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Mitochondrial metabolic states and membrane potential modulate mtNOS activity

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Abstract

The mitochondrial metabolic state regulates the rate of NO release from coupled mitochondria: NO release by heart, liver and kidney mitochondria was about 40–45% lower in state 3 (1.2, 0.7 and 0.4 nmol/min mg protein) than in state 4 (2.2, 1.3 and 0.7 nmol/min mg protein). The activity of mtNOS, responsible for NO release, appears driven by the membrane potential component and not by intramitochondrial pH of the proton motive force. The intramitochondrial concentrations of the NOS substrates, L-arginine (about 310 μM) and NADPH (1.04–1.78 mM) are 60–1000 times higher than their K_M values. Moreover, the changes in their concentrations in the state 4–state 3 transition are not enough to explain the changes in NO release. Nitric oxide release was exponentially dependent on membrane potential as reported for mitochondrial H_2O_2 production [S.S. Korshunov, V.P. Skulachev, A.A. Satarkov, High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett. 416 (1997) 15–18]. Agents that decrease or abolish membrane potential minimize NO release while the addition of oligomycin that produces mitochondrial hyperpolarization generates the maximal NO release. The regulation of mtNOS activity, an apparently voltage-dependent enzyme, by membrane potential is marked at the physiological range of membrane potentials.

Keywords: Mitochondrial NO; NO release; mtNOS; State 4–state 3 transition; Voltage-dependent enzyme activity; Mitochondrial membrane potential

1. Introduction

Nitric oxide (NO) production by the co-oxidation of L-arginine and NADPH by O_2 has been observed in mitochondrial membranes isolated from a series of mammalian organs: liver [1,2], heart [3,4], kidney [5], brain [6], diaphragm [7], and thymus [8]. The similar rates of NO production by mitochondria isolated from different organs strongly argue against the measured activity being caused by non-mitochondrial contaminants [9]. The responsible enzyme has been named mitochondrial nitric oxide synthase (mtNOS) [1,2,10] referring to its intracellular localization, in contrast to nNOS, iNOS and eNOS that were defined according to the cell type of origin, and later recognized as coded by different genes. The mitochondrial NOS isoenzyme is a constitutive protein of the mitochondrial inner membrane [11,12] that generates NO in a Ca^{2+}-dependent reaction [11–13]. Liver mtNOS has been sequenced and characterized as the α-isoform of nNOS, miristoylated in a different position from the one at eNOS and phosphorylated at the C-terminal region [12].

The operational concepts of mitochondrial metabolic states and respiratory control are based upon the rates of O_2 uptake by isolated mitochondria [14]. 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 biochemical mechanism of oxidative phosphorylation involves two main steps: the transduction of chemical redox potentials into an electrochemical H^+ gradient across the inner mitochondrial membrane and the ATP synthesis by the H^+ driven molecular rotor of F_1-ATPase. At the quasi-equilibrium of state 4, the electrochemical gradient regulates the rate of electron transfer

Abbreviations: mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; O_2, superoxide anion; ONOO, peroxynitrite; L-NMMA, L-N^G-monomethyl-L-arginine; Rh-123, Rhodamine 123; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; GSNO, S-nitrosoglutathione; DTT, dithiothreitol; SOD, superoxide dismutase

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by the reversibility of the vectorial reactions that increases H^+ into the P side. 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 deeply modifies the rates of electron transfer, the redox level of the components of the mitochondrial respiratory chain, the transmembrane potential and the sensitivity of cytochrome oxidase to NO [15].

It had been early recognized that coupled respiring mitochondria supplemented with substrates release NO to the reaction medium [1,2]. However, the relationship between the rates of NO release and mitochondrial energetics is not clear. In this context, the aim of this work was to determine the rates of NO production and release in mitochondrial metabolic states 4 and 3 and their relationship with mitochondrial membrane potential.

2. Materials and methods

2.1. Mitochondria isolation and mitochondrial membranes preparation

Heart, liver and kidney from Sprague–Dawley female rats (180–220 g) were excised and placed in an ice-cold homogenization media consisting of 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4 (MSTE). Heart, liver and renal cortex (separated from medulla and papilla) were homogenized at a ratio of 1 g tissue/9 ml of MSTE. The homogenate was centrifuged at 700×*g* for 10 min to discard nuclei and cell debris and the supernatant centrifuged at 7000×*g* for 10 min to precipitate mitochondria that were washed with MSTE [16]. Mitochondrial membranes were obtained by twice freezing and thawing the mitochondrial preparation, and were homogenized by passage through a tuberculin syringe with a needle.

2.2. Oxygen consumption

Oxygen uptake was determined polarographically with a Clark-type electrode in a 1.5 ml chamber at 37 °C, in an air-saturated respiration medium consisting of 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, 5 mM phosphate buffer, 4 or 2 mM or 0 $MgCl_2$ (for liver, kidney or heart mitochondria, respectively), 20 mM Tris–HCl (pH 7.0, 7.4 and 7.8) and 1 mg of mitochondrial protein. Respiratory rates were determined with either 6 mM malate and 6 mM glutamate or 8 mM succinate as respiratory substrates for complex I or complex II, respectively. State 3 active respiration was established by addition of 0.5 mM ADP. Oxygen uptake was expressed in ng at O/min mg protein [16].

2.3. Measurement of nitric oxide production and release

Nitric oxide generation was measured in coupled mitochondria and in mitochondrial membranes by following the oxidation of oxyhemoglobin (HbO_2) to methemoglobin at 37 °C, in air saturated reaction medium (185 μM O_2). The NO assay was performed using a Beckman DU 7400 diode array spectrophotometer in which the active wavelength is set at 577 nm and the reference wavelength at the isosbestic point at 591 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) [17,18]. The method is based on the original assay developed by Murphy and Noack [17] for perfused organs in which the HbO_2 γ band is used to follow NO production and it has been adapted to the hemoprotein α band for its use with neutrophils and lymphocytes [19]. The α band is more suitable for 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 [18].

The suspending medium for coupled mitochondria (0.2–0.5 mg protein/ml) was the respiration medium added with 30 μM HbO_2 heme, with respiratory substrates and ADP as described for respiration measurements. Experiments including the addition of 4 μM Cu,Zn-SOD (to avoid interference by O_2^-) and/or 0.1 μM catalase (to avoid HbO_2 oxidation by H_2O_2) were carried out to establish the specificity of the NO reaction with HbO_2. Recovery experiments using 5 μM S-nitrosoglutathione (GSNO) and 50 μM dithiothreitol (DTT) were also performed.

The reaction medium used with mitochondrial membranes (0.5–0.8 mg protein/ml) was 50 mM KOH–HEPES (pH 4.5 to 5.5) or 50 mM phosphate (pH 6.5 to 7.5) or 50 mM Tris–HCl (pH 7.5 to 8.5), 1 mM L-arginine, 1 mM $CaCl_2$, 100 μM NADPH, 10 μM DTT, 4 μM Cu,Zn-SOD, 0.1 μM catalase and 30 μM HbO_2. Controls adding 1 mM L-N^G-monomethyl-L-arginine (L-NMMA) as inhibitor of mtNOS were performed in all cases to consider only the L-NMMA-sensitive HbO_2 oxidation as due to NO formation. Addition of L-NMMA inhibited by about 90% the rate of HbO_2 oxidation [18–21]. The absorbance changes that were inhibitable by L-NMMA were expressed as nmol NO/min mg protein, considering the stoichiometric reaction of one HbO_2 with one NO.

2.4. Spectrofluorometric determination of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_{mit}$) was determined by measuring Rhodamine 123 (Rh-123) fluorescence at 503 nm→527 nm with a Hitachi F-3010 spectrofluorometer at 37 °C [22–24]. Rhodamine-123 was dissolved in ethanol and its concentration assayed spectrophotometrically at 507 nm ($\varepsilon = 101\ mM^{-1}\ cm^{-1}$). The ethanol concentration in the assayed mitochondrial preparations was kept below 0.2% (v/v).

The fluorescence of the media (150 mM sucrose, 4 mM $MgCl_2$, 5 mM potassium phosphate, 30 mM KOH–HEPES pH 7.4) containing 0.1 μM Rh-123 was determined before addition of mitochondria. This measurement was used as an indication of the total dye concentration ($[Rh\text{-}123]_{total}$, in nmol/μl). Rat liver mitochondria (0.5–0.7 mg/ml) were added to the media in the presence of 8 mM succinate, 0.5 mM ADP, 2 μM rotenone, 1 μM antimycin, 1 μM oligomycin, or 3 μM CCCP. After an equilibrium had been reached, the fluorescence of the suspension was measured and the contents of the cuvette were centrifuged at 15000×*g* to pellet the mitochondria. The Rh-123 concentration remaining in the media ($[Rh\text{-}123]_{out}$, in nmol/μl) was calculated from the fluorescence values of the supernatant. The initial total amount of Rh-123 in the cuvette ($[Rh\text{-}123]_{total}$) and the amount remaining in the media ($[Rh\text{-}123]_{out}$) were used to calculate by subtraction the total amount of Rh-123 taken up by mitochondria ($[Rh\text{-}123]_{mit}$, in nmol/mg protein). The concentration of free Rh-123 in the matrix ($[Rh\text{-}123]_{in}$, in nmol/μl) was calculated using the following equation, and the binding partition coefficients at 37 °C ($K_i = 26$ μl/mg, $K_o = 120$ μl/mg) [24]:

$$[Rh{-}123]_{mit} = Ki\,[Rh{-}123]_{in} + Ko\,[Rh{-}123]_{out}$$

Mitochondrial membrane potentials (negative inside) were calculated by the electrochemical Nernst–Guggenheim equation: $\Delta\psi = 59 \log ([Rh\text{-}123]_{in}/[Rh\text{-}123]_{out})$.

2.5. Chemicals

All the used reagents were from Sigma Chemical Co., St. Louis, MO, USA.

2.6. Statistics

Results are reported as mean values ± S.E.M. of three to six independent experiments.

3. Results

3.1. The state 4 to state 3 transition regulates mitochondrial NO release

The NO release from coupled isolated mitochondria was determined by following the oxidation of HbO_2 without exogenous supplementation of Ca^{2+}. The intramitochondrial concentration of Ca^{2+} in mitochondrial preparations is sufficient to maintain a mtNOS activity [2,13]. Fig. 1 illustrates the rate of HbO_2 oxidation during the 2 initial minutes of the reaction

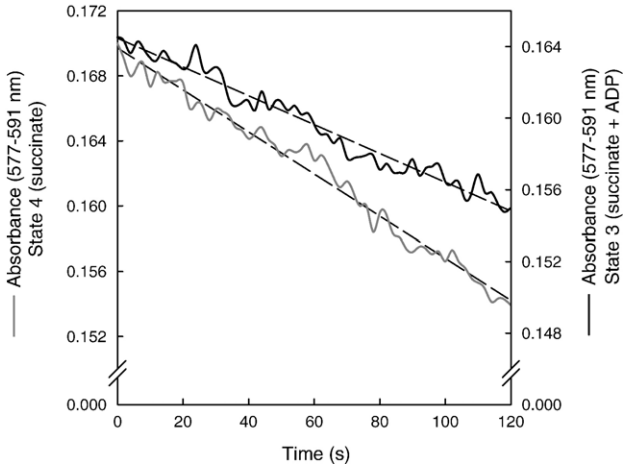

Fig. 1. Determination of NO release from rat heart mitochondria in states 4 (gray line) and 3 (black line) by measuring HbO_2 oxidation (577–591 nm): mitochondria (0.3 mg protein/ml) were suspended in the respiration medium and added with 30 μM HbO_2, 8 mM succinate and 0.5 mM ADP.

when heart mitochondria were added with succinate (state 4) or with succinate and ADP (state 3). The rate of NO release from coupled 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 transition from state 4 to state 3 (Table 1). Similarly, in liver and kidney mitochondria the rates of NO release were 40–45% lower in state 3 than in state 4 (Table 1). The addition of 4 μM Cu,Zn-SOD and 0.1 μM catalase to the reaction medium did not modify the rate of NO release by heart, liver and kidney mitochondria both in state 4 and state 3, using succinate or malate-glutamate as substrates (data not shown).

The addition of 5 μM GSNO with 50 μM DTT to the reaction medium [25] produced a sustained HbO_2 oxidation due to NO release (about 1.8 μM/min) (Table 2). When mitochondria were added, the total NO release rate measured was lower than the one expected from the addition of rates of mitochondrial NO release and of NO production by the GSNO system: about 45% with mitochondria in state 4 and about 50% with mitochondria in state 3 (Table 2). These values indicate an active NO diffusion to the mitochondria and an efficient utilization in the matrix.

Table 1
Nitric oxide release and oxygen consumption in coupled heart, liver and kidney mitochondria

	NO release (nmol/min mg protein)			Oxygen consumption (ng at O/min mg protein)		
	State 4	State 3	NOR [a]	State 4	State 3	RCR [b]
Heart						
Mal-Glu	2.20±0.07	1.25±0.04	1.8	45±4	248±11	5.5
Succ	2.23±0.06	1.22±0.05	1.8	98±8	394±35	4.0
Liver						
Mal-Glu	1.32±0.02	0.72±0.1	1.8	24±2	162±10	6.8
Succ	1.35±0.05	0.73±0.1	1.8	58±5	274±20	4.7
Kidney						
Mal-Glu	0.52±0.05	0.31±0.02	1.7	42±4	190±11	4.5
Succ	0.73±0.04	0.41±0.05	1.8	67±7	268±20	4.0

Additions: 6 mM Malate (Mal) – 6 mM Glutamate (Glu) or 8 mM Succinate, 0.5 mM ADP. Values are means±S.E.M. of six independent experiments.
[a] NOR: nitric oxide release ratio=(state 4/state 3) NO release.
[b] RCR: respiratory control ratio=(state 3/state 4) O_2 consumption.

Table 2
Nitric oxide release from rat heart mitochondria in the absence or in the presence of a NO generating system

Addition	NO release (μM/min)
Mitochondria in state 4	1.07±0.02
Mitochondria in state 3	0.61±0.01
GSNO+DTT	1.79±0.09
Mitochondria in state 4+GSNO+DTT	1.28±0.01
Mitochondria in state 3+GSNO+DTT	1.19±0.02

Values are means±S.E.M. of three independent experiments.
Additions: 8 mM succinate, 0.5 mM ADP, 5 μM GSNO, 50 μM DTT.

The ratio of NO release in states 4 and 3 was calculated to express the relationship between mitochondrial metabolic state and NO release, in a way similar to the classic concept of respiratory control. The values were 1.7–1.8 for mitochondrial preparations that exhibited respiratory control ratios in the range of 4.0 to 6.8 and high rates of O_2 uptake (Table 1).

3.2. Mitochondrial membrane potential and NO release from liver mitochondria

Fig. 2 illustrates the time course of Rh-123 fluorescence upon consecutive additions using rat liver mitochondria. Addition of succinate to rotenone de-energized mitochondria established state 4 and produced a rapid decrease of about 45% in fluorescence due to probe uptake by mitochondria (Fig. 2A). The following ADP addition produced a moderate increase in fluorescence, corresponding to mitochondrial state 3. Then, the addition of oligomycin (an inhibitor of ATP synthase) produced a decrease in Rh-123 fluorescence higher than the one produced by the addition of succinate. This effect corresponds to an hyperpolarization due to the blockade of H^+ flow through F_0-ATPase. Rhodamine-123 fluorescence was also recorded (Fig. 2B) in the presence of antimycin (an inhibitor of complex III), and CCCP (an uncoupler of oxidative phosphorylation). Antimycin allowed a partial recovery of fluorescence caused by the membrane potential decrease as a consequence of $\Delta\mu_{H+}$ dissipation, and the consecutive supplementation with CCCP allowed an almost total recovery of fluorescence. The corresponding membrane potentials (Table 3) were calculated by the Nernst–Guggenheim equation, as described in Materials and methods.

In order to correlate the changes in mitochondrial membrane potential with the mitochondrial NO production, NO release was determined in the same experimental conditions that Rh-123 fluorescence was measured. Table 3 shows that agents that decrease or abolish membrane potential are able to minimize NO release (0.48 nmol NO/min mg protein), while the addition of oligomycin that produces mitochondrial hyperpolarization generates the maximal measured NO release: 1.4 nmol NO/min mg protein.

Nitric oxide release from liver mitochondria shows a very strong and exponential dependence on $\Delta\psi$ (Fig. 3). This

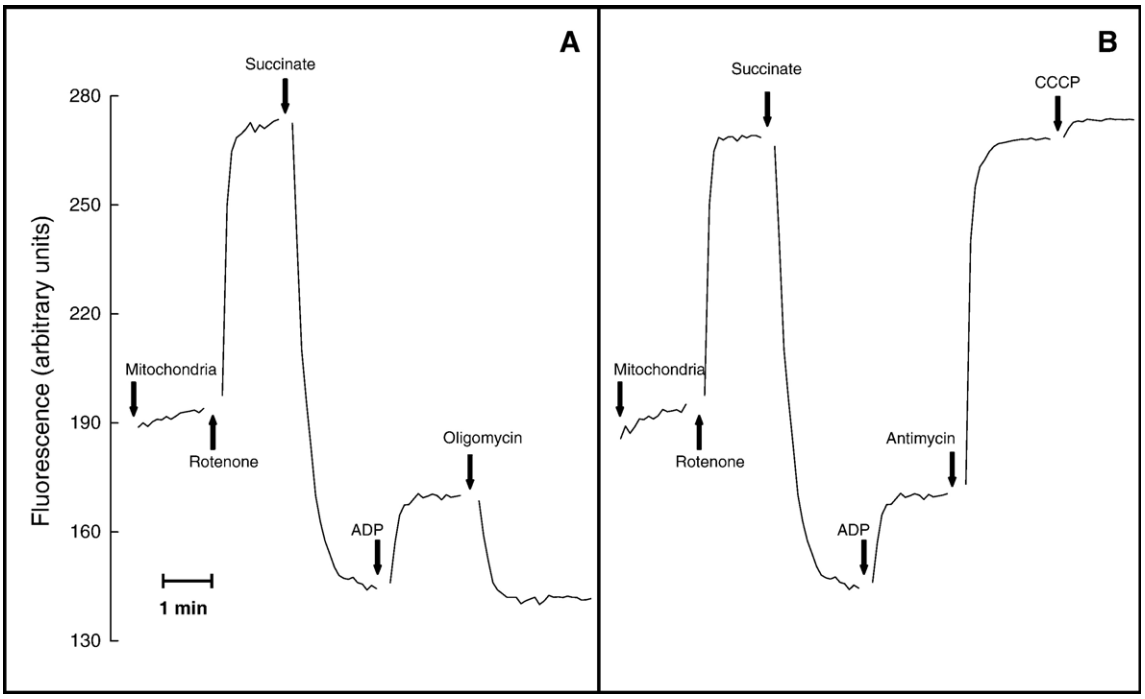

Fig. 2. Time course of Rhodamine 123 (503 nm→527 nm) fluorescence upon several additions. Mitochondria suspension (0.72 mg protein/ml) was added to the reaction medium containing 0.1 μM Rh-123. Further additions were 2 μM rotenone, 8 mM succinate and 0.5 mM ADP, and 1 μM oligomycin (A) and 1 μM antimycin and 3 μM CCCP (B).

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.

3.3. Effect of pH on the NO production by liver and kidney mitochondrial membranes

The pH dependence of the mtNOS activity of liver and kidney mitochondrial membranes was determined to evaluate an eventual effect of the intramitochondrial pH change in the rates of NO release in states 4 and 3. The highest mtNOS activity was observed at pH 7.4 with a NO production of 1.37±0.16 and 0.96±0.05 nmol/min mg protein for liver and kidney mitochondrial membranes, respectively (Fig. 4).

Table 3
Nitric oxide release and membrane potential of coupled rat liver mitochondria in states 4 and 3 and in the presence of inhibitors of mitochondrial function

Experimental condition	NO release (nmol/min mg protein)	Membrane potential (mV)
Liver mitochondria (state 1)	0.64±0.10	92±4
+mal-glu (state 4)	1.32±0.02	167±6
+mal-glu+ADP (state 3)	0.72±0.10	152±7
+succ (state 4)	1.35±0.05	174±6
+succ+ADP (state 3)	0.73±0.10	161±5
+succ+oligomycin	1.41±0.05	182±6
+succ+antimycin+CCCP	0.48±0.04	59±5

Additions: 8 mM succinate (succ), 6 mM malate (mal) – 6 mM glutamate (glu), 0.5 mM ADP, 1 μM oligomycin, 1 μM antimycin, and 3 μM CCCP.
Values are means±S.E.M. of four or six independent experiments.

4. Discussion

This study deals with two related phenomena: the regulation of mtNOS activity by mitochondrial membrane potential and the NO release from mitochondria. The NO produced by mtNOS is partially metabolized in the organelle and released to the surrounding medium. The rate of mitochondrial NO release directly depends on intramitochondrial NO steady state levels, following a simple diffusion process with the mitochondrial membrane permeable to NO. Intramitochondrial NO steady state levels are understood as sustained by the NO production of mtNOS activity (in the absence of extramitochondrial NO

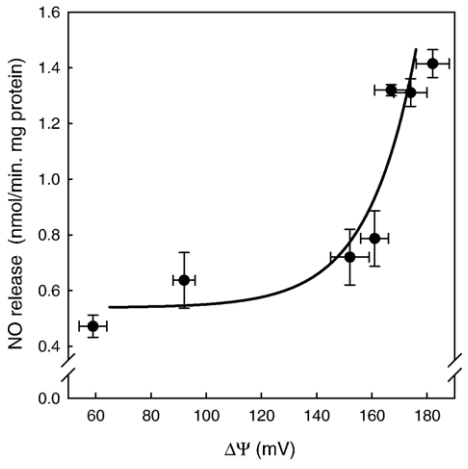

Fig. 3. Exponential relationship between membrane potential ($\Delta\psi$) and NO release in liver mitochondria. Values are means±S.E.M. of mitochondrial NO release (in nmol NO/min mg protein) and $\Delta\psi$ (mV).

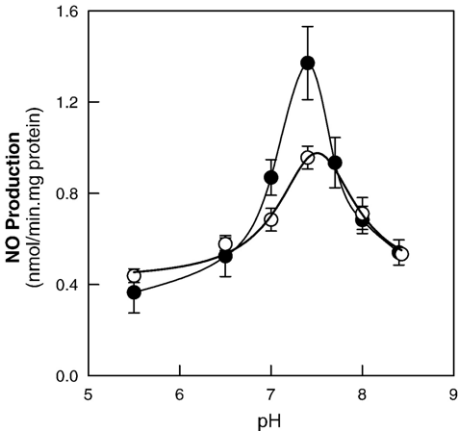

Fig. 4. pH dependence of mtNOS activity in mitochondrial membranes from rat liver (●) and kidney (○). Values (in nmol NO/min mg protein) are means ± S.E. M. of four independent experiments.

sources, as previously considered [26]). For an enzyme located at the inner mitochondrial membrane [11,12] as mtNOS, the changes in activity in the transition from state 4 to state 3 ought to be analyzed taking into account both the electrical and pH terms of the chemiosmotic equation ($\Delta P = \Delta\psi + 59\ \Delta$pH).

Mitochondrial NO release rates were 40–45% lower in state 3 than in state 4. This fact is consistent with the findings published by Brookes et al. [27] who showed that mitochondrial respiration is more sensitive to exogenous NO in state 3 than in state 4, at physiological O_2 levels. In the present study, recovery experiments using GSNO as a NO generating system showed an active NO consumption 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 [28], being this latter a species capable to consume NO in a diffusion-controlled reaction to yield $ONOO^-$ [26].

It is worth noting that the decrease in NO release in the state 4 to state 3 transition is opposite to what could be expected from the slight matrix acidification that follows the considered transition and the pH dependence of mtNOS activity. 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 [29]. Taking into account matrix pH values at state 3 (about 7.5) and state 4 (about 7.8), liver and kidney mtNOS show a 57% and 17% higher activity at pH 7.5 than at pH 7.8 (Fig. 4). Additionally, heart mtNOS activity was also observed higher (15%) at pH 7.5 than at pH 7.8 [7]. These results indicate that the higher NO release in state 4 is not due to pH changes.

Moreover, the difference observed in NO release in states 4 and 3 cannot 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. Regarding NADPH concentration, Giulivi and co-workers [2] determined a NADPH content of 6–8 nmol/mg liver mitochondrial protein by HPLC analysis (about 3–4 mM NADPH). Considering a total NADP(H) content of 3.91 nmol/mg protein and the content of this nucleotide in the reduced form in state 4 (91%) and in state 3 (53%) [14,30], liver mitochondria NADPH concentrations in state 4 and 3 were calculated assuming an intramitochondrial volume of 2 μl/mg protein. The values obtained were 1.78 mM in state 4 and 1.04 mM in state 3. In view of NOS K_M value for NADPH (0.1–1 μM) [31] and the content of this reduced nucleotide, the intramitochondrial NADPH concentration is not rate limiting and cannot explain the difference observed in the NO release rate in state 4 and 3. Mitochondrial L-arginine availability is neither rate limiting NO release by mitochondria. Considering an intramitochondrial volume of 2 μl/mg protein and the content of mtNOS substrate L-arginine in the mitochondrial matrix is about 0.62 nmol/mg [32], L-arginine concentration in the mitochondrial matrix results 310 μM, 60 times higher than the reported K_M of liver mtNOS (5 μM) [33].

In addition, the difference observed in the HbO_2 oxidation rates (NO release from mitochondria) in state 4 and state 3 (Fig. 1) is not due to inadequate HbO_2 concentration in the situation of active respiration (state 3). Coupled rat heart mitochondria supplemented with succinate or succinate and ADP showed the highest rates of O_2 consumption both in state 4 and state 3: 98 ng at O/min mg protein = 49 nmol O_2/min mg protein, and 394 ng at O/min mg protein = 197 nmol O_2/min mg protein, respectively. Nitric oxide release experiments were performed using 0.3 mg heart mitochondrial protein/ml and considering HbO_2 oxidation in the 2 initial minutes. In these conditions, heart mitochondria consumed 29.4 nmol O_2/ml in state 4 and 118 nmol O_2/ml in state 3. Considering that the O_2 dissolved in the reaction media at 37 °C is 185 μM, and that the O_2 bound to hemoglobin is 30 μM, the total O_2 content in the media is 215 nmol O_2/ml. This O_2 concentration is 1.8 times higher than the O_2 consumed in state 3 by heart mitochondria in 2 min. Under our experimental conditions, the O_2 concentration is high enough and the observed differences in HbO_2 oxidation rates are not due to O_2 exhaustion in the reaction medium and to O_2 depletion from HbO_2 as a consequence of mitochondrial respiration.

In the present report, the NO release was strongly increased, by a factor of about 2, in the transition from state 3 to state 4. Similarly, Boveris and Cadenas (1977; 1980) showed that rat liver and pigeon heart mitochondria release H_2O_2 at rates that markedly depend on the metabolic state [34,35]. The rate of H_2O_2 release was 4–6 times higher in state 4, which is characterized by a high reduction of the components of the respiratory chain, and lower in state 3, where the carriers are largely oxidized [34]. This fact indicated that a component of the respiratory chain, markedly changing its redox steady state level in the state 4–state 3 transition, is the H_2O_2 generator [34]. In agreement, ion movements through the inner mitochondrial membrane have been recognized to modify the rates of H_2O_2 production, in antimycin-supplemented mitochondria which supports the concept that there is a double regulation by the membrane potential and by the Complex III reduction level that modulate ubisemiquinone auto-oxidation and O_2^- production [35].

Considering the found relationship between NO release from intact liver mitochondria and mitochondrial membrane potential (Fig. 3), the regulation of mtNOS activity appears to be more

important at physiological membrane potential, where small changes in mitochondrial membrane potential produce significant changes in NO release from intact mitochondria. Additionally, in a previous observation of the regulation of an inner membrane enzymatic activity by membrane potential, Korshunov et al. (1997) 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 $\Delta\psi$ [36]. The observed regulation showed an exponential dependence of the 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. The marked decrease of NO release observed when mitochondrial membrane potential was collapsed by the uncoupler CCCP is a strong evidence for the location of mtNOS in the mitochondrial inner membrane. This concept is in agreement with the report by Dedkova et al. [37] who showed that collapsing the mitochondrial membrane potential with FCCP or blocking mitochondrial Ca^{2+}-uniporter with ruthenium red, inhibited NO production.

It is then clear that a high H^+ electrochemical potential across the inner membrane is linked to high production rates of NO and O_2^-, this latter the stoichiometric precursor of 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 notion that mitochondria-specific NOS (mtNOS) is associated to the inner membrane and is regulated by mitochondrial membrane potential, as a voltage-dependent enzyme activity.

Acknowledgements

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