Research Paper

Sites of reactive oxygen species generation by mitochondria oxidizing different substrates

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A R T I C L E   I N F O

Article history:
Received 22 March 2013
Received in revised form 4 April 2013
Accepted 5 April 2013

Keywords:
Superoxide
Hydrogen peroxide
Respiratory complexes
NADH
Ubiquinone
Cytochrome b

A B S T R A C T

Mitochondrial radical production is important in redox signaling, aging and disease, but the relative contributions of different production sites are poorly understood. We analyzed the rates of superoxide/H2O2 production from different sites in rat skeletal muscle mitochondria using a variety of conventional substrates in the absence of added inhibitors: succinate; glycerol 3-phosphate; palmitoyl-carnitine plus carnitine; or glutamate plus malate. In all cases, the sum of the estimated rates accounted fully for the measured overall rates. There were two striking results. First, the overall rates differed by an order of magnitude between substrates. Second, the relative contribution of each site was very different with different substrates. During succinate oxidation, most of the superoxide production was from the site of quinone reduction in complex I (site IQ), with small contributions from the flavin site in complex I (site IF) and the quinol oxidation site in complex III (site IIIQ). However, with glutamate plus malate as substrate, site IQ made little or no contribution, and production was shared between site IF, site IIIQ, and 2-oxoglutarate dehydrogenase. With palmitoyl carnitine as substrate, the flavin site in complex II (site II) was a major contributor (together with sites IF and IIIQ), and with glycerol 3-phosphate as substrate, five different sites all contributed, including glycerol 3-phosphate dehydrogenase. Thus, the relative and absolute contributions of specific sites to the production of reactive oxygen species in isolated mitochondria depend very strongly on the substrates being oxidized, and the same is likely true in cells and in vivo.

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Introduction

An increasing number of hypotheses propose that production of mitochondrial reactive oxygen species (ROS) plays a crucial role in different areas of physiology and pathology [1–4]. Despite this, we know very little about which mitochondrial sites in the electron transport chain and associated metabolic enzymes are responsible for physiological or pathological ROS production under native conditions (i.e. in the absence of added inhibitors).

Abbreviations: IF, flavin site of complex I; IQ, quinone-binding site of complex I; II, flavin site of complex II; IIIQ, quinol oxidation site of complex III; CDNB, 1-chloro-2,4-dinitrobenzene; Ep, redox potential; ETF, electron transferring flavoprotein; ETF-QOR, ETF:ubiquinone oxidoreductase; mGPDH, mitochondrial glycerol 3-phosphate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; Q, ubiquinone; QH2, ubiquinol; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.redox.2013.04.005

It is well established that isolated mitochondria can produce hydrogen peroxide (H2O2) in vitro [5,6]. Since the earliest observations by Chance and colleagues [7], this field has expanded considerably and many characteristics of mitochondrial superoxide/H2O2 production have been revealed. ROS are produced by the leak of electrons from donor redox centers to molecular oxygen. The mitochondrial electron transport chain (Fig. 1) consists of several complexes containing multiple redox centers that normally facilitate transfer of electrons to their final acceptor, molecular oxygen, which is reduced by four electrons to water at complex IV. Premature single electron reduction of molecular oxygen earlier in the chain forms the superoxide radical (O2•−) and divalent reduction forms H2O2. Superoxide dismutase-2 in the matrix converts superoxide to H2O2, which can escape and be assayed in the surrounding medium. The general term ROS can refer to several different species, but in this context we use it to refer only to superoxide or H2O2.

Using inhibitors to manipulate the redox states of particular sites and prevent superoxide generation from others, at least ten different sites of superoxide/H2O2 production in the electron transport chain and associated enzymes (Krebs cycle, β-oxidation etc.) have been identified in mammalian mitochondria (Fig. 1).
Each of these sites has been at least partially characterized. The sites that are often invoked as the most important mitochondrial superoxide producers are in respiratory complexes I and III [5,6]. In complex I there are two sites: the flavin in the NADH-oxidizing site (site I_F) and the ubiquinone-reducing site (site I_Q) [8]. In complex III, the superoxide is thought to arise from the quinol oxidizing site (site IIIQo) [9-11]. However, other sites of superoxide/H₂O₂ production have also been defined, including 2-oxoglutarate dehydrogenase (OGDH) [12,13]; pyruvate dehydrogenase (PDH) [14]; complex II (site IIF) [15]; and glyceral 3-phosphate dehydrogenase (mGPDH) [16]. In addition, there are suggestions that other less-well-described sites may also be involved in H₂O₂ production: the electron transferring flavoprotein/ETF:Q oxidoreductase (ETF/ETF:QOR) system of fatty acid β-oxidation [17,18]; proline dehydrogenase [19]; and dihydroorotate dehydrogenase [16,20].

Despite our understanding of the superoxide/H₂O₂-producing capacities of mitochondrial enzymes in vitro, we know very little about the native ROS-producing behavior of mitochondria in vitro or in situ. This is because the standard way to specify an ROS-producing site in a particular phenotype is to inhibit or genetically modify the site, and observe the change in ROS signal or in the downstream phenotype. However, this approach is fundamentally flawed, because blocking a site of electron transport will invariably interrupt normal electron flow and alter the redox states of other sites in the electron flow pathway, which can dramatically alter their downstream phenotype. This raises the question: how can the individual contributions from a complex suite of superoxide/H₂O₂-producing sites be assessed within intact mitochondria under native conditions? To address this question, we developed a novel method of estimating the rates of superoxide generation from two specific sites (I_F and IIIQo) by determining the dependence of superoxide production from each site (defined using inhibitors) on the redox state of its electron donor (reported by the redox states of NAD(P)H and cytochrome b₅₆₆, respectively), then measuring the redox state of the reporter under native conditions in the absence of added inhibitors to predict the contribution of the reported site to overall H₂O₂ production [21].

In the present study, we extend this approach of using endogenous reporters under native conditions to encompass many more superoxide/H₂O₂-producing sites and a greater variety of substrates. We determine the contributions of each site to overall H₂O₂ production by isolated skeletal muscle mitochondria oxidizing four different substrate combinations in the absence of inhibitors: (a) succinate, (b) glycerol 3-phosphate, (c) palmitoyl-carnitine plus carnitine, and (d) glutamate plus malate. The results show that the absolute and relative contribution of each site differs greatly with different substrates.

Materials and methods

Animals, mitochondrial preparation, and reagents

Female Wister rats (Harlan Laboratories), age 5–8 weeks, were fed chow ad libitum and given free access to water. Mitochondria from hind limb skeletal muscle were isolated at 4 °C in Chappel–Perry buffer (CP1; 100 mM KCl, 50 mM Tris, 2 mM EGTA, pH 7.1 at 25 °C) by standard procedures [22]. The animal protocol was approved by the Buck Institute Animal Care and Use Committee, in accordance with IACUC standards. All reagents were from Sigma except Amplex UltraRed, which was from Invitrogen.

Superoxide/H₂O₂ production

Rates of superoxide/H₂O₂ production were measured collectively as rates of H₂O₂ production, as two superoxide molecules are dismutated by endogenous or exogenous superoxide dismutase to yield one H₂O₂. H₂O₂ was detected using the horseradish peroxidase
and Amplex UltraRed detection system [22]. Mitochondria (0.3 mg protein ml\(^{-1}\)) were suspended under non-phosphorylating conditions in medium at 37 °C containing 120 mM KCl, 5 mM Hepes, 5 mM K₂HPO₄, 2.5 mM MgCl₂, 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\(^{-1}\) superoxide dismutase, 50 μM Amplex UltraRed and, except for experiments with palmitoylcarnitine, 1 μg ml\(^{-1}\) oligomycin. Reactions were monitored fluorometrically in a Shimadzu RF5301-PC or Varian Cary Eclipse spectrofluorometer (\(λ_{\text{excitation}}=365\) nm, \(λ_{\text{emission}}=590\) nm). NAD(P)H was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with 5 mM malate plus 5 mM glutamate and 4 μM rotenone. Intermediate values were determined as % reduced NAD(P)H relative to the 0% and 100% values.

Cytochrome b₅₆₆ redox state

Experiments were performed at 1.5 mg mitochondrial protein ml\(^{-1}\) in parallel with measurements of H₂O₂ production and NAD(P)H redox state in the same medium. The reduction state of endogenous cytochrome b₅₆₆ was measured with constant stirring at 37 °C in an Olis DW-2 dual wavelength spectrophotometer as the absorbance difference at 566–575 nm [11,21]. Cytochrome b₅₆₆ was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with saturating substrates plus antimycin A. Intermediate values were determined as % reduced b₅₆₆ relative to the 0% and 100% values.

Definition of sites and calibration of endogenous reporters

Site Iᵢ was defined as the site producing superoxide (measured as H₂O₂) in the presence of malate to reduce NAD to NADH, and rotenone to inhibit reoxidation of complex I by the Q-pool. Any H₂O₂ arising from reverse flow from the NDH pool into NAD oxidoreductases such as OGDH or PDH will appear in the analysis as a component of site Iᵢ (Fig. 2a). To decrease the contributions of forward electron flow at OGDH and PDH to H₂O₂ production, for full disclosure we use “NAD(P)H” [21,23] using a Shimadzu RF3501-PC or Varian Cary Eclipse spectrofluorometer at \(λ_{\text{excitation}}=590\) nm, \(λ_{\text{emission}}=560\) nm. NAD(P)H was assumed to be 0% reduced after 5 min without added substrate and 100% reduced with 5 mM malate plus 5 mM glutamate and 4 μM rotenone. Intermediate values were determined as % reduced NAD(P)H relative to the 0% and 100% values.

![Fig. 2](image-url)

**Fig. 2.** Calibration of endogenous reporters in isolated rat skeletal muscle mitochondria. (a) Definition of site Iᵢ (red dot) using malate as substrate in the presence of rotenone to inhibit site Iᵢ and cause reduction of upstream sites and oxidation of all downstream sites. The definition includes any small contribution from backflow of electrons from NADH into other sites, e.g. OGDH and PDH, shown in pink. Pᵢᵢ denotes the flavin/lipoate of pyruvate dehydrogenase. (b) Dependence of superoxide production (measured as extramitochondrial appearance of H₂O₂) from site Iᵢ on the redox state of NAD(P)H (measured by autofluorescence). Malate was titrated from 20 μM to 5 mM in the presence of 4 μM rotenone, 1.5 mM aspartate and 2.5 mM ATP. (c) Definition of site IIIQₒ (red dot) using succinate as substrate, rotenone to inhibit site Iᵢ, and sufficient succinate+malate (5 mM) to suppress site Iᵢ. The rate of superoxide/H₂O₂ production from site IIIQₒ was defined as the rate that was inhibited by 2 μM myxothiazol (after correction for changes at site Iᵢ using the calibration curve in (b)). The definition includes any small contribution from backflow of electrons from QH₂ into other sites, e.g. mGPDH or other Q-oxidoreductases, shown in pink. (d) Dependence of myxothiazol-sensitive superoxide production from site IIIQₒ on the redox state of cytochrome b₅₆₆ (measured by the absorbance spectroscopy). Electron input was titrated by adding different ratios of succinate:malonate (sum=5 mM) in the presence of 4 μM rotenone. Data are means ± SEM (n=4) recalculated from [21] using the new calibration in (b). For curve-fitting see Materials and methods.
assigned to site I$_F$, we added 1.5 mM aspartate to remove endo-
genous 2-oxoglutarate by transamination and 2.5 mM ATP to decrease carbon flows at various points in the Krebs cycle, particularly succinate thiokinase. This change to our previous protocol [18,21] strongly decreased our earlier estimates of the rates from site I$_F$. The rate of superoxide/H$_2$O$_2$ production from site I$_F$ defined by inhibitors in this way was measured as a function of the redox state of NAD(P)H at different malate concentrations between 0.02 mM and 5 mM (Fig. 2b). The data were arbitrarily fitted by non-linear regression to a single exponential, to give the parameter values in the following equation:

$$v_{H_2O_2} \times \text{NAD(P)H} = 0.88 \times e^{(0.04/\text{NAD(P)H})} + 26.5$$

where $v_{H_2O_2}$ is the rate of H$_2$O$_2$ production. This equation was used to predict the rate of superoxide/H$_2$O$_2$ production from site I$_F$ at any observed NAD(P)H redox state in the absence of inhibitors in subsequent experiments.

Superoxide production by site III$_{Q_0}$ was measured as H$_2$O$_2$ production in the presence of succinate to reduce the Q pool, malonate to keep total succinate plus malonate at 5 mM and inhibit superoxide/H$_2$O$_2$ formation from site II$_F$ without fully inhibiting succinate oxidation [15], and rotenone to inhibit superoxide formation from site I$_Q$ (Fig. 2c). Site III$_{Q_0}$ was defined as the component of the observed H$_2$O$_2$ production under these conditions that was sensitive to myxothiazol (a specific inhibitor of site III$_{Q_0}$) after correction for the small difference in rates from site I$_F$ before and after myxothiazol addition (calculated from parallel measurements of NAD(P)H and application of the calibration curve in Fig. 2b). The rate of superoxide/H$_2$O$_2$ production from site III$_{Q_0}$ defined by inhibitors in this way was measured as a function of the redox state of cytochrome b$_{566}$ at different succinate: malonate ratios ranging from 75% to 100% succinate at 5 mM increments (total dicarboxylate concentration 5 mM) (Fig. 2d). The data were arbitrarily fitted in the same way as for site I$_F$ to give the parameter values using the following equation:

$$v_{H_2O_2} \times \text{b}_{566} = 5.22 \times e^{(0.0064/\text{b}_{566})} - 5.22$$

This equation was used to predict the rate of superoxide/H$_2$O$_2$ production from site III$_{Q_0}$ at any observed cytochrome b$_{566}$ redox state in the absence of inhibitors in subsequent experiments.

See Ref. [21] for more extensive discussion and descriptions.

Statistics

When using the calibration curves in Fig. 2 to calculate rates of H$_2$O$_2$ production at a given reduction state of the reporter, the error in the measurements during calibration was taken into account. This error was calculated by standard methods of error propagation through all the steps, as detailed in [21].

The significance of differences between reported and experimentally measured rates of H$_2$O$_2$ production in each experimental condition was tested using Welch’s t-test. Because error propagation was used to capture uncertainty in the calibration curves, individual data-points could not be used for statistical analysis. Instead we used the traits describing the population of data (mean, SEM based on error propagation and number of observations) to calculate if differences were significant ($p < 0.05$).

Results and discussion

The aim of the present study was to determine the contributions of different sites of superoxide/H$_2$O$_2$ production to the total observed H$_2$O$_2$ generation by mitochondria during oxidation of different substrates in the absence of inhibitors. Electrons leak from the respiratory chain to generate superoxide or H$_2$O$_2$ at two different redox potentials ($E_0$): at the isopotential group of redox carriers around the NADH/NAD$^+$ pool at $E_0$ --280 mV and at the isopotential group of redox carriers around the QH$_2$/Q pool at $E_0$ +20 mV (Fig. 1). At each isopotential group, an important determinant of the rate of superoxide or H$_2$O$_2$ production is the redox state: a more reduced carrier generally leads electrons to oxygen at a faster rate. We exploited this relationship to create assays of the rate of superoxide/H$_2$O$_2$ production from different sites, either by defining the site precisely with inhibitors and determining its dependence on the redox state of the appropriate pool as described in Materials and methods (Fig. 2), or by inhibiting the site and determining the change in superoxide/H$_2$O$_2$ production after correction for secondary changes in the redox states of the two pools. Specifically, to predict rates from each site with different respiratory substrates in the absence of inhibitors, we measured the redox states of the endogenous reporters NAD(P)H and cytochrome b$_{566}$ as proxies of the redox states of the two isopotential groups, and determined the rates of superoxide/H$_2$O$_2$ production from sites I$_F$ and III$_{Q_0}$ respectively, using the calibration curves in Fig. 2. Assessing the contribution of other sites was a little more complicated, so we first provide an example of how the endogenous reporter calibration curves and the careful use of inhibitors can be used to quantify the sites of H$_2$O$_2$ production during oxidation of the substrate succinate.

Sites of superoxide/H$_2$O$_2$ production during oxidation of succinate: a worked example

When succinate is oxidized in the absence of rotenone and other electron transport chain inhibitors, electrons flow through complex II into the Q pool. Next, there are two options (Fig. 3a). The electrons can flow forward to complex III and thermodynamically downhill to more oxidized isopotential groups at cytochrome $c$ and H$_2$O$_2$O$_2$O$_2$, pumping protons to complexes III and IV and generating protonotive force. Alternatively, they can flow in reverse to complex I, driven thermodynamically uphill to the more negative isopotential group by reversal of the proton pumps in complex I driven by the protonotive force generated by proton pumping at complexes III and IV. In the process, a number of possible sites of superoxide/H$_2$O$_2$ production may be engaged, particularly sites I$_F$, III$_{Q_0}$, I$_Q$ and I$_P$ (Fig. 3a). However, it is generally agreed that the primary mechanism of H$_2$O$_2$ production during succinate oxidation is reverse electron transport into complex I, because this H$_2$O$_2$ production is very sensitive to rotenone, the classic Q-site inhibitor of complex I [24,25]. What is not usually discussed is that when an inhibitor such as rotenone is added there are subsequent shifts in electron distribution that invariably change the rates of superoxide/H$_2$O$_2$ production by sites both upstream and downstream of the inhibition site [21]. Therefore, the decrease in rate observed after the addition of an inhibitor is not an accurate indication of the rate from its target site before inhibition. The endogenous reporter method described here circumvents this problem: by monitoring the changes in the NADH isopotential group (through NAD(P)H) and the Q isopotential group (through cytochrome b$_{566}$), the changes in electron distribution after inhibitors are added can be quantified and corrected for.

In this worked example, we quantified the sites of H$_2$O$_2$ production during succinate oxidation by measuring the redox states of the endogenous reporters, and the changes in these redox states after addition of rotenone. From this information, we could predict the contribution of each site to the total H$_2$O$_2$ production observed during succinate oxidation. To begin, we measured the rate of H$_2$O$_2$ production during succinate oxidation in the absence of rotenone (891 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$) (Fig. 4a). In parallel, in different cuvettes, we measured the reduction states of the reporters. We observed that NAD(P)H was 89% reduced and cytochrome b$_{566}$ was 46% reduced (Fig. 4b, Table 1). From
this information, we could predict using the equations describing the
the changes in the reduction state of the reporters (Figs. 4a and b, Table 1).
To identify and quantify these sites, we added rotenone to inhibit
inhibitor of complex I: site IIF is the dominant superoxide/H₂O₂ producer when
The approach described above as a worked example using
the contributions of specific sites to superoxide/H₂O₂ production
during oxidation of different substrates
The contributions of specific sites to superoxide/H₂O₂ production
during oxidation of different substrates
There are no surprises in these conclusions (although they are
more accurate than simply assigning the rotenone-sensitive signal to
There was no unaccounted-for rate of
H₂O₂ production, either before (891 pmol H₂O₂ min⁻¹ mg protein⁻¹ observed
versus 886 pmol H₂O₂ min⁻¹ mg protein⁻¹ assigned, N.S.) or after addition of
rotenone (131 pmol H₂O₂ min⁻¹ mg protein⁻¹ observed versus 171 pmol H₂O₂ min⁻¹
mg protein⁻¹ assigned, N.S.), no other site made a substantial
certainty of the total observed rate. The reason site IIF did not contribute is that the conventional succinate concentration used (5 mM) was sufficiently high to effectively
abolish production of superoxide/H₂O₂ by site IIF [15].
There are no surprises in these conclusions (although they are
more accurate than simply assigning the rotenone-sensitive signal to
complex I: site IIF is the dominant superoxide/H₂O₂ producer when
caria and glutamate plus malate.
The contributions of specific sites to superoxide/H₂O₂ production
during oxidation of different substrates
The approach described above as a worked example using
succinate as substrate was used to define the contributions of
three additional important conventional substrates: glycerol 3-phosphate, palmitoylcarnitine plus carnitine, and glutamate plus malate. In each condition, the reduction state of the reporters was measured in parallel with H₂O₂ production. The changes in reduction state of the reporters after addition of inhibitors of complex I or complex II were measured and used to correct for consequent changes in electron distribution and superoxide/H₂O₂ production at other sites. Table 1 details the observed redox states.
of the reporters with each substrate before and after addition of the inhibitors rotenone or malonate.

Fig. 5 shows the central result of the present paper: the contributions of each of the individual sites of superoxide/H\textsubscript{2}O\textsubscript{2} production to overall H\textsubscript{2}O\textsubscript{2} generation by isolated skeletal muscle mitochondria with each of the four substrate combinations under native conditions in the absence of added inhibitors. The first striking result is that the overall rates of H\textsubscript{2}O\textsubscript{2} generation were very different with different substrates, as previously observed by [27] and others [28–30]. The second, crucial, observation is that the relative contribution of each site was very different with different substrates. Thus, the relative and absolute contributions of specific sites to the production of reactive oxygen species in isolated mitochondria depend very strongly on the substrates being oxidized.

To determine the contributions of individual sites to overall H\textsubscript{2}O\textsubscript{2} generation with each substrate, the rates of H\textsubscript{2}O\textsubscript{2} production at site I\textsubscript{F} and IIIQ\textsubscript{o} were first estimated from the redox states of NAD(P)H and cytochrome b\textsubscript{566} using the calibration curves in Fig. 2. With succinate and glycerol 3-phosphate, the contribution of site I\textsubscript{Q} was then estimated from the decrease in the rate of H\textsubscript{2}O\textsubscript{2} production after addition of rotenone followed by correction for changes in the rate from site I\textsubscript{F} (calculated from the redox state of NAD(P)H before and after rotenone addition) and from site IIIQ\textsubscript{o} (calculated from the redox state of cytochrome b\textsubscript{566} before and after rotenone addition). The contributions of mGPDH (with glycerol 3-phosphate as substrate) and OGDH (with glutamate plus malate as substrate) were estimated as the difference between the sum of the rates estimated for the other sites and the observed total rate.

Fig. 4. Sites of superoxide/H\textsubscript{2}O\textsubscript{2} production during succinate oxidation: a worked example. (a) Representative trace of Amplex UltraRed/resorufin fluorescence during oxidation of 5 mM succinate by rat skeletal muscle mitochondria before and after addition of 4 \textmu M rotenone. Numbers by the traces represent average calibrated rates in pmol H\textsubscript{2}O\textsubscript{2} min\textsuperscript{−1} mg protein\textsuperscript{−1}. (b) Reduction states of the endogenous reporters NAD(P)H and cytochrome b\textsubscript{566} before and after rotenone addition. The difference in reduction state before and after rotenone was significant for NAD(P)H (p = 0.01), but not for cytochrome b\textsubscript{566} (p = 0.11) by Student’s t-test. (c) Rates of superoxide/H\textsubscript{2}O\textsubscript{2} production from sites I\textsubscript{F} and IIIQ\textsubscript{o} before and after rotenone addition, predicted from (b) using the calibration curves in Fig. 2. Results are means ± SEM (n = 4). To account for error in both the measurements and the calibration curves, a Welch’s t-test was used. The difference in rates before and after rotenone did not fall within the 95% confidence interval (p = 0.09 for site I\textsubscript{F} and 0.15 for IIIQ\textsubscript{o}). (d) Observed total rate of H\textsubscript{2}O\textsubscript{2} production during succinate oxidation (before addition of rotenone) and assigned rates of superoxide/H\textsubscript{2}O\textsubscript{2} production from sites I\textsubscript{F}, IIIQ\textsubscript{o}, and I\textsubscript{Q} during succinate oxidation. Results are means ± SEM (n = 4); the error bars on the sum column show the combined propagated errors in the total sum value. There was no significant difference between the observed rate and the sum of the assigned rates (Welch’s t-test; p < 0.05).

Sites of superoxide/H\textsubscript{2}O\textsubscript{2} production during oxidation of glycerol 3-phosphate

Glycerol 3-phosphate oxidation by mGPDH results directly in Q pool reduction (Fig. 3b). From the Q pool, electrons may flow both forward (to complex III and beyond, generating protonmotive force) and in reverse (to complex I and complex II). In the process, a number of possible sites of superoxide/H\textsubscript{2}O\textsubscript{2} production may be engaged, particularly mGPDH and sites II\textsubscript{Q}, IIIQ\textsubscript{o}, I\textsubscript{Q} and I\textsubscript{F} (Fig. 3b). Protonmotive force is required for reverse electron flow to complex I [25], but not for reversal into complex II [16]. Complex II has...
been shown to generate superoxide in both the forward and reverse reactions [15].

With glycerol 3-phosphate as substrate, the total observed rate of H2O2 production was 622 pmol H2O2 min⁻¹ mg protein⁻¹. Sites Iₘ (6%) and IIIQₒ (4%) made only modest contributions to this total rate (Fig. 5). The reductions of sites Iₘ (33%) and IIₘ (26%) (after correction for changes in sites Iₘ and IIIQₒ following the addition of inhibitors) were more substantial. The absolute rates from sites Iₘ, IIIQₒ and Iₘ were lower with glycerol 3-phosphate than with succinate, because the two isopotential pools were less reduced (Table 1), presumably because succinate was a better substrate than glycerol 3-phosphate in this experiment, which had sub-optimal glycerol 3-phosphate and calcium concentrations. After these assignments, 192 pmol H2O2 min⁻¹ mg protein⁻¹ (31% of the total) remained unaccounted for. mGPDH itself is known to generate ROS (predominantly superoxide), which are released to both the matrix and the intermembrane space [16]. In this case, we assume that the remaining observed rates of H2O2 production must have arisen from mGPDH, since all other known sites were already accounted for, and we assign this site by difference (31%). It is unlikely that any of the unassigned H2O2 generation in this condition was from uncharacterized sites, since such sites would have to be engaged during oxidation of glycerol 3-phosphate but not during oxidation of succinate (where there was no unassigned H2O2 production, Fig. 5), despite the less extensive reduction of the two isopotential groups with glycerol 3-phosphate as substrate (Table 1).

Sites of superoxide/H2O2 production during oxidation of palmitoylcarnitine plus carnitine

Carnitine enhances oxidation of palmitoylcarnitine by removing inhibitory acetyl-CoA in the form of acetyl carnitine and by promoting entry of palmitoylcarnitine into the mitochondrial matrix [18]. When palmitoylcarnitine is metabolized by the β-oxidation pathway, electrons enter the respiratory chain at two sites: from acyl-CoA dehydrogenase through the electron transferring flavoprotein (ETF) and ETF:ubiquinone oxidoreductase (ETF:QOR) to the Q-pool, and from 3-hydroxyacyl-CoA dehydrogenase to NADH (Fig. 3c). Oxidation of the end product, acetyl-CoA, in the citric acid cycle leads to further electron input through the NAD-linked dehydrogenases and through complex II during oxidation of succinate. Electron entry by more than one route suggests that

<table>
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<th>Substrates and inhibitors</th>
<th>Reduced NAD(P)H (%)</th>
<th>Reported rate of H2O2 production</th>
<th>Reduced cytochrome b₅₅₆ (%)</th>
<th>Reported rate of H2O2 production</th>
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<td>38 ± 5</td>
<td>54 ± 18</td>
</tr>
</tbody>
</table>

Fig. 5. Native rates of superoxide/H2O2 production by mitochondria oxidizing different substrates. Observed total rate of H2O2 production (gray bars) and sum of assigned rates of superoxide/H2O2 production from different sites (colored stacked bars) in the presence of different substrates as indicated: 5 mM succinate; 27 mM disodium rac-α/β-glycerol phosphate (25% active optical isomer sn-glycerol 3-phosphate); 15 μM palmitoyl-L-carnitine plus 2 mM L-carnitine; and 5 mM L-glutamate plus 5 mM L-malate. With each substrate, the reduction states of NAD(P)H and cytochrome b₅₅₆ were measured in parallel with H2O2 production and the calibration curves in Fig. 2 were used to predict the rates of production from sites Iₘ and IIIQₒ. With succinate, 4 μM rotenone was subsequently added to allow calculation of the rate from site Iₘ as described in the text (data from Fig. 4d). With glycerol 3-phosphate, 1 mM malonate and 4 μM rotenone were subsequently added to allow calculation of the rates from sites Iₘ and IIIQₒ, respectively. The rate assigned to mGPDH was calculated by difference. With palmitoylcarnitine plus carnitine, 1 mM malonate was subsequently added to allow calculation of the rate from site Iₘ (data recalculated from [18]). With glutamate plus malate, the rate assigned to OGDH was calculated by difference (data recalculated from [21]). Results are means ± SEM (n=4–6); the error bars on the sum columns show the combined propagated errors in the total sum value. There was no significant difference between observed and assigned rates with succinate or with palmitoylcarnitine plus carnitine (Welch’s t-test; p < 0.05).
fatty acid oxidation may generate superoxide/H$_2$O$_2$ from several different sites, particularly ETF/ETF-QOR and sites II$_{QO}$, I$_Q$ and I$_F$ (Fig. 3c). Site II$_F$ may also contribute by reverse flow from the Q pool, or by forward flow from succinate. Importantly, under this condition the acetyl-CoA generated by β-oxidation will tend to deplete inhibitory oxaloacetate through the action of citrate synthase, making site II$_F$ more prone to generate superoxide/H$_2$O$_2$ [18].

With palmitoylcarnitine plus carnitine as substrate, the total observed rate of H$_2$O$_2$ production was 199 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. From the redox states of the reporters and the corrected effects of malonate on H$_2$O$_2$ production, we were able to account for the entirety of this observed H$_2$O$_2$ production. There were approximately equal contributions from sites II$_F$ (38%) and III$_{QO}$ (36%), with site I$_F$ producing slightly less (25%) (Fig. 5). The absence of unaccounted-for H$_2$O$_2$ production indicated that neither site I$_Q$ nor the ETF/ETF-QOR system generated substantial superoxide/H$_2$O$_2$. Indeed, the ETF/ETF: QOR system does not generate measurable superoxide/H$_2$O$_2$ except under very specific conditions and in the presence of respiratory chain inhibitors [18].

Sites of superoxide/H$_2$O$_2$ production during oxidation of glutamate plus malate

In the final condition, the substrate pair glutamate plus malate was used to generate NADH (Fig. 3d). During oxidation of this substrate combination, malate is oxidized to oxaloacetate by malate dehydrogenase and NADH is generated. Glutamate is used to transaminase the oxaloacetate to form aspartate and 2-oxoglutarate (and some glutamate may be oxidized to 2-oxoglutarate by glutamate dehydrogenase and NADH is generated). Glutamate is used to transaminase the oxaloacetate to form aspartate and 2-oxoglutarate (and some glutamate may be oxidized to 2-oxoglutarate by glutamate dehydrogenase). The aspartate is exchanged for glutamate on the glutamate–aspartate antiporter, and much of the 2-oxoglutarate is exchanged for malate on the oxoglutarate–malate antiporter (although some may be oxidized by 2-oxoglutarate dehydrogenase). In this way, removal of oxaloacetate maximizes NADH generation by malate dehydrogenase [31].

With glutamate plus malate as substrate, the total observed rate of H$_2$O$_2$ production was 182 pmol H$_2$O$_2$ min$^{-1}$ mg protein$^{-1}$. Under this condition, sites I$_Q$ and III$_{QO}$ contributed equally (30% each) [21] (Fig. 5). Malonate did not significantly inhibit the rate (3% inhibition ± 5%), so site I$_Q$ was not a significant contributor, presumably because it was inhibited by oxaloacetate or malate under these conditions [15]. A significant proportion (41%) of the total H$_2$O$_2$ production was unassigned, indicating that another site also contributed under this condition. We assume this was from forward flow through OGDH (Fig. 5) because some 2-oxoglutarate was likely formed during transamination of glutamate, but we cannot exclude a contribution from site I$_Q$ or uncharacterized other sites in this complex metabolic condition. In principle the contribution of site I$_Q$ could be assessed by the decrease in rate observed following addition of rotenone after correction for secondary changes in other defined sites, but definitive conclusions could not be drawn because of the relatively small rates of unassigned H$_2$O$_2$ production, large changes in redox states of the reporters following addition of rotenone, relatively large errors involved, and possible changes in other uncharacterized sites following addition of rotenone.

Advantages and limitations of the approach used

The great strength of the approach we have used here to measure the contributions of different sites to overall mitochondrial H$_2$O$_2$ production [21] is that, unlike all previous approaches, it reports rates under native conditions in the absence of added inhibitors. This gives it great potential for future studies with physiological substrate mixes in mitochondria and in intact cells and organisms. Its main limitation is the assumption that the calibration curves measured in the presence of inhibitors and particular substrates apply under native conditions using other substrates. The good agreement between calculated and measured total rates (Fig. 5) supports the validity of this assumption.

Potential pathological and physiological implications

The results obtained using the four different substrates described in Fig. 5 provide a very clear illustration of the remarkably different H$_2$O$_2$–producing profiles that can be attained by isolated muscle mitochondria oxidizing conventional substrates. When taken as a whole, two results are striking. (i) The native rates differed greatly between substrates. This implies that mitochondrial superoxide/H$_2$O$_2$ production rates in vivo likely depend critically on the substrate being oxidized, so physiological or pathological changes in substrate may be very important determinants of rates of radical production, even at the same overall rate of oxygen consumption. (ii) The contribution of each site differed markedly between substrates. With succinate, site I$_Q$ dominated, with relatively small contributions from sites I$_F$ and III$_{QO}$. However, with palmitoylcarnitine plus carnitine, site I$_F$ was an important contributor, and with glycerol 3-phosphate, five sites contributed, including site I$_F$ and mGPDH. Thus, which sites contribute to superoxide and H$_2$O$_2$ production in mitochondria, in cells, and in vivo under both physiological and pathological conditions likely depends critically on the substrates being utilized.

Notably, the sites are known to differ markedly in the topology of superoxide production [6,16,27,32,33]. Essentially all superoxide/H$_2$O$_2$ from sites I$_F$, I$_Q$ and I$_F$ is directed to the matrix, but about half the superoxide from site III$_{QO}$ and mGPDH appears in the intermembrane space. Thus, the strength of mitochondrial superoxide signaling in the cytosol (and also the amount of oxidative damage caused by superoxide and H$_2$O$_2$ in the matrix) will differ substantially between substrates, even at identical total rates of mitochondrial superoxide/H$_2$O$_2$ production.

Acknowledgments

Supported by National Institutes of Health grants P01 AG025901, PL1 AG032118, R01 AG03354 and TL1 AG032116 (M.D.B., C.L.Q.) and The Ellison Medical Foundation, grant AG-SS-2288-09 (M.D.B., I.V.P.). Fellowship support was from The Glenn Foundation (I.V.P) and The Carlsberg Foundation (M.H-M).

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