

Mitochondrial nitric oxide production supported by reverse electron transfer



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FMN

flavin mononucleotide

mtNOS

mitochondrial nitric oxide synthase

RET

reverse electron transfer

HbO₂

oxyhemoglobin

Δp

proton motive force

ΔμH⁺

proton electrochemical potential

O₂^{•-}

superoxide anion

ABSTRACT

Heart phosphorylating electron transfer particles (ETPH) produced NO at 1.2 ± 0.1 nmol NO·min⁻¹ mg protein⁻¹ by the mtNOS catalyzed reaction. These particles showed a NAD⁺ reductase activity of 64 ± 3 nmol min⁻¹ mg protein⁻¹ sustained by reverse electron transfer (RET) at expenses of ATP and succinate. The same particles, without NADPH and in conditions of RET produced 0.97 ± 0.07 nmol NO·min⁻¹ mg protein⁻¹. Rotenone inhibited NO production supported by RET measured in ETPH and in coupled mitochondria, but did not reduce the activity of recombinant nNOS, indicating that the inhibitory effect of rotenone on NO production is due to an electron flow inhibition and not to a direct action on mtNOS structure. NO production sustained by RET corresponds to 20% of the total amount of NO released from heart coupled mitochondria. A mitochondrial fraction enriched in complex I produced 1.7 ± 0.2 nmol NO·min⁻¹ mg protein⁻¹ and reacted with anti-75 kDa complex I subunit and anti-nNOS antibodies, suggesting that complex I and mtNOS are located contiguously. These data show that mitochondrial NO production can be supported by RET, and suggest that mtNOS is next to complex I, reaffirming the idea of a functional association between these proteins.

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1. Introduction

In mitochondria, nitric oxide (NO) is synthesized from L-arginine, NADPH and O₂ in a reaction catalyzed by mitochondrial nitric oxide synthase (mtNOS) [1,2]. Giulivi and coworkers [3] sequenced rat liver mtNOS and identified the enzyme as an inner

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membrane integral protein and as the α-splice variant of the nNOS transcript, myristoylated and phosphorylated. Decisive evidence was provided by Kanai et al. [4] with the electrochemical determination of Ca²⁺-induced NO release from a single mouse heart mitochondrion, a process that was abolished in nNOS^{-/-} knockout mice. NO production by heart mitochondria has been observed in mitochondrial membranes [5,6] and in coupled mitochondria [7,8]. Finocchietto et al. [9] have shown that the NO produced by translocated nNOS (mtNOS) is the insulin-dependent signaling molecule that controls mitochondrial O₂ utilization. Mitochondrial NO production accounts for about 60% of total heart NO generation [6] and it is produced by mitochondrial membranes

at a rate of 1.0–1.5 nmol NO·min⁻¹ mg protein⁻¹ and kept at a steady state level of 200–350 nM in the mitochondrial matrix [7,8,10]. At sub-micromolar concentrations, NO is a physiological regulator of mitochondrial functions [1,2,8] through two main effects on the mitochondrial respiratory chain: the competitive inhibition of cytochrome oxidase (complex IV) [11–13] and the inhibition of electron transfer between cytochromes *b* and *c* at complex III [14–16]. In addition, NO participates in the intramitochondrial diffusion limited reaction in which NO itself and superoxide anion (O₂⁻) react to produce peroxynitrite (ONOO⁻) [17].

Nitric oxide production by rat liver and brain mtNOS has been associated with complex I activity [18]. This finding agrees with the results obtained by Franco et al. [19] who have shown that complex I proteins from rat liver mitochondria co-immunoprecipitate with mtNOS. The association between mtNOS and complex I and IV proteins is compatible not only with the concept of respiratory chain supercomplexes formation with strong protein-protein interactions [20–23], but also with the dependence of mtNOS activity on the metabolic states and membrane potential [7,8,10].

Mitochondrial complex I (NADH-ubiquinone oxidoreductase) catalyzes the transfer of two electrons from NADH, via flavin mononucleotide (FMN) and a series of iron-sulfur centers (Fe-S) to ubiquinone (UQ) in a reaction associated with proton translocation across the inner membrane, contributing to the proton-motive force (Δp) [24,25]. Mitochondrial complex I reaction is reversible: the complex is able to reduce NAD⁺ in the presence of succinate, in a thermodynamically non spontaneous reaction, associated to the energy of ATP hydrolysis. The physiological occurrence of the reaction was early recognized by Chance and Hollunger [26] and by Klingenberg and colleagues [27] and was termed reverse electron transfer (RET). Complex I is then a reversible enzyme [28,29] that switches sharply between the forward and the reverse reactions through a point at which the free energy of the redox reaction is equal and opposite to that for proton translocation [30]. The point of equilibrium makes the reaction as thermodynamically reversible and energetically efficient.

Considering that complex I is a reversible enzyme and that mtNOS could use the electrons derived from succinate dehydrogenase to produce NO, the aim of this work was to study the NO production supported by reverse electron flow, using phosphorylating electron transfer particles (ETPH). In these bovine heart inside-out vesicles, mitochondrial inner membrane has a reverse orientation with the NADH dehydrogenase center of complex I and the F₁-ATPase exposed to the solutes in the surrounding medium. Taking into account that mtNOS is located at the inner mitochondrial membrane [2,31,32], this enzyme is exposed to the surrounding medium in ETPH particles. Thus, these vesicles are suitable for the study of the mtNOS activity supported by reverse electron transfer.

2. Materials and methods

2.1. Chemicals

Cu,Zn-superoxide dismutase (S7571, from bovine erythrocytes), catalase (C40, from bovine liver), recombinant nNOS (N3033, from rat brain) and other chemicals were from Sigma Chemical Co (St. Louis, MO). Anti-nitric oxide synthase antibodies (anti-nNOS antibody H-299) were from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-75 kDa complex I subunit antibodies (anti-Ndfs1 antibody ab22094) were from Abcam (Cambridge, UK). Other reagents were of analytical grade.

2.2. Bovine heart mitochondria isolation

Bovine heart mitochondria were obtained from slaughterhouse material according to the general procedure described by Blair [33] and Cadenas [34]. Beef heart left ventricles were cut in slices with a knife and chopped for 15 s. About 100 g of left ventricle were added with 200 ml of homogenization medium containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.80 (MSTE) and homogenized for 30 s at maximum speed (six times of 5 s each) with a manual blender (Braun, Minipimer MR 400). The pH was continuously maintained at 7.50 by addition of 1 M Trizma base and the left ventricle was homogenized in a glass-Teflon homogenizer. The homogenates were centrifuged in a refrigerated centrifuge (Sorvall-Instruments-Du Pont, Model RC5S) at 1200g for 15 min to discard nuclei and cell debris. The supernatant containing mitochondria was passed through a double layer of cheesecloth and the sediment was discarded. The supernatant was centrifuged at 16 000g for 15 min and the pellet was suspended in MSTE medium (1:4), homogenized and centrifuged at 16 000g for 20 min. The pellet obtained was suspended in a medium containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, pH 7.50 (MST), 1 mM ATP, and 15 mM MgCl₂, in a 1:4 proportion. This suspension was stored at -20 °C up to the following day. All the operations were performed at 0–4 °C [35,36].

2.3. Bovine heart inside-out submitochondrial particles (ETPH) preparation

Phosphorylating electron transfer particles were prepared by sonicating the thawed mitochondria (20 mg protein·ml⁻¹) in MST added with 1 mM ATP and 15 mM MgCl₂, six times for 10 s with 30 s intervals. The suspension was centrifuged at 15 500g for 10 min and the supernatant was centrifuged at 140 000g for 40 min [37] using an ultracentrifuge (Beckman Optima XL-90 ultracentrifuge, Beckman, USA). The inside-out particles were suspended in MST and kept at -80 °C until enzymatic determinations. This preparation is named “ETPH” in this paper. Protein concentration was determined with the Folin reagent using bovine serum albumin (BSA) as standard.

2.4. Preparation of complex I enriched mitochondrial fraction

Complex I was isolated following the technique described by Hatefi and Rieske [36] with some modifications [34]. According to these techniques, complex I was isolated following four steps: washing of the mitochondrial fraction, separation of the cytochrome oxidase fraction, dialysis and fractionation with deoxycholate-ammonium acetate. All the steps were carried out at 0–4 °C. After all these procedures were completed, the reddish-brown sediment, which is enriched in complex I, was obtained and suspended in 0.67 M sucrose, 0.05 M Tris-HCl, 1 mM histidine, pH 8.00 and stored at -80 °C. Under our experimental conditions, this fraction showed a rotenone sensitive-NADH-Q₁ reductase activity of 260 ± 25 nmol NADH oxidized·min⁻¹ mg protein⁻¹.

2.5. Rat heart mitochondria isolation

Rat heart mitochondria were obtained from heart homogenates by differential centrifugation in a Sorvall RC5C centrifuge (Sorvall-Instruments-Du Pont, Model RC5S). Care and handling of animals were performed according to international animal experimentation guidelines. Rats were anesthetized in a CO₂ atmosphere; the hearts were isolated, washed and weighed. Hearts were minced in an ice-cold medium containing 250 mM sucrose, 2 mM EGTA, 5 mM Tris-HCl, pH 7.40 (STE). This procedure was repeated and then the

sample was divided in two petri dishes, in one of them the buffer was removed and filled with a buffer containing 250 mM sucrose, 2 mM EGTA, 0.5% (w/v) BSA, 5 mM MgCl₂, 1 mM ATP, 2.5 U. ml⁻¹ type XXIV bacterial proteinase, 5 mM Tris-HCl, pH 7.40 (STE-2). After 4 min of incubation at 4 °C, both samples were homogenized in 1:10 STE buffer in a glass-Teflon homogenizer and centrifuged at 8000g for 10 min. The obtained pellet was resuspended in ice-cold STE buffer and centrifuged at 700g for 10 min. The sediment was discarded and both supernatants were mixed and centrifuged at 8000g for 10 min. The pellet of this centrifugation was resuspended in STE buffer and centrifuged at 8000g for 10 min. The obtained pellet containing the mitochondrial fraction was rinsed and resuspended in STE buffer. All the operations were performed at 0–2 °C [38].

2.6. Oxygen consumption by ETPH

Oxygen uptake was measured polarographically using a Clark type electrode (Oroboros Oxygraph, Graz, Austria) located in a 1.5-ml chamber at 30 °C. ETPH suspension (0.2–0.4 mg protein. ml⁻¹) were added to a medium consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM KH₂PO₄/K₂HPO₄, 2 mM MgCl₂, pH 7.40, in an air-saturated reaction medium (220 μM O₂), using 6 mM malate and 6 mM glutamate, 0.4 mM NADH or 7 mM succinate. The effect of 20 μM cytochrome c³⁺ was tested. Respiration rates are expressed as ng-at O. min⁻¹ mg protein⁻¹ [39].

2.7. Mitochondrial electron transfer activities

NADH-O₂ oxidoreductase, NADH-cytochrome c reductase (complex I-III), succinate-cytochrome c reductase (complex II-III), and cytochrome oxidase (complex IV) activities were determined spectrophotometrically using a Beckman DU 7400 diode array spectrophotometer, at 30 °C [40,41]. Complex I-III, II-III and IV were followed at 550–540 nm ($\epsilon = 19 \text{ mM}^{-1} \text{ cm}^{-1}$). NADH-cytochrome c reductase and succinate-cytochrome c reductase were determined by following the reduction of cytochrome c³⁺ to cytochrome c²⁺, in a reaction medium containing MST, inside-out particles (0.01–0.02 mg protein. ml⁻¹), 0.2 mM NADH or 7 mM succinate as substrates, 25 μM cytochrome c³⁺ and 0.5 mM KCN. Enzymatic activities are expressed as nmol reduced cytochrome c. min⁻¹ mg protein⁻¹. Cytochrome oxidase was determined in the same buffer supplemented with 60 μM cytochrome c²⁺, this latter prepared by reduction of cytochrome c³⁺ with Na₂S₂O₄, followed by Sephadex G-25 chromatography. The rate of cytochrome c²⁺ oxidation was determined as the pseudo-first order reaction constant *k*. mg protein⁻¹ [40,42] and expressed as nmol cytochrome c oxidized at 20 μM cytochrome c per minute per milligram protein, that is in accordance with physiological electron transfer rates [12,43]. NADH-O₂ oxidoreductase activity was determined by following the oxidation of NADH at 340–380 nm ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) with NADH (0.2 mM) and ETPH (0.03–0.04 mg protein. ml⁻¹) suspended in MST. Results are expressed as nmol. min⁻¹ mg protein⁻¹ [30].

2.8. Succinate-NAD⁺ reductase activity

Energy-dependent succinate-NAD⁺ reductase activity was measured spectrophotometrically at 340–380 nm ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) using a Beckman DU 7400 diode array spectrophotometer, at 37 °C. ETPH (0.3 mg protein. ml⁻¹) suspended in MST were pre-incubated at 37 °C for 3 min in the presence of 11 mM succinate. Then 0.25–3.0 mM MgCl₂, 0.05–0.6 mM KCN and 1.25 mM NAD⁺ were added. The reaction was initiated by addition of 1.5 mM ATP [37,44]. The effects of 1 μM rotenone, 0.25 μM

oligomycin or 1 μM carbonyl cyanide-3-chlorophenylhydrazone (m-CCCP) were tested. Results are expressed as nmol NADH. min⁻¹ mg protein⁻¹.

2.9. Nitric oxide production

Nitric oxide production was measured by following the oxidation of oxyhemoglobin to methemoglobin at 577–591 nm ($\epsilon = 11.2 \text{ mM}^{-1} \text{ cm}^{-1}$) using a Beckman DU 7400 diode array spectrophotometer, at 37 °C [5,45–47].

Nitric oxide generation sustained by mtNOS activity was measured in ETPH (0.15–0.40 mg of protein. ml⁻¹) and in the complex I enriched fraction (0.01–0.15 mg protein. ml⁻¹) using MST, 1 mM CaCl₂, 100 μM NADPH, 10 μM dithiothreitol (DTT), 4 μM Cu,Zn-SOD, 0.1 μM catalase, 1 mM L-arginine and 20 μM oxyhemoglobin heme (HbO₂).

The production of NO by rat heart coupled mitochondria (0.15–0.20 mg mitochondrial protein. ml⁻¹) was performed in a medium consisting of 120 mM KCl, 5 mM KH₂PO₄, 1 mM EGTA, 3 mM HEPES, 1 mg ml⁻¹ BSA, pH 7.20, where the samples were pre-incubated at 37 °C for 1 min in the absence or in the presence of 1 μM rotenone or 1 μM rotenone plus 1 μM oligomycin. Then 1 mM CaCl₂, 4 μM Cu,Zn-SOD, 0.1 μM catalase, 7 mM succinate and 20 μM oxyhemoglobin heme (HbO₂) were added.

The assay of NO production sustained by RET was performed using ETPH (0.15–0.50 mg protein. ml⁻¹) suspended in MST and pre-incubated at 37 °C for 3 min in the presence of 11 mM succinate. Then, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 μM DTT, 4 μM Cu,Zn-SOD, 0.1 μM catalase, 0.3 mM KCN, 1 mM L-arginine, 20 μM HbO₂ and 1.5 mM ATP were added. The effects of 1 μM rotenone, 0.25 μM oligomycin or 1 μM m-CCCP were tested.

Nitric oxide production by rat recombinant nNOS was measured in a reaction medium consisting of 50 mM HEPES, pH 7.40, 1 mM CaCl₂, 100 μM NADPH, 170 μM DTT, 12 μM tetrahydrobiopterin (BH₄), 1 μM calmodulin, 50 μM L-arginine, 0.05–0.18 Units ml⁻¹ nNOS and 5 μM HbO₂. Experiments were carried out with or without addition of 1 μM rotenone or 1 μM calmodulin as cofactor of the enzyme.

In order to consider only the HbO₂ oxidation by NO, the difference between the slopes of HbO₂ oxidation traces obtained in the presence of L-arginine and in the presence of the NOS inhibitor L-N^ω-monomethyl-L-arginine (L-NMMA; 1 mM) were used to calculate NO production. The absorbance changes are expressed as nmol NO. min⁻¹ mg protein⁻¹ or nmol NO. min⁻¹. U⁻¹.

2.10. Complex I and nitric oxide synthase expression

Complex I and nitric oxide synthase expression was assayed in three mitochondrial preparations: heart beef mitochondria, ETPH particles and in a complex I enriched mitochondrial fraction. A positive control using different dilutions (1/2000; 1/1000 and 1/500) of the recombinant nNOS from rat brain (N3033; Sigma) was included in the NOS Western blot assay. The proteins were subjected to 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Then, they were blotted into nitrocellulose films and probed with 1/500 diluted rabbit polyclonal anti-neuronal NOS (H-299; Santa Cruz Biotechnology) or 1/1000 diluted mouse polyclonal anti-75 kDa subunit of complex I (anti-Ndfus1 antibody ab22094; Abcam) and secondary goat anti-rabbit IgG antibodies (1:7500) or rabbit anti-mouse IgG antibodies (1:5000) [48]. The secondary antibodies were conjugated with horseradish peroxidase and revealed using an ECL assay. The chemiluminescence signal was captured using a high performance chemiluminescence film (Amersham Hyperfilm™ ECL, GE Healthcare Limited, Japan). In order to semiquantify the protein expression, the radiographic

films were digitalized and the images were analyzed using the Image J 1.45s software (Wayne Rasband, National Institute of Health, USA), for the determination of densitometric units. Results are expressed as densitometric units per μg of protein (%), taking as 100% the values obtained for mitochondrial fraction.

2.11. Statistics

Results included in tables and figures are expressed as means \pm SEM and represent replicated measurements on at least 3–6 independent experiments. The differences were analyzed by one-way ANOVA and by Dunnett's post-hoc test. Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and highly significant, respectively. Statistical analysis was done using GraphPad Instat 4 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Catalytic properties of bovine ETPH particles

The O_2 consumption rate with NADH as substrate measured in the presence or absence of cytochrome c^{3+} was used to estimate the percentage of inside-out particles (Table 1) in the ETPH preparation. In our experimental conditions, 60% of the particles are in reverse status. These data agrees with the percentage of inside-out particles calculated from NADH- O_2 oxidoreductase activity (60%) followed spectrophotometrically and performed in the absence or presence of cytochrome c^{3+} in the reaction medium (Table 1). These results are in agreement with the percentage of inside-out particles obtained by Esposito and Lenaz [49] of about 60–75%, using similar mitochondrial preparations.

Other catalytic properties of ETPH are included in Table 2. ETPH particles exhibited NADH and succinate oxidation rates of 228 ± 20 and 176 ± 13 ng-at O. min^{-1} mg protein $^{-1}$, respectively, measured through O_2 uptake. The oxidation of malate and glutamate by ETPH was 34 ± 1 ng-at O. min^{-1} mg protein $^{-1}$, a value which is comparable to state 4 O_2 uptake of coupled rat heart mitochondria [7,40]. In addition, NADH-cytochrome c reductase (349 ± 6 nmol min^{-1} mg protein $^{-1}$), succinate-cytochrome c reductase (222 ± 4 nmol min^{-1} mg protein $^{-1}$) and cytochrome oxidase (33 ± 2 min^{-1} mg protein $^{-1}$; 660 ± 40 nmol min^{-1} mg protein $^{-1}$) rates are in accordance to the reported activities for this type of preparation [14]. Taking these results into account, the particles were deemed suitable for further determinations.

3.2. Energy-dependent succinate-NAD⁺ reductase activity

3.2.1. Effect of rotenone, oligomycin and m-CCCP

In the presence of succinate and ATP, ETPH showed a succinate-NAD⁺ reductase activity of 64 ± 3 nmol NADH. min^{-1} mg protein $^{-1}$ (Fig. 1 and Table 3). This energy-dependent activity was abolished by rotenone (99%) as a result of the interruption of electron transfer to NAD⁺, i.e. the final electron acceptor at complex I. Moreover,

Table 2

Catalytic properties of ETPH: O_2 consumption and respiratory chain complexes activities.

	Rate
Oxygen consumption	(ng-at O. min^{-1} mg protein $^{-1}$)
NADH	228 ± 20
Succinate	176 ± 13
Malate-glutamate	34 ± 1
Mitochondrial activities	(nmol. min^{-1} mg protein $^{-1}$)
NADH-cytochrome c reductase	349 ± 6
Succinate-cytochrome c reductase	222 ± 4
Cytochrome oxidase	660 ± 40

O_2 consumption, n = 4; Mitochondrial Activities, n = 6.

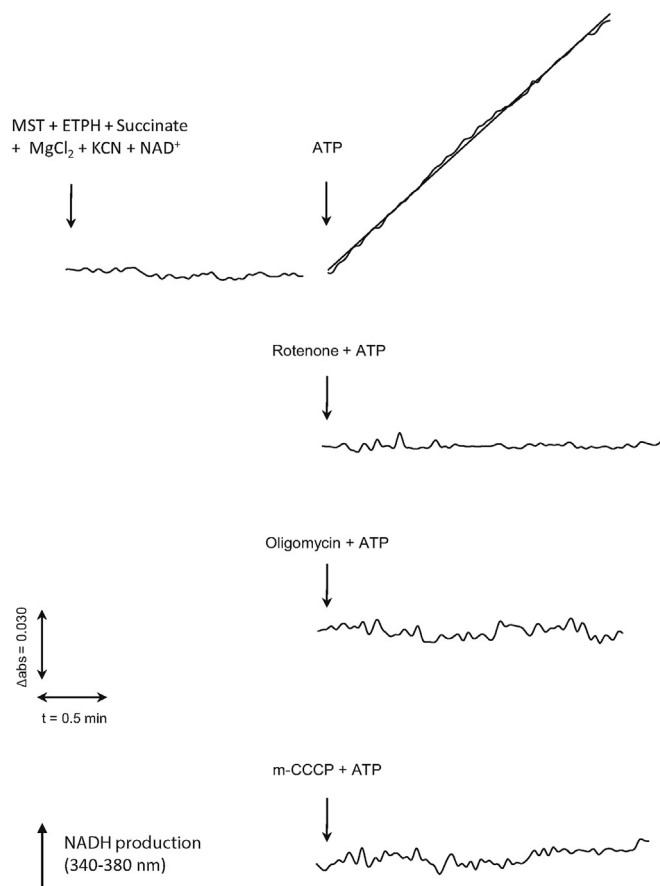


Fig. 1. Representative traces of ATP-dependent succinate-NAD⁺ reductase activity of ETPH and the effect of 1 μM rotenone, 0.25 μM oligomycin, or 1 μM m-CCCP. ETPH (0.3 mg protein. ml^{-1}) suspended in MST were pre-incubated at 37 °C for 3 min in the presence of 11 mM succinate. Then, 3.0 mM MgCl_2 , 0.5 mM KCN, 1.25 mM NAD^+ and 1.5 mM ATP were added.

oligomycin totally inhibited (99%) NAD⁺ reduction due to the inhibition of the energy yielded by the F_0F_1 -catalyzed ATP hydrolysis

Table 1

Percentage of inside-out particles estimated through O_2 consumption and NADH: O_2 oxidoreductase activity.

	O_2 consumption (ng-at O. min^{-1} mg protein $^{-1}$)	NADH: O_2 oxidoreductase activity (nmol min^{-1} mg protein $^{-1}$)
State 4	228 ± 20	203 ± 18
+ cytochrome c^{3+}	377 ± 28	340 ± 26
% inside-out particles ^a	60	60

n = 4.

^a The percentage of inside-out particles was calculated as follows:

% inside-out = activity without cytochrome c^{3+} \times 100/activity after addition of cytochrome c^{3+} .

Table 3
Effect of rotenone, oligomycin and m-CCCP on succinate-NAD⁺ reductase activity of ETPH.

Experimental condition	Succinate-NAD ⁺ reductase (nmol NADH. min ⁻¹ mg protein ⁻¹)
ETPH in MST	64 ± 3
+1 μM rotenone	0.41 ± 0.03**
+0.25 μM oligomycin	0.33 ± 0.07**
+1 μM m-CCCP	0.96 ± 0.10**

**p < 0.01 vs. ETPH in MST (ANOVA; Dunnett's post-hoc test), n = 5.

and of Δp generation. Similarly, the protonophore m-CCCP fully impaired (98%) NAD⁺ reduction through the collapse of Δp (Table 3).

3.2.2. Effect of MgCl₂ and KCN concentrations on succinate-NAD⁺ reductase activity

Succinate-NAD⁺ reductase activity has an absolute requirement of Mg²⁺ (Fig. 2). In the absence of this cation in the reaction medium, this activity became undetectable. The addition of 0.5–1.5 mM MgCl₂ in the reaction medium allowed the determination of succinate-NAD⁺ reductase activity without substantial differences to that detected with 3.0 mM MgCl₂, which is taken as the optimal experimental condition. However, it has been recognized that Mg²⁺ inhibits mtNOS activity in heart mitochondria [50]. The supplementation of the medium with 0.25 mM MgCl₂ allowed to assay succinate-NAD⁺ reductase activity, but with values significantly lower (55%) than the ones detected in the presence of 3.0 mM MgCl₂. Thus, 0.5 mM MgCl₂ was the concentration selected to assay mtNOS activity sustained by RET in ETPH.

In addition, the absence of KCN in the reaction medium yielded a succinate-NAD⁺ reductase activity lower than 3.0 nmol NADH. min⁻¹ mg protein⁻¹ (Fig. 3), being the presence of KCN essential to maximize mitochondrial RET.

However, the presence of KCN interferes with the oxidation of oxyhemoglobin to methemoglobin in the measurement of NO production by inside-out particles [46]. For this reason,

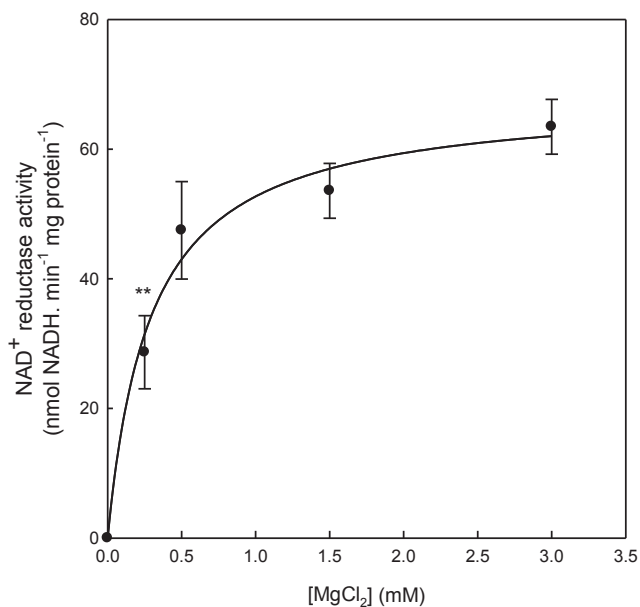


Fig. 2. Effect of MgCl₂ concentrations on reverse electron flow supported-NAD⁺ reductase activity assessed in ETPH, in the presence of 0.5 mM KCN. **p < 0.01 vs. 3 mM MgCl₂ (ANOVA; Dunnett's post-hoc test), n = 3.

0.05 mM–0.6 mM KCN were tested in the reaction medium of succinate-NAD⁺ reductase activity assay using two fixed MgCl₂ concentrations (0.5 and 3.0 mM). In both cases this activity showed a hyperbolic increase with the enhancement in KCN concentration, reaching a plateau at 0.3–0.4 mM KCN. As a consequence, 0.3 mM KCN was selected as a suitable concentration to measure mtNOS activity in ETPH particles, because it supported an acceptable reverse electron flow, in the presence of 0.5 mM MgCl₂

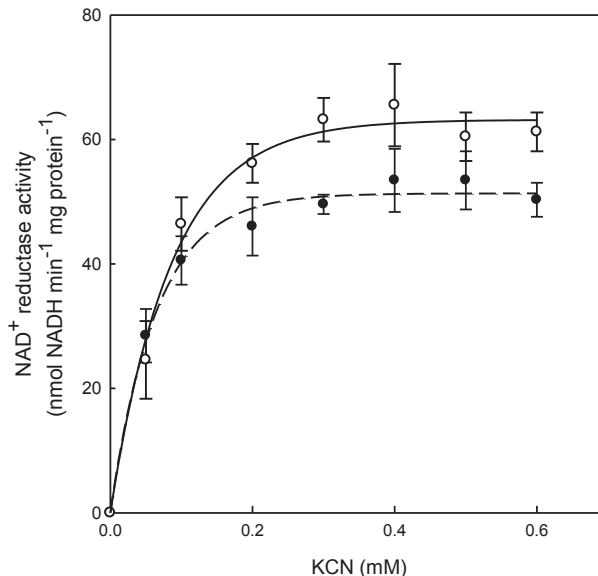


Fig. 3. Effect of KCN concentrations on NAD⁺ reductase activity performed in ETPH, in the presence of 0.5 mM (dotted line) and 3 mM (full line) MgCl₂, n = 3.

(47.6 ± 1.6 nmol NADH. min⁻¹ mg protein⁻¹).

3.3. Nitric oxide production

NO production by ETPH in the presence of the classical NOS electron donor, *i.e.* NADPH, and in conditions of RET; and by complex I enriched fraction was measured by following spectrophotometrically the oxidation of HbO₂ (Fig. 4A, C and E).

3.3.1. NO production by ETPH using NADPH as electron donor

The HbO₂ oxidation found in the presence of L-arginine was partially inhibited by the addition of the NOS inhibitor L-NMMA (Fig. 4), corresponding the remaining HbO₂ oxidation to non specific reactions. Thus, only the L-NMMA-sensitive HbO₂ oxidation was considered to calculate the NO formation rate. Table 4 shows that ETPH produce NO at a rate of 1.2 ± 0.1 nmol min⁻¹ mg protein⁻¹. Fig. 4B shows the specific HbO₂ oxidation, *i.e.* NO production as a function of the amount of ETPH protein. This plot shows a linear relationship (r² = 0.972) between NO production and protein content in the reaction cuvette.

3.3.2. NO production supported by reverse electron transfer

Fig. 4C shows the typical recording of HbO₂ oxidation by ETPH supported by RET in the presence of L-arginine or L-NMMA. The assay was performed using ETPH suspended in MST and pre-incubated at 37 °C for 3 min in the presence of 11 mM succinate. In these experimental conditions, ETPH produced a change in the rate of HbO₂ oxidation that was partially reduced by the addition of L-NMMA (Fig. 4C) resulting in a NO production of 0.97 ± 0.07 nmol NO. min⁻¹ mg protein⁻¹ (Table 4). These data

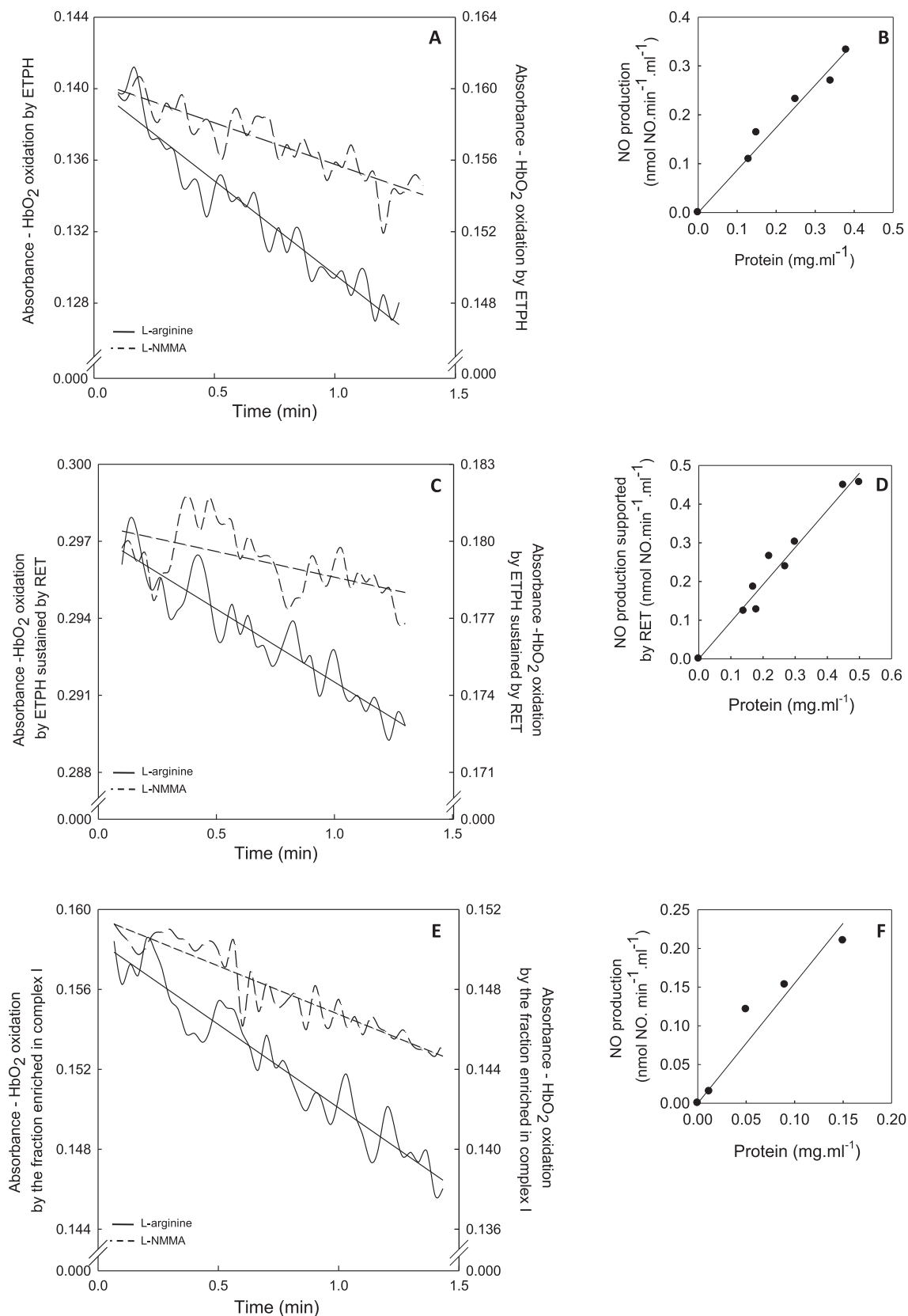


Fig. 4. Representative traces showing the HbO₂ oxidation (577–591 nm) in the presence of L-arginine or L-NMMA, determined in ETPH supported by electrons derived from (A) NADPH or (C) reverse electron transfer; or measured in mitochondrial complex I enriched fraction (E). Linear correlations (B, $r^2 = 0.972$; D, $r^2 = 0.960$; F, $r^2 = 0.920$) between specific NO production and protein content in the reaction cuvettes.

Table 4
Nitric oxide production by ETPH and by a mitochondrial fraction enriched in complex I.

Mitochondrial preparation	Source of electrons for mtNOS activity	NO production (nmol NO. min ⁻¹ mg protein ⁻¹)
ETPH	NADPH	1.20 ± 0.10
	Reverse electron flow	0.97 ± 0.07
	+1 μM rotenone	ND
	+0.25 μM oligomycin	ND
	+1 μM m-CCCP	ND
Complex I enriched fraction	NADPH	1.72 ± 0.18

Experimental conditions are detailed in Section 2.9. n = 5. ND: Not detectable.

indicate that mtNOS activity can be sustained by electrons derived from the low isopotential group of the respiratory chain, *i.e.* succinate dehydrogenase and ubiquinol, for NO generation. A linear correlation ($r^2 = 0.961$) was found between the NO production sustained by RET and the protein content in the reaction medium (Fig. 4D). Moreover, mtNOS activity supported by reverse electron flow was abolished by rotenone, oligomycin and m-CCCP (Table 4).

3.3.3. NO production by mitochondrial complex I enriched fraction

A mitochondrial fraction enriched in complex I was obtained using the Hatefi and Rieske technique [36] and used to measure NO production (Table 4). The HbO₂ oxidation by complex I enriched fraction was partially inhibited by L-NMMA (Fig. 4E), resulting in a NO production of 1.72 ± 0.18 nmol NO. min⁻¹ mg protein⁻¹ (Table 4). Moreover, NO production in this fraction depends on the amount of protein in the reaction medium ($r^2 = 0.920$; Fig. 4F).

3.3.4. NO production by coupled heart mitochondria

In order to know if NO production sustained by RET also occurs in intact mitochondria in physiological conditions, rat heart coupled mitochondria were isolated and NO production was measured according to Valdez et al. [7]. Table 5 shows that rat heart coupled mitochondria supplemented with succinate released 1.65 ± 0.19 nmol NO. min⁻¹ mg protein⁻¹. This production was inhibited (18%) by rotenone addition, suggesting that in physiological conditions 18% of the total NO released corresponds to the NO production sustained by RET. Moreover, an inhibition of 20% was observed when mitochondria were pre-incubated with rotenone plus oligomycin, that abolish RET. Thus, from the total amount of NO generated by coupled heart mitochondria in physiological conditions, about 80% is produced as a result of mtNOS activity using the endogenous NADPH (~1.3 nmol NO. min⁻¹ mg protein⁻¹), while the remaining NO is produced as a result of the electrons derived from RET towards mtNOS (~0.30 nmol NO. min⁻¹ mg protein⁻¹).

3.3.5. NO production by recombinant nNOS

Nitric oxide production by rat recombinant nNOS was assayed in an attempt to distinguish if the effect of rotenone on NO production by ETPH and by intact mitochondria under RET is due to its inhibitory action on RET or to an effect on mtNOS structure. In optimal conditions, the NO production rate was 0.93 ± 0.05 nmol NO. min⁻¹ U⁻¹ (Table 6). The absence of calmodulin in the reaction medium inhibited NO production by

Table 5
NO production and release by coupled heart mitochondria.

Experimental condition	NO production (nmol NO. min ⁻¹ mg protein ⁻¹)
+Succinate	1.65 ± 0.19
+1 μM rotenone	1.35 ± 0.26
+1 μM oligomycin +1 μM rotenone	1.31 ± 0.19

n = 3.

Table 6
NO production by recombinant nNOS.

Experimental condition	NO production (nmol NO. min ⁻¹ U ⁻¹)
Complete reaction medium	0.93 ± 0.05
- calmodulin	0.29 ± 0.05**
+1 μM rotenone	1.10 ± 0.10

**p < 0.01 vs. complete reaction medium (ANOVA; Dunnett's post-hoc test), n = 4.

69%, showing that the addition of this protein is essential for the isolated nNOS activity. In contrast, rotenone did not significantly modify nNOS activity (1.10 ± 0.10 nmol NO. min⁻¹ U⁻¹). Therefore, the results appear to indicate that the inhibitory effect of rotenone on NO production by ETPH (Table 6) and by intact mitochondria (Table 5) under RET is attributable to the electron flow inhibition and not to the direct action on mtNOS structure.

3.4. NOS expression in bovine heart mitochondria, ETPH particles and complex I enriched mitochondrial fraction

Due to previously published evidences that complex I proteins immunoprecipitate with mtNOS [19], Western Blot analysis of bovine heart mitochondria, ETPH and complex I enriched fraction were performed using anti-complex I and anti-NOS antibodies. All these mitochondrial preparations were recognized by anti-complex I antibodies that react with the 75 kDa subunit of complex I (Fig. 5A-B).

In addition, considering that mtNOS is the α -variant of the nNOS isoform with post-translational modifications [3], an anti-nNOS antibody with specific reactivity was used with heart mitochondrial fractions [6,48]. Previous results of our laboratory have shown that the anti-nNOS (Santa Cruz; H-299) directed to an epitope corresponding to aminoacids 2–300 mapping at the N-terminus of NOS 1 recognized a band with a molecular weight between 116 and 205 kDa in heart mitochondrial preparations [48]. Different dilutions of the recombinant nNOS from rat brain (N3033, Sigma Chemical Co) were performed and tested with the same anti-nNOS antibody as a positive control. The main band was observed at 130–160 kDa (Fig. 5C). Then, we observed that not only heart mitochondrial fraction and ETPH, but also the complex I enriched fraction, were recognized by anti-nNOS antibodies, an indication that mtNOS was present in the three mitochondrial preparations (Fig. 5D). Moreover, the values of the nNOS densitometric units per μ g protein (Fig. 5E) detected in the complex I enriched fraction are 65% higher than the ones observed in mitochondria, suggesting that when the mitochondrial preparation is enriched in complex I, it is also enriched in mtNOS (Fig. 5D and E). These data suggest that mtNOS enzyme is next to complex I, reaffirming the idea of a functional association between them that allows mtNOS enzyme to use electrons derived from the electron transfer chain for its enzymatic activity.

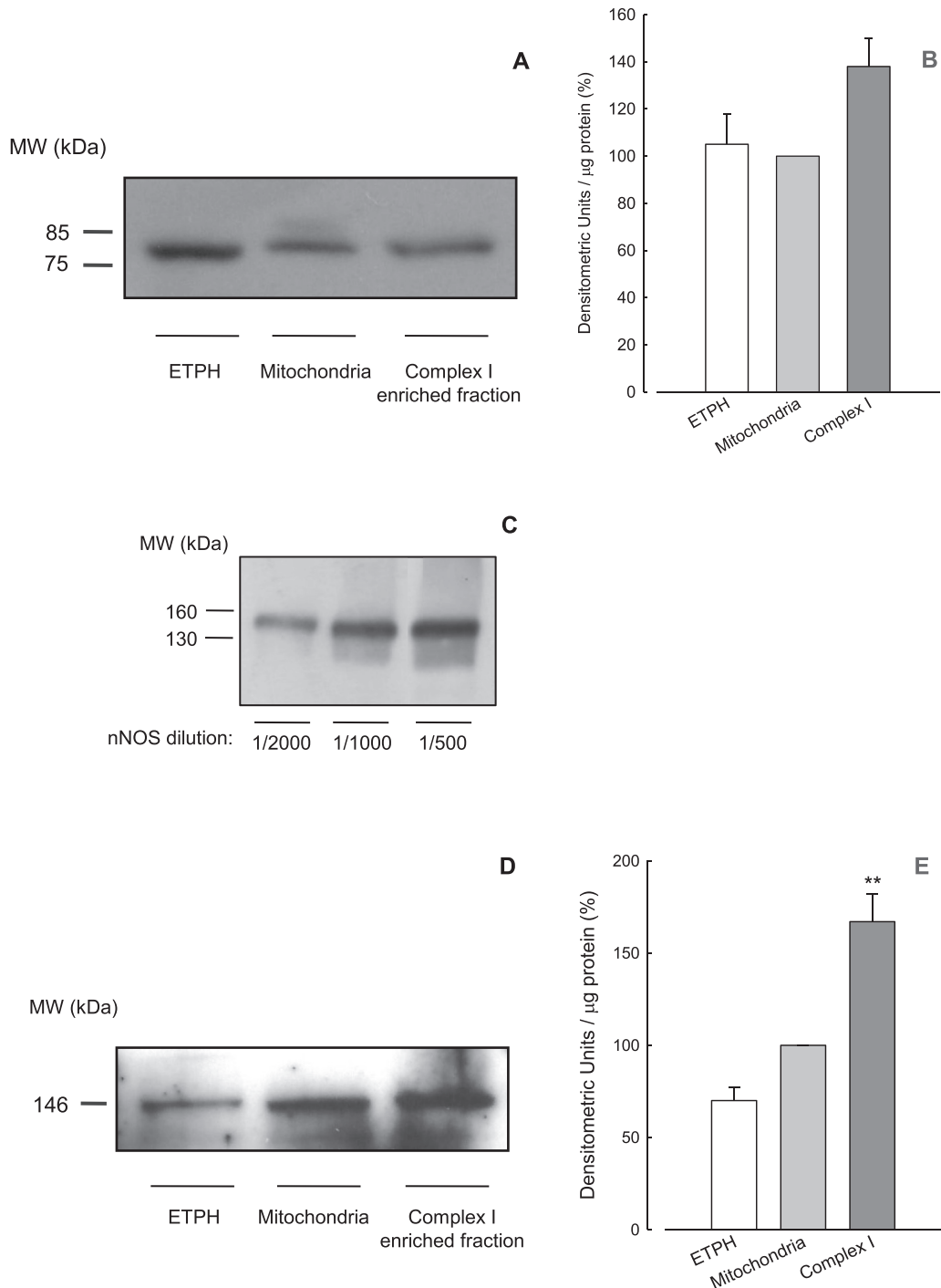


Fig. 5. Representative Western blot analysis (**A**, **C** and **D**) and Densitometric units/ μg protein expressed as percentage respect to mitochondrial membranes (100%) (**B** and **E**) of ETPH particles, beef heart mitochondrial membranes and complex I enriched mitochondrial fraction, using anti-75 kDa complex I subunit (**A** and **B**, $n = 3$) and anti-nNOS (**D** and **E**, $n = 4$, ** $p < 0.01$ complex I vs. mitochondrial fraction) antibodies. **C.** Representative Western blot analysis of dilutions of rat recombinant nNOS protein (N3033, from rat brain, Sigma Chemical Co) used as a positive control for the anti-nNOS antibodies (H-299, Santa Cruz Biotechnologies).

4. Discussion

The results presented in this paper provide evidence that bovine heart submitochondrial particles (ETPH) produce NO both by using NADPH, the conventional electron donor of NOS [5,7,10], and also supported by mitochondrial reverse electron transfer (RET) derived from succinate. The NO production detected in the complex I enriched mitochondrial fraction, together with the reactivity of that

fraction both with anti-complex I and anti-nNOS antibodies, suggest that complex I and mtNOS enzyme are located contiguously.

In this study, the use of inside-out particles which expose the NADH binding site of complex I, the F_1 -ATPase and mtNOS to the surrounding medium, has been a useful experimental strategy because potential interferences of mitochondrial matrix, inter-membrane space, or outer membrane are excluded. The reaction catalyzed by complex I is physiologically reversible because

coupled mitochondria or submitochondrial particles are capable of $\Delta\mu_{\text{H}^+}$ -dependent reduction of NAD^+ in the presence of the substrates that provide the reduction of ubiquinone, i.e. succinate. In our experimental conditions, RET was generated by succinate oxidation, in the presence of KCN to inhibit cytochrome oxidase, and energized by the proton-motive force coupled to ATP hydrolysis. Under these conditions the electrons are transferred from succinate to the FMN of complex I and are able to reduce NAD^+ or to participate in the production of NO by mtNOS (Scheme 1).

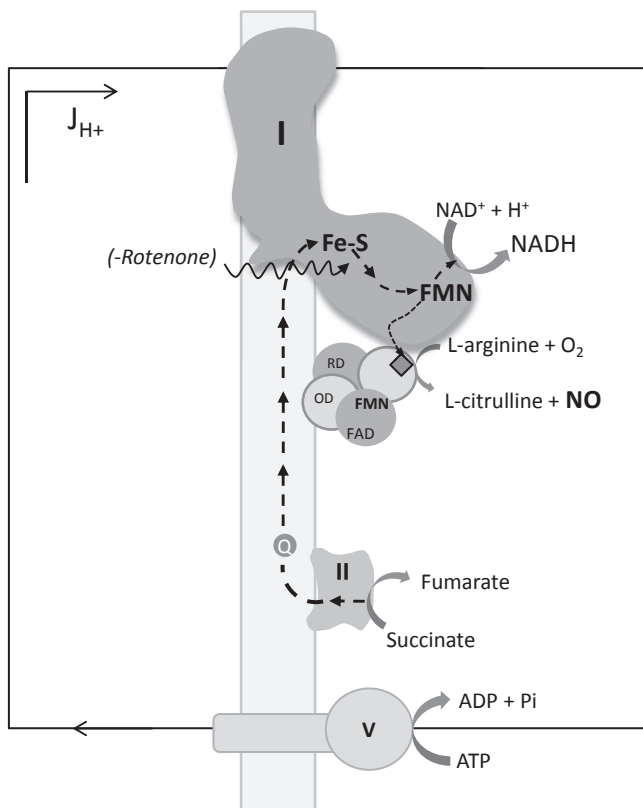
The mtNOS is an integral protein of the inner mitochondrial membrane [1,3,51] where respiratory chain complexes are embedded. Nitric oxide synthase isoenzymes are dimeric oxidoreductase proteins comprising an oxygenase domain and a reductase domain [52]. Each NOS monomer homodimerizes in a configuration that allows the reductase domain of each monomer to be in close contact with the oxygenase domain of the coupling monomer. The electron flow in the NOS dimer is driven via $\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{FMN}$ in the reductase domain of one monomer to the heme iron in the oxygenase domain of the other monomer, according to the standard redox potentials [53]. In the ETPH, NO production supported by NADPH and catalyzed by mtNOS was $1.2 \text{ nmol NO} \cdot \text{min}^{-1} \text{ mg protein}^{-1}$ (Table 4). The same particles were able to produce a similar rate of $0.97 \text{ nmol NO} \cdot \text{min}^{-1} \text{ mg protein}^{-1}$ supported by RET. In this experimental situation, rotenone addition abolished NO production (Table 4), showing that when electrons

derived from RET do not arrive at the FMN of complex I, they can not be transferred to the oxygenase domain of NOS (Scheme 1). These data agree with the results published by Parihar et al. [54] who showed in rat brain mitochondria and human dopaminergic neuroblastoma SHSY cells that the inactivation of complex I by rotenone produces a decrease in mitochondrial NO production. In our experimental conditions, rotenone inhibited NO production sustained by RET in ETPH and in coupled mitochondria but it did not inhibit the enzymatic activity of recombinant rat nNOS (Table 6), a fact consistent with the effect of rotenone on electron flow and not to an action on mtNOS structure. Similar results concerning the absence of a direct effect of rotenone on rat heart mtNOS activity measured in mitochondrial membranes have been previously published [55]. In addition, other inhibitors that effectively interfere with the energy supply for RET, such as oligomycin and m-CCCP, inhibited both NAD^+ reduction (Table 3) and NO production (Table 4) showing that electrons do not reach complex I and mtNOS oxygenase domain, because there is no Δp to drive electrons energetically uphill (Scheme 1).

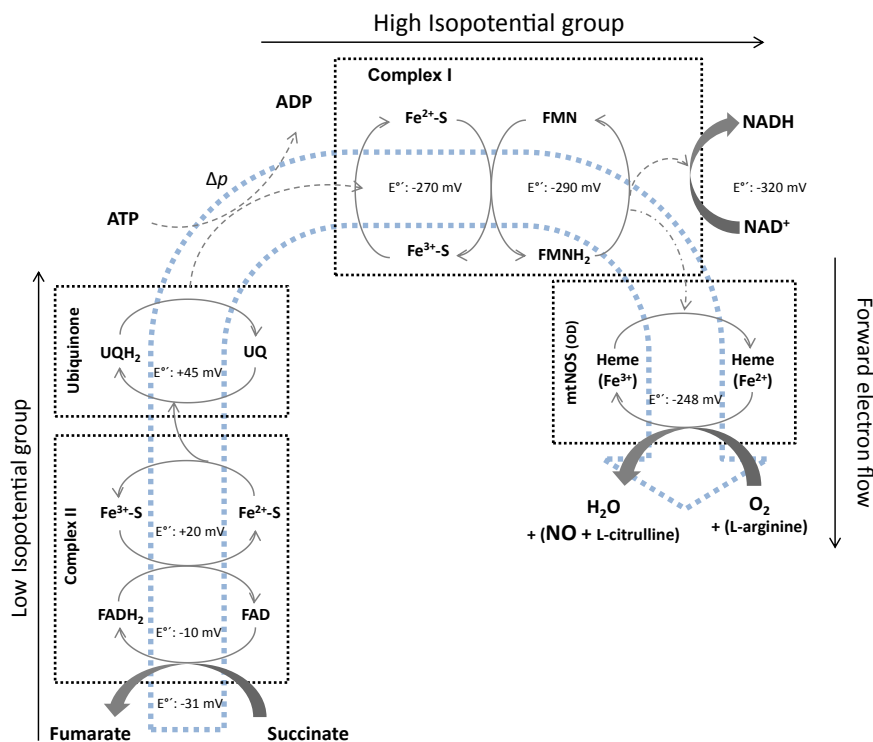
Interestingly, the mitochondrial fraction enriched in complex I produced $1.7 \text{ nmol NO} \cdot \text{min}^{-1} \text{ mg protein}^{-1}$ (Table 4), a value 45% higher than the one detected in ETPH using NADPH as electron donor (Table 4) and 51% higher than the NO production rate measured in beef heart mitochondria ($1.14 \pm 0.10 \text{ nmol NO} \cdot \text{min}^{-1} \text{ mg protein}^{-1}$; data not shown). Moreover, the fraction enriched in complex I reacted not only with an anti-complex I antibodies (Fig. 5A), but also with an anti-nNOS antibodies (Fig. 5D), being the NOS expression per μg protein in this fraction 65% higher than in the mitochondrial preparation. Altogether, these data suggest that when the mitochondrial preparation is enriched in complex I, it is also enriched in mtNOS. Regarding this point, Poderoso's group [19] showed that both complex IV and complex I proteins immunoprecipitate with mtNOS. This association agrees with the dependence of mitochondrial NO production on metabolic state and membrane potential [7,8,10], and its involvement in the regulatory mechanism of mitochondrial oxygen consumption.

Considering the potentials for the redox couples in NOS enzyme ($E'^{\circ}_{\text{NAD(P)}^+/\text{NAD(P)H}} = -320 \text{ mV}$; $E'^{\circ}_{\text{FAD}/\text{FADH}} = -280 \text{ mV}$, $E'^{\circ}_{\text{FMN}/\text{FMNH}} = -274 \text{ mV}$, $E'^{\circ}_{\text{Fe}^{3+}/\text{Fe}^{2+}} = -248 \text{ mV}$ [50,51]), and taking into account our results that support the notion of a functional association and an adjoining location of complex I and mtNOS, the FMN of complex I could transfer electrons ($E'^{\circ}_{\text{FMN}/\text{FMNH}} = -290 \text{ mV}$) to the heme group of the oxygenase domain of mtNOS ($E'^{\circ}_{\text{Fe}^{3+}/\text{Fe}^{2+}} = -248 \text{ mV}$) allowing the production of NO by the enzyme, in accordance with the thermodynamic reduction potentials (Scheme 2).

It is worth to note that functional association between mtNOS and complex I in brain mitochondria has been linked to the development of neurodegenerative diseases [56,57], in which the impairment of complex I activity and mitochondrial respiration is parallel to the reduction of mtNOS activity [58]. Evidence concerning mitochondrial dysfunction with a comparable decline in complex I and in biochemical and functional mtNOS activities was also observed in an ischemia/reperfusion model in isolated rabbit heart [40]. Recently, Chouchani and coworkers [59] have shown that the accumulation of succinate during ischemia is responsible for mitochondrial $\text{O}_2^{\cdot-}$ production upon reperfusion. Accumulated succinate is rapidly re-oxidized by succinate dehydrogenase in order to maintain the Q pool reduced, thereby sustaining a large protonmotive force by conventional electron transfer through complexes III and IV to O_2 . The situation seems to drive also an extensive $\text{O}_2^{\cdot-}$ generation by RET at mitochondrial complex I. Rotenone abolished the enhancement of ischaemic succinate oxidation and of $\text{O}_2^{\cdot-}$ generation through complex I upon reperfusion. In this physiopathological scenario, the NO production under RET leads to



Scheme 1. Diagram proposed to explain NO production under RET, a condition obtained when ETPH are incubated in the presence of succinate, NAD^+ , ATP and KCN. This latter inhibits cytochrome oxidase and forward electron flow. Succinate is the source of electrons, ATP is the energy source and NAD^+ is the electron acceptor. Succinate- NAD^+ oxidoreduction is supported by the Δp from ATP hydrolysis. The heme group of the oxygenase domain (OD) of mtNOS accepts electrons from the FMN of complex I. I (NADH-ubiquinone oxidoreductase complex); II (succinate-dehydrogenase complex); Q (ubiquinol-ubiquinone) and V (ATP synthase). OD: oxygenase domain of mtNOS; RD: reductase domain of mtNOS.



Scheme 2. Redox potentials and direction of electron flow under RET in ETPH: succinate-NAD⁺ oxidoreduction in the presence of KCN and Δp generated by ATP hydrolysis. In the absence of NADPH as mtNOS electron donor, the heme group of the mtNOS oxygenase domain (OD) could accept electrons derived from the FMNH₂ of complex I, according to the increasing reduction potential ($E^{\circ}_{\text{FMN}/\text{FMNH}_2} = -290 \text{ mV}$; $E^{\circ}_{\text{heme Fe}^{3+}/\text{Fe}^{2+}} = -248 \text{ mV}$).

generation of ONOO⁻, resulting in mitochondrial dysfunction through damage of complex I.

This mitochondrial dysfunction has been described as the “complex I syndrome” [60] and involves the formation of ONOO⁻, which is produced through the intramitochondrial reaction between NO and O₂⁻ at the vicinity of NADH-dehydrogenase active center, leading to complex I inactivation [61,62]. There is agreement that complex I operating in electron forward mode produces O₂⁻ by autooxidation of FMNH^{*}, when downstream electron transfer is partially or fully blocked. It is also accepted that O₂⁻ is generated by autooxidation of FMNH^{*} or at the ubiquinone binding site during RET [30,37,63,64]. Moreover, Bleier et al. [65] have reported that when O₂⁻ is generated by complex I during reverse electron flow, sixteen protein targets located in the mitochondrial matrix or inner membrane are oxidized. In this scenario, the NO production sustained by RET contributes to ONOO⁻ formation close to the complex I structure, which is vulnerable to oxidative and nitrosative damage and to inactivation as it has been observed in sepsis [66,67], Parkinson's disease [68,69], and heart ischemia/reperfusion [40].

To conclude, the data presented in this study show that heart mtNOS may produce NO supported by RET and functionally interacts with complex I. The results suggest that mtNOS could be physically associated with complex I proteins as part of a super-complex, being able to use electrons derived from the respiratory chain for its enzymatic activity. This association might be part of regulatory mechanisms involved in the regulation of mitochondrial NO production under physiopathological conditions. This may also partially explain the molecular mechanism involved in the pathogenesis of diseases associated to complex I syndrome, in which not only complex I but also mtNOS dysfunctions were observed.

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