BIOCELL
2016 40(1): 27-30

ISSN 1667-5746
ELECTRONIC

Superoxide and hydrogen peroxide productions by NO-inhibited complex III

Darío E. IGLESIAS*, Silvina S. BOMBICINO, Alberto BOVERIS, Laura B. VALDEZ

Key words: S-nitrosoglutathione (GSNO), spermine-NONOate (SPER -NO), electron paramagnetic resonance (EPR), ubisemiquinone.

ABSTRACT: Complex III plays a central role in the mitochondrial respiratory chain transferring electrons from ubiquinol to cytochrome *c* and pumping protons to the intermembrane space, contributing to the protonmotive force. Furthermore, complex III can act as a source of $O_2^{\bullet-}$ in the presence of ubiquinol and antimycin, an expermiental condition in which the oxidation of the cytochrome b hemes is blocked. The $O_2^{\bullet-}$ dismutation catalyzed by superoxide dismutase produces H_2O_2, a known second messenger in redox signalling. Results from our laboratory have shown that NO, released from GSNO or from SPER -NO or generated by mtNOS, inhibits electron transfer at ubiquinone-cytochrome *b* area producing antimycin-like effects. Thus, both antimycin- and NO-inhibited complex III showed a high content of cytochromes *b* in the reduced state (79 and 71%, respectively) and an enhancement in the ubisemiquinone EPR signal at g=1.99 (42 and 35%, respectively). As consequence, $O_2^{\bullet-}$ and H_2O_2 productions were increased, being the $O_2^{\bullet-}/H_2O_2$ ratio equal to 1.98 in accordance with the stoichiometry of the $O_2^{\bullet-}$ disproportionation. The interruption of the oxidation of cytochromes *b* by NO leads to an enhancement of the steady-state concentration of $UQH^{\bullet}$, allowing cytochrome bc_1 complex to act as a source of reactive oxygen species in physiological conditions.

The mitochondrial oxidative phosphorylation system utilizes the energy derived from the oxidation of metabolic substrates to drive the synthesis of ATP. Electron transfer through mitochondrial respiratory complexes is coupled to proton translocation across the mitochondrial inner membrane, generating a protonmotive force (Δp) consisting of a membrane potential and a pH gradient that leads the synthesis of ATP by the ATP synthase (Nicholls and Ferguson, 2002). Complex III (cytochrome bc_1 complex or ubiquinol:cytochrome *c* oxidoreductase) plays a central role in the mitochondrial respiratory chain. Its reaction mechanism, known as protonmotive Q cycle (Mitchell, 1975), leads to the transfer of electrons from ubiquinol to cytochrome *c* with the concomitant pumping of protons from the mitochondrial matrix to the intermembrane space, contributing to Δp. In the catalytic Q_o site of cytochrome bc_1 complex, ubiquinol (UQH_2) is oxidized by a bifurcated electron transfer reaction that steers the two electrons down divergent paths: the first electron to the Rieske cluster (high-potential chain) and the second electron to the heme b_L (low potential chain). The net translocation of $4H^+/2e^-$ 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 membrane sides, and by the vectorial transfer of electrons through cytochrome *b* towards the negative membrane side (Iwata *et al.,* 1998; Nicholls and Ferguson, 2002). As a consequence of the Q-cycle turnover, intermediate ubisemiquinone radicals ($UQH^{\bullet}$) are formed at both Q_o and Q_i sites. The $UQH^{\bullet}$ generated in the Q_o site has been postulated as the reductant for O_2, converting it to superoxide anion ($O_2^{\bullet-}$) (Boveris *et al.,* 1976; Turrens *et al.,* 1985; Murphy, 2009).

*Address correspondence to: Darío E. Iglesias,
diglesias@ffyb.uba.ar
Received: October 30, 2015. Accepted: April 16, 2016

Reactive oxygen species, such as $O_2^{\bullet-}$ or hydrogen peroxide (H_2O_2), are critical mediators in a broad range of cellular signalling processes. The mitochondrial respiratory chain is a major cellular source of reactive oxygen species and complex III has long been regarded as a source of $O_2^{\bullet-}$: when mitochondria are supplied with UQH_2 and when Q_i site is inhibited by antimycin, blocking the oxidation of the cytochrome *b* hemes in the low potential chain, complex III produces large amounts of $O_2^{\bullet-}$ (3-5 nmol $O_2^{\bullet-}$/ min . mg protein) (Boveris and Cadenas, 1975; Turrens *et al.,* 1985; Quinlan *et al.,* 2011). 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 $O_2^{\bullet-}$ (Boveris *et al.,* 1976; Turrens *et al.,* 1985; Bleier and Drose, 2013; Guillaud *et al.,* 2014). Superoxide dismutase (SOD) catalyzes the $O_2^{\bullet-}$ disproportionation producing stoichiometrically H_2O_2. This latter species easily diffuses to the cytosol acting as second messenger (Boveris and Cadenas, 2000; Sies, 2014; Yin *et al.,* 2014; Bleier and Drose, 2013; Bleier *et al.,* 2015).

In 1996, Poderoso *et al.* showed that nitric oxide (NO) inhibits electron transfer at ubiquinone-cytochrome *b* area, increasing $O_2^{\bullet-}$ production in rat heart submitochondrial particles. This effect of NO on mitochondrial respiration was added to the NO inhibitory interaction with cytochrome oxidase (Cleeter *et al.,* 1994; Brown and Cooper, 1994; Antunes *et al.,* 2004). In mammalian cells, NO is synthesized from L-arginine, NADPH, and O_2 in a reaction catalyzed by nitric oxide synthases (NOS). The mitochondrial isoform (mtNOS) is located in the inner mitochondrial membrane and it was identified as the α-nNOS with post-translational modifications (Ghafourifar and Richter, 1997; Giulivi *et al.,* 1998; Elfering *et al.,* 2002). Recently, results from our laboratory have shown that NO interacts with complex III producing antimycin-like effects. Accordingly, Fig. 1A shows that NO, released from GSNO or from SPER-NO or generated by mtNOS, inhibits succinate-cytochrome *c* reductase activity (complex II-III) and does not modify succinate-Q reductase activity (complex II), indicating that NO produces the inhibition of electron transfer at the ubiquinone-cytochrome *b* area with effects centred at complex III. These effects imply 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 (Iglesias *et al.,* 2015).

It is known that the inhibition of complex III increases $O_2^{\bullet-}$ production as a result of the autoxidation of $UQH^{\bullet}$. Quinlan *et al.* (2011) 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, bovine heart submitochondrial particles (SMP) added with succinate showed an EPR signal at g=1.99, attributable to $UQH^{\bullet}$ implicated in the Q cycle. Antimycin addition enhanced by 42% this ubisemiquinone EPR signal. Similarly, SMP incubated in the presence of GSNO or SPER-NO as NO sources, showed an EPR signal higher (~35%) than in the presence of succinate. Thus, not only antimycin but also NO produced an increase in the steady-state concentration of $UQH^{\bullet}$ (Iglesias *et al.,* 2015).

The intermediate $UQH^{\bullet}$ can be formed in two ways: as a part of the forward reaction toward one electron oxidation of ubiquinol at the Q_o site by the oxidized $[Fe_2S_2]$ center (*semiforward mechanism*) or as a part of the reverse reaction toward one electron reduction of quinone bound at Q_o site by the reduced heme b_L (*semireverse mechanism*) (Sarewicz *et al.,* 2010; Guillaud *et al,* 2014). Sarewicz *et al.* (2010) have shown that $O_2^{\bullet-}$ production by cytochrome bc_1 complex can be consequence of the combination of both *semiforward* and *semireverse mechanisms*. However, experimental evidence combined with modelling revealed that *semireverse mechanism* dominates the steady state of $UQH^{\bullet}$. Consequently, $O_2^{\bullet-}$ 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 (Sarewicz *et al.,* 2010; Guillaud *et al,* 2014; Quinlan *et al.,* 2011). This observation agrees with the content of cytochromes *b* in the reduced state registered by us in both antimycin- and NO-inhibited complex III (~71-79%) (Iglesias *et al.,* 2015).

Moreover, our results show that the inhibition of electron transfer at ubiquinol-cytochrome *b* area by NO correlates with the generation of $O_2^{\bullet-}$ by SMP: about 0.25 μM NO (100 μM GSNO) produces a half maximal inhibition of succinate-cytochrome *c* activity and also a half maximal increase in $O_2^{\bullet-}$ production rate. Superoxide anion is the stoichiometric precursor of H_2O_2, in accordance with the reaction $2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$, which involves the activity of the mitochondrial SOD.

In this way, SMP pre-incubated with GSNO showed a concentration dependent and hyperbolic increase not only in $O_2^{\bullet-}$ but also in H_2O_2 production rates. Considering that the equation $Y = c + aX/(b + X)$ fitted to the experimental data of enhancement of both $O_2^{\bullet-}$ and H_2O_2 productions (Y) as a function of [GSNO] (X), a confidence region analysis, to determine the relationship between the estimated parameters, was performed. When the adjusted parameters related to the maximal H_2O_2 production (a_H) and the basal H_2O_2 production (c_H) rates are multiplied by 2 (the stoichiometric coefficient of the dismutation reaction), the calculated confidence regions matched to the ones of the parameters that explain the $O_2^{\bullet-}$ hyperbolic increase (a_S and c_S). Thus, $2\ a_H = a_S$ and $2\ c_H = c_S$ considering their confidence areas. Furthermore, a linear correlation between both production rates (r^2= 0.993) was observed, with a slope of 1.98 (Iglesias *et al.,* 2015). These observations are in accordance with the

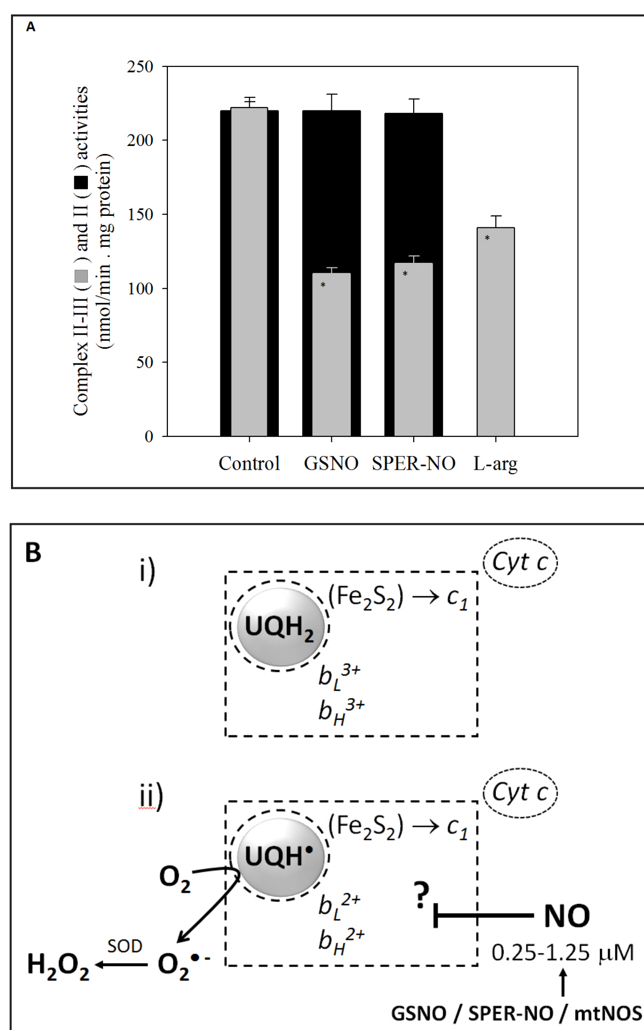

FIGURE 1. A. Effect of GSNO (500 µM), SPER-NO (30 µM), and mtNOS substrates and cofactors (1 mM L-arg, 1 mM Ca^{2+}, and 0.1 mM NADPH) on succinate-cytochrome *c* reductase (complex II-III) and succinate-Q reductase (complex II) activities. Control, SMP (0.01-0.03 mg/ml protein) added with 7 mM succinate. * Statistically different ($p<0.01$) respect to control. B. Scheme illustrating (i) the normal electron transfer events that follow the oxidation of ubiquinol (UQH_2) at the Q_o-site by both oxidized Fe_2S_2 centre and heme b_L, allowing the reduction of cytochrome *c* via cytochrome c_1. (ii) In the presence of NO, released from GSNO or from SPER NO or generated by mtNOS, the electron transfer to cytochrome *c* is inhibited, cytochromes *b* are accumulated in the reduce form (b^{2+}) and the steady-state concentration of ubisemiquinone ($UQH^•$) is enhanced. This latter species reduces O_2 converting it to $O_2^{•-}$, which generates H_2O_2 by SOD-catalyzed dismutation.

stoichiometry of $O_2^{•-}$ disproportionation, which governs the physiological H_2O_2 production by complex III (Cadenas *et al.,* 1977; Bleier and Drose, 2013; Sies, 2014).

The enhancement of H_2O_2 production (72-74%) was also observed when heart coupled mitochondria were incubated in the presence of 500 µM GSNO or 30 µM SPER-NO (~1.25 µM NO) (Iglesias *et al.,* 2015). In physiological conditions, the mtNOS-produced NO is involved in the generation and metabolism of reactive oxygen species (Valdez *et al.,* 2005). Accordingly, the difference in H_2O_2 production rate between the experimental conditions of maximal (L-arginine addition) and minimal (NOS inhibitor addition) NO generation 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 (Valdez *et al.,* 2005).

To conclude, the NO-inhibited complex III, as well as antimycin-inhibited complex III, is able to produce $O_2^{•-}$ and, as consequence, H_2O_2. The interruption of the oxidation of cytochromes *b* by NO leads to an enhancement of $[UQH^•]$ Ss llowing cytochrome bc_1 complex to act as a source of reactive oxygen species in physiological conditions (Fig. 1B). Further characterization of this effect is crucial for the understanding of the regulatory mechanisms of NO on the respiratory chain, its impact on $O_2^{•-}$ and H_2O_2 mitochondrial metabolism, and the signalling processes involved.

Acknowledgement

This work was supported by research grants from the University of Buenos Aires (UBACYT 200-201-101-00140 and 200-201-301-00731), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2012-0964), and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 112-201-101-00444).

References

Antunes F, Boveris A, Cadenas E (2004). On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide. *Proceedings of the National Academy of Sciences (USA)* **101:** 16774-16779.

Bleier L, Dröse S (2013). Superoxide generation by complex III: from mechanistic rationales to functional consequences. *Biochimica et Biophysica Acta* **1827:** 1320-1331.

Bleier L, Wittig I, Heide H, Steger M, Brandt U, Dröse S (2015). Generator-specific targets of mitochondrial reactive oxygen species. *Free Radical Biology and Medicine* **78:** 1-10.

Boveris A, Cadenas E (1975). Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Letters* **54:** 311-314.

Boveris A, Cadenas E, Stoppani AO (1976). Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochemical Journal* **156:** 435-444.

Boveris A, Cadenas E (2000). Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. *IUBMB Life* **50:** 245-250.

Brown GC, Cooper CE (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Letters* **356:** 295-298.

Cadenas E, Boveris A, Ragan CI, Stoppani AO (1977). Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Archives of Biochemistry and Biophysics* **180:** 248-257.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH (1994). Reversible inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Letters* **345:** 50-54.

Elfering SL, Sarkela TM, Giulivi C (2002). Biochemistry of mitochondrial nitric-oxide synthase. *Journal of Biological Chemistry* **277:** 38079-38086.

Ghafourifar P, Richter C (1997). Nitric oxide synthase activity in mitochondria. *FEBS Letters* **418:** 291-296.

Giulivi C, Poderoso JJ, Boveris A (1998). Production of nitric oxide by mitochondria. *Journal of Biological Chemistry* **273:** 11038-11043.

Guillaud F, Dröse S, Kowald A, Brandt U, Klipp E (2014). Superoxide production by cytochrome bc1 complex: a mathematical model. *Biochimica et Biophysica Acta* **1837:** 1643 1652.

Iglesias DE, Bombicino SS, Valdez LB, Boveris A (2015). Nitric oxide interacts with mitochondrial complex III producing antimycin-like effects. *Free Radical Biology and Medicine* **89:** 602-613.

Iwata S, Lee JW, Okada K, Lee JK, Iwata M, Rasmussen B, Link TA, Ramaswamy S, Jap BK (1998). Complete structure of the 11-subunit bovine mitochondrial cytochrome bc_1 complex. *Science* **281:** 64–71.

Mitchell P (1975). The protonmotive Q cycle: a general formulation. *FEBS Letters* **59:** 137 139.

Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal* **417:** 1-13.

Nicholls D, Ferguson SJ (2002). In: *Bioenergetics 3*, 2nd ed., Academic Press, London, UK.

Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A (1996). Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Archives of Biochemistry and Biophysics* **328:** 85-92.

Quinlan CL, Gerencser AA, Treberg JR, Brand MD (2011). The mechanism of superoxide production by the antimycin-inhibited mitochondrial Q-cycle. *Journal of Biological Chemistry* **286:** 31361-31372.

Sarewicz M, Borek A, Cieluch E, Świerczek M, Osyczka A (2010). Discrimination between two possible reaction sequences that create potential risk of generation of deleterious radicals by cytochrome bc_1. Implications for the mechanism of superoxide production. *Biochimica et Biophysica Acta* **1797:** 1820-1827.

Sies H (2014). Role of metabolic H_2O_2 generation. *Journal of Biological Chemistry* **289:** 8735-8741.

Turrens JF, Alexandre A, Lehninger AL (1985). Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Archives of Biochemistry and Biophysics* **237:** 408-414.

Valdez LB, Zaobornyj T, Boveris A (2005). Functional activity of mitochondrial nitric oxide synthase. *Methods in Enzymology* **396:** 444-455.

Yin F, Boveris A, Cadenas E (2014). Mitochondrial energy metabolism and redox signalling in brain aging and neurodegeneration. *Antioxidants and Redox Signaling* **20:** 353-371.