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Sites of superoxide and hydrogen peroxide production during fatty acid oxidation in rat skeletal muscle mitochondria

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ABSTRACT

H_2O_2 production by skeletal muscle mitochondria oxidizing palmitoylcarnitine was examined under two conditions: the absence of respiratory chain inhibitors and the presence of myxothiazol to inhibit complex III. Without inhibitors, respiration and H_2O_2 production were low unless carnitine or malate was added to limit acetyl-CoA accumulation. With palmitoylcarnitine alone, H_2O_2 production was dominated by complex II (44% from site II_F in the forward reaction); the remainder was mostly from complex I (34%, superoxide from site I_F). With added carnitine, H_2O_2 production was about equally shared between complexes I, II, and III. With added malate, it was 75% from complex III (superoxide from site III_{Qo}) and 25% from site I_F. Thus complex II (site II_F in the forward reaction) is a major source of H_2O_2 production during oxidation of palmitoylcarnitine ± carnitine. Under the second condition (myxothiazol present to keep ubiquinone reduced), the rates of H_2O_2 production were highest in the presence of palmitoylcarnitine ± carnitine and were dominated by complex II (site II_F in the reverse reaction). About half the rest was from site I_F, but a significant portion, ~40 pmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$, was not from complex I, II, or III and was attributed to the proteins of β-oxidation (electron-transferring flavoprotein (ETF) and ETF-ubiquinone oxidoreductase). The maximum rate from the ETF system was ~200 pmol $H_2O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$ under conditions of compromised antioxidant defense and reduced ubiquinone pool. Thus complex II and the ETF system both contribute to H_2O_2 productionduring fatty acid oxidation under appropriate conditions.

Fatty acid β-oxidation by skeletal muscle mitochondria is a major source of ATP under physiological conditions [1,2], and impaired lipid metabolism is associated with several pathological conditions, such as multi-acyl-CoA dehydrogenase deficiency, obesity-related insulin resistance, and type 2 diabetes [3]. Changes in reactive oxygen species (ROS)[1] production in these pathological conditions have been reported [4–8].

Palmitoylcarnitine is a major substrate for β-oxidation. It enters mitochondria on the carnitine–acylcarnitine translocase and is converted to palmitoyl-CoA. The fatty acid β-oxidation spiral involves four sequential enzymes: acyl-CoA dehydrogenase, 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase [9]. In contrast to peroxisomal β-oxidation, in which molecular oxygen serves as the electron acceptor of acyl-CoA oxidases, mitochondrial acyl-CoA dehydrogenases transfer single electrons to electron-transferring flavoprotein (ETF) [10]. Singly reduced ETF^- is then oxidized by ETF-ubiquinone oxidoreductase (ETFQOR), which donates electrons directly to the ubiquinone (Q) pool in the mitochondrial inner membrane [11–13], to be passed to complex III of the respiratory chain, cytochrome *c*, complex IV, and finally molecular oxygen. The second dehydrogenation reaction in the β-oxidation spiral is catalyzed by hydroxyacyl-CoA dehydrogenase, which uses NAD^+ as its electron acceptor. The reduced NADH is then oxidized by complex I, reducing the Q pool. The end product of β-oxidation, acetyl-CoA, condenses with oxaloacetate to form citrate, which is then oxidized by the Krebs cycle. Thus fatty acid oxidation in mitochondria is linked to oxidative phosphorylation and ATP production.

In contrast to many other substrates, such as malate, glutamate, succinate, or glycerol 3-phosphate, oxidation of fatty acids requires four enzymatic reactions and donates electrons at multiple points in the electron transport chain: complex I, ETFQOR, and complex II

Abbreviations: PC, palmitoylcarnitine; ETF, electron-transferring flavoprotein; ETFQOR, electron-transferring flavoprotein ubiquinone oxidoreductase; Q, ubiquinone, QH_2, ubiquinol; ROS, reactive oxygen species; Site I_F, flavin site of complex I; Site I_Q, ubiquinone-binding site of complex I; Site II_F, flavin site of complex II; Site III_{Qo}, outer ubiquinone-binding site of complex III; GPDH, glycerol 3-phosphate dehydrogenase; SOD, superoxide dismutase; DCPIP, dichlorophenolindophenol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; CDNB, 1-chloro-2,4-dinitrobenzene

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(via formation of succinate in the Krebs cycle). This makes fatty acid oxidation a good candidate for high rates of superoxide or H_2O_2 formation due to possible leaks of electrons to molecular oxygen at several different sites.

There are several sites of superoxide or H_2O_2 production in the Krebs cycle and electron transport chain [14–16]. In order of maximum capacity in skeletal muscle mitochondria, they are the ubiquinol-oxidizing site of complex III (site III_{Qo}), the ubiquinone-reducing site of complex I (site I_Q) and the flavin site of complex II (site II_F), the flavin site of complex I (site I_F), and the Q-binding site of glycerol 3-phosphate dehydrogenase (GPDH) [17,18]. Other sites include the dihydrolipoate moieties of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase, and the ETF/ETFQOR system, but their maximum rates have not been established. The native rates from various sites in the absence of inhibitors have been measured only during oxidation of glutamate and malate, when sites I_F and III_{Qo} dominate [19]; oxidation of succinate, when site I_Q dominates [20,21]; and oxidation of glycerol 3-phosphate, when site I_Q dominates and sites II_F and GPDH also contribute [17,18].

The sites of H_2O_2 production during oxidation of palmitoylcarnitine by skeletal muscle mitochondria have been addressed in earlier studies in isolated mitochondria [20,22–25]. Although mitochondria isolated from the complex cellular environment lose some inputs of metabolic control, this widely used model still provides a detailed understanding of mechanisms of metabolic regulation, including those related to mitochondrial ROS production. These insights then feed forward our understanding of more complex systems such as intact cells, tissues, or animals. Prior studies in isolated mitochondria have identified several different components of the respiratory chain and β-oxidation pathway as sources of H_2O_2 during palmitoylcarnitine oxidation by isolated mitochondria: site I_Q [20,23], site III_{Qo} [20,22–24], ETF/ETFQOR [20,22–24], and acyl-CoA dehydrogenase [22,25]. One possible source of disagreement is the use of site-specific inhibitors of the respiratory chain. Such inhibitors are great tools for understanding the capacities and mechanisms of H_2O_2 production at sites of interest. However, inhibition of one center disrupts normal electron flow and may lead to changes in the reduction states of other centers far from the site of inhibition, with consequent changes in their production of superoxide or H_2O_2. In the presence of inhibitors it becomes impossible to measure changes in native rates of H_2O_2 production in different metabolic states or disease models, which is crucial for the investigation of ROS-related physiology and pathology. Therefore it is of high importance to resolve the contradicting conclusions and perform measurements in the absence of respiratory chain inhibitors (native rates) that more closely match physiological conditions in vivo.

The disadvantages of site-specific inhibitors can be partially avoided by using endogenous reporters to predict rates of superoxide formation from sites I_F and III_{Qo}[19]. This approach assumes a unique relationship between the reduction state of the superoxide-producing species within the site and its rate of reaction with oxygen to generate superoxide (or H_2O_2). The reduction state of the superoxide producer can be detected by measuring the reduction state of an endogenous "reporter," a species that is close to equilibrium with the superoxide-producing moiety. NADH was established as a reporter of rates of superoxide production at site I_F (plus any other sites that respond to NADH reduction state) and cytochrome b_{566} as a reporter of superoxide production at site III_{Qo}[19].

For the first time we provide a complete analysis and dissection of the specific sites of superoxide/H_2O_2 production during fatty acid oxidation in the absence of respiratory chain inhibitors. We also investigate the contribution of the ETF/ETFQOR system to the rates of H_2O_2 production in the absence and presence of complex III inhibitors that lead to reduction of the Q pool and estimate the maximum rate of H_2O_2 production from this system in skeletal muscle mitochondria. We identify complex II as a new site of superoxide and/or H_2O_2 production that was not recognized in earlier studies and whose H_2O_2 production was previously wrongly attributed to other sites.

Experimental procedures

Animals, reagents, and mitochondrial preparation

Female Wistar rats (Harlan Laboratories), age 5–8 weeks, were fed chow ad libitum with free access to water. Skeletal muscle mitochondria were isolated in Chappell–Perry buffer (100 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.1 at 25 °C) by standard procedures [26]. Protein concentration was determined by the biuret method. The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with IACUC standards. 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). The L-isomer of palmitoylcarnitine was used in all experiments.

H_2O_2 production

Rates of superoxide production were measured indirectly as rates of H_2O_2 production after conversion of superoxide to H_2O_2 by endogenous superoxide dismutase (SOD) in the matrix. H_2O_2 was detected using horseradish peroxidase oxidizing Amplex UltraRed to its fluorescent resorufin product [19,27]. Exogenous SOD was added to convert any superoxide released in the medium. Mitochondria (0.3 mg protein · ml^{-1}) were suspended in a medium containing 120 mM KCl, 5 mM Hepes, 5 mM K_2HPO_4, 1 mM EGTA, and 0.3% (w/v) bovine serum albumin (pH 7.0 at 37 °C), together with 5 U · ml^{-1} horseradish peroxidase, 25 U · ml^{-1} SOD, and 50 μM Amplex UltraRed. The fluorescence signal was recorded using a Varian Cary Eclipse spectrofluorimeter (λ_{ex}560 nm, λ_{em}590 nm) with constant stirring. Rates of fluorescence change were calibrated with known amounts of H_2O_2 and normalized to the amount of protein [27]. The rates of H_2O_2 production in the calibration curve in Fig. 6B (see below) were obtained by titrating rotenone in the presence of 4 μM FCCP and 5 mM malate [28].

NAD(P) reduction state

Experiments were performed using 0.3 mg mitochondrial protein · ml^{-1} at 37 °C in the same medium as for H_2O_2 measurements. The reduction state of endogenous NAD(P) was determined by autofluorescence [28] using a Shimadzu RF5301-PC spectrofluorimeter at λ_{ex} 365 nm, λ_{em} 450 nm. NAD(P) was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with 5 mM malate and 4 μM rotenone (Fig. 2A). Intermediate values were determined as %NAD(P)H relative to the 0 and 100% values. Although this technique measures contribution from both mitochondrial NADH and mitochondrial NADPH, the content of NAD^+ plus NADH in skeletal muscle mitochondria is much greater than the combined $NADP^+$ and NADPH [28,29]. Moreover the enhancement of NADH fluorescence in mitochondria is two- to fourfold greater than it is for mitochondrial NADPH [30]. The higher content and greater fluorescence enhancement of NADH make the autofluorescence signal predominantly a measure of NADH. The $NAD(P)^+$ reduction states in the calibration curve in Fig. 6B (see below) were manipulated by varying the concentration of rotenone in the presence of 5 mM malate and 4 μM FCCP [28].