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Mitochondrial nitric oxide production supported by reverse electron transfer

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List of abbreviations:
FMN
flavin mononucleotide
mtNOS
mitochondrial nitric oxide synthase
RET
reverse electron transfer
HbO_2
oxyhemoglobin
Δp
proton motive force
$\Delta\mu H^+$
proton electrochemical potential
$O_2^{\bullet-}$
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.

1. Introduction

In mitochondria, nitric oxide (NO) is synthetized from L-arginine, NADPH and O_2 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 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 Ca^{2+}-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 O_2 utilization. Mitochondrial NO production accounts for about 60% of total heart NO generation [6] and it is produced by mitochondrial membranes

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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 ($O_2^{\bullet-}$) 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 protonmotive force (Δp) [24,25]. Mitochondrial complex I reaction is reversible: the complex is able to reduce NAD^+ in the presence of succinate, in a thermodinamically 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 tranlocation [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 F_1-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-Ndfus1 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 1200*g* 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 16 000*g* for 15 min and the pellet was suspended in MSTE medium (1:4), homogenized and centrifuged at 16 000*g* 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 $MgCl_2$, 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 $MgCl_2$, six times for 10 s with 30 s intervals. The suspension was centrifuged at 15 500*g* for 10 min and the supernatant was centrifuged at 140 000*g* 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-Q_1 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 CO_2 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