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Mitochondrial nitric oxide inhibits ATP synthesis Effect of free calcium in rat heart

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Summary. Nitric oxide is a small potentially toxic molecule and a diatomic free radical. We report the interaction of L-arginine, oxygen and calcium with the synthesis of nitric oxide in heart mitochondria. Nitric oxide synthesis is increased in broken rat heart mitochondria compared with intact and permeabilized mitochondria. Intact mitochondria subjected to hypoxia-reoxygenation conditions accumulated nitric oxide that inhibits oxygen consumption and ATP synthesis. ATPase activity is not affected during this augment of nitric oxide. Physiological free calcium concentrations protected mitochondria from the damage caused by the accumulation of nitric oxide. Higher concentrations of the divalent cation increase the damage exerted by nitric oxide.

Keywords: Heart – Mitochondria – Nitric oxide – Hypoxia-reoxygenation – Calcium – ATP synthesis

Introduction

Nitric oxide (NO) is an important reactive molecule in many organisms. This chemical specie is classified as a free radical, because it possesses a non-paired electron in its electronic configuration (Stamler et al., 1992). NO is generated in biological systems from oxygen and L-arginine in the reaction catalyzed by nitric oxide synthase (NOS) enzymes (NOS, EC 1.14.13.39) (Griffith and Stuehr, 1995; Mayer, 1995). NO has many functions, for instance as an intercellular messenger, vasodilatation, inhibition of platelet aggregation and neurotransmission (Ignarro, 1989; Bredt and Zinder, 1990), among others. However, it can also be toxic under certain conditions such as inhibitor of respiration in a variety of biological systems (Cleeter et al., 1994; Schweizer and Richter, 1994; Lizasoain et al., 1996; Brown and Cooper, 1994; Poderoso et al., 1996). The inhibition of cytochrome c oxidase by extramitochondrial NO may play a normal role in controlling mitochondrial oxygen consumption (Brown, 1995; Dai et al., 2001). Cytochrome oxidase (complex IV) is the terminal complex of the mitochondrial respiratory chain, and passes electrons from cytochrome c to oxygen. It has been suggested that within cytochrome oxidase NO binds to the reduced form of cytochrome a_3 and probably also to Cu_B (Brown and Cooper, 1994). This is the same site and same form of the enzyme to which oxygen binds; therefore NO inhibits cytochrome oxidase in an apparently competitive form with oxygen (Torres et al., 1995).

Studies have demonstrated the presence of NOS in the perinuclear region, in discrete regions of the plasma membrane of cultured endothelial cells, and in intact blood vessels (Liu et al., 1995; García-Cardeña et al., 1996). Immunocytochemical studies have revealed the presence of NOS, or an antigenically related protein, in mitochondria isolated from different tissues (Bates et al., 1995; Bates et al., 1996; Kanai et al., 2001). The predominant association of this mitochondrial NOS (mtNOS) with the mitochondrial inner membrane, co-localized with succinate dehydrogenase, suggests a particulate localization (Bates, 1996). Recently, a constitutively expressed and continuously active mtNOS have demonstrated a Ca^{2+}-dependence, that exerts substantial control on mitochondrial respiration, on transmembrane potential and ATP synthesis (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Giulivi, 1998; Manzo-Ávalos et al., 2002) and also with an intramitochondrial superoxide interaction for the formation of peroxynitrite (Ghafourifar et al., 1999).

Tissue ischemia results in the depletion of intracellular ATP stores, which subsequently compromises the function of membrane-associated ATP-dependent ionic pumps in cells allowing entry of calcium, sodium and water (Granger and Korthuis, 1995). The resultant accumulation of calcium and other ions in the cell can result in cell swelling and the inappropriate activation of cellular enzymes (Granger and Korthuis, 1995). On restoration of blood flow (reperfusion) to the tissue and with the reintroduction of molecular oxygen these are conditions to produce free radicals, nitric oxide and superoxide that increased greatly and can readily interact with each other to yield peroxynitrite (Packer et al., 1996). In ischemic heart, a number of effects on mitochondria contribute to subsequent heart pathology (Borutaité et al., 1996). It has been demonstrated that 30–45 min of ischemia of isolated rat heart causes an irreversible inhibition of state-3 respiration rate and a stimulation of state-4 respiration rate of the subsequently isolated mitochondria, therefore, incubation times of ischemic conditions less than 30 min maintain in a reversible manner the mitochondrial functions normally (Borutaité et al., 1996). The inhibition of mitochondrial respiration is mainly due to the loss of cytochrome c and can be largely reversed by adding external cytochrome c (Borutaité et al., 1996).

Cytosolic calcium levels rise up to 5 μM in ischemic-reperfusion hearts and mitochondria isolated from irreversibly damaged, ischemic hearts contain high levels of calcium (Borutaité et al., 1999). In this study, we arise the possibility that intramitochondrial NO synthesis in rat heart can be accumulated during a period of an incomplete absence of oxygen, as in hypoxia, process that should affect Ca^{2+} homeostasis as well as energy production, i.e., ATP synthesis. The latter process requires membrane integrity to continue most of the physiological functions normally, which in our understanding needs specific and detailed research to explain how the respiratory chain through the cytochrome oxidase is affected by nitric oxide when Ca^{2+} is present. Besides, the interaction between NO and superoxide both from intramitochondrial source, as well as calcium in an elevated condition, will increase the damage. In fact, little is known about the factors that establish responses to mitochondrial NO as protective or pathological. Here we propose the interaction of calcium with the respiratory chain in the presence of mitochondrial nitric oxide to explain the relationship between NO and the inhibition of ATP synthesis. Previous results from our laboratory (Ramírez-Emiliano and Saavedra-Molina, 1999; Manzo-Ávalos et al., 2002; Aguilera-Aguirre et al., 2002) and others (Brookes et al., 1999), have demonstrated the presence of an mtNOS activity in rat heart mitochondria.

Materials and methods

Isolation of intact mitochondria

Male adult Wistar rats of 4 months of age were used, fed *ad libitum* and kept under controlled conditions of light:darkness cycles (Departamento de Farmacobiología, Cinvestav-IPN, Sede Sur. D.F.). All animal procedures were conducted in accordance with our *Federal Regulations for Use and Care of Animals* (Ministry of Agriculture, SAGAR, 2001. México). Heart mitochondria were isolated by differential centrifugation as described in (Pérez-Vázquez et al., 2002) with slight modifications (Manzo-Ávalos et al., 2002). Briefly, rat hearts were excised, fragmented and washed with SHE medium (0.25 M sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4). The heart fragments were incubated with the protease Nagarse, washed with buffer and homogenized (1/10, w/v). The homogenate was centrifuged at 700 $\times$ *g*; the pellet was centrifuged at 7168 $\times$ *g*. The mitochondrial pellet was resuspended with 1 ml of SHE medium and incubated with 4 mg/ml BSA and 2.88 mM ADP. The mitochondrial suspension was diluted by adding SHE and centrifuged at 9072 $\times$ *g*. The pellet was resuspended in 0.5 ml of the same buffer. All the centrifugations were carried out during 10 minutes at a temperature of 4°C. Mitochondrial protein concentration was obtained by a slight modification of the biuret method (Gornall et al., 1949). As a control, all mitochondria used had a respiratory control higher than 4 using 5 mM glutamate and malate as substrates.

Rupture of mitochondria

Once mitochondria were isolated, the suspension was subjected to sonication in a Branson sonifier; three pulses of 30 sec each with an intensity of 4.5 pps were applied with intervals of 1 min plus the addition of 0.5 mM PMSF (proteases inhibitor) since the beginning.

Incubation media

Intact mitochondria were incubated in a medium containing KCl (120 mM), MOPS (20 mM), pH 7.4, EGTA (0.5 mM) (KME medium) plus the addition of glutamate-malate (10 mM), NaCl (10 mM), NaH_2PO_4 H_2O (5 mM), the substrate L-arg (1 mM), and the cofactors NADPH (0.35 mM), FAD (7 μM), tetrahydrobiopterin (BH_4) (10 μM) at 25°C. Permeabilized mitochondria were incubated as described for intact mitochondria plus the addition of Triton X-100 (0.0013%). Broken mitochondria were incubated in KME medium and added with L-arg (1 mM), NADPH (0.35 mM), FAD (7 μM), BH_4 (10 μM) and free Ca^{2+} (1 mM) as described (Ghafourifar and Richter, 1997; Giulivi et al., 1998). For hypoxia-reoxygenation mitochondria, we used the same conditions as for intact mitochondria, except that the mitochondrial suspension was incubated in a closed chamber (2.7 ml total volume) with two holes, one to receive fresh incubation medium, the other to discard it depending of the experiment.

NO determination

The reaction between NO and oxyhemoglobin (oxyHb) is based in the oxidation of NO to nitrate by oxyHb plus methemoglobin (metHb) as described (Feelisch et al., 1996).

ATP and ATPase determination

ATP determination was according to the $NADP^+$-coupled system at 340 nm and using the extinction molar coefficient of 6.22×10^3 M/cm as described (Castrejón et al., 1997). The standard ATPase assay used was as described by Guerra et al. (1995).

Statistical analysis

Data were subjected to ANOVA and Student's *t* tests to determine the statistical significance ($p < 0.05$). Points represent the mean ± SEM with the number of experiments in parenthesis in duplicate and separate.

Results

Intramitochondrial nitric oxide in intact and permeabilized mitochondria

As a result that the mitochondrial NOS, mtNOS, was localized attached to the inner membrane and oriented through the matrix (Ghafourifar and Richter, 1997) we aimed to test NO synthesis in rat heart using intact mitochondria (Fig. 1). NO production presented a saturation kinetic curve with an initial rate at 1 min a maximum velocity (V_{max}) of 0.185 ± 0.03 nmoles NO/min/mg, amount that increased significantly to a V_{max} of 1.58 ± 0.25 nmoles NO/min/mg ($p < 0.05$) when permeabilized heart mitochondria were used. The increment of 7.5-fold compared to control values is due to the accessible transport of both the cofactors and the substrate to the active site of the mtNOS in the permeabilized mitochondria. The increment in NO synthesis in permeabilized mitochondria diminished to a V_{max} of 0.56 ± 0.2 nmoles NO/min/mg when the competitive inhibitor N^w-monomethyl-L-arginine (L-NMMA) (Boucher et al., 1999; Tsikas et al., 2000) was added (Fig. 1). The inhibition of NO synthesis values in permeabilized mitochondria continued augmented 2.8 times when the values were compared with the basal (0.185 ± 0.03 nmoles NO/min/mg control values of NO synthesis from intact heart mitochondria (Fig. 1).

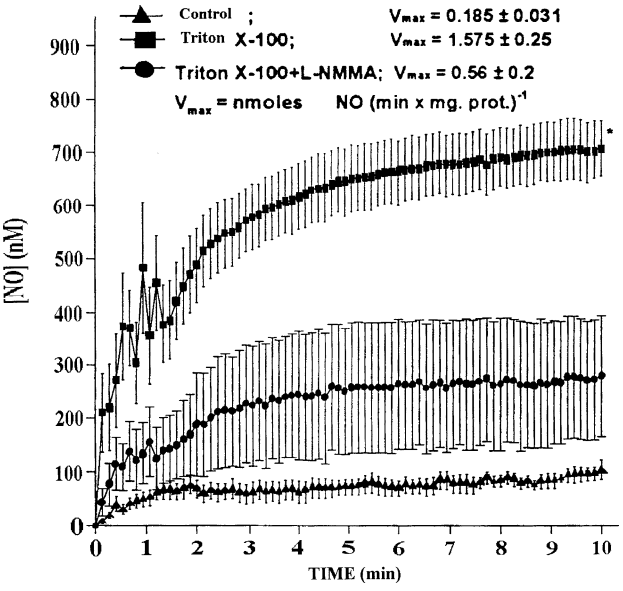

Fig. 1. Effect of nitric oxide on intact and permeabilized mitochondria. Heart mitochondria (0.3 mg/ml) were incubated in KME (120 mM KCl, 20 mM MOPS, 0.5 mM EGTA, pH 7.4) medium added with L-arginine (1 mM), NADPH (0.35 mM), BH_4 (10 μM), FAD (7 μM) as described (Ghafourifar and Richter, 1997; Giulivi et al., 1998). Permeabilized mitochondria were added with Triton X-100 (0.0013%). Other details as in Materials and methods section. Points represent the mean ± SEM (n = 4). *$p < 0.05$

Intramitochondrial nitric oxide is calcium-dependent

Recently, it was found that liver mitochondria have an mtNOS calcium-dependent activity enzyme (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Giulivi, 1998). In this report, we aimed to study how the presence of calcium could affect initial velocities of the mtNOS from heart. Therefore, nitric oxide synthesis was assayed using broken heart mitochondria (Fig. 2). A linear kinetic curve with a V_{max} of 2.74 ±

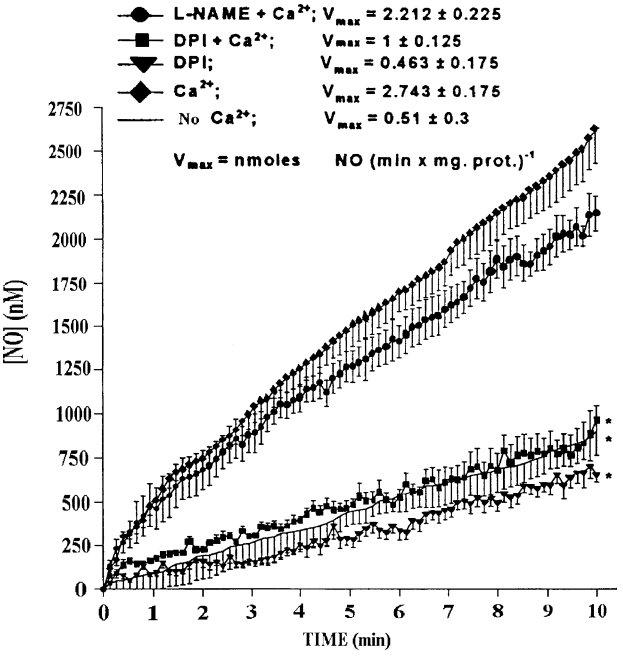

Fig. 2. Effect of nitric oxide on broken mitochondria. Heart mitochondria (0.3 mg/ml) were incubated in KME medium as in Fig. 1. Other details as in Materials and methods section. Points represent the mean ± SEM (n = 4). *$p < 0.05$

0.17 nmoles NO/min/mg was obtained when $CaCl_2$ (1 mM) was present compared with the V_{max} of 0.51 ± 0.3 nmoles NO/min/mg obtained in the absence of calcium. When we tested the addition of the competitive inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME) (Boucher et al., 1999; Tsikas et al., 2000) plus $CaCl_2$ the NO production diminished to a V_{max} of 2.2 ± 0.22 nmoles NO/min/mg and a diminution of 19% was obtained compared with the addition of calcium and without inhibitor (Fig. 2). When the addition of the uncompetitive inhibitor Diphenyleneiodonium (DPI) plus calcium was tested, NO synthesis diminished significantly to a V_{max} of 1.0 ± 0.12 nmoles NO/min/mg ($p < 0.05$). In rat heart broken mitochondria (Fig. 2) added with the substrate and the cofactors BH_4 and NADPH, NO synthesis was assayed, a V_{max} of 0.51 ± 0.3 nmoles NO/min/mg was obtained, which diminished to a V_{max} of 0.46 ± 0.1 nmoles NO/min/mg when DPI was present. The results of Fig. 2 clearly demonstrate that the rat heart mtNOS has a Ca^{2+}-dependent activity and probably suggest the same mechanism as for liver mitochondria (Ghafourifar and Richter, 1997; Tatoyan and Giulivi, 1998).

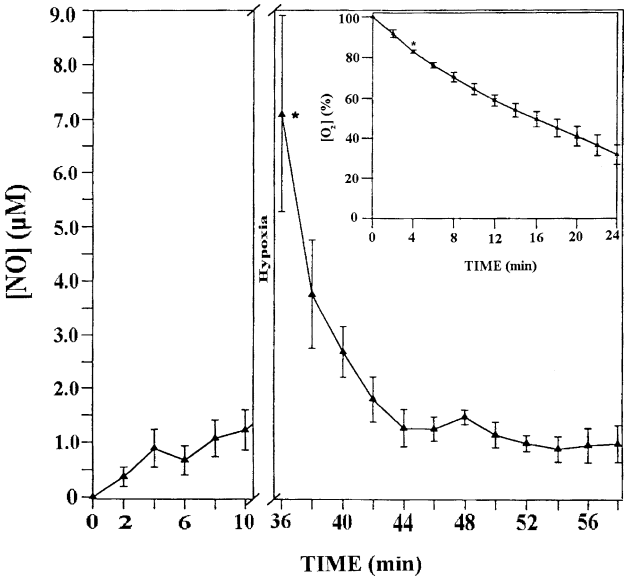

Fig. 3. Effect of nitric oxide on hypoxia-reoxygenation mitochondria. Intact heart mitochondria (0.3 mg/ml) were incubated as in Figure 1 added with glutamate-malate (10 mM), NaH_2PO_4 (5 mM), NaCl (10 mM). Insert represents oxygen concentration in percentage during hypoxia. Points represent the mean ± SEM (n = 4). *$p < 0.05$

Hypoxia-reoxygenation and intramitochondrial nitric oxide

When is reduced or cessation of blood occurs to a vascular bed and then rapidly is reperfused, a severe perturbation occurs to the organ perfused (McCord, 1987). This reperfusion injury to the organ is accompanied by synthesis and release of reactive oxygen species (ROS) (Cadenas and Davies, 2000; Jaimes et al., 2001). Contrary results of the ROS damage were described in heart mitochondria where oxygen radicals may serve a beneficial role in the regulation of metabolic function (Gabbita et al., 2000; Finkel, 2000; Nulton-Persson and Szweda, 2001). In this context, mitochondrial NO synthesis should have a crucial role due to the interaction between ROS and NO in ischemic conditions as described (Manzo-Ávalos et al., 2002). In the present study, we hypothesized that the intramitochondrial nitric oxide is accumulated in hypoxia conditions, due to the partial presence of one of the substrates, i.e., oxygen in rat heart (Fig. 3). Intact heart mitochondria were incubated in a solution that contained the respiratory substrates glutamate plus malate, condition that produced 1 μM NO after 10 min incubation; then the flux of the incubation medium was stopped and the period of hypoxia (24 min) was started. After the hypoxic period, NO synthesis reached an accumulation of about 7 μM ($p < 0.05$), amount that decreased to the initial basal values (1 μM) when reoxygenation was started (Fig. 3). Oxygen concentrations measured for 24 min in the incubation medium decreased about 25% during the hypoxic period (see insert of Fig. 3) suggesting that heart mitochondria did not have irreversible damage during the experiment.

ATP synthesis and intramitochondrial nitric oxide

To correlate the effect of the mitochondrial NO accumulated during the hypoxic period on ATP synthesis, this mitochondrial function was measured under three NO concentrations chosen (1, 3.5 and 7 μM) with the use of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (Table 1). Control ATP values (142 ± 15 nmoles/min/mg) from heart mitochondria represent 100%. In the presence of 1 μM NO, ATP synthesis decreased 62%. A significant diminution to 44% ($p < 0.05$) in the presence of 3.5 μM NO was obtained, and a more pronounced diminution to 23% when it was added 7 μM NO ($p < 0.05$) (Table 1). Since an inhibition on ATP synthesis in the presence of NO was obtained, we aimed to test the activity of the enzyme

involved in its synthesis, i.e., ATPase, as the possible target of the mitochondrial nitric oxide accumulated (Table 1). The ATPase activity was not affected in whenever the concentrations of NO-SNAP produced tested, as is shown in Table 1. Positive control of inhibition was assayed with the addition of oligomycin.

Table 1. Effect of nitric oxide on ATP synthesis and ATPase activity in heart mitochondria[a,b,c,d]

NO	ATP	ATPase
0	142 ± 15	0.28 ± 0.02
1.0	88 ± 4	0.24 ± 0.04
3.5	63 ± 3	0.25 ± 0.03
7.0	33 ± 9	0.27 ± 0.02
–	–	0.03 ± 0.001[d]

[a] NO (μM) concentrations was achieved by using the SNAP donor
[b] ATP (nmoles/mg/min) was measured using the method described in Castrejón et al., 1997
[c] ATPase activity (μmoles/mg/min) was measured using the method described in Guerra et al. (1995)
[d] Oligomycin was used as a positive control and added at a final concentration of 0.05 mg/ml

Intramitochondrial nitric oxide and respiratory control ratio

Mitochondrial electron transport chain supplies the membrane potential necessary to maintain functioning the transport mechanisms involved and required in normal conditions. Calcium homeostasis is one element that requires that integrity. The earlier NO concentrations chose and obtained in hypoxia-reoxygenation conditions (Fig. 3) were used to test our main hypothesis where should be an interaction between mitochondrial NO and matrix free calcium on the respiratory chain, which represent both state 3 and state 4 respiration. For this experiment, free Ca^{2+} was assayed from physiological (nanomolar) to micromolar concentrations. Figure 4 shows the respiratory control ratio (RCR) as 100% in the absence of calcium (Fig. 4A). Here in the presence of 1 μM NO a significant ($p < 0.05$) diminution to 51 ± 10% in the RCR was obtained. When we tested 3.5 or 7.0 μM NO, again, a significant ($p < 0.05$) decrease to 38 ± 5% and 40 ± 7% on the RCR, respectively, was observed. In the presence of physiological Ca^{2+} concentration (0.1 μM) (Fig. 4B), the RCR values were maintained between 59 ± 1% to 65 ± 0.8% but without the critical

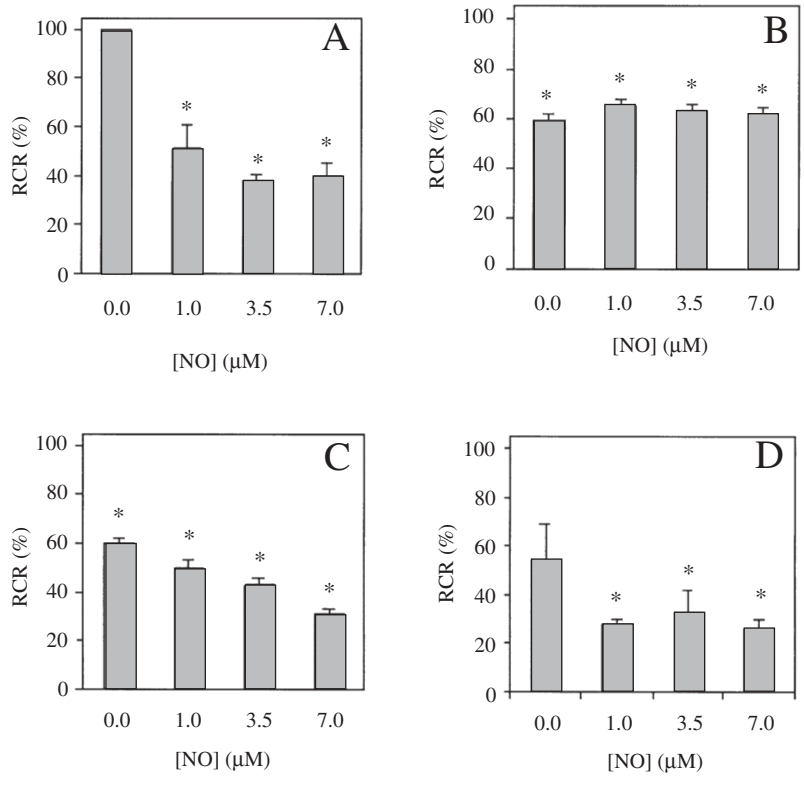

Fig. 4. Effect of nitric oxide on the respiratory control ratio. Oxygen consumption was measured in heart mitochondria (0.3 mg/ml) incubated in KME medium as in Fig. 3 in a final volume of 2.5 ml. Respiration was stimulated with 800 μmoles ADP. nmoles O_2/min/mg for state 3 respiration of mitochondria incubated in absence of calcium and the nitric oxide donor was 79 ± 8; state 4 was 10 ± 3. **A**= 0 Ca^{2+}; **B** = 0.1 μM Ca^{2+}; **C** = 1.0 μM Ca^{2+}; **D** = 2.0 μM Ca^{2+}. The free concentration of Ca^{2+} in the incubation medium was calculated with the computer program WinMax 2.0 (Patton, 1999). Data represent the mean ± SEM. n = 3. *$p < 0.05$

and irreversible inhibitory effects of the NO added (Fig. 4A). In this experiment (Fig. 4B), state 3 respiration was maintained, phosphorylating ADP even in the presence of the different NO concentrations tested (data not shown). This result suggests that a dual interaction between mitochondrial NO and matrix free Ca^{2+} takes place, controlling the oxygen consumption by heart mitochondria. When an upper but still physiological concentration of free Ca^{2+} (1 μM) was tested (Fig. 4C), a 60 $\pm$ 1% in the RCR was obtained. The addition of the three different concentrations of NO inhibited significantly ($p < 0.05$) the RCR to 50 $\pm$ 2% (1 μM NO), 43 $\pm$ 0.4% (3.5 μM NO) and 31 $\pm$ 0.9% (7 μM NO), respectively. The influence of the highest concentration of free Ca^{2+} (2 μM) started inhibiting the RCR to 54 $\pm$ 14%, as expected. This inhibition on the RCR was potentate significantly ($p < 0.05$) when NO was tested at the three different concentrations, i.e., 1 μM = 28 $\pm$ 2%; 3.5 μM = 32 $\pm$ 8%; 7 μM = 26 $\pm$ 5%.

Discussion

In the previous study, we determined that heart mitochondria are sensitive to matrix free Mg^{2+} concentrations and to a clear response of inhibition of the mtNOS activity by the L-arginine analogs L-NMMA and L-NAME (Manzo-Ávalos et al., 2002). In the present study, the response exhibited in Fig. 1 by the mtNOS against the specific inhibitors L-NMMA or DPI (Fig. 2) confirmed the presence of this isoform in the mitochondrial milieu, as observed by others (Kanai et el., 2001; Aguilera-Aguirre et al., 2002; Hardy and May, 2002; Manzo-Ávalos et al., 2002). The cofactor BH_4 can diffuse constantly and there are not transport mechanisms in mitochondria described yet, but the requirement for NADPH is not necessary in mitochondria because is synthesized in the organelle (Lerner et al., 2001). In most cell types, L-arginine transport is energy-independent between extracellular compartments (Hardy and May, 2002), but in intracellular compartments is regulated by transmembrane potential (Baydoun et al., 1999; Hattori et al., 1999), which is the case for intact mitochondria. In addition, the influence of L-arginine transport in the heart-permeabilized mitochondria was not a problem due to the lack of the membrane potential barrier that sense the concentration of the L-amino acid to be transported into the mitochondria for NO synthesis (Hardy and May, 2002). Nitric oxide measurement monitoring the oxyhemoglobin conversion to methemoglobin by difference absorption spectroscopy (Feelisch et al., 1996) is a well-recognized technique (Kanai et al., 2001; Aguilera-Aguirre et al., 2002). The low concentration of NO detected in intact mitochondria is an important specific conclusion obtained in this study. The oxyhemoglobin assay allows detection of only the NO synthesized outside of the intact mitochondria, which suggests that intramitochondrial NO is required only for mitochondrial purposes. In addition, in our experimental approach we showed that heart mitochondria mtNOS is a Ca^{2+}-dependent activity as described by others (Kanai et al., 2001; Aguilera-Aguirre et al., 2002; Manzo-Ávalos et al., 2002). Besides, the demonstration of the initial maximum velocity values for this enzyme correlated well with the specific experimental approach, i.e., the presence of inhibitors or substrates. Regarding the results of the substrates, the presence of oxygen (insert Fig. 3) was a clue in the understanding of the NO accumulation. After the 24 min hypoxia conditions, heart mitochondria accumulated NO (Fig. 3). This fact is not contrary with the results of Manzo-Ávalos et al. (2002) where NO concentrations were lowered in ischemia conditions due to the lack of oxygen, a substrate for the mtNOS. In our results, oxygen still present as showed (insert of Fig. 3) with heart mitochondria respiring, allowing mitochondria to continue synthesizing NO. This accumulation inhibited ATP synthesis as shown by others (Bates et al., 1996; Brookes et al., 1999; Dai et al., 2001). The mechanism of inhibition was not on the ATPase activity (Table 1), but on the other system involved in energy production, i.e., respiratory electrons transport chain. There are few studies suggesting the specific inhibition site, which involves the cytochrome c oxidase activity (Brown and Cooper, 1994; Cleeter et al., 1994; Giulivi, 1998; Kanai et al., 2001). In this study we detailed that mitochondrial free calcium is involved in the interaction between NO and its regulatory effect on the respiratory electrons transport chain (Fig. 4). In the absence of calcium, mitochondrial NO diminished RCR that indicates uncoupling of phosphorylation and the electrons transport chain systems; however, Ca^{2+} under physiological concentrations protected mitochondria from the inhibitory effects observed when NO accumulated (Fig. 4B). This protective effect at physiological concentrations is due to free Ca^{2+}, which stimulate oxidative phosphorylation (Hansford, 1985) or citrulline synthesis (Saavedra-Molina and Devlin, 1997), among other

effects, where small oscillations in free calcium affected those mitochondrial metabolic pathways. This condition is not present when free Ca^{2+} increased to supraoptimal concentrations (Figs. 4C, 4D), where the addition of the different NO concentrations tested invariably inhibited state 3 respiration and increase the state 4 respiration, allowing an RCR of inhibition. We concluded that mitochondrial free Ca^{2+} plays an important role as part of the mechanism on the inhibition of the electron transport chain by nitric oxide. Inhibition of ATP production by nitric oxide in heart mitochondria becomes an important issue due to the importance in pathologic myocardium and myocardial contractility as well as in hypertension.

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