Superoxide and hydrogen peroxide productions by NO-inhibited complex III

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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\(_2^−\) in the presence of ubiquinol and antimycin, an experimental condition in which the oxidation of the cytochrome b hemes is blocked. The O\(_2^−\) dismutation catalyzed by superoxide dismutase produces H\(_2\)O\(_2\), 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\(_2^−\) and H\(_2\)O\(_2\) productions were increased, being the O\(_2^−\)/H\(_2\)O\(_2\) ratio equal to 1.98 in accordance with the stoichiometry of the O\(_2^−\) disproportionation. The interruption of the oxidation of cytochromes b by NO leads to an enhancement of the steady-state concentration of UQH\(_2\), 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 (\(\Delta p\)) 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 \(\Delta p\). In the catalytic Q\(_s\) 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\(_p\)) and ubiquinone-reduction site (N center or Q\(_N\)), 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\(^+\)) are formed at both Q\(_p\) and Q\(_N\) sites. The UQH\(^+\) generated in the Q\(_p\) site has been postulated as the reductant for O\(_2^−\), converting it to superoxide anion (O\(_2^−\)) (Boveris et al., 1976; Turrens et al., 1985; Murphy, 2009).
Reactive oxygen species, such as $O_2^•$ 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 $O_2^•$: when mitochondria are supplied with UQH$_2$ and when Q$_s$ site is inhibited by antimycin, blocking the oxidation of the cytochrome $b$ hemes in the low potential chain, complex III produces large amounts of $O_2^•$ ($3$-$5$ nmol $O_2^•$ / 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$_s$ site and allows it sufficient time to interact with and reduce molecular O$_2$ to generate $O_2^•$ (Boveris et al., 1976; Turrens et al., 1985; Bleier and Drose, 2013; Guillaud et al., 2014). Superoxide dismutase (SOD) catalyzes the $O_2^•$ 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 $O_2^•$ 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 a-nNOS with post-translational modifications (Ghafoorifar 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$^•$] which, in turn, leads to an increase in $O_2^•$ and $H_2O_2$ mitochondrial production rates (Iglesias et al., 2015).

It is known that the inhibition of complex III increases $O_2^•$ production as a result of the autoxidation of UQH$^•$. 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$^•$ 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$^•$ (Iglesias et al., 2015).

The intermediate UQH$^•$ can be formed in two ways: as a part of the forward reaction toward one electron oxidation of ubiquinol at the Q$_s$ site by the oxidized [Fe$_2$S$_2$] center (semiforward mechanism) or as a part of the reverse reaction toward one electron reduction of quinone bound at Q$_s$ site by the reduced heme $b_1$ (semireverse mechanism) (Sarewicz et al., 2010; Guillaud et al., 2014). Sarewicz et al. (2010) have shown that $O_2^•$ production by cytochrome $b_1c_1$ 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$^•$. Consequently, $O_2^•$ production depends on the reduction state of the $b_1$ heme in the superoxide-generating Q$_s$ site, with the highest rates at 70-80% reduction of $b_1$ (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 $O_2^•$ by SMP; about 0.25 $\mu$M NO (100 $\mu$M GSNO) produces a half maximal inhibition of succinate-cytochrome $c$ activity and also a half maximal increase in $O_2^•$ production rate. Superoxide anion is the stoichiometric precursor of $H_2O_2$, in accordance with the reaction $2O_2^• + 2H^+ \rightarrow H_2O_2 + O_n$, 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 $O_2^•$ 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 $O_2^•$ 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 ($a_0$) and the basal $H_2O_2$ production ($c_i$) 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 $O_2^•$ hyperbolic increase ($a_i$ and $c_i$). Thus, $2a_0 = a_i$ and $2c_i = c_i$ 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
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The enhancement of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ production”, and it is explained by the intramitochondrial [NO] 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$_2$O$_2$. The interruption of the oxidation of cytochromes b by NO leads to an enhancement of [UQH$^+$]Ss allowing 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$_2$O$_2$ mitochondrial metabolism, and the signalling processes involved.

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