Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions

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Mitochondrial production of superoxide and hydrogen peroxide is potentially important in cell signaling and disease. Eleven distinct mitochondrial sites that differ markedly in capacity are known to leak electrons to oxygen to produce $O_2^{\cdot-}$ and/or H_2O_2. We discuss their contributions to $O_2^{\cdot-}/H_2O_2$ production under native conditions in mitochondria oxidizing different substrates and in conditions mimicking physical exercise and the changes in their capacities after caloric restriction. We review the use of S1QELs and S3QELs, suppressors of mitochondrial $O_2^{\cdot-}/H_2O_2$ generation that do not inhibit oxidative phosphorylation, as tools to characterize the contributions of specific sites *in situ* and *in vivo*.

Superoxide ($O_2^{\cdot-}$)[2] and hydrogen peroxide (H_2O_2) are produced by leaks of electrons from donor redox centers of the mitochondrial electron transport chain and associated metabolic enzymes to cause either one-electron or two-electron reduction of oxygen (1, 2). To date, at least 11 sites that produce $O_2^{\cdot-}$ and/or H_2O_2 ($O_2^{\cdot-}/H_2O_2$), namely sites O_F, B_F, A_F, P_F, I_F, I_Q, II_F, III_{Qo}, G_Q, E_F, and D_Q, have been identified in mammalian mitochondria (Fig. 1), with each of them exhibiting distinct properties (3–33). The majority of the work on site-specific mitochondrial $O_2^{\cdot-}/H_2O_2$ production has focused on measurement of the maximum capacities of these sites under conditions in which concentrations of substrates were optimal (usually saturating) and inhibitors of electron transport were used to induce optimal (usually maximal) reduction of redox centers (16, 34, 35). However, maximum capacities, which are mainly dependent on the abundance of the relevant proteins or complexes in the mitochondria, are not necessarily related to the importance of particular mitochondrial sites in physiological or pathological $O_2^{\cdot-}/H_2O_2$ production *in vivo*. The measurement of "native" rates of $O_2^{\cdot-}/H_2O_2$ production from mitochondrial sites under physiological conditions in the absence of inhibitors of electron transport is required to answer such questions. Rates of mitochondrial $O_2^{\cdot-}/H_2O_2$ production from sites I_F and III_{Qo} in any condition can be assessed indirectly by measuring endogenous reporters (36). Such endogenous reporters allow assessment of native rates following specific inhibition of the site of interest by measurement of the decrease in overall rate after correction for the secondary changes in the rates from other sites (assessed using the endogenous reporters) (36–38).

Although excessive production of reactive oxygen species (ROS) from mitochondria is associated with a broad spectrum of pathologies (39–41), there is growing evidence supporting a role of mitochondrial ROS as signaling molecules in physiological pathways (42–45). In this context, mitochondrial ROS-mediated signaling cascades have been proposed to be involved in the response or adaptation to bioenergetic changes (46, 47). Therefore, the characterization of mitochondrial $O_2^{\cdot-}/H_2O_2$ production in response to changes in bioenergetic states allows a better understanding of the physiological roles of $O_2^{\cdot-}/H_2O_2$ as signaling molecules and may illuminate potential therapeutic target(s) for related pathologies.

In the following sections we discuss the contributions of different mitochondrial sites to physiological $O_2^{\cdot-}/H_2O_2$ production under different bioenergetic conditions. We also discuss the use of S1QELs and S3QELs, site-specific suppressors of mitochondrial $O_2^{\cdot-}/H_2O_2$ generation that have no effect on oxidative phosphorylation, as tools for such characterization *in situ* and *in vivo*.

Why native rates?

Understanding of the origins and rates of mitochondrial $O_2^{\cdot-}/H_2O_2$ production under physiological conditions is important for understanding normal cellular behavior and signaling, and it is an important prerequisite for investigating the roles of mitochondrial $O_2^{\cdot-}/H_2O_2$ production in pathology. Conventionally, the characterization of mitochondrial $O_2^{\cdot-}/H_2O_2$ production involves the use of specific combinations of conventional substrates and electron transport inhibitors to provide information about the kinetics and the capacities of particular sites of interest. However, the impairment of electron flow by these inhibitors and the subsequent redistribution of electrons to other redox centers lead to misinterpretation when studying the contributions of particular mitochondrial sites to physiological $O_2^{\cdot-}/H_2O_2$ production. Instead, the measurement of native rates of $O_2^{\cdot-}/H_2O_2$ production is required when evaluating the contribution of each site under different experimental conditions.

Quinlan *et al.* (36) introduced measurement of the redox states of endogenous reporters as an indirect way to estimate the rates of $O_2^{\cdot-}/H_2O_2$ production from mitochondrial sites. They identified unique relationships between the reduction

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[2] The abbreviations used are: $O_2^{\cdot-}$, superoxide; ROS, reactive oxygen species; ETF, electron-transferring flavoprotein.

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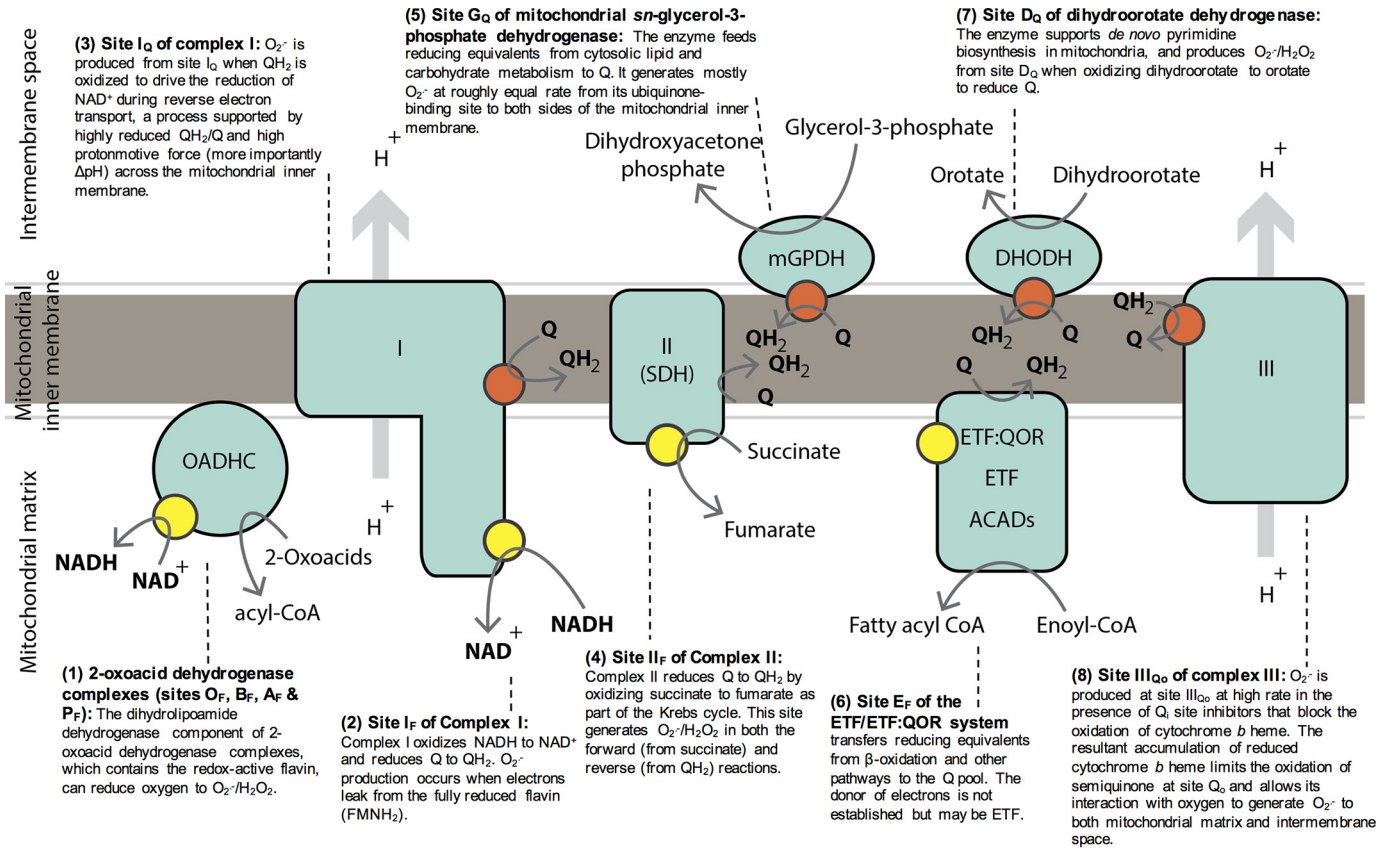

Figure 1. Mitochondrial sites of $O_2^{\cdot-}/H_2O_2$ production. Eleven distinct mitochondrial sites that leak electrons to oxygen to produce superoxide and/or hydrogen peroxide during substrate oxidation have been identified (21, 25–28, 30–33). *Yellow circles* indicate flavin groups, and *orange circles* indicate quinone-binding sites of enzymes and complexes. *ETF,* electron-transferring flavoprotein; *ETF:QOR*, electron-transferring flavoprotein-ubiquinone oxidoreductase; *QH_2,* ubiquinol; *Q*, ubiquinone. Sites O_F, B_F, A_F, P_F, I_F, I_Q, II_F, III_{Qo}, G_Q, E_F, and D_Q refer to the flavin (subscript F) or ubiquinone-binding sites (subscript Q) of the 2-oxoglutarate (O), branched chain 2-oxoacid (B), 2-oxoadipate (A), and pyruvate (P) dehydrogenase complexes, of complex I, complex II, and the outer ubiquinone site of complex III (III_{Qo}), and of mitochondrial glycerol-3-phosphate dehydrogenase (G), the ETF/ETF:QOR system (E), and dihydroorotate dehydrogenase (D).

state of the mitochondrial NAD pool and the rate of $O_2^{\cdot-}$ or H_2O_2 production at the flavin site of mitochondrial complex I (site I_F). The relationship between the reduction state of cytochrome b_{566} of mitochondrial complex III and the rate of $O_2^{\cdot-}$ production at the Q_o site of mitochondrial complex III (site III_{Qo}) was also elucidated. Calibration curves of measured H_2O_2 production arising from site I_F against the mitochondrial NAD(P)H redox state and measured H_2O_2 production arising from site III_{Qo} against the cytochrome b_{566} redox state allow the quantification of the contribution of sites I_F and III_{Qo} to total H_2O_2 production under various experimental conditions. These endogenous reporters also serve as a tool to correct for secondary changes in the redox state of the NAD pool and cytochrome b_{566} when specific inhibitors or omissions of particular substrates are used to shut down other sites, allowing the quantitative determination of $O_2^{\cdot-}/H_2O_2$ production rates from these other mitochondrial sites as well (36–38, 48).

Mitochondrial $O_2^{\cdot-}/H_2O_2$ production and substrate oxidation

Mitochondrial enzyme complexes oxidize a wide variety of substrates to support mitochondrial respiration. The origin of $O_2^{\cdot-}/H_2O_2$ production when mitochondria oxidize different substrates mostly depends on the redox levels at which the substrates donate their electrons. Quinlan *et al.* (38) demonstrated the following contributions of different mitochondrial sites to H_2O_2 production during oxidization of the following: (i) complex I-linked substrates, glutamate plus malate; (ii) the complex II-linked substrate, succinate; (iii) a substrate for fatty acid oxidation, palmitoylcarnitine plus carnitine; and (iv) the substrate for mitochondrial glycerol-3-phosphate dehydrogenase (an enzyme abundantly expressed in skeletal muscle), glycerol 3-phosphate (Fig. 2). The overall rates of mitochondrial H_2O_2 production differed when mitochondria were oxidizing different substrates as fuel molecules. The oxidation of succinate led to the highest rate of $O_2^{\cdot-}/H_2O_2$ production. Succinate is a classical substrate that can be used to study $O_2^{\cdot-}/H_2O_2$ production from complex I during reverse electron transport (site I_Q). Because of its high sensitivity to inhibitors of the quinone-binding site of complex I, the $O_2^{\cdot-}/H_2O_2$ production of site I_Q is defined as the rotenone-sensitive signal when mitochondria are oxidizing succinate. After correcting for changes in $O_2^{\cdot-}/H_2O_2$ production from site I_F and site III_{Qo} upon rotenone inhibition (using endogenous reporters), 83% of the overall rate of mitochondrial $O_2^{\cdot-}/H_2O_2$ production during succinate oxidation was attributed to site I_Q (38). The total rate of $O_2^{\cdot-}/H_2O_2$ production with glutamate plus malate as substrate was considerably lower, only 20–25% of the rate with succinate, showing the importance of the source of

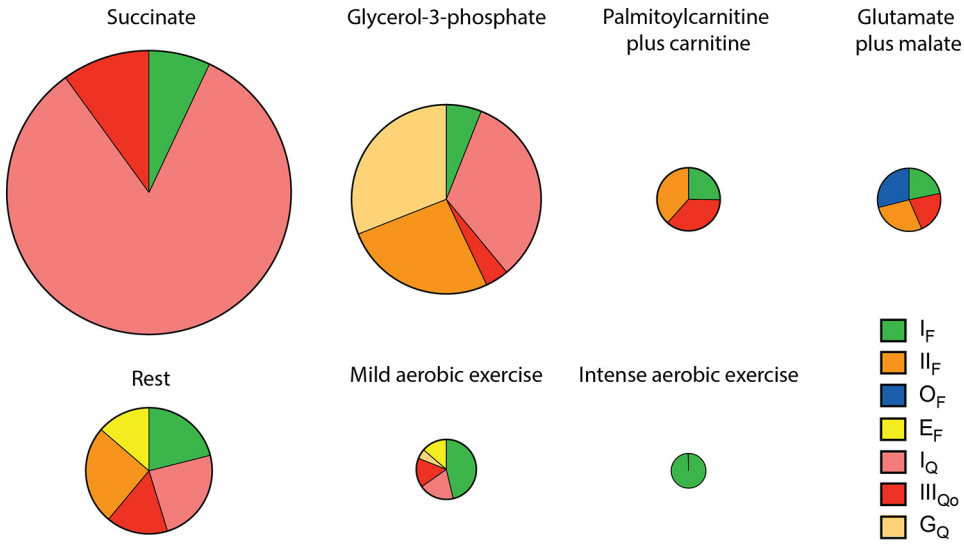

Figure 2. Relative contributions of specific sites of $O_2^{\cdot-}/H_2O_2$ production to total H_2O_2 production by isolated rat muscle mitochondria under different bioenergetic conditions. The diameters of the *pie charts* are proportional to the total rates of mitochondrial H_2O_2 production. Total rates of mitochondrial H_2O_2 production (pmol of H_2O_2 min^{-1} mg $protein^{-1}$) were ~890 (succinate), ~620 (glycerol 3-phosphate), ~200 (palmitoylcarnitine), ~180 (glutamate plus malate), ~340 (rest), ~80 (mild aerobic exercise), and ~50 (intense aerobic exercise). Data are from Refs. 38 and 48.

reducing equivalents in determining the rate of mitochondrial $O_2^{\cdot-}/H_2O_2$ production.

Importantly, the engagement of particular mitochondrial sites changes with different substrates (Fig. 2), due mostly to the different entry points of reducing equivalents during metabolism and the resulting different redox states of distinct sets of enzymes and complexes. Taking complete oxidation of fatty acids as an example, fatty acids, or more specifically palmitoylcarnitine, have three entry points into the electron transport chain as follows: complex I (via the reduction of NAD^+ by hydroxyacyl-CoA dehydrogenase and the dehydrogenases of the tricarboxylic acid cycle); electron-transferring flavoprotein (ETF-ubiquinone oxidoreductase via its reduction by ETF and mitochondrial acyl-CoA dehydrogenases); and to a minor extent complex II (via the formation of succinate formed during operation of the tricarboxylic acid cycle) (37). During oxidation of palmitoylcarnitine, site I_F, site II_F (the flavin site of mitochondrial complex II), and site III_{Qo} were the main contributors to mitochondrial $O_2^{\cdot-}/H_2O_2$ production under non-phosphorylating conditions; the rate of $O_2^{\cdot-}/H_2O_2$ production from the ETF system was negligible. The contribution of site II_F was caused by backflow of electrons from the ubiquinone pool into complex II rather than by forward flow from succinate formed in the tricarboxylic acid cycle. It is worth noting that sites I_F and III_{Qo} contributed to $O_2^{\cdot-}/H_2O_2$ production during oxidization of all tested substrates. This is because the redox states of the NAD and ubiquinone pools differ little between substrates; they are common entry points for most substrates to the electron transport chain, and their redox states determine the rates of $O_2^{\cdot-}/H_2O_2$ production from these two sites.

Mitochondrial substrate oxidation plays an important role in determining the origin and rates of $O_2^{\cdot-}/H_2O_2$ production. Changes in bioenergetic states, for example, by physical exercise or caloric restriction, influence mitochondrial substrate availability and lead to the resultant alterations in the rates and the contributions of different sites of mitochondrial $O_2^{\cdot-}/H_2O_2$ production.

Mitochondrial $O_2^{\cdot-}/H_2O_2$ production during physical exercise

As mentioned above, individual mitochondrial sites produce $O_2^{\cdot-}/H_2O_2$ at different rates depending on the substrate being oxidized (38, 48). *In vivo* and in whole cells, many substrates are metabolized simultaneously, and the availability of these substrates can change. In skeletal muscle, for example, the concentrations of respiratory substrates, effectors such as Ca^{2+} and intracellular pH, and energy demand (reflected in the concentrations of ADP and phosphate) change dramatically between states of rest, mild exercise, and exhaustive exercise (48).

Sites I_F and III_{Qo}, or more generally mitochondrial complexes I and III, are conventionally recognized as the major sites of mitochondrial production of $O_2^{\cdot-}/H_2O_2$. However, sites II_F and I_Q also have the potential to produce $O_2^{\cdot-}/H_2O_2$ at high rates in isolated mitochondria oxidizing low concentrations of succinate under non-phosphorylating conditions in which protonmotive force is high and rapid reoxidation of the ubiquinone pool is prevented. Such conditions seem relevant to resting muscles (30), where the rates of mitochondrial respiration and ATP synthesis are low (49). When skeletal muscle mitochondria were incubated with physiological concentrations of substrates and effectors under conditions mimicking rest *ex vivo*, sites II_F and I_Q were found to be major sites of mitochondrial $O_2^{\cdot-}/H_2O_2$ production, each contributing a quarter of the total rate of mitochondrial $O_2^{\cdot-}/H_2O_2$ production; the remainder was mostly from sites I_F and III_{Qo} for the reason discussed previously (Fig. 2) (48).

Exercise is associated with an increase in the generation of free radicals (50, 51). With the use of electron paramagnetic resonance spectroscopy, a rise in free radicals was seen in intact muscles during exercise, and mitochondria were proposed to be the major source (52). In contrast, an increasing body of evidence suggests a decreased production rate of these reactive species from mitochondria during muscular contractile activity (53). During exercise, the concentrations of ADP and mito-

chondrial substrates and effectors all increase, and the mitochondria respire rapidly to produce ATP. It is not obvious whether the increased substrate supply will raise mitochondrial $O_2^{\cdot-}/H_2O_2$ production or the decreased protonmotive force and reduction state of mitochondrial redox centers will lower it. When skeletal muscle mitochondria were incubated with physiological concentrations of substrates and effectors under conditions mimicking exercise *ex vivo*, the resultant decrease in protonmotive force and the more oxidized ubiquinone pool led to decreased rates of $O_2^{\cdot-}/H_2O_2$ production despite the increased substrate availability (48). This decrease was particularly marked for $O_2^{\cdot-}/H_2O_2$ production from site I_Q, which is sensitive to the pH gradient (ΔpH) across the mitochondrial inner membrane, and for site III_{Qo}, which responds to a decreased steady-state concentration of semiquinone in the electron transport chain (21, 26, 48, 54). Under *ex vivo* conditions that mimic mild aerobic exercise in skeletal muscles, the overall rate of mitochondrial $O_2^{\cdot-}/H_2O_2$ production was found to be ~50% lower than in the resting state, whereas the low-capacity site I_F remained as the major contributor. Sites I_Q, III_{Qo}, and probably E_F and G_Q also generated some $O_2^{\cdot-}/H_2O_2$ during mild exercise (48). With an increase in the intensity of aerobic exercise, the protonmotive force (and more importantly ΔpH) is lower and the ubiquinone pool is further oxidized, which leads to further decreases in $O_2^{\cdot-}/H_2O_2$ production from sites I_Q and III_{Qo}. As a consequence, site I_F contributed most of the overall $O_2^{\cdot-}/H_2O_2$ production under conditions mimicking intense exercise (Fig. 2) (48).

Despite the lack of *in vivo* evidence, the *ex vivo* findings demonstrate the heterogeneity of the sources of mitochondrial $O_2^{\cdot-}/H_2O_2$ in skeletal muscle during rest and mild and intense aerobic exercise. An overall decrease in $O_2^{\cdot-}/H_2O_2$ production in response to conditions mimicking increasing intensities of physical exercise was also observed in skeletal muscle mitochondria.

Mitochondrial $O_2^{\cdot-}/H_2O_2$ production during caloric restriction

In parallel to physical exercise, caloric restriction is considered to be a health-promoting intervention. Caloric restriction is defined as a reduction of food intake with adequate intake of protein and micronutrients to avoid malnutrition (55–57). Although caloric restriction was found to extend the life span of several animal species (58), its effect on mitochondrial $O_2^{\cdot-}/H_2O_2$ production is still controversial. Caloric restriction decreased the maximum capacity of $O_2^{\cdot-}/H_2O_2$ production when mitochondria isolated from brain, heart, kidney, liver, and skeletal muscle of rodents oxidized pyruvate and malate in the presence of rotenone (59–61). A similar trend in rotenone-sensitive H_2O_2 production from liver mitochondria energized with succinate was also observed in mice subjected to a 2-month course of caloric restriction (60). These findings suggest a decreased capacity for $O_2^{\cdot-}/H_2O_2$ production from site I_F and site I_Q of the mitochondrial electron transport chain as well as from the upstream dehydrogenases, particularly pyruvate dehydrogenase, after caloric restriction. However, none of these studies included the crucial measurement of native rates.

As reviewed by Walsh *et al.* (62), the beneficial effects of caloric restriction on cellular redox homeostasis are mainly mediated by a more reduced glutathione pool, rather than by decreased mitochondrial $O_2^{\cdot-}/H_2O_2$ production, with a resultant lower extent of oxidative damage to macromolecules (62). Interestingly, at the cellular and tissue level, the exposure of cells to low glucose could lead to a more oxidized mitochondrial NAD pool and a concomitant decrease in mitochondrial protonmotive force (63, 64). The oxidation of the NAD pool and the drop in mitochondrial protonmotive force (and ΔpH) may decrease rates of mitochondrial $O_2^{\cdot-}/H_2O_2$ production from site I_F, site I_Q, and upstream dehydrogenases under these conditions (21, 27, 32).

In contrast, overnutrition was suggested to be associated with a more reduced NAD pool, which may in turn lead to higher mitochondrial $O_2^{\cdot-}/H_2O_2$ production at site I_F of the electron transport chain and by upstream dehydrogenases (36, 64–67). Enhanced substrate supply may also increase protonmotive force (68), which may in turn favor the production of superoxide/H_2O_2 from site I_Q. Availability of nutrients can also impact mitochondrial dynamics. How such changes affect formation of mitochondrial $O_2^{\cdot-}/H_2O_2$ production remains to be investigated (69).

The contributions (under native conditions in mitochondria oxidizing different substrates and in conditions mimicking physical exercise) and capacities (after caloric restriction) of mitochondrial sites to $O_2^{\cdot-}/H_2O_2$ production in response to the changes in bioenergetic state have been mainly investigated in isolated mitochondria. Despite the effort to characterize the involvement of mitochondrial enzyme complexes in $O_2^{\cdot-}/H_2O_2$ production in cells and tissues (mostly by genetic or pharmacological modifications) under pathological conditions, the contributions of specific mitochondrial sites to physiological $O_2^{\cdot-}/H_2O_2$ production and their roles in cell signaling *in situ* and *in vivo* have not yet been elucidated.

Novel tools to characterize contributions of mitochondrial sites to $O_2^{\cdot-}/H_2O_2$ production *in vitro* and *in situ*

In experiments with isolated mitochondria, $O_2^{\cdot-}/H_2O_2$ are released and can be detected relatively easily in the surrounding assay medium. The measurement of mitochondrial $O_2^{\cdot-}/H_2O_2$ production, and more specifically $O_2^{\cdot-}/H_2O_2$ production from particular mitochondrial sites, is far more problematic in cells and tissues because of the ambiguous specificity and cellular localization of current probes as well as the impaired bioenergetics upon genetic modifications (70–72). A novel way around the problem of direct measurements in cells is to use molecules that suppress electron leak at specific sites. High-throughput chemical screening has identified molecules that specifically suppress $O_2^{\cdot-}/H_2O_2$ production from particular mitochondrial sites without influencing mitochondrial oxidative phosphorylation. S1QELs (Suppressors of site I_Q Electron Leak) are small molecules that suppress mitochondrial $O_2^{\cdot-}$ production from site I_Q during reverse electron transport (73). S3QELs (Suppressors of site III_{Qo} Electron Leak) are small molecules that suppress mitochondrial $O_2^{\cdot-}$ production from site III_{Qo} (74). These compounds are very potent (effective at nanomolar concentrations) and inhibit phenotypes dependent on $O_2^{\cdot-}$ production from sites I_Q and III_{Qo} in several cell and tissue models (73, 74). The high potency, high specificity, and lack of inhibition of mitochondrial electron flow and oxidative phosphorylation make them

promising tools to study the engagement of these mitochondrial sites in physiological $O_2^{\cdot-}/H_2O_2$ production *in situ* and *in vivo*. A good example is the use of S1QELs to examine the involvement of mitochondrial $O_2^{\cdot-}$ production from site I_Q in primary astrocytes under an unstressed condition (74). In this study, the activity of succinate dehydrogenase, which has iron–sulfur centers that are susceptible to oxidative damage by $O_2^{\cdot-}$ in the mitochondrial matrix (75), was used as an indirect measure of mitochondrial $O_2^{\cdot-}$ production. S1QELs preserved succinate dehydrogenase activity in primary astrocytes cultured at 3% oxygen, showing the generation of $O_2^{\cdot-}$ from site I_Q at physiologically relevant rates in resting, unstimulated cells in the absence of overt oxidative stress. The finding was further supported by a parallel protective effect of S1QELs on cellular aconitase activity (74).

Summary

In summary, the rates and the relative contributions of different mitochondrial sites of $O_2^{\cdot-}/H_2O_2$ production change in response to changes in bioenergetic states, as a consequence of alterations of mitochondrial substrate availability and the reduction states of mitochondrial redox centers. Characterizing mitochondrial $O_2^{\cdot-}/H_2O_2$ production in isolated mitochondria *in vitro* and in cells and tissues *in situ* and *in vivo* may help us understand its roles in cell signaling under both physiological and pathological conditions.

References

1. Halliwell, B., and Gutteridge, J. M. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219,** 1–14
2. Fridovich, I. (1999) Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann. N.Y. Acad. Sci.* **893,** 13–18
3. Loschen, G., Flohé, L., and Chance, B. (1971) Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. *FEBS Lett.* **18,** 261–264
4. Boveris, A., and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134,** 707–716
5. Loschen, G., Azzi, A., Richter, C., and Flohé, L. (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Lett.* **42,** 68–72
6. Boveris, A., and Cadenas, E. (1975) Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett.* **54,** 311–314
7. Loschen, G., and Azzi, A. (1975) On the formation of hydrogen peroxide and oxygen radicals in heart mitochondria. *Recent Adv. Stud. Cardiac. Struct. Metab.* **7,** 3–12
8. Boveris, A., Cadenas, E., and Stoppani, A. O. (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem. J.* **156,** 435–444
9. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Arch. Biochem. Biophys.* **180,** 248–257
10. Ksenzenko, M., Konstantinov, A. A., Khomutov, G. B., Tikhonov, A. N., and Ruuge, E. K. (1983) Effect of electron transfer inhibitors on superoxide generation in the cytochrome bc_1 site of the mitochondrial respiratory chain. *FEBS Lett.* **155,** 19–24
11. Cadenas, E., and Boveris, A. (1980) Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem. J.* **188,** 31–37
12. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch. Biochem. Biophys.* **237,** 408–414
13. Dileepan, K. N., and Kennedy, J. (1985) Complete inhibition of dihydroorotate oxidation and superoxide production by 1,1,1-trifluoro-3-thenoylacetone in rat liver mitochondria. *Biochem. J.* **225,** 189–194
14. Nohl, H., and Jordan, W. (1986) The mitochondrial site of superoxide formation. *Biochem. Biophys. Res. Commun.* **138,** 533–539
15. Sekhar, B. S., Kurup, C. K., and Ramasarma, T. (1987) Generation of hydrogen peroxide by brown adipose tissue mitochondria. *J. Bioenerg. Biomembr.* **19,** 397–407
16. Hansford, R. G., Hogue, B. A., and Mildaziene, V. (1997) Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *J. Bioenerg. Biomembr.* **29,** 89–95
17. St-Pierre, J., Buckingham, J. A., Roebuck, S. J., and Brand, M. D. (2002) Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* **277,** 44784–44790
18. Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552,** 335–344
19. Cadenas, E. (2004) Mitochondrial free radical production and cell signaling. *Mol. Aspects Med.* **25,** 17–26
20. Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L., and Parker, N. (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic. Biol. Med.* **37,** 755–767
21. Lambert, A. J., and Brand, M. D. (2004) Superoxide production by NADH: ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.* **382,** 511–517
22. Andreyev, A. Y., Kushnareva, Y. E., and Starkov, A. A. (2005) Mitochondrial metabolism of reactive oxygen species. *Biochemistry* **70,** 200–214
23. Kowaltowski, A. J., de Souza-Pinto, N. C., Castilho, R. F., and Vercesi, A. E. (2009) Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.* **47,** 333–343
24. Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem. J.* **417,** 1–13
25. Brand, M. D. (2010) The sites and topology of mitochondrial superoxide production. *Exp. Gerontol.* **45,** 466–472
26. Quinlan, C. L., Gerencser, A. A., Treberg, J. R., and Brand, M. D. (2011) The mechanism of superoxide production by the antimycin-inhibited mitochondrial Q-cycle. *J. Biol. Chem.* **286,** 31361–31372
27. Treberg, J. R., Quinlan, C. L., and Brand, M. D. (2011) Evidence for two sites of superoxide production by mitochondrial NADH-ubiquinone oxidoreductase (complex I). *J. Biol. Chem.* **286,** 27103–27110
28. Orr, A. L., Quinlan, C. L., Perevoshchikova, I. V., and Brand, M. D. (2012) A refined analysis of superoxide production by mitochondrial *sn*-glycerol 3-phosphate dehydrogenase. *J. Biol. Chem.* **287,** 42921–42935
29. Dröse, S., and Brandt, U. (2012) in *Mitochondrial Oxidative Phosphorylation* (Kadenbach, B., ed) pp. 145–169, Springer, New York
30. Quinlan, C. L., Orr, A. L., Perevoshchikova, I. V., Treberg, J. R., Ackrell, B. A., and Brand, M. D. (2012) Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions. *J. Biol. Chem.* **287,** 27255–27264
31. Hey-Mogensen, M., Goncalves, R. L., Orr, A. L., and Brand, M. D. (2014) Production of superoxide/H_2O_2 by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria. *Free Radic. Biol. Med.* **72,** 149–155
32. Quinlan, C. L., Goncalves, R. L., Hey-Mogensen, M., Yadava, N., Bunik, V. I., and Brand, M. D. (2014) The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I. *J. Biol. Chem.* **289,** 8312–8325
33. Brand, M. D. (2016) Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic. Biol. Med.* **100,** 14–31
34. Votyakova, T. V., and Reynolds, I. J. (2001) $\Delta\Psi_m$-dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J. Neurochem.* **79,** 266–277
35. Tahara, E. B., Navarete, F. D., and Kowaltowski, A. J. (2009) Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. *Free Radic. Biol. Med.* **46,** 1283–1297
36. Quinlan, C. L., Treberg, J. R., Perevoshchikova, I. V., Orr, A. L., and Brand, M. D. (2012) Native rates of superoxide production from multiple sites in isolated mitochondria measured using endogenous reporters. *Free Radic. Biol. Med.* **53,** 1807–1817
37. Perevoshchikova, I. V., Quinlan, C. L., Orr, A. L., Gerencser, A. A., and Brand, M. D. (2013) Sites of superoxide and hydrogen peroxide produc-

tion during fatty acid oxidation in rat skeletal muscle mitochondria. *Free Radic. Biol. Med.* **61,** 298–309
38. Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L., and Brand, M. D. (2013) Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biol.* **1,** 304–312
39. Lenaz, G. (2001) The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. *IUBMB Life* **52,** 159–164
40. Squier, T. C. (2001) Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol.* **36,** 1539–1550
41. Bergamini, C. M., Gambetti, S., Dondi, A., and Cervellati, C. (2004) Oxygen, reactive oxygen species and tissue damage. *Curr. Pharm. Des.* **10,** 1611–1626
42. Le Bras, M., Clément, M.-V., Pervaiz, S., and Brenner, C. (2005) Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histol. Histopathol.* **20,** 205–219
43. Starkov, A. A. (2008) The role of mitochondria in reactive oxygen species metabolism and signaling. *Ann. N.Y. Acad. Sci.* **1147,** 37–52
44. Hamanaka, R. B., and Chandel, N. S. (2010) Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem. Sci.* **35,** 505–513
45. Sena, L. A., and Chandel, N. S. (2012) Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* **48,** 158–167
46. Pi, J., Bai, Y., Zhang, Q., Wong, V., Floering, L. M., Daniel, K., Reece, J. M., Deeney, J. T., Andersen, M. E., Corkey, B. E., and Collins, S. (2007) Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes* **56,** 1783–1791
47. Dodson, M., Darley-Usmar, V., and Zhang, J. (2013) Cellular metabolic and autophagic pathways: traffic control by redox signaling. *Free Radic. Biol. Med.* **63,** 207–221
48. Goncalves, R. L., Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., and Brand, M. D. (2015) Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed *ex vivo* under conditions mimicking rest and exercise. *J. Biol. Chem.* **290,** 209–227
49. Tonkonogi, M., and Sahlin, K. (2002) Physical exercise and mitochondrial function in human skeletal muscle. *Exerc. Sport Sci. Rev.* **30,** 129–137
50. Kanter, M. (1998) Free radicals, exercise and antioxidant supplementation. *Proc. Nutr. Soc.* **57,** 9–13
51. Davies, K. J., Quintanilha, A. T., Brooks, G. A., and Packer, L. (1982) Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* **107,** 1198–1205
52. Jackson, M. J., Edwards, R. H., and Symons, M. C. (1985) Electron spin resonance studies of intact mammalian skeletal muscle. *Biochim. Biophys. Acta* **847,** 185–190
53. Pearson, T., Kabayo, T., Ng, R., Chamberlain, J., McArdle, A., and Jackson, M. J. (2014) Skeletal muscle contractions induce acute changes in cytosolic superoxide, but slower responses in mitochondrial superoxide and cellular hydrogen peroxide. *PLoS ONE* **9,** e96378
54. Nicholls, D. G. (2004) Mitochondrial membrane potential and aging. *Aging Cell* **3,** 35–40
55. Hursting, S. D., Lavigne, J. A., Berrigan, D., Perkins, S. N., and Barrett, J. C. (2003) Calorie restriction, aging, and cancer prevention: mechanisms of action and applicability to humans. *Annu. Rev. Med.* **54,** 131–152
56. Trepanowski, J. F., Canale, R. E., Marshall, K. E., Kabir, M. M., and Bloomer, R. J. (2011) Impact of caloric and dietary restriction regimens on markers of health and longevity in humans and animals: a summary of available findings. *Nutr. J.* **10,** 10.1186/1475–2891-10–107
57. Rizza, W., Veronese, N., and Fontana, L. (2014) What are the roles of calorie restriction and diet quality in promoting healthy longevity? *Ageing Res. Rev.* **13,** 38–45
58. Weiss, E. P., and Fontana, L. (2011) Caloric restriction: powerful protection for the aging heart and vasculature. *Am. J. Physiol. Heart Circ. Physiol.* **301,** H1205–H1219
59. Gredilla, R., Sanz, A., Lopez-Torres, M., and Barja, G. (2001) Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. *FASEB J.* **15,** 1589–1591
60. Hagopian, K., Chen, Y., Simmons Domer, K., Soo Hoo, R., Bentley, T., McDonald, R. B., and Ramsey, J. J. (2011) Caloric restriction influences hydrogen peroxide generation in mitochondrial sub-populations from mouse liver. *J. Bioenerg. Biomembr.* **43,** 227–236
61. Lambert, A. J., and Merry, B. J. (2004) Effect of caloric restriction on mitochondrial reactive oxygen species production and bioenergetics: reversal by insulin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286,** R71–R79
62. Walsh, M. E., Shi, Y., and Van Remmen, H. (2014) The effects of dietary restriction on oxidative stress in rodents. *Free Radic. Biol. Med.* **66,** 88–99
63. Sinclair, D. A. (2005) Toward a unified theory of caloric restriction and longevity regulation. *Mech. Ageing Dev.* **126,** 987–1002
64. Fulco, M., Cen, Y., Zhao, P., Hoffman, E. P., McBurney, M. W., Sauve, A. A., and Sartorelli, V. (2008) Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev. Cell* **14,** 661–673
65. Price, N. L., Gomes, A. P., Ling, A. J., Duarte, F. V., Martin-Montalvo, A., North, B. J., Agarwal, B., Ye, L., Ramadori, G., Teodoro, J. S., Hubbard, B. P., Varela, A. T., Davis, J. G., Varamini, B., Hafner, A., *et al.* (2012) SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab.* **15,** 675–690
66. Teodoro, J. S., Gomes, A. P., Varela, A. T., Duarte, F. V., Rolo, A. P., and Palmeira, C. M. (2013) Uncovering the beginning of diabetes: the cellular redox status and oxidative stress as starting players in hyperglycemic damage. *Mol. Cell. Biochem.* **376,** 103–110
67. Kim, H.-J., Kim, J. H., Noh, S., Hur, H. J., Sung, M. J., Hwang, J.-T., Park, J. H., Yang, H. J., Kim, M.-S., Kwon, D. Y., and Yoon, S. H. (2011) Metabolomic analysis of livers and serum from high-fat diet induced obese mice. *J. Proteome Res.* **10,** 722–731
68. Görlach, A., Dimova, E. Y., Petry, A., Martínez-Ruiz, A., Hernansanz-Agustín, P., Rolo, A. P., Palmeira, C. M., and Kietzmann, T. (2015) Reactive oxygen species, nutrition, hypoxia and diseases: Problems solved? *Redox Biol.* **6,** 372–385
69. Liesa, M., and Shirihai, O. S. (2013) Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab.* **17,** 491–506
70. Bartosz, G. (2006) Use of spectroscopic probes for detection of reactive oxygen species. *Clin. Chim. Acta* **368,** 53–76
71. Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O_2 sensing. *J. Biol. Chem.* **275,** 25130–25138
72. Lefort, N., Glancy, B., Bowen, B., Willis, W. T., Bailowitz, Z., De Filippis, E. A., Brophy, C., Meyer, C., Højlund, K., Yi, Z., and Mandarino, L. J. (2010) Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1b protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. *Diabetes* **59,** 2444–2452
73. Orr, A. L., Vargas, L., Turk, C. N., Baaten, J. E., Matzen, J. T., Dardov, V. J., Attle, S. J., Li, J., Quackenbush, D. C., Goncalves, R. L., Perevoshchikova, I. V., Petrassi, H. M., Meeusen, S. L., Ainscow, E. K., and Brand, M. D. (2015) Suppressors of superoxide production from mitochondrial complex III. *Nat. Chem. Biol.* **11,** 834–836
74. Brand, M. D., Goncalves, R. L., Orr, A. L., Vargas, L., Gerencser, A. A., Borch Jensen, M., Wang, Y. T., Melov, S., Turk, C. N., Matzen, J. T., Dardov, V. J., Petrassi, H. M., Meeusen, S. L., Perevoshchikova, I. V., Jasper, H., *et al.* (2016) Suppressors of superoxide-H_2O_2 production at site I_Q of mitochondrial complex I protect against stem cell hyperplasia and ischemia-reperfusion injury. *Cell Metab.* **24,** 582–592
75. Powell, C. S., and Jackson, R. M. (2003) Mitochondrial complex I, aconitase, and succinate dehydrogenase during hypoxia-reoxygenation: modulation of enzyme activities by MnSOD. *Am. J. Physiol. Lung Cell Mol. Physiol.* **285,** L189–L198