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Oxidative stress caused by blocking of mitochondrial Complex I H^+ pumping as a link in aging/disease vicious cycle

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Abstract

Vulnerability of mitochondrial Complex I to oxidative stress determines an organism's lifespan, pace of aging, susceptibility to numerous diseases originating from oxidative stress and certain mitopathies. The mechanisms involved, however, are largely unknown. We used confocal microscopy and fluorescent probe MitoSOX to monitor superoxide production due to retarded forward electron transport in HEPG2 cell mitochondrial Complex I in situ. Matrix-released superoxide production, the un-dismuted surplus (J_m) was low in glucose-cultivated cells, where an uncoupler (FCCP) reduced it to half. Rotenone caused a 5-fold J_m increase (AC_{50} 2 μM), which was attenuated by uncoupling, membrane potential ($\Delta\Psi_m$), and ΔpH-collapse, since addition of FCCP (IC_{50} 55 nM), valinomycin, and nigericin prevented this increase. J_m doubled after cultivation with galactose/glutamine (i.e. at obligatory oxidative phosphorylation). A hydrophobic amiloride that acts on the ND5 subunit and inhibits Complex I H^+ pumping enhanced J_m and even countered the FCCP effect (AC_{50} 0.3 μM). Consequently, we have revealed a new principle predicting that Complex I produces maximum superoxide only when both electron transport and H^+ pumping are retarded. H^+ pumping may be attenuated by high protonmotive force or inhibited by oxidative stress-related mutations of ND5 (ND2, ND4) subunit. We predict that in a vicious cycle, when oxidative stress leads to higher fraction of, e.g. mutated ND5 subunits, it will be accelerated more and more. Thus, inhibition of Complex I H^+ pumping, which leads to oxidative stress, appears to be a missing link in the theory of mitochondrial aging and in the etiology of diseases related to oxidative stress.

Keywords: In situ mitochondrial superoxide production; MitoSOX; Proton-pumping NADH:quinone oxidoreductase; HEPG2 cells; Oxidative stress; Aging

1. Introduction

Complex I (H^+-pumping NADH:quinone oxidoreductase) is an essential component of the mitochondrial respiratory chain (Brandt, 2006; Lenaz, Baracca, Fato, Genova, & Solaini, 2006; Lenaz, Fato, Formiggini, & Genova, 2007; Yagi & Matsuno-Yagi, 2003), participating not only in cell respiration, but also in cellular/organismal reactive oxygen species homeostasis (Brand et al., 2004; Ježek & Hlavatá, 2005), apoptosis initiation or modulation (Ott, Gogvadze Orrenius, & Zhivotovsky, 2007), and O_2 sensing (Piruat & Lopez-Barneo, 2005). This huge 46 subunit mammalian complex is vulnerable to oxidative stress, hence it is one of the factors that determines lifespan, pace of aging, susceptibility to oxidative stress-related diseases,

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and certain mitopathies (Bai et al., 2004; Bourges et al., 2004; Chomyn & Attardi, 2003; Smigrodzki & Khan, 2005). This is so because Complex I comprises 7 of 13 proteins encoded by the mitochondrial genome (membrane arm subunits ND1-6, and ND4L, among which ND2, ND4, and ND5 act in H^+ pumping; Gemperli, Schaffitzel, Jakob, & Steuber, 2007; Nakamaru-Ogiso, Boo Seo, Yagi, & Matsuno-Yagi, 2003; Nakamaru-Ogiso, Sakamoto, Matsuno- Yagi, Myioshi, & Yagi, 2003). Thus, mtDNA mutations in regions encoding Complex I subunits, induced by continuous inevitable mitochondrial superoxide ($O_2^{\bullet-}$) production, might initiate a vicious cycle due to further enhanced $O_2^{\bullet-}$ production brought on by the now impaired function of Complex I or other respiratory machinery elements (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). The progressive increase in mitochondria-derived oxidative stress may also contribute to numerous diseases including atherosclerosis, hypertension, ischemia-reperfusion injury, inflammation, cystic fibrosis, cancer, type-2 diabetes, and neurodegenerative diseases (e.g. multiple sclerosis, Parkinson's and Alzheimer's disease) (Brand et al., 2004; Ježek & Hlavatá, 2005). Despite the suggestion that mitochondria-derived oxidative stress impacts many disease states and aging, it is not understood why Complex I-related mtDNA mutations lead to oxidative stress. Notably, we lack a high-resolution structure of Complex I, a detailed mechanism of $O_2^{\bullet-}$ production within the Complex I, and a detailed pathway of electron transport and H^+ pumping (Brandt, 2006; Brand et al., 2004; Grivennikova & Vinogradov, 2006; Ohnishi & Salerno, 2005).

Scant direct evidence exists for the dependence of mitochondrial $O_2^{\bullet-}$ formation in intact cells on the mitochondrial inner membrane potential ($\Delta\Psi_m$) or on the entire protonmotive force ($\Delta p = \Delta\Psi_m + \Delta pH$, in mV) (Ježek & Hlavatá, 2005). There are no indications of attenuation of in situ mitochondrial $O_2^{\bullet-}$ production by uncoupling or by other means of Δp modulation despite clear demonstrations of such phenomenon in isolated mitochondria (Brand et al., 2004; Ježek & Hlavatá, 2005). Rather, when uncouplers like carbonyl cyanide *p*-(trifluoro-methoxy)phenylhydrazone (FCCP) are added to culture cells, remodeling (De Vos, Allan, Grierson, & Sheetz, 2005) or fragmentation/fission of mitochondrial network (Benard et al., 2007; Duvezin-Caubet et al., 2006; Ishikara, Fujita, Oka, & Mihara, 2006; Lyamzaev et al., 2004; Pletjushkina et al., 2006), apoptosis (Aronis et al., 2003; Dispersyn, Nuydens, Connors, Borgers, & Geerts, 1999), or changes in gene expression (Desquiret et al., 2006; Kuruvilla et al., 2003) are observed.

It is still un-clear whether Complex I derived $O_2^{\bullet-}$ generation is sensitive to $\Delta\Psi_m$. Complex I derived $O_2^{\bullet-}$ production due to both forward (Lambert & Brand, 2004a) and reverse electron transport diminishes with decreasing ΔpH in isolated skeletal muscle mitochondria (Lambert & Brand, 2004a, 2004b). The reverse electron transport is inhibited by rotenone. Complex III (ubiquinol-cytochrome c oxidoreductase) can also produce $O_2^{\bullet-}$ as shown in vitro in isolated glutathione-depleted nonphosphorylating rat heart mitochondria respiring with succinate at state 4. These mitochondria exhibited a 55% decrease in H_2O_2 formation when $\Delta\Psi_m$ decreased by only 10% (Korshunov, Skulachev, & Starkov, 1997). About 30–43% (Starkov & Fiskum, 2003; Starkov et al., 2004) of the levels of state 4 H_2O_2 generation was maintained in the phosphorylating state, i.e. state 3. Thus, H^+ backflux to the matrix, ensured either by ATP synthase or by uncoupling, attenuates $O_2^{\bullet-}$ production. Still, the relative contributions of Complexes I and III to overall $O_2^{\bullet-}$ formation in mitochondria is not known (Ježek & Hlavatá, 2005; Muller, Liu, & Van Remmen, 2004). Nevertheless, almost 100% of Complex I $O_2^{\bullet-}$ production is released to the matrix (Brand et al., 2004; Muller et al., 2004).

In this work, we used confocal microscopy with the mitochondrial superoxide indicator MitoSOX Red to assess excessive matrix $O_2^{\bullet-}$ release in situ. We found that Complex I $O_2^{\bullet-}$ production is enhanced only and exclusively when both electron transport and H^+ pumping are retarded. Since inhibition of Complex I H^+ pumping usually results from oxidative stress-induced mutations in mtDNA encoding subunits ND2, ND4, and ND5, the fact that this inhibition produces further oxidative stress represents a missing link in a vicious cycle of aging or oxidative stress-related diseases.

2. Materials and methods

2.1. Cell cultivation

The human hepatocellular carcinoma cell line HEPG2 (ECACC 85011430) was cultivated at 37 °C in humidified air with 5% CO_2 in DMEM (Gibco, cat. no. 11995-065; contains no glucose) supplemented with 3 mM glutamine, 5% FCS (Biochrome, cat. no. S0113), 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The added carbon source was either 25 mM glucose or 10 mM galactose (with glucose-free dialyzed FCS, PAA, cat. no. A15-107). The latter is referred to as oxphos conditions (cells), because cells rely largely on oxidative phosphorylation (Rossignol et al., 2004). The doubling time for glc and oxphos cells was