^{-6} M s^{-1} for state 3 and of 1.66×10^{-6} M s^{-1} for state 4, with a six times higher mtNOS activity in state 4 than in state 3, in a process likely driven by membrane potential. Similar results were obtained when liver-mitochondria NO production was considered (12).

Then, it is appropriate to think of mtNOS as a voltage-dependent enzyme, whose activity is regulated by mitochondrial membrane potential. It is interesting to note that up to the moment two mitochondrial activities, the enzymatic activity of mtNOS and the production of H_2O_2 (likely produced by the auto-oxidation of ubisemiquinone bound to a complex III protein and by disproportionation of O_2^-) have been recognized as sensitive to mitochondrial membrane potential. It seems that the electric field is able to regulate the conformation and reactivity of enzyme that are membrane constitutive proteins.

4.6. Heart mtNOS functional activity

Nitric oxide is an intercellular messenger and a regulator of respiration (14,57,68-70) and cellular processes. At nanomolar concentrations, NO exhibits two main effects on the mitochondrial respiratory chain: the competitive inhibition of cytochrome oxidase (14,68-70) and the stimulation of O_2^- production by inhibition of electron transfer at complex III (57). The ability of mtNOS activity to modulate by its product NO, mitochondrial O_2 uptake and H_2O_2 production is known as *mtNOS functional activity* (32,34,43,65). The biological situations in which the regulation of mitochondrial respiration by NO are considered relevant include hypoxia, ischemia-reperfusion, hypothyroidism, inflammation, apoptosis and aging. In the consideration of the effects of NO on mitochondrial functions, the production of NO by mtNOS is important by the vicinity of the NO source (mtNOS) and of the NO targets: cytochrome oxidase and ubiquinol-cytochrome c reductase.

Table 4 shows heart mtNOS functional activity in the inhibition of O_2 uptake and in the enhancement of H_2O_2 production. The effect of mtNOS activity on mitochondrial O_2 uptake is assayed at the maximal physiological rate of O_2 uptake and ATP synthesis (state 3 respiration). Respiratory rates are determined at maximal and minimal intramitochondrial NO concentrations. The first condition is achieved by supplementation with L-arginine and SOD,

Table 4. Heart mitochondrial NOS functional activity in the inhibition of O_2 uptake and in the enhancement of H_2O_2 production

Conditions	O_2 consumption [1] (ng-at O/min. mg protein)	H_2O_2 production [2] (nmol H_2O_2/min. mg protein)
Control	234 ± 11	0.70 ± 0.04
+ L-arginine (a)	199 ± 9	0.90 ± 0.03
+ NOS Inhibitor (b)	257 ± 12	0.68 ± 0.03
mtNOS functional activity	58 ± 15	0.22 ± 0.05
mtNOS functional activity (%)	25 %	31 %

6 mM malate and 6 mM glutamate were used as substrates [1] The data were taken from Valdez *et al* (64). Experiments were carried out with mitochondria in metabolic state 3 (0.5 mM ADP). Mitochondria were supplemented with 0.1 mM L-arginine and 1 μM SOD (a), or with 1 mM L-NNA and 25 μM HbO_2 (b). Mitochondrial NOS functional activity is defined as [(b) - (a)] or {[(b - a)/Control] x 100} [2] Data were taken from Boveris *et al.* (42) and Valdez *et al.* (64). Experiments were carried out with mitochondria in metabolic state 4. Mitochondria were supplemented with 0.1 mM L-arginine (a), or with 1 mM L-NNA. Mitochondrial NOS functional activity is defined as [(a) - (b)] or {[(a - b)/Control] x 100}

and the second by addition of a NOS inhibitor and of HbO_2. Supplementation of state 3 heart mitochondria with the mtNOS substrate and with SOD decreases the respiration rate by 15 %. L-arginine and SOD have synergistic effects in maximizing the intramitochondrial steady-state level of NO by providing the mtNOS substrate and by removing extramitochondrial $O_2^{\cdot-}$. Supplementation of the mitochondrial preparation with a NOS inhibitor (L-NNA) and HbO_2, a condition that minimize intramitochondrial NO levels, increase the O_2 consumption by 10 % in heart mitochondria. These effects are explained by the continuous production of NO by mtNOS and by the inhibition of cytochrome oxidase by NO. In this process, NO binds to the enzyme in its reduced form in a mechanism that is competitive with O_2 (14,68-70). The difference between the state 3 O_2 uptake with L-arginine and SOD and with NOS inhibitor and HbO_2 indicates the mtNOS functional activity in the inhibition of cytochrome oxidase activity. Concerning H_2O_2 production, the addition of L-arginine increases H_2O_2 production by 30 % in heart mitochondria in state 4, whereas the supplementation of the same preparation with L-NNA decreases H_2O_2 generation by 3 % (Table 4). The difference in H_2O_2 production between the conditions of maximal and minimal NO levels is known as the functional activity of mtNOS on the regulation H_2O_2 production. The effects on H_2O_2 production are again explained by the intramitochondrial NO steady-state concentrations and, in this case, by the NO inhibition of ubiquinol-succinate-cytochrome c reductase activity that enhances H_2O_2 production (65). The functional activity of mtNOS can be expressed as a percentage or fraction of state 3 O_2 consumption or state 4 H_2O_2 production.

Heart mtNOS functional activity is modified under pathological situations and after pharmacological treatments (65). Mitochondrial NOS functional activity changes reveal modifications of NO production by mtNOS. Treatment with the drug enalapril produced a marked increase (1.9-5.4 times) in NO production by heart, liver and kidney mitochondria and an enhancement in mtNOS functional activity, measured both through the O_2 uptake (1.5-2.6 times) and H_2O_2 production (1.9-3.5 times) (65).

5. CONCLUSIONS AND PERSPECTIVES

In summary, the mitochondrial metabolic state regulates the production of both H_2O_2 and NO. Interestingly, the redox state of the respiratory chain, particularly the level of ubisemiquinone, regulates H_2O_2 production, whereas mitochondrial membrane potential regulates NO production. Due to the fact that NO and H_2O_2 are diffusible molecules, these species jointly with ATP report the existence of a high mitochondrial energy charge to the cytosol. The regulation of mtNOS activity, an apparently voltage-dependent enzyme, at the physiological range of membrane potentials, makes mtNOS a regulable enzyme that in turn regulates mitochondrial O_2 uptake and H_2O_2 production (Figure 1). In this way, mitochondrial NO and H_2O_2 may signal and modulate genetic expression and cell metabolism in physiological and pathological conditions.

This view is in agreement with Lacza hypothesis that considers that mtNOS is a mitochondrial NOS-like oxygenase enzyme that uses the electron transport chain as an electron source instead of its own reductase domain (71). According to an emergent number of studies, the respiratory chain complexes are assembled into supercomplexes (72, 73). Recently, Schäfer *et al* (73) have confirmed the stoichiometries of I_1III_2 and $I_1III_2IV_1$ supercomplexes that had been proposed based on biochemical data, and they suggested that the supercomplexes $I_1III_2IV_1$ is a major physiological component of the respiratory chain in mammalian mitochondria. In addition, Persichini *et al* (74) reported that mtNOS is associated with cytochrome oxidase using electron microscopic immunolocalization and co-immunoprecipitation studies, and found that the association is due to the physical interaction of mtNOS with the C-terminal peptide of the Va subunit of cytochrome oxidase. The proximity between mtNOS and cytochrome oxidase is adequate to increase the functional coupling between the mitochondrial NO production and the cytochrome oxidase, as the sensing system and leading to a fine-tuning of cytochrome oxidase activity by NO. Moreover, the group of Poderoso (75) showed that not only complex IV proteins but also complex I proteins immunoprecipitate with intramitochondrial and translocated nNOS, which

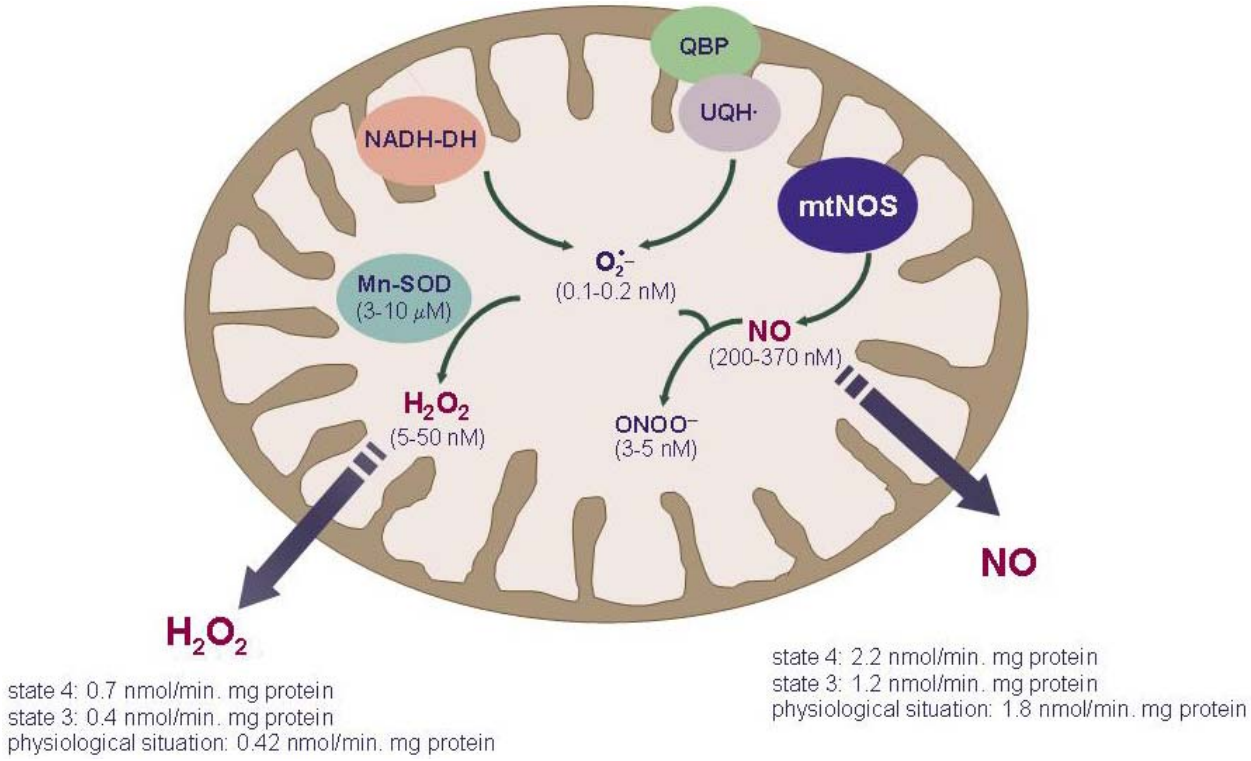

Figure 1. Diagram showing the metabolism of NO and H_2O_2 in rat heart mitochondria. The values below the symbols indicate physiological steady state concentrations. The arrows reaching outside the mitochondria indicate diffusion of H_2O_2 and NO to the cytosol and the values below H_2O_2 and NO represent rates of diffusion in the indicated conditions. QBP: ubiquinone binding protein; NADH-DH: NADH dehydrogenase; MnSOD: Mn-superoxide dismutase. Reproduced with permission from (12) and adapted to physiological situation of heart mitochondria.

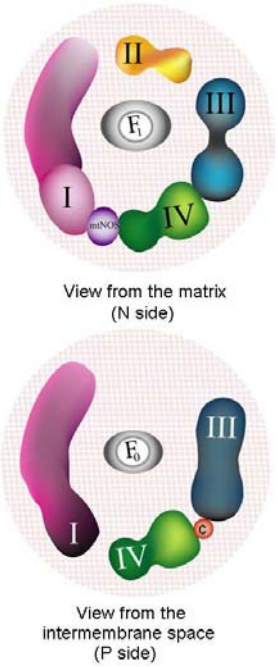

Figure 2. Illustration showing one of the likely position of mtNOS in the inner mitochondrial membrane, interacting with both Complex I and with Complex IV.

indicates direct molecular interactions between mtNOS and complexes I and IV proteins. Figure 2 shows a schematic representation of mtNOS probable location in the inner mitochondrial membrane, interacting with both Complex I and with Complex IV, and all three included in one of the respiratory chain supercomplexes.

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