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Production of superoxide/H_2O_2 by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria

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ABSTRACT

Dehydrogenases that use ubiquinone as an electron acceptor, including complex I of the respiratory chain, complex II, and glycerol-3-phosphate dehydrogenase, are known to be direct generators of superoxide and/or H_2O_2. Dihydroorotate dehydrogenase oxidizes dihydroorotate to orotate and reduces ubiquinone to ubiquinol during pyrimidine metabolism, but it is unclear whether it produces superoxide and/or H_2O_2 directly or does so only indirectly from other sites in the electron transport chain. Using mitochondria isolated from rat skeletal muscle we establish that dihydroorotate oxidation leads to superoxide/H_2O_2 production at a fairly high rate of about 300 pmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ when oxidation of ubiquinol is prevented and complex II is uninhibited. This H_2O_2 production is abolished by brequinar or leflunomide, known inhibitors of dihydroorotate dehydrogenase. Eighty percent of this rate is indirect, originating from site II_F of complex II, because it can be prevented by malonate or atpenin A5, inhibitors of complex II. In the presence of inhibitors of all known sites of superoxide/H_2O_2 production (rotenone to inhibit sites in complex I (site I_Q and, indirectly, site I_F), myxothiazol to inhibit site III_{Qo} in complex III, and malonate plus atpenin A5 to inhibit site II_F in complex II), dihydroorotate dehydrogenase generates superoxide/H_2O_2, at a small but significant rate (23 pmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$), from the ubiquinone-binding site. We conclude that dihydroorotate dehydrogenase can generate superoxide and/or H_2O_2 directly at low rates and is also capable of indirect production at higher rates from other sites through its ability to reduce the ubiquinone pool.

The biosynthesis of pyrimidine is an essential part of cell proliferation. Most mammalian cells have two pathways for pyrimidine synthesis, de novo and salvage. The de novo pathway involves six enzymatic steps, five in the cytosol and one in the mitochondria (Fig. 1), where dihydroorotate is converted to orotate in a reversible reaction catalyzed by dihydroorotate dehydrogenase [1]. There are also salvage pathways in which uracil is regenerated from various compounds in nucleotide metabolism. Proliferating cells have a great requirement for de novo pyrimidine biosynthesis; therefore dihydroorotate dehydrogenase is a pharmaceutical target for small-molecule inhibitors to treat cancer and autoimmune diseases and for immunosuppression. The two best-known inhibitors of dihydroorotate dehydrogenase are brequinar and leflunomide. Both have been extensively tested, but only leflunomide is currently available as a pharmaceutical and is licensed for the treatment of psoriatic and rheumatoid arthritis [1].

The dihydroorotate dehydrogenases can be divided into two broad families based on alignment of sequences from different organisms, subcellular location, and preference for electron acceptor [2]. Eukaryotes and most gram-negative bacteria, including *Escherichia coli,* have dihydroorotate dehydrogenases belonging to family 2. In eukaryotes, dihydroorotate dehydrogenase is located in the mitochondrial inner membrane with the dihydroorotate binding site facing the intermembrane space and a hydrophobic tail inserted in the membrane [3]. Family 2 dihydroorotate dehydrogenases contain two redox-active sites: a flavin mononucleotide prosthetic group, which accepts two electrons from dihydroorotate, and a ubiquinone in the quinone binding site, which subsequently accepts the electrons and joins the pool of ubiquinone in the membrane (the Q-pool)[1] [4]. Quinone reduction involves two single-electron transfers and it is likely that a semireduced flavin intermediate is formed during the catalytic cycle [5]. The midpoint potential of the flavin in purified *E. coli* dihydroorotate dehydrogenase is sufficiently negative (−310 mV) to make this enzyme a

Abbreviations: I_F, flavin of complex I; I_Q, quinone binding site of complex I; II_F, flavin of complex II; III_{Qo}, quinone binding site on the outer/cytosolic face of complex III; ETF:QOR, electron-transferring flavoprotein:ubiquinone oxidoreductase; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; Q-pool, pool of ubiquinone in the mitochondrial inner membrane; SOD, superoxide dismutase

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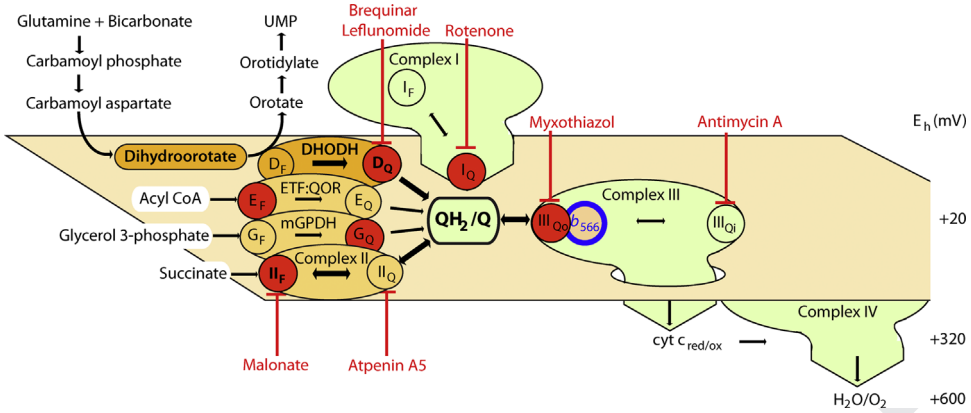

Fig. 1. The QH_2/Q isopotential group and its component superoxide/H_2O_2-producing enzymes, including dihydroorotate dehydrogenase. For comparison, see also the sites of superoxide/H_2O_2 production in the NADH/NAD^+ isopotential group [32] and the overall scheme of electron flow and superoxide/H_2O_2 production in the various mitochondrial isopotential groups [33]. The background plane represents the QH_2/Q isopotential group of redox centers operating at a redox potential (E_h) of approximately +20 mV. Their redox state is reported by the redox state of cytochrome b_{566} [15]. Normal electron flow from complex I through sites I_F and I_Q to ubiquinone (QH_2/Q) and then through site III_{Qo} of complex III, cytochrome *c* (cyt c), and complex IV to oxygen at E_h +600 mV is indicated by large green arrows. Ovals represent other enzymes that reduce Q; circles denote their flavin (F)- and ubiquinone (Q)-linked redox sites—complex II (sites II_F and II_Q) [12], mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) (sites G_F and G_Q) [11], the electron-transferring flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QOR) system of acyl CoA oxidation (sites E_F and E_Q) [13], and dihydroorotate dehydrogenase (DHODH) (sites D_F and D_Q). Sites in the QH_2/Q isopotential group that generate superoxide/H_2O_2 are indicated in red. Sites of action of relevant inhibitors are shown by red blunted arrows. The de novo pyrimidine biosynthesis pathway from glutamine and bicarbonate to UMP is shown associated with dihydroorotate dehydrogenase. With dihydroorotate as substrate, electron flow was controlled using inhibitors: in Fig. 2 rotenone blocked site I_Q (and access from QH_2 to site I_F), antimycin A blocked site III_{Qi}, and atpenin A5 and malonate blocked complex II, leaving site III_{Qo} open so electrons from dihydroorotate reduced cytochrome b_{566} unless brequinar or leflunomide was added to inhibit site D_Q. In Figs. 3 and 4 sites I_Q and III_{Qo} were blocked by rotenone, antimycin A, and myxothiazol, so electrons from dihydroorotate passed through sites D_F and D_Q, producing superoxide and/or H_2O_2, into the QH_2/Q pool, and then back into complex II through site II_Q to site II_F, producing superoxide/H_2O_2. Addition of malonate blocked site II_F directly, and addition of atpenin A5 blocked access to site II_F through site II_Q (Fig. 3), whereas addition of brequinar or leflunomide blocked site D_Q and reduction of Q, indirectly preventing superoxide/H_2O_2 production by site II_F (Fig. 4). In Fig. 6 sites I_Q, III_{Qo}, and II_F were inhibited by rotenone, antimycin A, myxothiazol, atpenin A5, and malonate, and sites E_F and G_Q were inactive because of lack of acyl-CoA and glycerol 3-phosphate, leaving only site D_Q active. Its production of superoxide/H_2O_2 was inhibited by brequinar and partially by leflunomide.

good electron donor to oxygen to generate superoxide and/or H_2O_2 [5] although the reactivity with quinone substrates is 14- to 58-fold higher [6].

In early studies, Forman and Kennedy concluded that superoxide was produced by isolated mitochondria during dihydroorotate oxidation [7,8]. However, the same group later concluded that the source of superoxide lay outside dihydroorotate dehydrogenase and was likely to be complex III [9]. In contrast, a recent study [10] investigating the reactive oxygen species-induced apoptotic effect of *N*-(4-hydroxyphenyl)retinamide concluded that the reactive oxygen species most likely originated from dihydroorotate dehydrogenase itself, because manipulating dihydroorotate dehydrogenase protein content and activity also affected cellular levels of reactive oxygen species. However, these results could be explained by changes in superoxide/H_2O_2 production from other Q-pool-linked sites when the reduction state of the Q-pool was altered by manipulating electron input through dihydroorotate dehydrogenase. Thus, they do not show that dihydroorotate dehydrogenase itself generates superoxide/H_2O_2 in situ.

The mitochondrial electron transport chain and matrix contain several protein complexes that have flavins and ubiquinone binding sites with the capacity to produce superoxide/H_2O_2. Fig. 1 shows the sites at the potential of the Q-pool. Our group is characterizing mitochondrial sites of superoxide/H_2O_2 production and developing methods for measuring the contribution of each site under different conditions [11–16]. In a recent study we found that mitochondria isolated from rat skeletal muscle produced superoxide/H_2O_2 when oxidizing dihydroorotate under conditions that should prevent contributions from other known sites, implicating dihydroorotate dehydrogenase as a direct generator of superoxide/H_2O_2 in isolated mitochondria [11]. The objective of the present study was to more fully characterize the sources of superoxide/H_2O_2 production during dihydroorotate oxidation. We found that dihydroorotate dehydrogenase itself can generate superoxide/H_2O_2 at a small rate of about 20–30 pmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ in rat skeletal muscle mitochondria and can drive superoxide/H_2O_2 production at 10-fold higher rates from other sites.

Materials and methods

Animals, mitochondrial preparation, and reagents

Female Wistar rats (Harlan Laboratories), age 5–8 weeks, were fed chow ad libitum and given free access to water. Skeletal muscle mitochondria were isolated in ice-cold Chappell–Perry medium (100 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.4 at 4 °C) by standard procedures [17]. Protein concentration was determined using the biuret method. The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with NIH guidelines for the care and use of laboratory animals. All reagents were from Sigma (St. Louis, MO, USA) except for Amplex UltraRed (Invitrogen, Carlsbad, CA, USA) and atpenin A5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Mitochondrial respiration

Oxygen consumption rate was measured using an XF24 extracellular flux analyzer (Seahorse Bioscience) [18]. Ten micrograms of mitochondria (2 μg when using succinate plus rotenone) was added to each well in a volume of 20 μl and centrifuged for 15 min at 2000 *g*. Total well volume was brought to 500 μl with KHEPMB medium, comprising 120 mM KCl, 5 mM Hepes, 5 mM K_2HPO_4, 1 mM EGTA, 2 mM $MgCl_2$, and 0.3% (w/v) bovine serum albumin (pH 7.0 at 37 °C). The uncoupled oxygen consumption rate was measured in the presence of 1 mM ADP, 1 μg ml^{-1} oligomycin, 1 μM carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and 20 μM leflunomide, 10 μM brequinar, or vehicle (dimethyl sulfoxide; DMSO). After the basal respiration rate was determined, substrates were added. Substrate combinations were 5 mM succinate plus

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