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Original Contribution

Nitric oxide interacts with mitochondrial complex III producing antimycin-like effects

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ABSTRACT

The effect of NO between cytochromes *b* and *c* of the mitochondrial respiratory chain were studied using submitochondrial particles (SMP) from bovine heart and GSNO and SPER-NO as NO sources. Succinate-cytochrome *c* reductase (complex II-III) activity (222 ± 4 nmol/min. mg protein) was inhibited by 51% in the presence of 500 μM GSNO and by 48% in the presence of 30 μM SPER-NO, in both cases at ~1.25 μM NO. Neither GSNO nor SPER-NO were able to inhibit succinate-Q reductase activity (complex II; 220 ± 9 nmol/min. mg protein), showing that NO affects complex III. Complex II-III activity was decreased (36%) when SMP were incubated with L-arginine and mtNOS cofactors, indicating that this effect is also produced by endogenous NO. GSNO (500 μM) reduced cytochrome b_{562} by 71%, in an [O_2] independent manner. Hyperbolic increases in $O_2^{\bullet-}$ (up to 1.3 ± 0.1 nmol/min. mg protein) and H_2O_2 (up to 0.64 ± 0.05 nmol/min. mg protein) productions were observed with a maximal effect at 500 μM GSNO. The $O_2^{\bullet-}/H_2O_2$ ratio was 1.98 in accordance with the stoichiometry of the $O_2^{\bullet-}$ disproportionation. Moreover, H_2O_2 production was increased by 72–74% when heart coupled mitochondria were exposed to 500 μM GSNO or 30 μM SPER-NO. SMP incubated in the presence of succinate showed an EPR signal ($g=1.99$) compatible with a stable semiquinone. This EPR signal was increased not only by antimycin but also by GSNO and SPER-NO. These signals were not modified under N_2 atmosphere, indicating that they are not a consequence to the effect of NOx species on complex III area. These results show that NO interacts with ubiquinone-cytochrome *b* area producing antimycin-like effects. This behaviour comprises the inhibition of electron transfer, the interruption of the oxidation of cytochromes *b*, and the enhancement of $[UQH^{\bullet}]_{ss}$ which, in turn, leads to an increase in $O_2^{\bullet-}$ and H_2O_2 mitochondrial production rates.

1. Introduction

Mitochondrial complex III (cytochrome bc_1 complex or ubiquinol:cytochrome *c* oxidoreductase) is a homodimer of identical monomers consisting of 11 different subunits. In each monomer, the functional core is composed by three subunits, containing all prosthetic groups involved in the redox reactions: the diheme cytochrome *b* (b_H and b_L), the cytochrome c_1, and the Rieske iron-sulfur protein containing a [Fe_2S_2] cluster [1–7]. Complex III plays a central role in the mitochondrial respiratory chain transferring electrons from ubiquinol to cytochrome *c*, and pumping H^+ to the intermembrane space through the protonmotive Q-cycle [8,9]. The catalytic Q_o site of cytochrome bc_1 is unique in that it changes the electronic stoichiometry by steering two electrons from ubiquinol (UQH_2) to two separate and different redox acceptors: it delivers one electron to the Rieske cluster in the high-potential chain and the second electron to the heme b_L in the low potential chain [8,9]. The net translocation of $2H^+/2e^-$ is achieved by a directed uptake and release of protons at topologically separated ubiquinol-oxidation site (P center or Q_o) and ubiquinone-reduction site (N center or Q_i), located at opposite sides of the membrane, and the vectorial transfer of electrons through cytochrome *b* towards the negative side of the membrane. At Q_o site, the intermediate ubisemiquinone radical ($UQH^{\bullet}$) can be formed in two ways: as a part of the forward reaction toward oxidation of quinol when the oxidized [Fe_2S_2] center withdraws one electron from quinol bound at that site (*semiforward mechanism*) or as a part of the reverse reaction toward reduction of quinone when the reduced heme b_L donates its electron to the quinone bound at the Q_o site (*semireverse mechanism*) [10,11]. The $UQH^{\bullet}$ formed during the oxidation of UQH_2 by complex III has been postulated as the reductant for O_2 converting it to superoxide anion ($O_2^{\bullet-}$) [12–15].

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Complex III produces $O_2^{\bullet-}$ at high rates (3–5 nmol $O_2^{\bullet-}$/ min. mg protein) [12,13] in the presence of the Q-cycle inhibitor antimycin, binding this latter in the Q_i site and blocking the oxidation of the cytochrome *b* hemes in the low potential chain. The backup of electrons on the cytochrome *b* hemes limits the oxidation of semiquinone in the Q_o site and allows it sufficient time to interact with and reduce molecular O_2 to generate $O_2^{\bullet-}$ [13,16,17]. Superoxide dismutase (SOD) catalyzes the $O_2^{\bullet-}$ disproportionation producing stoichiometrically hydrogen peroxide (H_2O_2) [14,15], this latter species involved in the regulation of redox sensitive signalling [18,19,20]. Isolated respiring mitochondria produce H_2O_2 at rates that depend on the redox state of the components of the respiratory chain and, consequently, on the mitochondrial metabolic state and the presence of inhibitors [21,22]. Moreover, in 1996, Poderoso and co-workers showed that nitric oxide (NO) inhibits electron transfer increasing $O_2^{\bullet-}$ production in rat heart submitochondrial particles [23]. This effect of NO on mitochondrial respiration was added to the NO inhibitory interaction with cytochrome oxidase (COX) [24,25]. In mammalian cells, NO is synthesized from L-arginine, NADPH and O_2 in a reaction catalyzes by nitric oxide synthases (NOS) [26,27]. The mitochondrial isoform (mtNOS) is located in the inner mitochondrial membrane and it was identified as the α-nNOS with post-translational modifications [28]. At submicromolar concentrations, NO regulates mitochondrial respiration under physiological conditions [29–31] through two main effects on the respiratory chain: the competitive inhibition of COX with O_2 [24,25] and the inhibition of electron transfer at complex III [23]. It is worth noting that the inhibition of COX occurs by a direct competition between NO and O_2 for the two-electron-reduced Cu_B/heme a_3 center of the enzyme [24,25]. Antunes and co-workers explained the reversible inhibition of COX by NO using a mathematical model based in experimental data [32,33]. Conversely, little is known about how NO interacts with the NO-reactive component of the ubiquinone-cytochrome *b* area of the mitochondrial respiratory chain.

Therefore, the aim of this work was to study the effect of NO on mitochondrial electron transfer between cytochromes *b* and *c* using heart submitochondrial particles (SMP) with emphasis on the redox state of cytochromes, the production of $O_2^{\bullet-}$ and H_2O_2, and the steady state concentration of $UQH^{\bullet}$. The consequence of the interaction of NO with complex III was also evaluated in heart coupled mitochondria.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were of analytical grade.

2.2. Isolation of heart mitochondria

Bovine heart mitochondria were obtained from slaughterhouse material according to the general procedure described by Cadenas et al. [15]. Left ventricles from beef hearts were cut in slices with knife and blended 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 mixer (Braun, Minipimer MR 400). The pH was continuously maintained in 7.50 by addition of 1 M Trizma base. The left ventricle was further 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. In order to prepare bovine submitochondrial particles (SMP), the mitochondrial suspensions were stored at −20 °C up to the following day. All the operations were performed at 0–2 °C.

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 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 $MgCl_2$, 1 mM ATP, 2.5 U/ml 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 8000*g* for 10 min. The obtained pellet was resuspended in ice-cold STE buffer and centrifuged at 700*g* for 10 min. The sediment was discarded and both supernatants were mixed and centrifuged at 8000*g* for 10 min. The pellet of this centrifugation was resuspended in STE buffer and centrifuged at 8000*g* 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 [34].

2.3. Preparation of bovine submitochondrial particles

Bovine submitochondrial particles (SMP) were prepared by sonication (Branson Sonifier 450) of bovine thawed mitochondria (20 mg protein/ml) in MST buffer added with 1 mM ATP and 15 mM $MgCl_2$, twelve 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 using an ultracentrifuge (Beckman Optima XL-90 ultracentrifuge, Beckman, USA). Submitochondrial particles were suspended in MST and stored at −80 °C until use [35].

2.4. Preparation of mitochondrial membranes

Bovine mitochondrial membranes were obtained by three cycles of freezing and thawing of the mitochondrial preparation, and homogenized by passage through a 25 G hypodermic needle [36].

2.5. Preparation of S-nitrosoglutathione (GSNO) synthesis and spermine-NONOate (SPER-NO) solution

S-nitrosoglutathione (GSNO) was prepared by mixing equal volumes of 100 mM glutathione in 100 mM KH_2PO_4/K_2HPO_4, pH 7.40, and of 100 mM $NaNO_2$ in 100 mM HCl, at room temperature, to promote thiol nitrosation. The solution was protected from light, kept at 4 °C, and used immediately. To test the effect of decomposed GSNO, GSNO solution was prepared 30–45 days before its use and it was conserved without protection from light, at 4 °C. Taking into account a $t_{1/2}=80$ h for the GSNO decomposition in the dark [37], more than 7 half-lives is an appropriate period to consider an inactivated GSNO solution.

Spermine-NONOate (SPER-NO; $t_{1/2}=39$ min at 37 °C) was prepared in 10 mM NaOH and its concentration was determined

spectrophotometrically at 250 nm (ε=8.0 mM^{-1} cm^{-1}) [38]. The effect of the decomposed adduct was evaluated using a spermine solution (10 mM) in 10 mM NaOH.

2.6. Determination of NO concentration in the reaction medium

Nitric oxide released from GSNO or SPER-NO was estimated using two experimental procedures: a NO sensor (ISO-NOP; World Precision Instruments, Sarasota, FL) and the HbO_2 assay [26,36].

The NO sensor was calibrated by the chemical generation of NO. A known amount of KNO_2 was added to a media containing KI and H_2SO_4 in great excess, to produce a known amount of NO, because the reaction goes to completion. A standard calibration curve was constructed between current output (ΔpA) and [NO]. In order to measure the rate of NO release from GSNO or SPER-NO, the sensor was immersed in a vial containing 100 mM KH_2PO_4/K_2HPO_4, pH 7.50, and 50–500 μM GSNO or 5–30 μM SPER-NO were injected. The slope gives the rate of NO release, in pA/min. The values were converted in μM NO/min using the calibration curve. The effective NO concentration achieved in the media when the NO-donors were incubated for 2 min (pre-incubation time used to expose SMP to NO-donors) was determined according to this approach. All measurements were carried out under stirring at room temperature.

Furthermore, NO released from GSNO or SPER-NO was determined following the HbO_2 oxidation to methemoglobin by NO ($k=3\times10^7$ M^{-1} s^{-1}) for 2 min. The NO assay was performed using a diode array spectrophotometer in which the active wavelength is set at 577 nm and the reference wavelength at the isosbestic point at 591 nm (ε=11.2 mM^{-1} cm^{-1}), at room temperature [26,36]. The reaction medium consisted of 100 mM KH_2PO_4/K_2HPO_4, pH 7.50, 100–500 μM GSNO or 5–30 μM SPER-NO, and 20 μM HbO_2. The difference between the absorbance changes registered in the presence and absence of NO-donors were expressed as μM NO/min.

2.7. SMP exposure to GSNO and SPER-NO

Submitochondrial particles (1 mg protein/ml) were pre-incubated for 2 min, at room temperature, in MST in the absence of substrates (*i.e.* reducing equivalents) and in the presence of 50–500 μM GSNO. After that, aliquots (4 to 10 μl) of pre-incubated SMP were used to measure mitochondrial enzymatic activities and $O_2^{\bullet-}$ and H_2O_2 production rates. In this way, the NO released from GSNO should be negligible during the measurement time.

To note, experimental controls in the presence of 0.6 μM Cu,Zn-SOD (to avoid $ONOO^-$ formation), in the absence of GSNO, or in the presence of decomposed GSNO in the pre-incubation medium were performed during the same incubation time. In the case of $O_2^{\bullet-}$ production assay, SOD addition was omitted in the pre-incubation medium.

On the other hand, SMP (0.01–0.03 mg protein/ml) were exposed to 2.5–30 μM SPER-NO, and mitochondrial electron transfer activities were measured. Experimental controls in the absence of SPER-NO or in the presence of spermine (in 10 mM NaOH) were also implemented.

In order to distinguish the semiquinone signal by EPR, SMP (25–35 mg protein/ml) suspended in MST buffer were exposed to GSNO (500 μM) or SPER-NO (30 μM), at room temperature.

2.8. Mitochondrial electron transfer activities

Complexes I-III (NADH-cytochrome *c* reductase), II-III (succinate-cytochrome *c* reductase) and II (succinate-Q reductase) activities were determined spectrophotometrically using a diode array spectrophotometer (Beckman DU 7400), at 30 °C. Submitochondrial particles exposed to SPER-NO or pre-incubated in the presence of GSNO were added to a medium consisting of MST (0.01–0.03 mg protein/ml), 0.2 mM NADH (complex I-III) or 7 mM succinate (complex II-III), 0.6 μM Cu,Zn-SOD, 0.5 mM KCN, and 25 μM cytochrome c^{3+} [39]. For complexes I-III and II-III, cytochrome *c* reduction was assayed at 550–540 nm (ε=19 mM^{-1} cm^{-1}). To assess the effect of endogenous NO on complex II-III activity, SMP (0.01-0.03 mg protein/ml) were pre-incubated for 3 min at 30 °C, in the presence of 1 mM L-arginine or 2 mM N^G-monomethyl-L-arginine (L-NMMA), 1 mM Cl_2Ca, and 100 μM NADPH. Then, 7 mM succinate, 0.6 μM Cu,Zn-SOD, 0.5 mM KCN, and 25 μM cytochrome c^{3+} were added to the reaction medium. In all cases, the reaction was initiated by addition of cytochrome c^{3+} and the mitochondrial enzymatic activity was expressed as nmol reduced cytochrome *c*/min. mg protein.

Succinate-Q reductase activity was followed at 600 nm (ε=21 mM^{-1} cm^{-1}) as 2,6-dichlorophenolindophenol (DCPIP) reductase activity, in a reaction medium containing MST, SMP (0.01–0.03 mg protein/ml), 16 mM succinate, 0.6 μM Cu,Zn-SOD, 0.5 mM KCN, 1.5 μM rotenone, 1.5 μM antimycin, and 100 μM DCPIP. The reaction was initiated by the addition of 50 μM decylubiquinone and results were expressed as nmol DCPIP reduced/min. mg protein [40].

To note, mitochondrial electron transfer activities were measured in the presence of 0.6 μM Cu,Zn-SOD because SOD activity in SMP was undetectable. The SOD concentration used in the assays is high enough to decompose the presumably formed $O_2^{\bullet-}$.

2.9. Cytochromes redox state

Absorbance spectra (λ=500–640 nm) of mitochondrial membranes (1 mg protein/ml) suspended in MST were obtained in the presence of 7 mM succinate. Additions of 1 mM KCN, 1.5 μM antimycin, and 500 μM GSNO were measured in air-saturated conditions (220 μM O_2). The effect of GSNO was also assessed at 30 μM O_2. This [O_2] was achieved by mixing nitrogen- and air-saturated buffer, and carrying out the measurements in rubber septum-sealed cuvettes. The cytochromes reduction state obtained in the presence of dithionite was considered as 100% of cytochromes reduction.

2.10. Superoxide and hydrogen peroxide production rates

Superoxide anion ($O_2^{\bullet-}$) production rate was measured by the SOD-sensitive reduction of acetylated ferricytochrome *c* [35]. Cytochrome *c* reduction was monitored at two wavelengths in a diode array spectrophotometer (Beckman DU 7400) at 550–540 nm (ε=19 mM^{-1} cm^{-1}), at 30 °C, in a reaction medium containing MST, 7 mM succinate, 0.1 μM catalase, and SMP (0.05–0.2 mg protein/ml) pre-incubated 2 min, in the absence or presence of 50–500 μM GSNO. The reaction was initiated by addition of 15 μM acetylated cytochrome c^{3+}.

Hydrogen peroxide (H_2O_2) production was determined fluorometrically at 365–450 nm (Hitachi F-3010 spectrofluorometer) using the scopoletin-horseradish peroxidase assay [41], at 30 °C. The reaction medium consisted of MST, 7 mM succinate, 0.6 μM Cu,Zn-SOD, 1 μM HRP, 1 μM scopoletin, and SMP (0.05–0.20 mg protein/ml) or heart coupled mitochondria (0.05–0.10 mg protein/ml), without or with the addition of 10 μM catalase. Submitochondrial particles (1 mg protein/ml) were pre-incubated 2 min in the absence or presence of GSNO (50–500 μM) and an aliquot of this pre-incubated particles suspension was added to the H_2O_2 reaction medium. To measure H_2O_2 production by heart coupled mitochondria, the reaction medium was added with 1 μM rotenone to inhibit the complex I-H_2O_2 production by reverse electron transfer. Heart coupled mitochondria (10 mg protein/ml)

were pre-incubated 2 min in the absence or presence of GSNO (500 μM) or SPER-NO (30 μM) and then they were added to the H_2O_2 reaction medium.

A calibration curve was performed using H_2O_2 (0.05–0.35 μM) to express the fluorescence changes as nmol H_2O_2/min. mg protein. Only the fluorescence change inhibited by catalase addition was considered to calculate H_2O_2 production.

2.11. Detection of semiquinones by Electron Paramagnetic Resonance (EPR)

EPR spectra were recorded using a Bruker EMX 080 spectrometer (Bruker Analytik GmbH, Rheinstetten, Germany) equipped with a TM 8810 microwave cavity. Spectra were carried out at room temperature at a microwave frequency of 9.80 GHz and 100 kHz field modulation [42,43].

In order to identify the EPR signal corresponding to ubisemiquinone ($UQH^{\bullet}$), the analogous durosemiquinone was used as standard. This semiquinone was generated by the *in vitro* reaction between duroquinol and NO. Duroquinol was previously obtained through the reduction of duroquinone with excess of $NaBH_4$. The medium consisted of 100 mM KH_2PO_4/K_2HPO_4, pH 7.50, 85 μM duroquinol, and 7.5 mM GSNO [43].

The EPR signals registered using SMP (25–35 mg protein/ml) were obtained adding 175 mM succinate to the medium (MST), in the absence or presence of 1 nmol antimycin/mg protein, 1 nmol myxothiazol/mg protein (a Q_o site inhibitor), 1 mM KCN, 500 μM GSNO, 500 μM GSH or 30 μM SPER-NO.

2.12. Protein concentration and statistics

Protein concentration was determined with the Folin reagent using bovine serum albumin as standard. Results are expressed as means ± SEM of four to six independent experiments. Dunnet's t test was used to analyze the significance of differences between paired determinations. The 0.01 or 0.05 probability levels were used as a criterion for biological significance. Statistical analysis was done using GraphPad Instat 4 (GraphPad Software, La Jolla, CA, USA).

3. RESULTS

3.1. Effect of GSNO on succinate-cytochrome c reductase, succinate-Q reductase and NADH-cytochrome c reductase activities

GSNO inhibited succinate-cytochrome *c* reductase activity (complex II-III; 222 ± 4 nmol/min. mg protein) of heart SMP in a concentration dependent manner (Fig. 1A). Complex II-III activity showed a hyperbolic decay as function of GSNO concentration. At 500 μM GSNO, a 51% inhibition of electron transfer from succinate to cytochrome *c* was registered. Control experiments using inactivated GSNO (in a concentration equivalent to 500 μM GSNO) have shown that when more than 7 half-lives have spent, GSNO was not able to inhibit complex II-III activity (226 ± 7 nmol/min. mg protein). In addition, Fig. 1A shows that the inhibitory effect of GSNO was not observed when succinate-Q reductase (complex II; 220 ± 9 nmol/min. mg protein) activity was measured, indicating that NO inhibits complex III. As well as the effect observed on complex II-III activity, a similar hyperbolic inhibition pattern was observed on NADH-cytochrome *c* reductase activity (complex I-III; 349 ± 6 nmol/min. mg protein), reaching a 63% inhibition at 500 μM GSNO (Fig. 1A inset). The absence of NO effect on succinate-Q reductase activity and the inhibition of complex II-III and I-III activities in a similar mode agree with the existence of a NO-sensitive site in the ubiquinone-cytochrome *b* area of the mitochondrial respiratory chain [23].

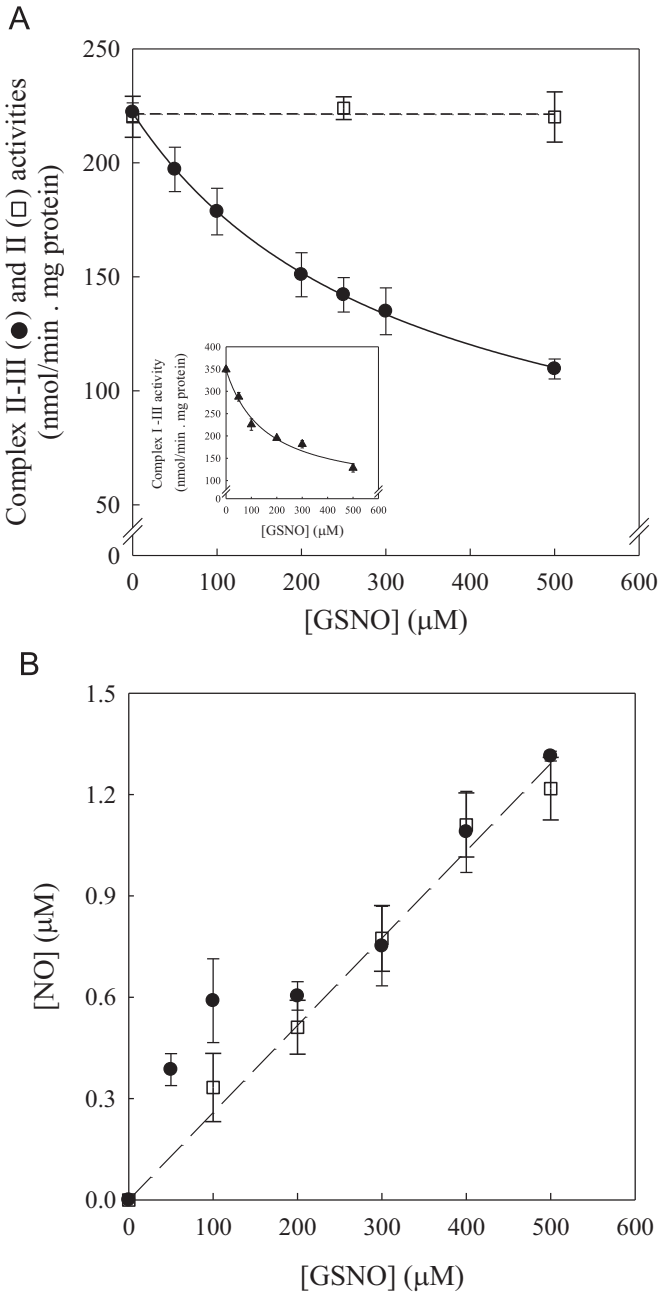


Fig. 1. A. Effect of GSNO on succinate-cytochrome *c* reductase (complex II-III), succinate-Q reductase (complex II) and (inset) NADH-cytochrome *c* reductase (complex I-III) activities. SMP (1 mg protein/ml) were pre-incubated for 2 min in the presence of 50–500 μM GSNO. Pre-incubated SMP (0.01–0.03 mg/ml protein) were transferred to the reaction medium as described in "Materials and Methods". Values obtained from 100 μM GSNO were statistically different ($p < 0.01$) respect to control (0 μM GSNO). **B.** Effective NO concentrations reached after 2 min of incubation of GSNO in the reaction medium, and measured using a NO-sensitive electrode (•) or the HbO_2 assay (□; $r^2 = 0.985$, $p < 0.0001$).

In order to estimate the NO concentration released from GSNO to the medium, a NO-sensitive electrode and the HbO_2 oxidation assay were performed. Fig. 1B shows the effective NO concentration achieved after 2 min in the reaction medium when 50–500 μM GSNO were used. At 2 min of incubation (pre-incubation time used to expose SMP to GSNO), the release of NO from 100–500 μM GSNO reached a plateau (data not shown). When [NO] was measured using the NO sensor, a non-linear behaviour was observed at low GSNO concentrations (< 200 μM GSNO). This

unusual result concerning to the NO release from low concentrations of GSNO and its electrochemical detection have been previously reported [37,44]. In contrast, when [NO] was measured through the HbO_2 assay, a linear regression ($r^2=0.985$; $p<0.0001$) was obtained throughout the GSNO concentration range studied. For this reason, and taking into account that similar NO concentrations have been detected at higher concentrations than 200 μM GSNO by both methods, this linear function was used to estimate the NO concentration in the assayed conditions. Accordingly, the maximal inhibitory effect (51%) on succinate-cytochrome *c* reductase activity was observed at 500 μM GSNO, this latter corresponding to ~1.3 μM NO. Moreover, a statistically significant inhibition of complex II-III activity of 20% was achieved by about 0.25 μM NO released from 100 μM GSNO.

3.2. Reversibility and effect of endogenous NO on succinate-cytochrome c reductase

In order to assess the reversibility of the inhibition of succinate-cytochrome *c* reductase activity, SMP were pre-incubated for 2 min in the presence of GSNO, and then exposed to 50 μM HbO_2, as a NO scavenger. The reaction between NO and HbO_2 is one of the most important processes for *in vivo* NO catabolism due to both the high rate constant between HbO_2 and NO ($k=3\times10^7\ M^{-1}\ s^{-1}$) and the blood HbO_2 concentration (2.5 mM as tetrameric form). Table 1 shows that there is not significant changes in the inhibition of complex II-III activity registered in the presence of 500 μM GSNO (~1.3 μM NO) in comparison to the values obtained in the presence of GSNO plus HbO_2 (110 ± 4 *vs.* 106 ± 7 nmol/min. mg protein), showing that the effect of NO on complex III is not readily reversible in the assayed experimental conditions. Moreover, succinate-cytochrome *c* reductase activity was 36% lower than control activity when SMP were incubated for 3 min at 30 °C, in the presence of mtNOS substrates (L-arginine and NADPH) and cofactors (Ca^{2+}). However, in the absence of L-arginine and in the presence of the competitive inhibitor of NOS L-NMMA, the inhibition on succinate-cytochrome *c* reductase activity was not detected (214 ± 6 *vs.* 222 ± 4 nmol/min. mg protein). These data suggest that the inhibitory effect observed can be triggered by mtNOS-produced NO.

3.3. Effect of GSNO on cytochromes redox state

The 500–640 nm absorbance spectra of mitochondrial membranes added with succinate were recorded in the presence of inhibitors of electron transfer (Fig. 2). The spectrum registered at air-saturated conditions (220 μM O_2) under inhibition of complex IV by KCN (Fig. 2A) showed the characteristic peaks of cytochromes *b* (562 nm), cytochrome *c* (550 nm), and cytochrome aa_3 (605 nm). The addition of antimycin (Fig. 2B) abolished the cytochromes *c* and aa_3 peaks and preserved the peak at about 562 nm, which corresponds to both cytochromes *b* (b_H and b_L; 562 and 566 nm, respectively) in the reduced states. Due to the fact that the inhibition of complex IV by NO is competitive with O_2 [24,25,32,33] the spectrum was registered in the presence of GSNO

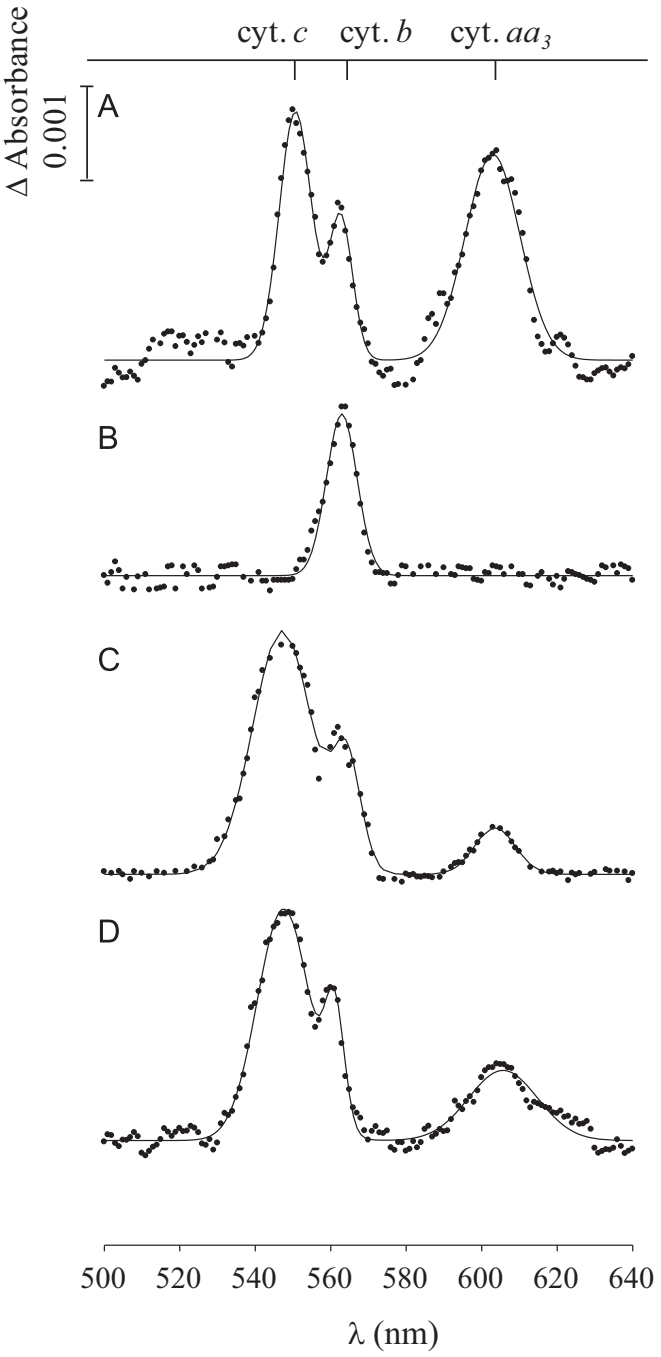


Fig. 2. Absorbance spectra (λ= 500-640 nm) of mitochondrial membranes (1 mg protein/ml) obtained in the presence of 7 mM succinate, in air-saturated conditions, *i.e.* 220 μM O_2 (**A-C**) or in the presence of 30 μM O_2 (**D**). Additions: 1 mM KCN (**A**), 1.5 μM antimycin (**B**), or 500 μM GSNO (**C** and **D**).

Table 1
Succinate-cytochrome *c* reductase activity of SMP exposed to GSNO, GSNO plus HbO_2 or mtNOS substrates, cofactors, and inhibitor

Condition	Complex II-III (nmol/min. mg protein)
SMP, control	222 ± 4
+ 500 μM GSNO	110 ± 4*
+ 500 μM GSNO+50 μM HbO_2	106 ± 7*
+ 1 mM L-arginine+1 mM Ca^{2+}+100 μM NADPH	141 ± 8*
+ 2 mM L-NMMA+1 mM Ca^{2+}+100 μM NADPH	214 ± 6

Statistically different (*$p<0.01$) respect to control

at two O_2 concentrations. At 220 μM O_2 and in the presence of 500 μM GSNO (~1.3 μM NO), the spectrum showed three characteristic peaks (550, 562, and 605 nm) in accordance to inhibition of cytochrome oxidase (COX) activity (Fig. 2C). At 30 μM O_2, an experimental condition that improves the inhibitory effect of NO on complex IV, cytochrome aa_3 (605 nm) and cytochrome *c* (550 nm) peaks were increased while cytochromes *b* (562 nm) peak was not modified (Fig. 2D). These results are expressed as percentage respect to maximal reduction (Table 2), this latter achieved after dithionite addition (100% of cytochromes reduction). The inhibition by KCN leads to a high reduction of the three cytochromes ($b=85\%$, $c=95\%$, and $aa_3=94\%$). This observation agrees

Table 2
Effect of KCN, antimycin, and GSNO on the cytochromes reduction of mitochondrial membranes supplemented with succinate

Inhibitor	$[O_2]$ (μM)	Reduction[#] (% ± SEM)		
		b_{562}	c_{550}	$aa_{3\ 605}$
KCN (1 mM)	220	85 ± 6	95 ± 5	94 ± 6
Antimycin (1.5 μM)	220	79 ± 5	–	–
GSNO (500 μM)	220	71 ± 5	78 ± 6	41 ± 3
GSNO (500 μM)	30	71 ± 4	89 ± 4	61 ± 6

[#] The reduction state in the presence of $Na_2S_2O_4$ was taken as 100% of cytochrome reduction.

with the inhibition of the terminal enzyme of the mitochondrial respiratory chain. When antimycin was used, cytochromes *b* located before the block-site of the inhibitor were highly reduced (79%), while cytochromes *c* and aa_3 remained in the oxidized state. The presence of GSNO at low O_2 concentration (30 μM) leads to a higher amount of reduced cytochrome aa_3 (61%) than the one obtained at air-saturated conditions (41%), whereas the content of cytochromes *b* in the reduced state remained unchanged (71%). These results confirm that the inhibition of COX depends on the $[O_2]$/[NO] relationship [24,25,45,32,33], and show that the inhibition of complex III by NO does not depend on O_2 concentration or the $[O_2]$/[NO] ratio. Moreover, these values verify the existence of two sites of NO interaction: the known Cu_B/heme a_3 center of COX and the bc_1 complex.

3.4. Effect of GSNO on superoxide and hydrogen peroxide production rates

Submitochondrial particles pre-incubated with GSNO showed a concentration dependent and hyperbolic increase in $O_2^{\bullet-}$ and H_2O_2 production rates (Fig. 3A), with significant enhancements from

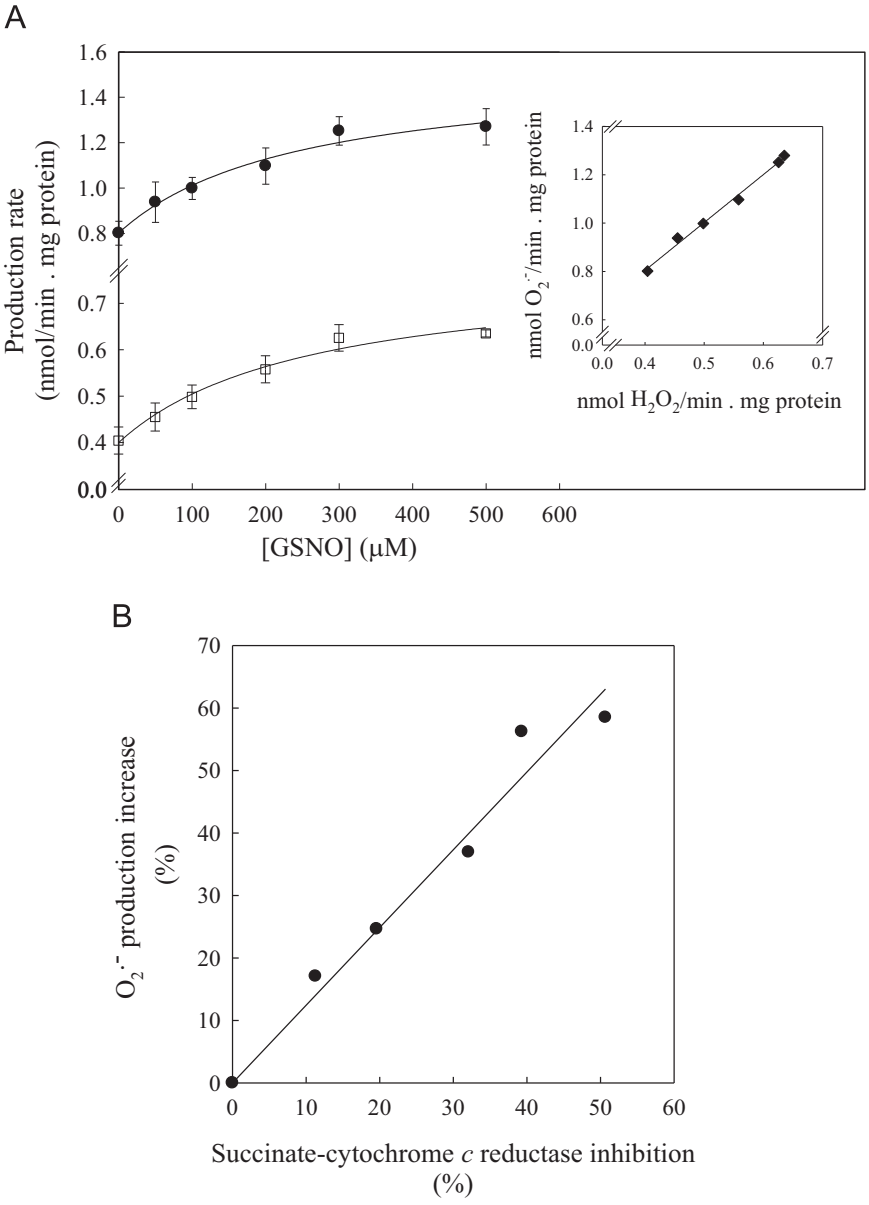


Fig. 3. A. Effect of GSNO on $O_2^{\bullet-}$ (●) and H_2O_2 (□) production rates. SMP (1 mg protein/ml) were pre-incubated for 2 min in the presence of 50-500 μM GSNO. Pre-incubated SMP (0.01–0.05 mg protein/ml) were transferred to the reaction medium as described in "Materials and Methods". Values from 200 μM GSNO were statistically different ($p < 0.05$) respect to control (0 μM GSNO). Inset. Linear correlation ($r^2 = 0.993$; $p < 0.0001$) between $O_2^{\bullet-}$ and H_2O_2 production rates. **B.** Linear correlation ($r^2 = 0.965$; $p < 0.0005$) between $O_2^{\bullet-}$ production rate increase and succinate-cytochrome *c* reductase inhibition, both expressed as percentage of the values determined in the absence of GSNO.

200 μM GSNO. Maximal production rates, 58% higher than in the absence of GSNO, were observed at 500 μM GSNO (~1.3 μM NO). In order to maximize the $O_2^{\bullet-}$ production rate by bc_1 complex, the addition of the classical Q_i site inhibitor antimycin was evaluated. In the presence of 1.5 μM antimycin, $O_2^{\bullet-}$ production rate was 2.4 ± 0.2 nmol/min. mg protein. Thus, in the assessed experimental conditions, the maximal $O_2^{\bullet-}$ production reached after GSNO addition represents a 53% of the total production registered in the presence of antimycin. Moreover, a linear correlation (Fig. 3A inset) between $O_2^{\bullet-}$ and H_2O_2 production rates ($r^2 = 0.993$; $p < 0.0001$) was observed. Since under physiological conditions H_2O_2 is produced via $O_2^{\bullet-}$ dismutation catalyzed by SOD, the stoichiometry between $O_2^{\bullet-}$ and H_2O_2 is close to 2.0. The slope of this plot of 1.98 is in accordance with the stoichiometry of $O_2^{\bullet-}$ dismutation reaction [46,15]. Interestingly, $O_2^{\bullet-}$ production increase and complex II-III inhibition, both expressed as percentage of the values in the absence of GSNO (Fig. 3B), show a linear correlation ($r^2 = 0.965$; $p < 0.0005$), suggesting the existence of a common intermediate in both processes. In this scenario, the $UQH^{\bullet}$ formation could be the intermediate involved, being this latter the main source of $O_2^{\bullet-}$ and H_2O_2 productions [14,41].

3.5. Effect of GSNO on ubisemiquinone formation

To recognize the EPR signal corresponding to $UQH^{\bullet}$, the reaction between NO and duroquinol (DQH_2) was taken as a prototype. The EPR spectrum (Fig. 4A) confirmed that the reaction between DQH_2 and NO produces a stable durosemiquinone radical ($DQH^{\bullet}$) that can be detected by EPR, at room temperature, with a signal centered at $g = 2.03$.

The steady state concentration of $UQH^{\bullet}$ in SMP was achieved adding succinate to the reaction medium (Fig. 4B), in the absence or presence of inhibitors of mitochondrial electron transfer chain. Fig. 4B and Table 3 show representative EPR spectra and the heights of EPR signals at $g = 1.99$, respectively, of 4 independent experiments. Thus, SMP incubated in the presence of succinate for

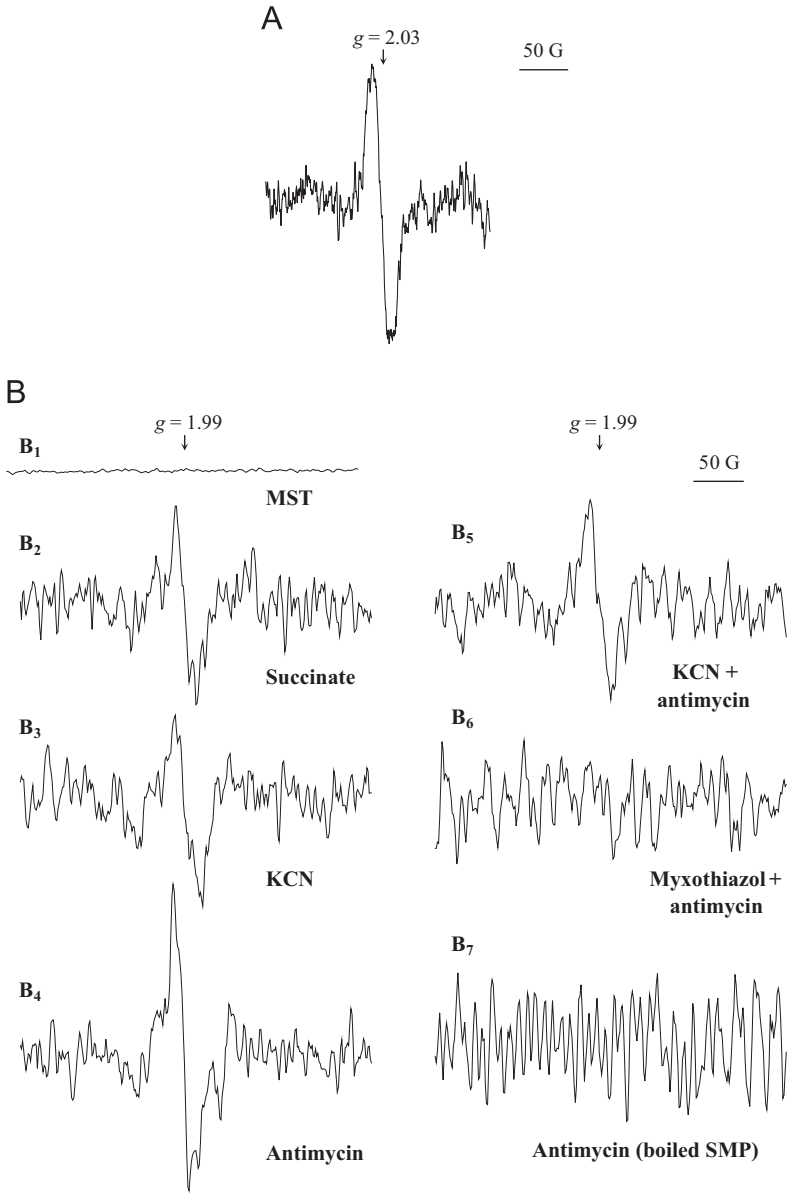


Fig. 4. A. EPR signal of $DQH^{\bullet}$ generated by reaction between 85 μM DQH_2 and NO released from 7.5 mM GSNO. **B.** EPR spectra of SMP (25–35 mg protein/ml) added with 175 mM succinate (**B_2**) and inhibitors of mitochondrial electron transfer chain (**B_3-B_7**). Additions: 1 mM KCN (**B_3**), 1 nmol antimycin/mg protein (**B_4**), 1 mM KCN plus 1 nmol antimycin/mg protein (**B_5**), 1 nmol myxothiazol/mg protein plus 1 nmol antimycin/mg protein (**B_6**). Controls: MST alone (**B_1**) and "boiled SMP" plus 1 nmol antimycin/mg protein (**B_7**). EPR instrument settings: 30 mW microwave power, 10 G modulation amplitude, 10.24 ms time constant, 20 scans (**A**) or 50 scans (**B**), room temperature.

Table 3
Effect of KCN, antimycin, and GSNO on the height of UQH• EPR signal

Condition	h_0 (10^4 AU/mg protein)
Succinate, control	6.18 ± 0.37
+ KCN	6.26 ± 0.22
+ antimycin	8.79 ± 0.41**
+ KCN+antimycin	6.52 ± 0.37
+ GSNO	8.23 ± 0.30*
+ GSNO (N_2 atmosphere)	7.74 ± 0.28*
+ GSH	4.95 ± 0.07

SMP (25-35 mg protein/ml) added with 175 mM succinate were incubated in the absence or presence of 1 mM KCN, 1 nmol antimycin/mg protein, 500 μM GSNO, or 500 μM GSH. Statistically different (**$p < 0.01$, *$p < 0.05$) respect to succinate.

1 min showed an EPR signal at $g=1.99$ (Fig. 4B_2; Table 3) that is attributed to UQH•, implicated in the Q-cycle of mitochondrial electron transfer at complex III [8,9]. This signal was not modified by KCN addition (Fig. 4B_3; Table 3), but it was 42% higher than the control one when the Q_i site inhibitor antimycin was added to the medium (Fig. 4B_4; Table 3). Moreover, when antimycin plus KCN were simultaneously added to the reaction medium, the signal centered at $g=1.99$ decreased by about 26% (Fig. 4B_5; Table 3) in comparison with the signal registered in the sole presence of antimycin (Fig. 4B_4). The attenuation of UQH• signal is explained considering the increase in steady state concentration of reduced cytochrome *c* reached by KCN addition and the role of cytochrome c^{3+} in the oxidation of UQH_2 to UQH• [14]. The incubation of SMP with antimycin plus myxothiazol avoided the UQH• formation (Fig. 4B_6), in agreement with the reported blockage of $O_2^{\bullet-}$ formation [12]. Control experiments were carried out promoting the denaturalization of proteins by heat. The UQH• signal was completely absent if SMP were previously boiled ("boiled SMP"), suggesting that this signal depend on the enzymatic activities (Fig. 4B_7).

In addition, when SMP were incubated in the presence of GSNO for 1 min, the EPR signal centered at $g=1.99$ obtained in the presence of succinate (Fig. 5A) was significantly increased by about 33% (Fig. 5B; Table 3). This signal could partially explain the NO-dependent increase of $O_2^{\bullet-}$ and H_2O_2 production rates showed in Fig. 3A. To discard any effect of GSH on EPR signal, addition of GSH was assessed during the same incubation time. Fig 5C and Table 3 show that the signal at $g=1.99$ obtained in the presence of GSH was not significantly different to the control one (Fig. 5A). Moreover, when GSNO plus myxothiazol were simultaneously added to the reaction medium, the signal centered at $g=1.99$ was not detected (Fig. 5D), similarly to the effect observed by the addition of myxothiazol plus antimycin (Fig. 4B_6). The UQH• signal obtained in the presence of GSNO was absent if the SMP were previously denatured by heat (Fig. 5E). To distinguish if the EPR signals might be consequence of the effect of any chemical species generated by the reaction between NO with molecular oxygen, EPR spectra were obtained under N_2 atmosphere. In this experimental condition, the EPR signal (Fig. 5F) was similar to the one observed in air-saturated situations (Fig. 5B), suggesting that they are not a consequence of the effect of NOx species on complex III.

3.6. Effect of SPER-NO on succinate-cytochrome c reductase and succinate-Q reductase activities and ubisemiquinone formation

Due to the fact that GSNO is not considered a strictly NO donor [37,47], succinate cytochrome *c* reductase and succinate-Q reductase activities and UQH• formation (EPR signal) were

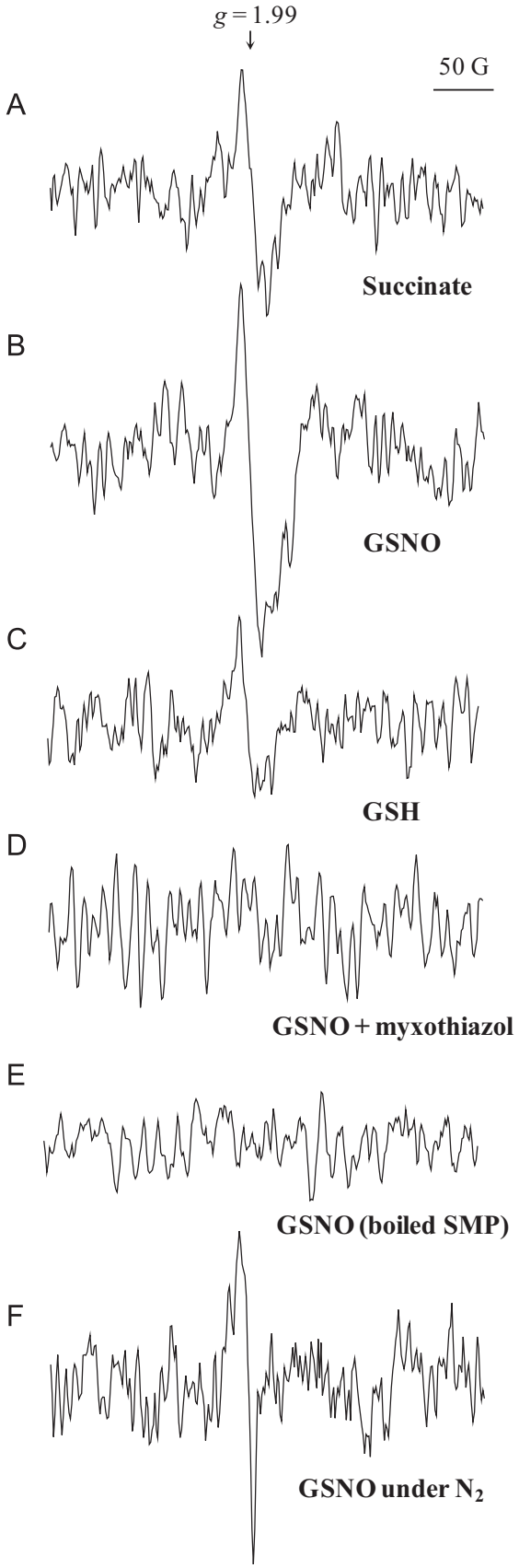


Fig. 5. EPR spectra of SMP (25–35 mg protein/ml) added with 175 mM succinate (**A**) in the presence of 500 μM GSNO (**B, D-F**) or 500 μM GSH (**C**), at atmospheric condition (**A-E**) or under N_2 atmosphere (**F**). Additions: 500 μM GSNO at air-saturated conditions (**B**); 500 μM GSH (**C**); 500 μM GSNO plus 1 nmol myxothiazol/mg protein (**D**); "boiled SMP" plus 500 μM GSNO (**E**); and 500 μM GSNO under N_2 atmosphere (**F**). EPR instrument settings: 30 mW microwave power, 10 G modulation amplitude, 10.24 ms time constant, 50 scans, room temperature.

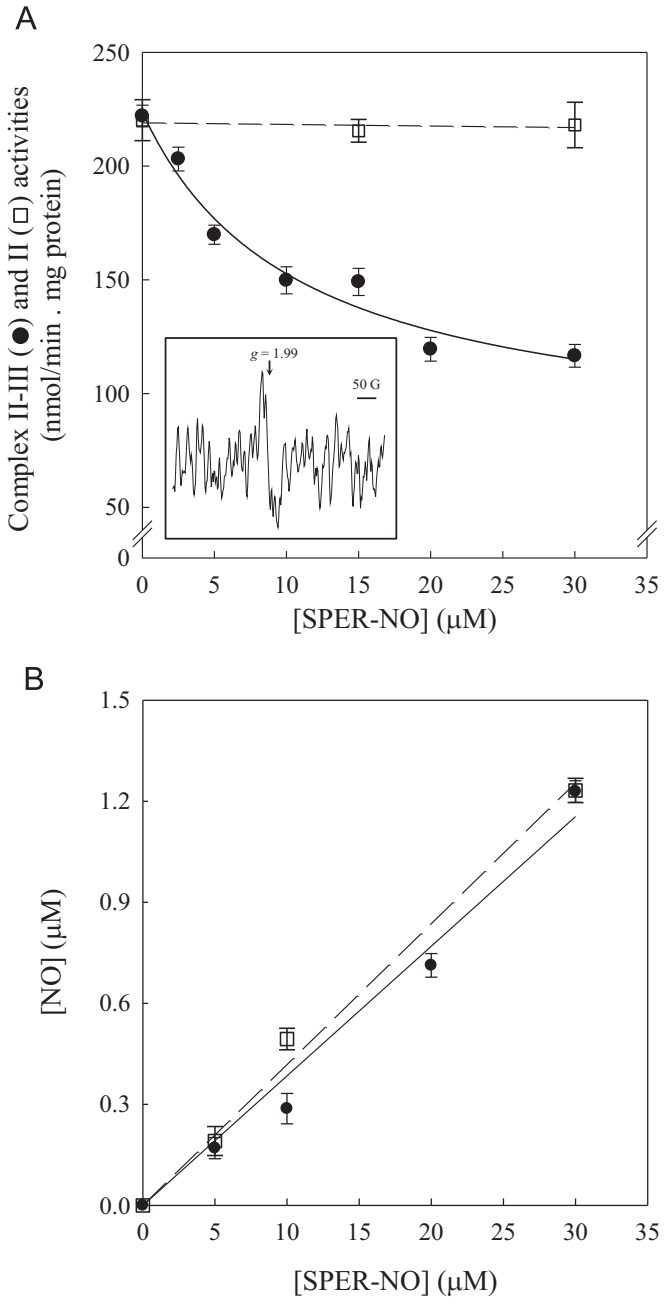


Fig. 6. A. Effect of SPER-NO on succinate-cytochrome *c* reductase (complex II-III) and succinate-Q reductase (complex II) activities. SMP (0.01–0.03 mg protein/ml) in the reaction medium (see "Materials and Methods"). Inset: ubisemiquinone EPR detection. SMP (25–35 mg protein/ml) suspended in MST buffer pH 7.50 and added with 175 mM succinate were exposed to 30 μM SPER-NO. EPR instrument settings: 30 mW microwave power, 10 G modulation amplitude, 10.24 ms time constant, 50 scans, room temperature. **B.** Effective NO concentrations reached after 2 min of incubation of SPER-NO in the reaction medium, measured using a NO-sensitive electrode (●; $r^2=0.981$, $p<0.001$) or the HbO_2 assay (□; $r^2=0.993$, $p<0.005$).

performed using the NO donor SPER-NO. Fig. 6A shows that the NO released from SPER-NO inhibited the succinate-cytochrome *c* reductase activity of heart SMP in a concentration dependent manner. At 30 μM SPER-NO, complex II-III activity was inhibited by 48%. Control experiments using 30 μM spermine have shown that this compound was not able to inhibit complex II-III activity (224 ± 6 nmol/min. mg protein). In addition, SPER-NO did not inhibit succinate-Q reductase activity (220 ± 9 nmol/min. mg protein; Fig. 6A) showing that NO interacts with complex III. SMP incubated in the presence of succinate and 30 μM SPER-NO show an EPR signal at $g=1.99$ (Fig. 6A inset) similar to the one obtained in the presence of antimycin (Fig. 4B_4) or GSNO (Fig. 5B). Thus, the addition of SPER-NO increased the EPR signal by 34% respect to the one observed in the presence of succinate, being the signal height of $(8.38 \pm 0.39) \times 10^4$ AU/mg protein.

Fig. 6B shows the NO concentration achieved after 2 min of incubation of 5–30 μM SPER-NO in the reaction medium. Linear relations between the effective NO and SPER-NO concentrations were observed using not only the NO-sensitive electrode ($r^2=0.981$; $p<0.001$) but also the HbO_2 oxidation assay ($r^2=0.993$; $p<0.005$). Furthermore, comparable NO concentrations were detected using both techniques. The maximal effect on succinate-cytochrome *c* reductase activity was observed at 30 μM SPER-NO, showing an inhibition of 48%, similarly to the one obtained in the presence of 500 μM GSNO (Fig. 1A). In these experimental conditions, the NO concentration reached was about 1.2 μM NO. Moreover, a statistically significant inhibition of complex II-III activity of 20% was achieved by about ~0.20 μM NO released from 5 μM SPER-NO (Fig. 6B).

3.7. *Effect of GSNO and SPER-NO on H_2O_2 production by heart coupled mitochondria*

In order to know if the inhibitory effect of NO on complex III observed in SMP also occurs in intact mitochondria, the effect of 500 μM GSNO or 30 μM SPER-NO on H_2O_2 production by heart coupled mitochondria was studied. Hydrogen peroxide production was determined in a reaction medium in which succinate and rotenone (to inhibit the complex I-H_2O_2 production by reverse electron transfer) were present. Under this experimental conditions, complex III of heart coupled mitochondria produced 0.40 ± 0.04 nmol H_2O_2/min. mg protein (Table 4). The pre-incubation of coupled mitochondria for 2 min with 500 μM GSNO or 30 μM SPER-NO increased H_2O_2 generation by 72–74%. This enhancement was similar to the one obtained when coupled mitochondria were pre-incubated in the presence of the complex III inhibitor antimycin (Table 4). Thus, these data agree with the results obtained using SMP (Fig. 3) and with the mtNOS functional activity concept [48].

4. Discussion

The present work shows that NO, released from GSNO or from SPER-NO or generated by mtNOS, inhibits electron transfer at complex III of the mitochondrial respiratory chain, producing antimycin-like effects. The results obtained in the presence of GSNO were confirmed using the strictly NO donor, SPER-NO. This finding is in accordance with the previous report of Poderoso and coworkers [23] who showed the inhibition of electron transfer and the enhancement of $O_2^{\bullet-}$ production in rat heart mitochondria by NO.

In our hands, complex II-III activity was inhibited by 20% at about 0.20–0.25 μM NO, obtained with 5 μM SPER-NO or with 100 μM GSNO (Fig. 6A; Fig. 1A). The maximal inhibitory effect (51–

Table 4
Effect of GSNO, SPER-NO, and antimycin on H_2O_2 production by heart coupled mitochondria

Condition	H_2O_2 production (nmol/min. mg protein)
Coupled mitochondria, control	0.40 ± 0.04
+ 500 μM GSNO	0.68 ± 0.07**
+ 30 μM SPER-NO	0.69 ± 0.03**
+ 1.5 μM antimycin	0.71 ± 0.06**

Statistically different (**$p<0.01$) respect to control

48%) was reached at ~1.25 μM NO, when 500 μM GSNO or 30 μM SPER-NO were the NO sources. Although Welter et al. [49] and Simonin and Galina [50] have reported the inhibition of complex II by NO, under our experimental conditions, neither GSNO nor SPER-NO were able to inhibit succinate-Q reductase activity (Figs. 1A and 6A). Thus, our results indicate that the [Fe_2S_2] cluster of complex II is protected against NO by the ubiquinol-cytochrome *c* reductase. On the other hand, a hyperbolic pattern of inhibition similar to complex II-III was observed when NADH-cytochrome *c* reductase (complex I-III; Fig. 1A inset) activity was assayed using GSNO. Taking all these results together, we confirm that sub-micromolar NO concentrations produce the inhibition of electron transfer at the ubiquinone-cytochrome *b* region [23,43] and that this effect is centered in a NO-reactive component at complex III. Furthermore, the reduction of succinate-cytochrome *c* reductase activity (36%) observed when SMP were exposed to mtNOS substrates and cofactors (Table 1) is consistent with a physiological role of mtNOS-produced NO in the regulation of mitochondrial energy production and signalling [31,36,51,22]. Thereby, taking into account that heart mitochondria produce about 1 nmol NO/min. mg protein [51] and considering that 0.03 mg of mitochondrial protein is incubated for 3 min, the NO concentration in the reaction medium would be about 0.1 μM, a value that is in agreement with the inhibition of complex II-III by NO released from NO-donors.

In addition, cytochrome spectra (Fig. 2) showed that the content of cytochromes *b* in the reduced state was equal (71%) at 220 μM and at 30 μM O_2, suggesting that the inhibition of complex III by NO (~1.3 μM) is not affected by the [O_2]/[NO] ratio (169 and 23, respectively). On the other hand, in the assessed experimental conditions, the inhibitory effect was not reversed by HbO_2 addition (Table 1), a powerful scavenger of NO ($k=3\times10^7\ M^{-1}\ s^{-1}$; [52]). Thus, the independence on [O_2] and the strong binding of NO to complex III even in the presence of HbO_2, are two differential properties to the ones reported for the inhibition of complex IV by NO [24,25,43,33].

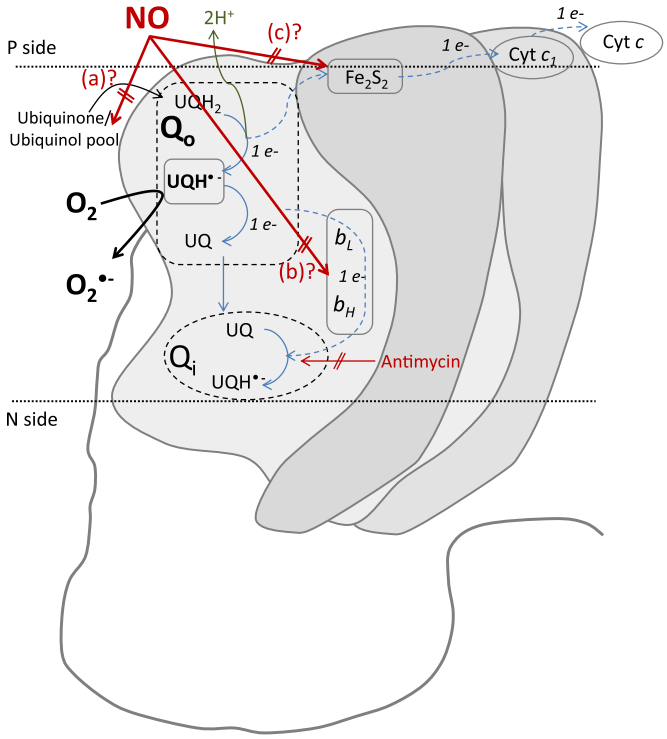
It is known that the inhibition of complex III increases $O_2^{\bullet-}$ production as a result of the autoxidation of $UQH^{\bullet}$. Although the $UQH^{\bullet}$ in the Q_o site is difficult to observe by EPR under conditions that should favour its formation, Quinlan et al. [53] have predicted that at subsaturating substrate concentration, detection of semiquinone by EPR may be possible even in the presence of oxygen. In our experimental conditions, antimycin enhanced (42%) the $UQH^{\bullet}$ signal centered at $g=1.99$ (Table 3). Similarly, when SMP were incubated in the presence of GSNO or SPER-NO the EPR signal ($g=1.99$) was higher (~35%) than in the presence of succinate, showing that NO produces an antimycin-like effect on the ubiquinone-cytochrome *b* area. Thus, not only antimycin but also NO, released from GSNO or from SPER-NO, produced an increase in the steady-state concentration of $UQH^{\bullet}$, which explains the significant enhancement in $O_2^{\bullet-}$ and H_2O_2 production rates. Moreover, myxothiazol, even in the presence of antimycin or GSNO, avoided $UQH^{\bullet}$ formation (Fig. 4B_6 and Fig. 5D, respectively), in agreement with the reported blockage of $O_2^{\bullet-}$ formation [12].

Superoxide anion production by the cytochrome bc_1 complex is originated upon $UQH^{\bullet}$ formation and its production occurs mainly within the Q_o pocket [11,13,16,20]. Mechanistic studies revealed that this intermediate is only formed at very low occupancy. Due to the fact that the production of $O_2^{\bullet-}$ by antimycin-inhibited complex III increases with the fraction of oxidized quinone, reaching a maximum at about 70% reduction of pool, $O_2^{\bullet-}$ can be formed in a reverse reaction involving transient reduction of ubiquinone by heme b_L. Sarewicz and co-workers [10] have shown that $O_2^{\bullet-}$ production by cytochrome bc_1 can be consequence of the combination of both *semiforward* and *semireverse mechanisms*. However, the experimental analysis of a broad set of conditions combined with modelling revealed that formation of $UQH^{\bullet}$ by the reverse heme b_L to quinone electron transfer (*semireverse mechanism*) dominates the steady state of $UQH^{\bullet}$, and, as a consequence, the $O_2^{\bullet-}$ production.

Ubisemiquinone may donate its electron to O_2 that access to the Q_o site with the formation of $O_2^{\bullet-}$ ($O_2+e^-\rightarrow O_2^{\bullet-}$, $E_{m,7}=-160$ mV; $UQH^{\bullet}\rightarrow UQ+e^-+H^+$, $E_{m,7}=+160$ mV) [13,12,6]. The oxidation of $UQH^{\bullet}$ bound at the Q_o site is dependent on the passage of the released electron across the membrane via the *b* cytochromes. Since this is opposed by the membrane potential ($\Delta\psi$), the actual $E_{h,7}$ of the $UQH^{\bullet}$/UQ couple will become more negative at high $\Delta\psi$. Therefore, in the absence of membrane potential, $O_2^{\bullet-}$ production depends on the reduction state of the b_L heme in the superoxide-generating Q_o site, with the highest rates at 70–80% reduction of b_L, independently of the substrates used [10,11,53]. This observation agrees with the content of cytochromes *b* in the reduced state obtained in the presence of antimycin and of GSNO (Table 2); in both situations, cytochrome b^{2+} is about 71–79%. However, in physiological situations, the rate of $O_2^{\bullet-}$ generation by $UQH^{\bullet}$ at cytochrome bc_1 depends on the magnitude of the membrane potential [54,10,11]. A dramatic enhancement in the generation of reactive oxygen species was observed when $\Delta\psi$ increases from a typical respiratory state 3 to state 4 [14,41,55,22], with the consequent increase in steady state of the autoxidable component ($UQH^{\bullet}$). Estimates of the production of $O_2^{\bullet-}$ in respiring mitochondria vary from 0.1% to as much as 4% of total O_2 consumption, depending on the mitochondrial state and on the local O_2 concentration.

Interestingly, a linear relationship between the increase in $O_2^{\bullet-}$ production rate and the inhibition of complex II-III activity by GSNO was observed (Fig. 3B). In this sense, the [GSNO] that produces the half maximal change not only on succinate-cytochrome *c* activity (Fig. 1A) but also in $O_2^{\bullet-}$ and H_2O_2 production rates (Fig. 3A) is about 100 μM, a [GSNO] that correspond to an effective [NO] of about 0.25 μM (Fig. 1B). The close association between the generation of reactive oxygen species and the inhibition of electron transfer at ubiquinol-cytochrome *b* area suggests the existence of a common mechanism with a common intermediate. Moreover, the $O_2^{\bullet-}$ derived from complex III and the subsequently produced H_2O_2 have been linked to cellular redox signalling pathways [18,20,56]. Concerning the inhibitory effect of NO on complex III area, an increase in H_2O_2 production was observed not only when SMP were exposed to GSNO (Fig. 3A) but also when heart coupled mitochondria were incubated in the presence of GSNO and SPER-NO (Table 4). These results agree with the data published by Poderoso et al. [23] who showed that about 1 μM NO produces an increase in H_2O_2 generation of about 0.35 nmol/min. mg protein. In physiological conditions, the mtNOS-produced NO is involved in the generation and metabolism of reactive oxygen species [48], through its effects on ubiquinol-cytochrome *b* area of mitochondrial electron transfer. The difference in H_2O_2 production rate between the experimental conditions of maximal (L-arginine addition) and minimal (NOS inhibitor addition) NO levels is known as "the functional activity of mtNOS on the regulation of mitochondrial H_2O_2 production", and it is explained by the intramitochondrial $[NO]_{ss}$ and by the NO inhibition of ubiquinol-cytochrome *c* reductase activity [48].

Under a NO-steady state condition, given by the release from NO-donors, together with the increase in $O_2^{\bullet-}$ production, the $ONOO^-$ formation is predictable. In the inside-out SMP, this should mainly occur at the vicinity of the complex III proteins exposed to the N side. Accordingly, these proteins should be nitrated and inactivated. The inactivation of the mitochondrial respiratory chain by $ONOO^-$ was reported by Cassina and Radi [57]. However, in the present work, SMP were pre-incubated in the absence of reduction equivalents and in the presence of GSNO, a situation that favours



Scheme 1. This scheme illustrates the electron transfer events that follow the oxidation of an ubiquinol (UQH_2) at the P-side of the inner mitochondrial membrane under conditions in which the N-side is initially either vacant or occupied by an ubiquinone (UQ) molecule, the inhibitory site of antimycin, and the hypothetical molecular mechanisms that lead to the inhibition of electron transfer by NO: (a) the direct one-electron transfer reaction between UQH_2 and NO, which involves the oxidation of UQH_2 to ubisemiquinone ($UQH^{\bullet}$) and the reduction of NO to nitroxyl anion (NO^-), and the interaction of NO (b) with the Fe in the heme groups of cytochromes *b* or (c) with the Fe in the Rieske [Fe_2S_2] cluster. In any case, the electron transfer from UQH_2 to cytochrome *c* results inhibited and the $O_2^{\bullet-}$ production is increased.

the increase in NO-steady state without mitochondrial-derived $O_2^{\bullet-}$ in the pre-incubation medium. In this experimental situation, the production of $ONOO^-$ should be negligible. The results obtained were confirmed by the addition of SOD in the pre-incubation medium, in a high enough concentration to decompose the presumably formed $O_2^{\bullet-}$. As a consequence, the primary effects described in this work are attributable to NO and not to $ONOO^-$. This latter species, as well as H_2O_2, could be generated as result of the interaction of NO with complex III and involved in secondary inhibition or signalling processes. Two main hypotheses, not mutually exclusive, could explain the molecular mechanisms that lead to the inhibition of electron transfer and the consequent enhancement in $O_2^{\bullet-}$ production by autoxidation of the $UQH^{\bullet}$: (i) the direct one-electron transfer reaction between ubiquinol (UQH_2) and NO (Scheme 1, a), which involves the oxidation of UQH_2 to $UQH^{\bullet}$ and the reduction of NO to nitroxyl anion (NO^-) [43]; and (ii) the interaction of NO with the Fe in the heme groups of cytochromes (Scheme 1, b) or in the Rieske [Fe_2S_2] cluster (Scheme 1, c), where one Fe is coordinated by two conserved Cys while the other is coordinated by two conserved Hys [7,4]. In any case, the electron transfer from ubiquinol to cytochrome *c* results inhibited and $O_2^{\bullet-}$ production is increased.

In conclusion, NO inhibits complex III, in an oxygen concentration independent manner, leading to an $[UQH^{\bullet}]_{ss}$ enhancement which, in turn, generates an increase in $O_2^{\bullet-}$ and H_2O_2 production rates. The effect of NO at complex III is antimycin-like (Scheme 1) regarding $UQH^{\bullet}$ EPR signal, cytochromes b^{2+} content and $O_2^{\bullet-}$ and H_2O_2 productions. Although further studies using NO gas are needed to clarify the inhibitory mechanisms of NO on complex III, the biochemical characterization of this effect is crucial for the understanding of the regulatory mechanisms of NO on the respiratory chain and its impact on $O_2^{\bullet-}$ and H_2O_2 mitochondrial metabolism.

Acknowledgements

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