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CELL BIOPHYSICS

The Generation of Superoxide Radicals by Complex III in Heart Mitochondria and the Antioxidant Effect of Dinitrosyl Iron Complexes at Different Partial Pressures of Oxygen

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Abstract—EPR spin trapping and EPR oximetry were used to study the superoxide radical generation in heart mitochondria from Wistar rats at various oxygen concentrations. Lithium phthalocyanine and TEMPONE-^{15}N-D_{16} were chosen to determine the oxygen content in a gas-permeable capillary containing mitochondria. TIRON was used as a spin trap. Several oxygen concentrations in the incubation mixture were tested; heart mitochondria were found to generate superoxide in complex III at various partial pressures of oxygen, including deep hypoxia (<5% O_2). Dinitrosyl iron complexes with glutathione (the drug Oxacom) exerted an antioxidant effect regardless of the partial pressure of oxygen; the magnitude and kinetic characteristics of the effect depended on the drug concentration.

Keywords: superoxide radicals, dinitrosyl iron complexes, heart, mitochondria, antioxidants, electron paramagnetic resonance

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INTRODUCTION

There is much evidence that mitochondria contribute far more to cell function in the cardiovascular system than their action as the major ATP producer in energy metabolism [1–3]. Mitochondria play a key role in signaling and regulatory events that arise in response to various physiological and biochemical influences on the cell [4–6]. Considerable interest is now attracted by superoxide anion radicals that are generated at the Qo site of complex III and released into both the intermembrane space and the cytosol [7–10]. These superoxide radicals or subsequently forming hydrogen peroxide have been found to play a regulatory role in cell processes that are related to hypoxia [11–13]. Mitochondrial superoxide production has been shown to occur not only at a normal partial pressure of oxygen, but also in hypoxia [14, 15]. However, it remains unclear how the kinetics of superoxide radical generation change depending on the severity of hypoxia and to what extent the partial pressure of oxygen can be reduced without stopping superoxide generation in heart mitochondria.

Abbreviations: DNIC, dinitrosyl iron complex.

Aqueous solutions of dinitrosyl iron complexes (DNICs) with glutathione ligands have been shown to exert a prolonged hypotensive effect in experimental animals [16]. Based on DNICs, the drug Oxacom has been developed in the Russian Cardiology Research and Production Complex, for use as a hypotensive agent [17]. We have observed that an antioxidant effect is exerted by DNICs with thiol ligands as they are degraded by superoxide anion radicals that are produced in the reaction mixture by mitochondria or model enzyme systems [18, 19].

In this work, EPR spin trapping and EPR oximetry were used to study the effect of hypoxia that varied in severity, anoxia, and reoxygenation on the superoxide production rate in isolated rat heart mitochondria and to evaluate the interaction of superoxide radicals with DNIC with glutathione ligands (Oxacom).

MATERIALS AND METHODS

Isolated mitochondria. All of the steps for isolating mitochondria were performed at 0–4°C. Wistar rats (body weight 250–300 g) were anesthetized intraperitoneally with urethane (1.8 g/kg body weight) or Avertin (250 mg/kg body weight). The heart was isolated, washed with a chilled isolation buffer (70 mM sucrose,

220 mM mannitol, 50 mM HEPES, 1 mM EDTA, pH 7.4), and cut with scissors. Heart muscle fragments were passed through a stainless steel mesh strainer (hole diameter 0.8 mm) and homogenized at a 1 : 8 tissue–isolation buffer ratio for 2–3 min. The homogenate was centrifuged at 700 *g* for 10 min. The supernatant, which contained mitochondria, was filtered and centrifuged in a K-24 centrifuge (Germany) at 14000 *g* for 10 min. The mitochondrial pellet was suspended in the isolation buffer with the addition of 3 mg/mL bovine serum albumin. The mitochondrial suspension (30–70 mg protein/mL) was stored on ice.

Functional activity of mitochondria. The oxygen uptake rate was measured using a Clark electrode and a YSI 53 polarograph (Yellow Spring Instruments, United States). The incubation medium contained 225 mM sucrose, 10 mM KCl, 20 mM HEPES (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$, 4 mM KH_2PO_4, and 2.5 μM rotenone; the temperature was 25°C; the protein concentration was 0.3–0.7 mg/mL. As a respiratory substrate, we used 4 mM succinate or 3 mM glutamate with 4 mM malate (state 4). To measure the respiration rate in state 3, ADP was added to 300 μM.

EPR spectra were recorded and analyzed using an E-109E spectrometer (Varian, United States) and a compact ESR 70-03 XD/2 spectrometer (Special Equipment Construction Bureau, Belarussian State University, Belarus). During the recording of the spectrum, the reaction mixture was in a gas-permeable PTFE 22 capillary (internal diameter 0.635 mm, wall thickness 0.051 mm) (Zeus Industrial Products, United States); the partial pressure of oxygen was varied. The oxygen content in the gas that was used to blow the sample was inferred from the width of the lithium phthalocyanine EPR signal; the oxygen concentration in the reaction mixture was measured by the spectral component widths of 4-oxo-2,2,6,6-tetramethylpiperidine-D_{16}-1-oxyl-^{15}N (TEMPONE-^{15}N-D_{16}) [20, 21].

Complex III-dependent superoxide radical production in isolated mitochondria was detected using 4,5-dioxybenzene-1,3-disulfonate (TIRON) as a spin trap. We have previously demonstrated that the superoxide generation rate in the reaction mixture is proportional to the squared EPR signal from $TIRON^{\bullet-}$ free radicals (semiquinones) [22, 23]. The incubation mixture for EPR measurements contained 250 mM sucrose, 20 mM HEPES, 1 mM EGTA, 4 mM KH_2PO_4, 3 mM $MgCl_2$, and 10 mM $TIRON^{\bullet-}$ pH 7.4. Mitochondria were added to 1–2 mg/mL to the incubation medium. The conditions of TIRON EPR spectroscopy were as follows: microwave power, 5 mW, microwave frequency, ~9.15 GHz in the case of the E-109E spectrometer and microwave power attenuation, 10 dB; microwave frequency, ~9.32 GHz; and modulation amplitude, 0.05 mT in the case of the ESR 70-03 XD/2 spectrometer.

The reagents were from Sigma-Aldrich (United States), ICN (United States), Serva (Germany), and other companies. The drug Oxacom was manufactured by the Experimental Production Plant of Biomedical Substances (Russian Cardiology Research and Production Center, Russia).

Result processing. Statistical analyses included the *t*-test and ANOVA and were performed using the Origin 7 program (OriginLab, United States). The results are shown as the mean ± standard error of the mean ($M \pm m$).

RESULTS AND DISCUSSION

Using EPR spin trapping and EPR oximetry, we studied the kinetics of the generation of short-lived oxygen-free radicals by isolated heart mitochondria while stringently controlling the sample oxygenation. Figure 1 shows the resultant EPR spectrum of TEMPONE-^{15}N-D_{16} nitroxyl radicals and $TIRON^{\bullet-}$ semiquinones as recorded for a mitochondrial suspension during complex III-dependent superoxide generation in the presence of succinate and Antimycin A. The reaction mixture was in a gas-permeable capillary at 25°C; the partial pressure of oxygen in the gas flow was maintained constant while recording the EPR spectra. EPR oximetry employs various paramagnetic probes whose EPR spectrum parameters depend on magnetic dipole–dipole interactions with molecular oxygen. A linear dependence on the environmental oxygen concentration has been demonstrated for the line width of an EPR singlet signal from lithium phthalocyanine and the component line widths of the doublet EPR spectrum of the fully deuterated TEMPONE nitroxyl radical in which ^{14}N is replaced by the ^{15}N isotope [15, 20, 21]. When a sample was blown with air (21% O_2, partial pressure 157.4 mm Hg, temperature ~25°C), the low-field component width ($m_I = +1/2$) of the TEMPONE-^{15}N-D_{16} EPR spectrum was 26.8 μT, which corresponds to a steady-state oxygen concentration of 238 μM in the mitochondrial incubation medium.

Many studies have employed TIRON as an efficient antioxidant that penetrates into the cell and acts as a superoxide radical scavenger (e.g., see [24, 25]). We have found that recording the kinetics of superoxide-dependent TIRON oxidation to free-radical intermediates ($TIRON^{\bullet-}$ semiquinones) provides a convenient method to measure the superoxide generation rate for both model enzyme systems and isolated mitochondria [22, 23]. The rate of succinate-dependent generation of superoxide radicals in mitochondrial complex III is low (approximately 0.01 nmol $O_2^{\bullet-}$/min/mg protein) and increases substantially (to

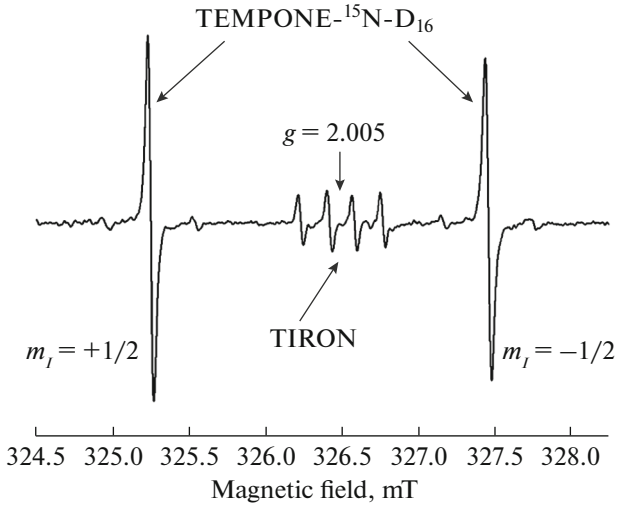

Fig. 1. The resultant EPR spectrum of TEMPONE-^{15}N-D_{16} nitroxyl radicals and TIRON spin trap as recorded for a rat heart mitochondrial suspension during complex III-dependent superoxide generation in the presence of succinate and Antimycin A. The sample was in a gas-permeable capillary, which was continuously blown with air (21% O_2). The EPR spectra were recorded at ~25°C.

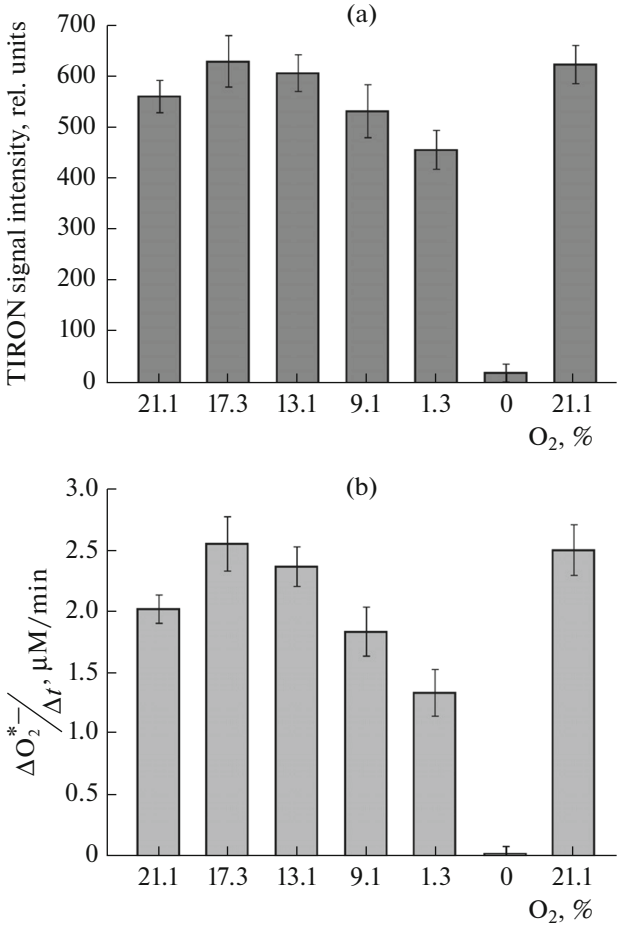

Fig. 2. (a) The TIRON EPR signal intensity and (b) superoxide radical generation rate as functions of the oxygen content in the gas flow that was used to blow the sample. The incubation mixture contained succinate and Antimycin A; the temperature was ~25°C.

0.4 nmol $O_2^{\bullet-}$/min/mg protein) in the presence of Antimycin A, which blocks electron transfer from cytochrome *b* to oxidized coenzyme Q. Complex III-dependent superoxide generation is completely suppressed by Q-cycle inhibitors (myxothiazole and stigmatellin), which block electron transfer from ubiquinol to the Rieske Fe–S center. When anoxia (a complete absence of oxygen in the incubation medium) was induced by replacing air with nitrogen in the gas flow, the EPR spectrum of TIRON$^{\bullet-}$ semiquinones gradually disappeared. Equilibrium between the external gas medium (nitrogen) and the mitochondrial suspension in a capillary was achieved in 4–5 min; the low-field component width of the TEMPONE-^{15}N-D_{16} EPR spectra decreased to 18.6 μT.

Figure 2a shows the manner in which the EPR signal intensity of TIRON$^{\bullet-}$ semiquinones that resulted from free-radical oxidation of the spin trap in the mitochondrial suspension depended on the oxygen content in the gas flow (that is, on the partial pressure of oxygen). A corresponding dependence of the superoxide generation rate in heart mitochondria was obtained using a superoxide-generating xanthine oxidase–xanthine model system [22] (Fig. 2b). As is seen from Fig. 2, although the TIRON EPR signal became somewhat lower with a decrease in O_2, complex III of the mitochondrial respiratory chain continued to produce superoxide radicals in spite of the decreasing oxygen concentration up to deep hypoxia (1.3% O_2). It was not until anoxia was achieved, i.e., oxygen was completely removed from the reaction mixture as air was replaced with nitrogen in the gas flow, that mitochondria ceased to generate superoxide radicals. Reoxygenation (a change from nitrogen to air) rapidly increased the EPR signal from TIRON. When equilibrium between the gas environment and mitochondrial suspension was achieved after a 5-min reoxygenation, the superoxide radical production rate was slightly higher than prior to anoxia. It is possible to believe that certain changes occur in membranes of isolated heart mitochondria upon exposure to deep hypoxia, anoxia, and reoxygenation to increase the superoxide-radical generation rate by electron transporters (coenzyme Q semiquinones) of complex III of the respiratory chain (see also [26]).

Apart from complex III substrate succinate, we used glutamate and malate, which are substrates of complex I. The kinetics of the changes in the TIRON EPR signal that were observed in the presence of the complex I substrates were similar to those in the presence of succinate (data not shown), but the complex I

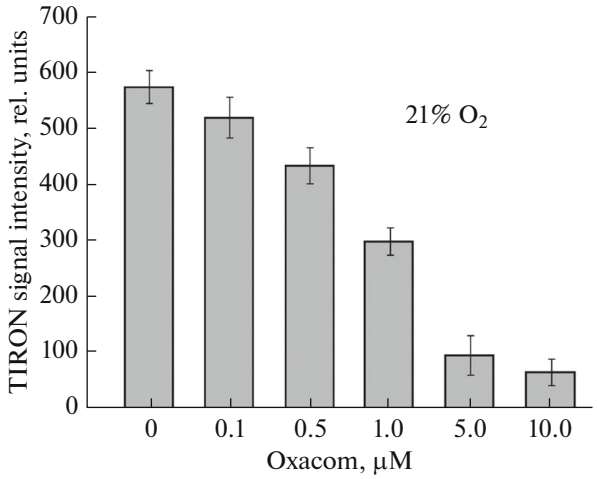

Fig. 3. The TIRON EPR signal intensity in a rat heart mitochondrial suspension as a function of the concentration of DNICs with glutathione (Oxacom) in the incubation medium. The oxygen content in the gas flow was 21%; the incubation mixture contained succinate and Antimycin A; the temperature was ~25°C.

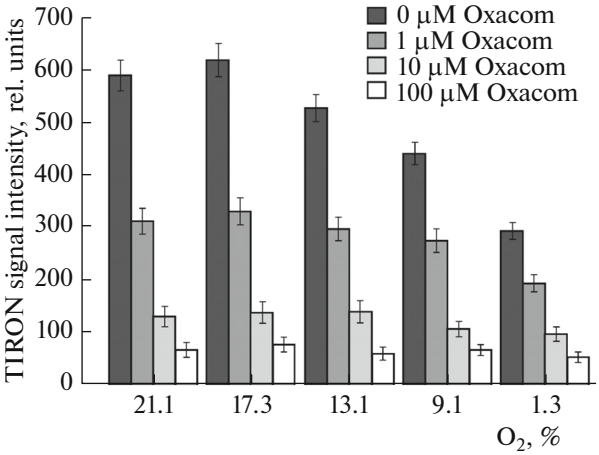

Fig. 4. The TIRON EPR signal intensity in a rat heart mitochondrial suspension as a function of the oxygen content in the gas flow and the concentration of DNICs with glutathione (Oxacom) in the incubation medium. The medium contained succinate, Antimycin A, and Oxacom (the Oxacom concentrations are specified in the figure).

inhibitor rotenone terminated superoxide generation by complex III in this case.

The EPR signal intensity of $TIRON^{\bullet-}$ was studied dependent on the concentration of DNICs with glutathione ligands (Oxacom) in the reaction mixture (Fig. 3). In normoxia (~21% O_2), the generation rate of spin trap TIRON free radicals significantly depended on the DNIC concentration in the mitochondrial suspension. When succinate was used as an oxidation substrate, 1–100 μM Oxacom did not exert any effect on the oxygen uptake rate of mitochondria. Oxacom used at ⩾1 mM almost completely suppressed the EPR signal from TIRON (data not shown). It should be noted that a high rate constant is characteristic of the interaction between the TIRON spin trap and superoxide radicals [22, 23]. Therefore, a comparable or even a higher rate constant of interactions with superoxide must be characteristic of Oxacom DNICs.

The EPR signal intensity of the TIRON that was contained in a rat heart mitochondrial suspension was plotted as a function of the oxygen content in the gas flow and the concentration of DNICs with glutathione (Oxacom) in the incubation medium (Fig. 4). DNICs with glutathione efficiently interacted with superoxide radicals at various partial pressures of oxygen, including deep hypoxia. In the presence of 0.1–1 μM Oxacom, the TIRON semiquinone EPR signal gradually increased over a prolonged incubation period (20–30 min). To explain the effect, we assumed that DNICs are degraded to a greater extent in the reaction with superoxide radicals at the above concentrations as compared with the conditions where DNIC regeneration is possible in the mitochondrial suspension. We observed that after the maximal inhibitory effect of DNICs is achieved, the TIRON EPR signal increases at a lower rate in a mitochondrial system compared with a xanthine oxidase–xanthine superoxide-generating model system [19]. This observation indicates that DNIC degradation is incomplete or DNIC regeneration occurs in the presence of mitochondria.

CONCLUSIONS

Thus, superoxide radicals in amounts that were detectable with spin traps occurred at the boundary between the inner mitochondrial membrane and the intermembrane space at various partial pressures of oxygen. Based on our findings and published data [2, 14, 20], heart mitochondria are capable of generating reactive oxygen species even when the oxygen content in their environment is low, as is the case in myocardial ischemia. Our experiments also showed that DNICs with glutathione ligands efficiently interact with superoxide radicals at various partial pressures of oxygen, including deep hypoxia.

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