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## The role of mitochondrial function and cellular bioenergetics in ageing and disease

M.D. Brand, A.L. Orr, I.V. Perevoshchikova, and C.L. Quinlan

The Buck Institute for Research on Aging, 8001 Redwood Blvd., Novato, CA 94945, U.S.A

### Summary

Mitochondria constitute an important topic of biomedical enquiry (one paper in every 154 indexed in PubMed since 1998 is retrieved by the keyword ‘mitochondria’) because of widespread recognition of their importance in cell physiology and pathology. Mitochondrial dysfunction is widely implicated in ageing and in the diseases of ageing, through dysfunction in adenosine triphosphate (ATP) synthesis, Ca<sup>2+</sup> homeostasis, central metabolic pathways or radical production. Nonetheless, the mechanisms and regulation of superoxide and hydrogen peroxide formation by mitochondria remain poorly described. Measurement of the capacities of different sites of superoxide and hydrogen peroxide production in isolated skeletal muscle mitochondria show that the maximum capacities of sites in complexes I, II and III and in several associated redox enzymes greatly exceed the native rates observed in the absence of respiratory chain inhibitors. *In vitro*, the native rates and the relative importance of different sites both depend on the substrate being oxidized, with sites I<sub>Q</sub>, II<sub>F</sub>, GPDH, I<sub>F</sub> and III<sub>Q<sub>o</sub></sub> each being important with particular substrates. The techniques involved in measuring rates from each site should become applicable to cell cultures and *in vivo* in the future.

### The importance of mitochondria in cell physiology and medicine

There has been explosive growth in the biomedical literature over the past few decades. It has grown tenfold from 100 000 papers per year in the 1950s to the current one million per year (Fig. 1). Studies of mitochondria had a golden age in the 1970s, when the mechanism of energy conservation and adenosine triphosphate (ATP) synthesis by mitochondria was first being worked out and the chemiosmotic theory of oxidative phosphorylation was being established and tested, culminating in the award of the Nobel Prize for Chemistry to Peter Mitchell in 1978. In 1973, at the peak of this revolution, 3229 papers on ‘mitochondria’ were published, comprising 1.4% of the biomedical literature that year, according to PubMed. You might expect that this intense focus on mitochondrial bioenergetics would have been the high water mark of the study of mitochondria, but since 1998 there has been a remarkable resurgence of activity and publications on mitochondria, as their roles in cell physiology, apoptosis, radical production, ageing and disease have become hot topics of

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Correspondence: Martin D. Brand., mbrand@buckinstitute.org.

#### Conflicts of interest

None declared.

research. So much so that there were nearly twice as many papers on mitochondria published in 2011 (5921) as there were in 1973. Indeed, the explosive growth of biomedical research has been matched and partly driven by papers on mitochondria: over the last 15 years an average of one paper in every 154 published in biomedicine was associated with 'mitochondria', that's one every 20 min of the working day, each working day, every year.

Of course, nobody has the time to read and digest all of these papers. Nevertheless, we can ask what topics the majority of them cover. A relatively simple way to do this is to see what other terms are linked to 'mitochondria' in PubMed. Table 1 shows that over the last 5 years, nearly all mitochondrial papers are also picked up by the keywords 'cell', 'function', 'physiology' and 'metabolism', indicating that their role in the general area of cell physiology is the main driver of the current huge interest in mitochondria. High on the list are terms showing that there is great interest in the relationship between mitochondria and 'pathology' and 'medicine', with 'human', 'drug', 'treatment', 'disease', 'therapeutic', 'clinical', 'injury', 'target' and 'pediatric' featuring very prominently. The role of mitochondria in programmed cell death is highlighted by the linkage to 'death', 'apoptosis', 'caspase' and 'survival', while the importance of shape and movement is picked out by the terms 'morphology' and 'translocation'. Their roles in 'signaling' and in 'growth' and 'development' are also high on the list. Continued interest in the basic mechanisms of mitochondrial biochemistry is suggested by 'enzyme', 'membrane', 'bioenergetics', 'electron', 'oxidoreductase', 'membrane potential', 'cytochrome', 'energy', 'ATP', 'structure', 'calcium' and so on, although many of these links may also indicate how these mechanisms play out in cell physiology and disease. The relationship of mitochondria to reactive oxygen species (ROS) production and damage is very strong and marked by terms including 'oxidative stress', 'reactive', 'antioxidant', 'redox', 'ROS', 'superoxide', 'peroxide', 'glutathione', 'disorder', 'dysfunction', 'DNA', 'mutation', 'damage' and 'toxicity'. Significantly, many specific diseases are linked to mitochondria, including 'cancer', 'diabetes', 'ischemia', 'reperfusion' (injury), 'Parkinson's', 'neurodegeneration', 'infection', 'obesity', 'dementia', 'trauma', 'Alzheimer', 'immunity', 'sepsis', 'epileptic' and 'Huntington'. The links to ageing are marked by 'ageing', 'elderly' and 'aged'; links to life-style are marked by 'alcohol', 'exercise' and 'training'. The species of interest include 'human', 'mouse', 'rat', 'bovine', 'fish', 'drosophila' and 'parasite', while the tissues and organs of interest include 'muscle', 'cerebral', 'brain', 'heart', 'liver', '*in vivo*', 'neuron', 'cardiovascular', 'fibroblast', 'nerve', 'lung', 'kidney', 'stem cell', 'cortex', 'hippocampus', 'embryo', 'striatum' and 'skin'. Overall, Table 1 gives a compelling snapshot of active current research that is considering roles of mitochondria in a wide range of physiological and pathological situations, as might be expected given their great functional importance in energy metabolism and other processes.

## Functions of mitochondria

The functions of mitochondria obviously include oxidative phosphorylation to produce cellular ATP, but they also have important roles in ion homeostasis, in several metabolic pathways, in apoptosis and programmed cell death, and in ROS production and consumption. All of these functions may be significant in ageing and/or disease. Damage may cause mitochondria to accumulate dysfunctional components. This damage may be

caused directly by radicals produced by the mitochondria themselves. It may be caused by sequence or regulatory errors following mutation of nuclear or mitochondrial DNA<sup>1,2</sup> as a result of a wide range of internal or environmental insults, such as exposure of the skin to ultraviolet radiation. These effects can be exacerbated by degradation of the quality control machinery that normally limits the build-up of dysfunctional mitochondria by targeting poorly performing constituents of the mitochondrial network for destruction.<sup>3,4</sup>

The classic role of mitochondria is oxidative phosphorylation, which generates ATP by utilizing the energy released during the oxidation of the food we eat. ATP is used in turn as the primary energy source for most biochemical and physiological processes, such as growth, movement and homeostasis. We turn over approximately our own body weight in ATP each day, and almost all of this is generated by mitochondria, primarily within muscle, brain, liver, heart and gastrointestinal tract.<sup>5</sup> The pre-eminent role of eating is to provide the fuel for mitochondria, and the pre-eminent role of breathing is to provide the oxygen and to remove the carbon dioxide produced during oxidative phosphorylation by mitochondria. Similarly, a major role of the cardiovascular system is to deliver the substrates (glucose, fatty acids, oxygen) and remove the products (carbon dioxide) of mitochondrial activity.

As a result of intensive study, particularly since the 1950s, the mechanism of oxidative phosphorylation is very well understood, both in general principle and detailed biochemistry. The general principle is chemiosmotic coupling (Fig. 2),<sup>6</sup> in which the oxidation of respiratory substrates by oxygen, catalysed by the mitochondrial electron transport chain, causes proton extrusion across the mitochondrial inner membrane. The proton-motive force set up by this proton pumping drives protons back into the mitochondrial matrix through the ATP synthase to generate ATP. The proton-motive force also drives the uptake of ADP and phosphate and the efflux of ATP to deliver the synthesized ATP to the cytosol where it is consumed. It is also crucial for uptake and efflux of Ca<sup>2+</sup>, and hence for ionic homeostasis in the cytosol and matrix and for Ca<sup>2+</sup>-related signalling pathways. The crystal structures of most of the electron transport chain complexes have been solved (Fig. 2) and the detailed mechanisms of the coupling of electron transport to proton pumping in complexes III and IV are well understood.<sup>7,8</sup> The mechanisms of proton pumping in complex I<sup>9</sup> and the ATP synthase<sup>10</sup> are known in outline but have yet to be worked out in molecular detail. In addition to ATP synthesis, the proton-motive force is coupled directly to uptake of substrates such as pyruvate, glutamate and ornithine and to export of products such as citrulline across the mitochondrial inner membrane,<sup>11</sup> to proton leak pathways through the adenine nucleotide translocase and specific uncoupling proteins that provide thermogenesis and regulation of radical production,<sup>6</sup> to calcium transporters that regulate matrix and cytosolic calcium concentrations,<sup>12</sup> and to the nicotinamide nucleotide transhydrogenase that maintains the reduction state of the matrix glutathione pool.<sup>13</sup>

Mitochondria have several critical roles in metabolism,<sup>14</sup> even in organisms that live anaerobically and do not use their mitochondria for ATP synthesis.<sup>15</sup> They are the central player in carbon metabolism. As well as their well-known catabolic role in oxidation of sugars (pyruvate), fats (palmitoylcarnitine) and proteins (glutamine, glutamate, alanine, and so on), they have a critical anabolic role, providing the carbon skeletons for the biosynthesis

of most biomolecules, particularly glucose, fatty acids and amino acids. They are a major player in 1-carbon metabolism. They are central in nitrogen metabolism, metabolizing the glutamate used in transamination reactions and the glutamine used to shuttle nitrogen around the body, as well as the site of half of the reactions of the urea cycle. They are also essential in the synthesis of haem and iron-sulphur clusters.

As reviewed extensively elsewhere,<sup>16–20</sup> mitochondria are central players in programmed cell death. They activate caspases in the cytosol through the release of cytochrome c and other factors from the intermembrane space when pro-apoptotic stimuli trigger Bcl-2 family members and the permeability transition pore.

Dysfunction in any of these pathways may contribute to the pathologies that develop with age and stress. In the following sections the mitochondrial sources of ROS that may contribute to such dysfunction will be examined.

## The mitochondrial free radical theory of ageing and disease

Mitochondria generate ROS during oxidative metabolism. In the mitochondrial free radical theory of ageing,<sup>21</sup> these ROS are the primary cause of damage to proteins, lipids and nucleic acids. Some damage is not repaired (perhaps because it is not repairable), causing failure of cellular machinery and leading to ageing-related diseases and to ageing itself. In the strictest version of the theory, the damage is self-reinforcing: damaged mitochondrial DNA codes for dysfunctional electron transport complexes that generate even more radicals than usual, leading to a vicious cycle of exponentially increasing damage and dysfunction. There is a substantial amount of evidence both supporting and against the theory.<sup>21–31</sup> The current consensus is best summarized by the view that radicals generated by mitochondria can be an important contributor to ageing, and are particularly important in age-related diseases, including Alzheimer disease,<sup>32,33</sup> cardiomyopathy<sup>34–36</sup> and cancer.<sup>37,38</sup> However, mitochondrial radical production is not the sole cause of ageing, and may be most prominent only in particular model organisms and conditions of husbandry. Mitochondrial radical production may contribute to many of the symptoms of ageing, such as frailty and loss of elasticity in skin.

To understand mitochondrial ROS generation and fully establish its true role in ageing and age-related diseases, it is necessary to identify, quantify and ultimately manipulate the specific mitochondrial electron transport chain sites that generate ROS within cells.

## The production of reactive oxygen species by mitochondria *in vitro*: maximum capacities of different sites

The generation of hydrogen peroxide by isolated mitochondria was first reported and characterized in the early 1970s.<sup>39,40</sup> Most of this hydrogen peroxide is produced initially as superoxide and is then converted to hydrogen peroxide by a very active superoxide dismutase in the mitochondrial matrix.<sup>41</sup> Subsequent work by many research groups<sup>42–46</sup> has identified a number of sites of superoxide and hydrogen peroxide production in the citric acid cycle and the electron transport chain of mammalian mitochondria (Table 2).

The maximum capacities of these sites under standard conditions in mitochondria isolated from skeletal muscle are illustrated in Figure 3. Site III<sub>Qo</sub> has the greatest capacity for superoxide/hydrogen peroxide production, followed by sites I<sub>Q</sub> and II<sub>F</sub>. Other sites have lower maximum capacities. Of course, these absolute maximum capacities and their relative importance will differ between tissues and species as the concentrations of the relevant redox centres differ. For example, the hydrogen peroxide-generating capacity of glycerol 3-phosphate dehydrogenase is much less than that of complex II in skeletal muscle mitochondria (Fig. 3), or in heart or brain mitochondria, but significantly exceeds the capacity of complex II in mitochondria from brown adipose tissue, where glycerol 3-phosphate dehydrogenase is much more highly expressed.<sup>47</sup>

Figure 3 also shows the native rates of hydrogen peroxide generated by skeletal muscle mitochondria during oxidation of the conventional respiratory substrates glutamate plus malate. Under resting conditions (state 4, with no ATP synthesis), the native rate is much lower than the maximum capacity of any of the individual major sites. Under active conditions (state 3, with ADP added to allow ATP synthesis), the native rate is even lower. Clearly, knowledge of the maximum capacities of different sites in the presence of respiratory inhibitors to allow full reduction of the site is important, but it is not sufficient to allow prediction of which sites are actually operating under native conditions in the absence of respiratory inhibitors, as they will normally be in cells or *in vivo*.

### The production of reactive oxygen species by mitochondria *in vitro*: rates from different sites under native conditions

The sites responsible for hydrogen peroxide production by mitochondria in cells or *in vivo* remain unknown<sup>44,45</sup> because inhibiting or genetically modifying a candidate site disrupts normal electron flow and alters the redox states of remaining sites, which can dramatically alter their rates of superoxide or hydrogen peroxide production. To help solve this problem, we have devised methods to evaluate the rates of superoxide and hydrogen peroxide production from different sites in isolated mitochondria. To do this, we use internally calibrated endogenous reporters of the redox states of the different sites [the redox state of endogenous NAD(P)H to report site I<sub>F</sub> and the redox state of cytochrome *b*<sub>566</sub> to report site III<sub>Qo</sub>] together with selective inhibition of specific subsidiary sites such as II<sub>F</sub>.<sup>48</sup>

Figure 4 shows the native rates of superoxide and hydrogen peroxide production from different sites during oxidation of glutamate plus malate under native conditions in state 4 in the absence of respiratory inhibitors, i.e. it dissects the small native rate shown in red in Figure 3 into its component sites.<sup>48</sup> Figure 4 also shows the same analysis during oxidation of other commonly used substrates: succinate, glycerol 3-phosphate and palmitoylcarnitine plus carnitine. There are two striking results. Firstly, the native rates differ greatly between substrates, suggesting that the rates of ROS production by mitochondria in cells and *in vivo* are likely to depend very strongly on what substrate is being oxidized. Secondly, the contributions of different sites are very different with different substrates. During succinate oxidation, the major site of superoxide production is site I<sub>Q</sub>, with small contributions from I<sub>F</sub> and III<sub>Qo</sub>. However, with glutamate plus malate as substrate, site I<sub>Q</sub> makes little contribution. With palmitoylcarnitine as substrate, site II<sub>F</sub> becomes a significant contributor,

and with glycerol 3-phosphate as substrate, five different sites all contribute, including glycerol 3-phosphate dehydrogenase. Thus, which sites contribute to ROS production in cells and *in vivo* is likely to depend very strongly on the substrates being oxidized.

It is important to note that the sites differ markedly in the proportion of the superoxide or hydrogen peroxide they produce to the matrix or to the intermembrane space,<sup>45,47,49,50</sup> with essentially all of the ROS from sites I<sub>F</sub>, I<sub>Q</sub> and II<sub>F</sub> being directed to the matrix, but about half that from sites III<sub>Q<sub>o</sub></sub> and GPDH being directed to the intermembrane space. Thus the amount of damage in the matrix compartment and the strength of cytosolic ROS signalling will be different with different substrates, even at identical overall rates of mitochondrial ROS production.

## The production of reactive oxygen species by mitochondria in cells and *in vivo*

Extension of the principles used above to measure the native rates of ROS production by different sites in isolated mitochondria may also prove useful in intact cells, whole tissues and *in vivo*. The reduction states of mitochondrial NAD and ETF can be measured in cells.<sup>51–53</sup> Measurement of the redox state of cytochrome *b*<sub>566</sub> in cells or *in vivo* is more difficult, but has been reported.<sup>54</sup> The ability to make such measurements suggests that assessment of the rates of ROS production from specific mitochondrial sites in cells and *in vivo* using endogenous reporters may ultimately be feasible. This would provide a method to quantify ROS production *in situ* from different mitochondrial sites in health, ageing and disease, and allow the efficacy of treatments designed to suppress oxidative stress originating at the mitochondria to be assessed.

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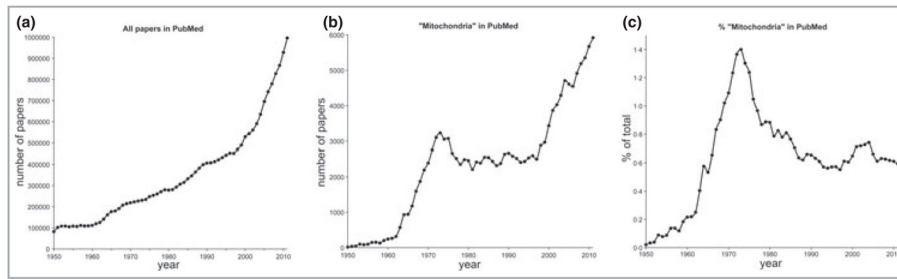
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**What's already known about this topic?**

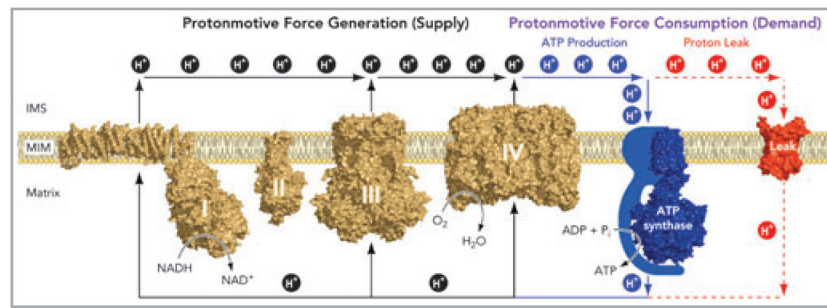
- Mitochondrial dysfunction is widely implicated in ageing and its diseases.
- Mitochondria are well understood; one paper in every 154 indexed in PubMed since 1998 has studied mitochondrial function.
- Nonetheless, the mechanisms and regulation of mitochondrial reactive oxygen species (ROS) formation remain poorly described.

**What does this study add?**

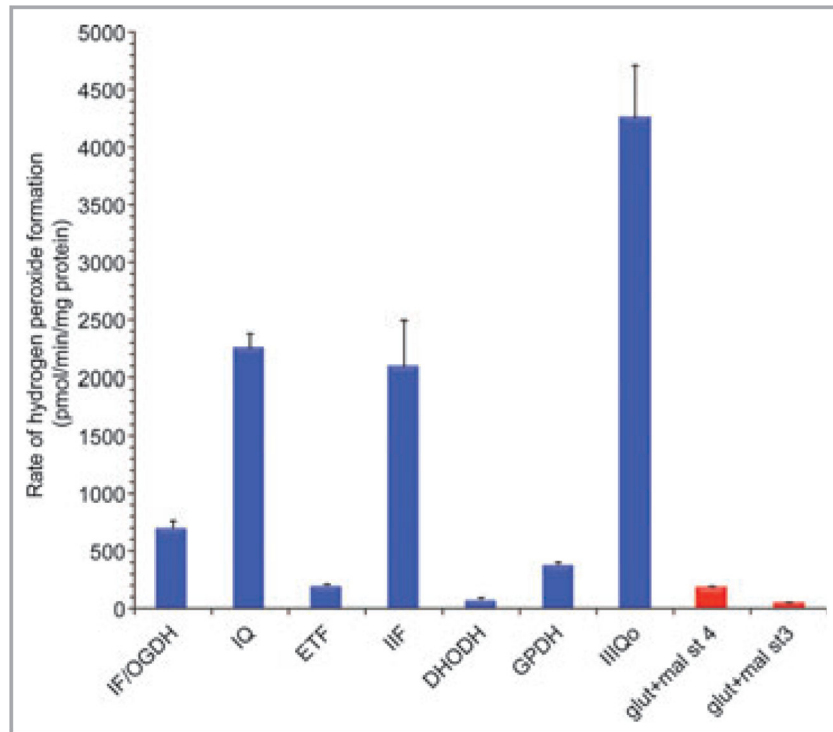
- The capacities and *in vitro* rates of different sites of mitochondrial ROS production are being established.
- The techniques involved are applicable to isolated cells.



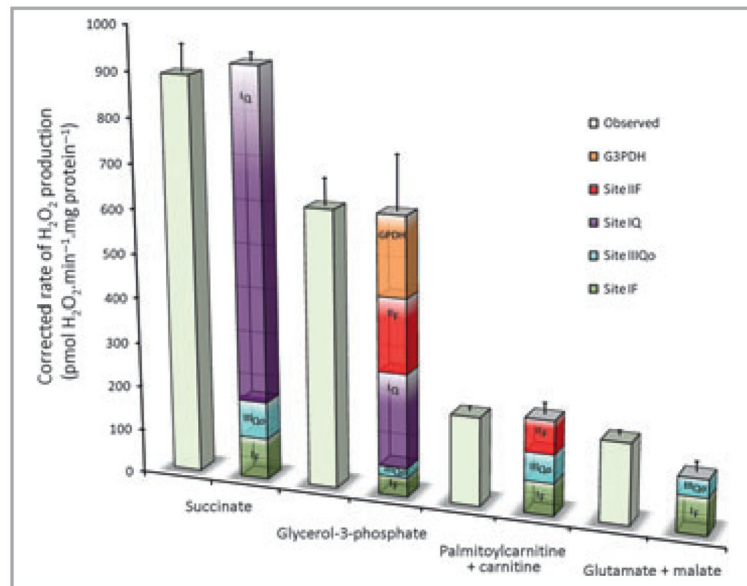
**Fig 1.** Papers in biomedicine and papers on 'mitochondria' 1950–2011. (a) All papers in PubMed each year. (b) Papers in PubMed each year retrieved using the keyword 'mitochondria'. (c) Mitochondrial papers as a percentage of all papers in PubMed each year. Search date October 2012.



**Fig 2.** Chemiosmotic coupling of oxidative phosphorylation in mitochondria. Electrons harvested from oxidizable substrates are passed through the respiratory chain in an exergonic process that drives proton pumping by respiratory complexes I, III and IV. The resulting electrochemical proton gradient across the mitochondrial inner membrane can be dissipated in two ways: (i) through the  $F_0F_1$ -ATP synthase, where relieving the proton-motive force drives ADP phosphorylation, and (ii) via proton leak pathways that do not generate ATP, but regulate physiological processes including nonshivering thermogenesis and perhaps glucose-stimulated insulin secretion and protection from oxidative damage. Proton leak pathways are structurally represented by ANT, which can mediate both basal and inducible proton conductance. The structures depicted are: complex I from *Thermus thermophilus* (PDB ID: 3M9S); complex II from porcine heart (PDB ID: 1Z0Y); dimeric complex III from bovine heart (PDB ID: 1BGY); dimeric complex IV from bovine heart (PDB ID: 2OCC); F1c10 ATP synthase complex from *Saccharomyces cerevisiae* (PDB ID: 2XOK) and carboxyatractyloside-inhibited ANT from bovine heart (PDB ID: 1OKC). Reproduced from Divakaruni and Brand.<sup>6</sup> ADP, adenosine diphosphate; ANT, adenine nucleotide translocase; ATP, adenosine triphosphate.



**Fig 3.** Hydrogen peroxide generation at different sites in isolated muscle mitochondria. Maximum capacities of sites defined using different combinations of substrates and inhibitors are in blue, actual overall rates during oxidation of glutamate plus malate in the absence of respiratory inhibitors are in red. All rates were either measured in the presence of 1-chloro-2,4-dinitrobenzene to greatly attenuate losses of hydrogen peroxide in the matrix by endogenous glutathione-linked peroxidases,<sup>65</sup> or corrected to such measurements using the equations in Quinlan *et al.*<sup>48</sup> and Treberg *et al.*<sup>65</sup> St 4, state 4 (no ATP synthesis); st 3, state 3 (maximum ATP synthesis); OGDH, 2-oxoglutarate dehydrogenase; ETF, electron-transferring flavoprotein and ETF:Q oxidoreductase; DHODH, dihydroorotate dehydrogenase; GPDH, glycerol 3-phosphate dehydrogenase; glut + mal, glutamate plus malate; IF, IQ, IIF, IIIQo, see Table 2. Data are from unpublished observations and references 47, 48 and 59. Values are means  $\pm$  SEM ( $n = 3$ ).



**Fig 4.** The contributions of different sites to native rates of hydrogen peroxide production in mitochondria isolated from muscle during oxidation of different substrates in state 4. All rates were either measured in the presence of 1-chloro-2,4-dinitrobenzene to greatly attenuate losses of hydrogen peroxide in the matrix by endogenous glutathione-linked peroxidases,<sup>65</sup> or corrected to such measurements using the equations in Quinlan *et al.*<sup>48</sup> and Treberg *et al.*<sup>65</sup> Open bars, observed total rates; coloured stacks of bars, calculated contributions of different sites to the observed rates, as indicated. Values are means  $\pm$  SEM ( $n = 3$ ). Data are from unpublished observations and references 47 and 48.



**Table 1**

Numbers of papers in the last 5 years associated with both 'mitochondria' and the terms listed (chosen using background knowledge; queried in PubMed in October 2012)

Mitochondria	28 275
Cell	26 032
Function	25 407
Physiology	25 568
Metabolism	23 064
Human	14 581
Enzyme	13 703
Morphology	11 428
Genetics	10 635
Drug	10 635
Membrane	9735
Treatment	8827
Bioenergetics	8153
Apoptosis	7875
Oxygen	7195
Oxidative	7050
Pathology	6880
Stress	6320
Mouse	5991
Medicine	5835
DNA	5707
Death	5682
Reactive	5578
Disorder	5556
Dysfunction	5514
Electron	5417
Disease	5303
Signaling	5238
Oxidative stress	4889
Cancer	4856
Therapeutic	4851
Oxidoreductase	4845
Membrane potential	4597
Cytochrome	4580
Translocation	4505
Growth	4340
Lipid	4301
Antioxidant	4228
Caspase	3982

Development	3895
Energy	3855
Muscle	3800
Damage	3708
Inhibition	3635
Survival	3606
ATP	3567
Redox	3453
RNA	3446
Phosphorylation	3285
Cerebral	3198
ROS	3180
Rat	3116
Brain	3102
Heart	2853
Liver	2851
Toxicity	2833
Oxidation	2768
Mutation	2658
Structure	2630
<i>In vivo</i>	2621
Calcium	2545
Neuron	2491
Cardiovascular	2463
Respiration	2436
Injury	2423
Clinical	2307
Target	2085
Ageing	2069
Superoxide	2019
Resistance	1884
Homeostasis	1849
Permeability	1841
Peroxide	1678
Deficiency	1627
Protective	1543
Phenotype	1438
Elderly	1406
Glutathione	1397
Diabetes	1365
Aged	1357
NADH	1333
Chronic	1330

Ischemia	1319
Evolution	1286
Biogenesis	1269
Acute	1240
Fibroblast	1202
Autophagy	1115
Reperfusion	1047
Coenzyme	1141
ATPase	1127
Pore	1117
Hypoxia	1076
Uncoupling	984
Radical	958
Parkinson's	919
Transgenic	916
Alcohol	880
Neurodegeneration	868
Nerve	863
Proton	833
Steroid	804
Lung	792
Movement	784
Infection	779
Kidney	771
Neuroprotective	717
Stem cell	700
Fusion	679
Cortex	654
Embryo	654
Cytoskeleton	629
Exercise	616
Obesity	602
Import	594
Efficiency	590
Catalase	569
Bovine	541
Dementia	549
Training	501
Fish	498
Ubiquinone	492
Hippocampus	488
Defective	487
Trauma	467

Pediatric	448
Drosophila	418
Parasite	377
Alzheimer	372
Estrogen	346
Preconditioning	342
Immunity	337
Striatum	230
Sepsis	164
Epileptic	150
Huntington	147
Skin	144

**Table 2**

Sites of superoxide and hydrogen peroxide production associated with oxidative phosphorylation in mitochondria

Site	References
Citric acid cycle	
Pyruvate dehydrogenase	55
2-oxoglutarate dehydrogenase	55–57
Dihydrolipoamide dehydrogenase	58
Ubiquinone reduction pathways	
Complex II flavin (site IIF)	59
Electron transferring flavoprotein/ETF:Q oxidoreductase	50
Glycerol 3-phosphate dehydrogenase	47,60
Dihydroorotate dehydrogenase	47
Electron transport chain	
Complex I flavin (site IF)	61
Complex I ubiquinone (site IQ)	61–63
Complex III outer ubiquinone binding site (site IIIQo)	40,64