The role of mitochondrial reactive oxygen species in insulin resistance

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Abstract

Insulin resistance is one of the earliest pathological features of a suite of diseases including type 2 diabetes collectively referred to as metabolic syndrome. There is a growing body of evidence from both pre-clinical studies and human cohorts indicating that reactive oxygen species, such as the superoxide radical anion and hydrogen peroxide are key players in the development of insulin resistance. Here we review the evidence linking mitochondrial reactive oxygen species generated within mitochondria with insulin resistance in adipose tissue and skeletal muscle, two major insulin sensitive tissues. We outline the relevant mitochondria-derived reactive species, how the mitochondrial redox state is regulated, and methodologies available to measure mitochondrial reactive oxygen species. Importantly, we highlight key experimental issues to be considered when studying the role of mitochondrial reactive oxygen species in insulin resistance. Evaluating the available literature on both mitochondrial reactive oxygen species/redox state and insulin resistance in a variety of biological systems, we conclude that the weight of evidence suggests a likely role for mitochondrial reactive oxygen species in the etiology of insulin resistance in adipose tissue and skeletal muscle. However, major limitations in the methods used to study reactive oxygen species in insulin resistance as well as the lack of data linking mitochondrial reactive oxygen species and cytosolic insulin signaling pathways are significant obstacles in proving the mechanistic link between these two processes. We provide a framework to guide future studies to provide stronger mechanistic information on the link between mitochondrial reactive oxygen species and insulin resistance as understanding the source, localization, nature, and quantity of mitochondrial reactive oxygen species, their targets and downstream signaling pathways may pave the way for important new therapeutic strategies.

Keywords

Insulin resistance, mitochondria, superoxide radical anion, hydrogen peroxide, coenzyme Q, redox signaling

Insulin-regulated glucose transport and insulin resistance

Insulin signals via its receptor in liver, muscle, and adipose tissues to lower circulating glucose by limiting hepatic glucose output and promoting glucose uptake and storage in muscle and fat. Appropriate and proportional insulin responses in these tissues are critical to glucose homeostasis and metabolic health [1-4]. Insulin resistance, a state where cells and tissues are less responsive to insulin, is a risk factor for the development of type 2 diabetes, other metabolic diseases such as cardiovascular disease and non-alcoholic fatty liver disease (NAFLD), and some cancers. Therefore, understanding the mechanisms of insulin resistance is likely key to finding new ways to mitigate type 2 diabetes and related metabolic disorders. Environmental stressors including a positive energy balance and obesity trigger insulin resistance. Yet, the precise mechanisms that link such stresses to insulin resistance in different tissues remain incompletely defined. Insulin resistance has been reported in a range of insulin-target tissues, including centrally (reviewed in [5]) and peripherally in liver (reviewed in [5, 6]), muscle and fat tissues (reviewed in [6, 7]) and in endothelial cells [8, 9]. There are other reviews that provide a perspective on different aspects of insulin resistance [6, 7, 10-13], but here we focus on the role of mitochondrial reactive oxygen species as key intracellular signals that cause insulin resistance in muscle and adipose tissue. We define insulin resistance here as impaired insulin-stimulated glucose transport into these tissues. Therefore, our review focusses on muscle and white adipose tissues (*e.g.*, visceral and subcutaneous fat), where these measures are more common. Throughout the review, we will draw on evidence from studies performed in humans, preclinical models, and cultured cells systems.

Insulin action in muscle and fat tissues

Insulin binding to its receptor on muscle and fat cells activates a signaling cascade that targets several cellular processes. Here we will focus on insulin-stimulated glucose uptake, as it plays a critical role in lowering blood glucose after a meal and majority of studies centered on reactive oxygen species in insulin resistance have focused on muscle/myocytes and/or adipose tissue/adipocytes. Muscle is the major site of post-prandial glucose disposal [14], and impaired insulin-stimulated glucose transport into muscle (muscle insulin resistance) is an early event in the progression to type 2 diabetes [14, 15]. Similarly, glucose uptake by adipose tissue is impaired in insulin resistance [16, 17]. Although fat tissue may contribute less to total glucose disposal than muscle, genetic models show that insulin-stimulated glucose uptake into fat tissue is critical to maintain whole body metabolic health [2].

Insulin increases glucose uptake into muscle and fat cells via redistribution of the facilitative glucose transport, GLUT4, to the plasma membrane (reviewed in [18]). Insulin binding to its receptor at the cell surface initiates a signaling cascade via the lipid kinase phosphatidylinositol-3-kinase (PI3K) and protein kinase Akt. Active Akt is sufficient for insulin-stimulated GLUT4 translocation [19]. While there are additional sites of insulin action, a key step in the mobilization of GLUT4 from intracellular membranes is Akt-mediated phosphorylation of the Rab-GAP protein TBC1D4, also known as AS160 [20]. Phosphorylation of TBC1D4 is thought to remove TBC1D4 from GLUT4-containing vesicles,

allowing Rab-mediated traffic and GLUT4 accumulation at the plasma membrane (reviewed in [18]). Delivery of GLUT4 to the plasma membrane is the rate-limiting step for glucose uptake into muscle and fat cells, and insulin-stimulated GLUT4 translocation is impaired in insulin resistant muscle and fat tissue [21, 22]. Although some studies have identified impaired insulin signaling in insulin resistance in these tissues, the point at which this insulin receptor-PI3K-Akt-TBC1D4-GLUT4 pathway is impaired in insulin resistance remains unknown (reviewed in [23]).

Mechanisms of insulin resistance in muscle and fat tissues

Insulin resistance in muscle and fat tissue is often coincident with obesity in humans [24], and diet interventions that induce a positive energy balance and increased fat mass in rodents are common experimental models for inducing and studying muscle and fat tissue insulin resistance [25]. Importantly, several preclinical studies in mice have shown that feeding diets high in fat for 3-5 days or diets high in sucrose for 14-21 days is sufficient to induce muscle and adipose tissue insulin resistance as measured by impaired glucose uptake into these tissues [26, 27]. Similarly, normal insulin sensitivity can be restored after 7-9 days return to a chow diet [28]. These data imply that the drivers of insulin resistance in these tissues are plastic and highly sensitive to the nutritional environment.

Although the molecular basis for impaired GLUT4 trafficking in insulin resistance in muscle and fat cells remains unclear, studies in cell models, rodents and humans have implicated an array of stressors that appear to 'translate' nutritional stress, or other systemic responses such as inflammation and hyperinsulinemia, to invoke insulin resistance. These include stress from specific lipids including DAGs (reviewed in [29]) and ceramides (reviewed in [10]), endoplasmic reticulum stress (reviewed in [30]), and mitochondrial dysfunction (reviewed in [13, 31, 32]) including altered mitochondrial biogenesis/degradation [33] and the balance of fusion/fission [34, 35]. It may be that these stressors are related and co-occur (proposed in [7, 23]), but studies to interrogate their inter-relationship in insulin resistance are lacking. Reactive oxygen species, particularly those in mitochondria, are also garnering considerable attention as causing insulin resistance in muscle and adipose tissue. The role of reactive oxygen species in causing insulin resistance in adipocytes was first proposed based on studies in the 3T3-L1 adipocyte cell line. These early studies reported that exogenous H_2O_2 treatment impaired insulin responses [36], and that small molecules with reducing or antioxidant activity such as *N*-acetylcysteine or MnTBAP (a cell-permeable mimetic of superoxide dismutase, SOD) or over-expression of Cu,Zn- and Mn-SOD, or peroxisome- or mitochondria-targeted catalase improved insulin sensitivity, albeit to varying degrees [37].

Notwithstanding the above, reactive oxygen species are not necessarily detrimental to insulin responses *per se* [38]. Indeed, reactive oxygen species play important role in signal transduction and maintenance of normal cell function. For example, NADPH oxidase (NOX), which along with DUOX and xanthine oxidase are considered major sources of superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), is reported to be required for maximal insulin signaling. H_2O_2 derived from NOX

inactivates phosphatases to activate insulin signaling, and blocking H_2O_2 production via NOX reduced insulin signaling ([39] and reviewed in [40]). The regulation of physiological signal transduction by reactive species relies on highly localized and compartmentalized redox process, often in response to transient environmental cues. For example, NOX isoforms are localized to specific cellular membrane domains facilitating H_2O_2 to act in spatially confined areas. Because of this, NOX activation can lead to an 'oxidizing environment' leading to plasma membrane localized phosphatase inhibition and insulin signaling. Once insulin levels and so NOX activity decrease, the redox environment at the plasma membrane is restored via the cellular redox buffering systems [40].

Despite their role in normal insulin signaling, a significant body of literature suggests that aberrant increases in cytosolic reactive oxygen species may promote metabolic disease (reviewed in [38, 41]). Changes in concentration of reactive species above or below a particular threshold may alter a localized redox environment to an extent that adversely affects signal transduction. Factors in addition to the concentration of reactive oxygen species may dictate their role as protective or harmful agents. As suggested by the results of Houstis and colleagues [37], the sub-cellular source and/or location of reactive oxygen species may play a key role in whether they confer insulin resistance. Indeed, several studies suggest that the concentrations of reactive oxygen species in mitochondria are altered in, and may cause, insulin resistance [42-45]. Indeed, mitochondria are a logical candidate for translating nutritional stress to the cell since they and are known signaling hubs for other processes such as the cell death pathways apoptosis [46] and ferroptosis [47].

In this review, we will examine this idea focusing on the concept of mitochondrial reactive species as redox signaling molecules [48] in insulin resistance rather than oxidative molecular damage as part of the most recent definition of 'oxidative stress' [49]. This is an important distinction as pathology is not always driven by direct oxidative damage or substantive changes to overall redox homeostasis and the initiation of an adaptive stress response. Rather, complex redox signaling pathways are initiated or re-routed by changes, potentially even small changes, in reactive species. For example, 'redox relays', *i.e.*, a specific protein-protein interaction followed by disulfide-thiol exchange reactions can provide for selectivity and spatial segregation of cell signaling, but do not necessarily result in substantive changes to the local or overall cellular thiol redox homeostasis given the large pool size of GSH and its link to energy production and the NADPH/$NADP^+$ redox couple.

To study their role in a biological process such as insulin resistance, it is important to understand which reactive oxygen species are produced in mitochondria, their chemical properties, and which antioxidant systems and regulatory pathways are in place that modulate their formation and metabolism. To address this, we will begin this review by outlining the sources and nature of various mitochondrial reactive oxygen species and techniques to measure or modulate these, including advantages and caveats to each approach. Following this, we discuss the challenges associated with assaying reactive oxygen species specifically in mitochondria in the context of insulin resistance. We then discuss the growing evidence that reactive oxygen species generated in mitochondria play a causal role in muscle and fat

insulin resistance, including the emerging role of mitochondrial lipids in insulin resistance. Finally, we reflect upon how causal links between mitochondrial reactive oxygen species and insulin resistance could be established in future studies and discuss the requirement for future studies to clarify the role that reactive species at this site play in muscle and fat tissue insulin resistance. Identifying the source, localization, chemical nature, and quantity of mitochondrial reactive oxygen species, their targets, and downstream signaling pathways in the pathogenesis of insulin resistance could reveal important new therapeutic strategies to treat or prevent insulin resistance and metabolic disease.

Mitochondrial reactive oxygen species

Overview

It is the intricate balance of production and removal that underpins how reactive oxygen species aid in the maintenance of normal cell physiology and, conversely, drive disease. Importantly, reactive oxygen species in biological systems are not generated in isolation. Rather they are dynamic chemical entities, responding rapidly to their environment, with the potential to transform into new species and/or to diffuse in and out of mitochondria. While the inner and outer membrane provide a level of spatial 'isolation' from other cellular components, there is strong evidence that mitochondrial redox signaling underpins an array of cellular processes outside mitochondria. Many oxidant species are generated in biological systems (**Fig. 1**) and the different chemical properties of one- versus two-electron redox processes (discussed below) may potentially result in distinct and specific cellular signaling. In the following section, we will discuss the main mitochondrial oxidants produced in mammalian cells and outline the processes that govern mitochondrial redox state.

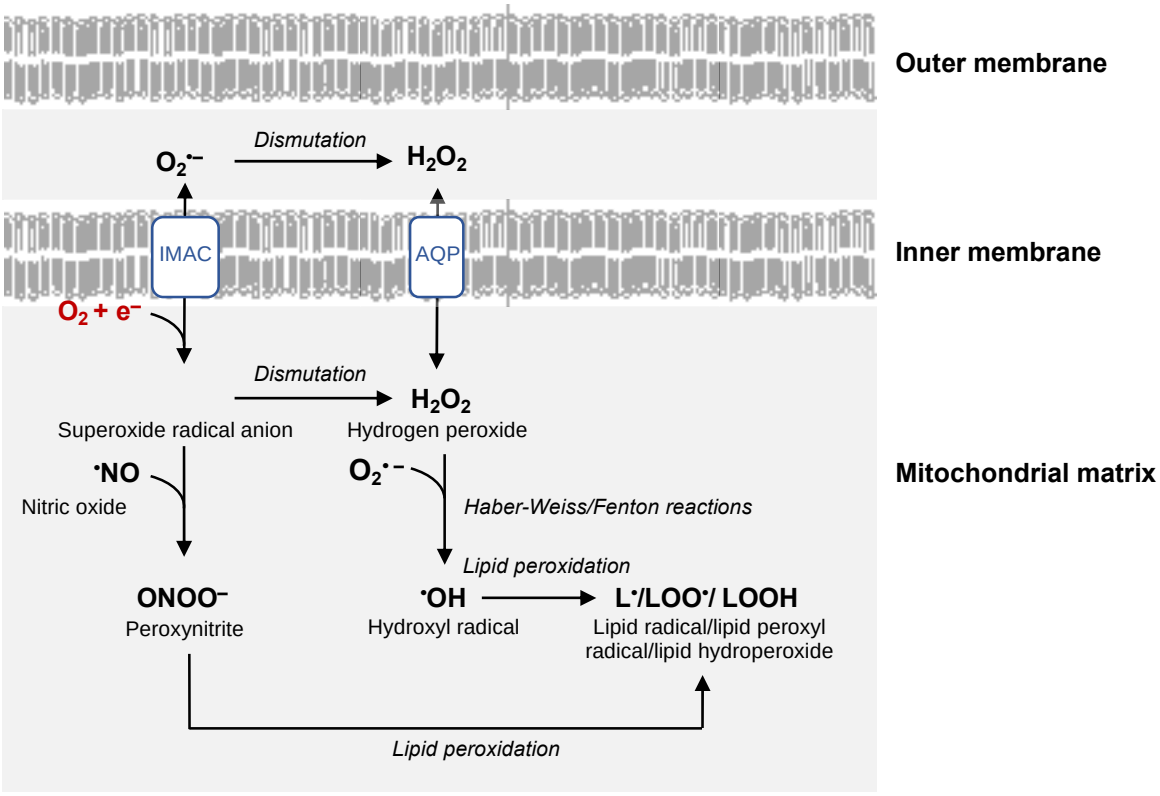

Fig. 1. Major mitochondrial pathways of reactive oxygen, nitrogen, and lipid species generation in mammalian cells. Generation of the superoxide radical anion ($O_2^{\bullet-}$) by one-electron reduction of molecular oxygen is the initiating step in the formation of reactive oxygen, nitrogen, and lipid species in mitochondria. $O_2^{\bullet-}$ can lead to the formation of peroxynitrite ($ONOO^-$) through reaction with nitric oxide ($^{\bullet}NO$) or H_2O_2 via dismutation. $O_2^{\bullet-}$ and H_2O_2 can be transported out of the matrix through the inner membrane anion channel (IMAC) and aquaporins (AQP), respectively, or remain in the matrix where it can lead to the formation of the hydroxyl radical ($^{\bullet}OH$) through the Haber-Weiss/Fenton reactions. Species such as $ONOO^-$ or $^{\bullet}OH$ can also lead to mitochondrial lipid peroxidation involving carbon-centered lipid radicals ($L^{\bullet}$), lipid peroxyl radicals ($LOO^{\bullet}$) and lipid hydroperoxides (LOOH).

Superoxide radical anion ($O_2^{\bullet-}$)

The superoxide radical anion is considered the proximal or primordial reactive oxygen species, as it can drive to the formation of other oxidants, especially H_2O_2. $O_2^{\bullet-}$ is a stronger reductant than oxidant [50] and is formed by the one-electron reduction of molecular oxygen (O_2). In mitochondria, the one electron is thought to be derived primarily from reduced coenzymes or prosthetic groups (*e.g.*, iron-sulfur clusters). Mitochondrial respiration is a significant source of $O_2^{\bullet-}$, with a small percentage (anywhere from 0.01-1 % depending on the reports, [51]) of the O_2 consumed 'hijacked' and released as $O_2^{\bullet-}$. This process is affected by the NADH/NAD^+ and the ubiquinol ($CoQH_2$)/ubiquinone (CoQ) redox couples. The reduction potential of the NADH/NAD^+ couple is -316 mV, implying that NADH is a highly reducing agent. However, the NADH/NAD^+ ratio in mitochondria is maintained between 1:10 and 1:1000, thereby favoring oxidation reactions that generate NADH [52], with electrons entering the NADH/NAD^+ pool through NAD^+-linked dehydrogenases into Complex I. Coenzyme Q is best known for its role in mitochondrial energy production via transfer of electrons and translocation of protons described in the Mitchell Cycle [53]. In this cycle, a series of one-electron transfer reactions occur transferring electrons from Complex I and II to Complex III. The ubisemiquinone radical generated as

part of this can transfer an electron to O_2, resulting in $O_2^{\bullet-}$ formation. In addition, electrons can be passed into the $CoQH_2$/CoQ pool by different dehydrogenases. Overall, the redox state of the $CoQH_2$/CoQ pool can determine forward or reverse electron transport (RET).

To date 11 sites of $O_2^{\bullet-}$ production have been identified in mammalian mitochondria [54, 55]. These sites can be broadly categorized as sites interacting with the NADH/NAD^+ or the $CoQH_2$/CoQ pool (see [54] for an in-depth review on the sites of mitochondrial $O_2^{\bullet-}$ formation). Most of these sites release $O_2^{\bullet-}$ into the mitochondrial matrix, either because they are situated in the matrix (*e.g.*, dehydrogenases), or because they are situated at the inner face of the inner membrane facing the matrix (*e.g.*, Complex I). However at least two sites (Q_o site of Complex III [56] and mitochondrial glycerol-phosphate dehydrogenase (mGPDH;[57]) are situated on the outer side of the mitochondrial inner membrane and can release $O_2^{\bullet-}$ into the matrix or the cytosolic face of the inner membrane. The $O_2^{\bullet-}$ formed in the matrix can move across the inner membrane via the inner membrane anion channel (reviewed in [58]), although more likely, it is metabolized by Mn-SOD (encoded by *Sod2*). $O_2^{\bullet-}$ in the intermembrane space may be carried to the cytoplasm via voltage-dependent anion channels [59]. The inner membrane space also contains Cu,Zn-SOD (encoded by *Sod1*) [60], suggesting that $O_2^{\bullet-}$ released vectorially into the inner membrane space may also be rapidly converted to H_2O_2.

Until recently, forward electron transfer through the respiratory chain was considered the physiologically relevant mode of electron transfer and thus $O_2^{\bullet-}$ production, despite observations in the 1960s that isolated mitochondria can produce NADH from NAD^+ [61]. There is now considerable data indicating that RET is an important contributor to physiological and pathological $O_2^{\bullet-}$ production. RET has been described to occur at Complex I [62, 63] and Complex II [57] in situations where the CoQ pool is highly reduced and a large proton motive force drives electrons back through the respective complex. A particularly intriguing aspect of RET is that it does not require damage to, or inhibition of, the respiratory chain [63].

The relative contribution of the different sites to the overall $O_2^{\bullet-}$ production varies from organ to organ, and the metabolic state of the mitochondria can determine where and how $O_2^{\bullet-}$ is formed (*e.g.*, actively respiring versus highly reduced [64]). Complex I is a good example of this. In situations where mitochondria are respiring on complex I substrates (glutamate and malate), the NADH/NAD^+ ratio is low and only small amounts of $O_2^{\bullet-}$ are produced by Complex I [65]. However, when the NADH/NAD^+ ratio is high or under RET, greater $O_2^{\bullet-}$ production at Complex I has been identified (reviewed in [66]). Importantly, the relative contribution of different respiratory chain sites to $O_2^{\bullet-}$ will vary in pathology. For example, pyruvate dehydrogenase has been identified as a source of $O_2^{\bullet-}$ during RET and may be associated with the development of insulin resistance and metabolic disorders [67], while $O_2^{\bullet-}$ derived from Complex I via RET has been well characterized in ischemia-reperfusion injury [68]. The high concentration of Mn-SOD in the matrix along with its high rate of reaction with $O_2^{\bullet-}$ (k ~2.3 x 10^9 M^{-1} s^{-1}) results in low steady-state concentration of intramitochondrial $O_2^{\bullet-}$. Dismutation of $O_2^{\bullet-}$ may also occur in the inner membrane space [60]. For these reasons, H_2O_2 efflux is frequently assessed as a

measure of mitochondrial oxidant production capacity especially in isolated mitochondria. $O_2^{\bullet-}$ has the thermodynamic capacity to act as an oxidant, but it is generally not reactive with common components of cells such as peptides, carbohydrates, nucleic acids or lipids, and thus is not considered an indiscriminate oxidant [69]. However, $O_2^{\bullet-}$ can directly react with enzymes important in energy production and amino acid metabolism. The classic example of this is $O_2^{\bullet-}$-mediated inactivation of aconitase, where $O_2^{\bullet-}$ oxidizes the Fe-S center, leading to iron release and the potential for secondary radical production via Fenton chemistry [70]. It is likely this ability of $O_2^{\bullet-}$ to react and give rise to secondary reactive species that underlies its importance in biological signaling and homeostasis.

Hydrogen peroxide

The stoichiometric conversion of $O_2^{\bullet-}$ to H_2O_2 by the action of Mn-SOD is a major determinant of H_2O_2 production in the mitochondrial matrix, although other sources of H_2O_2 exist. Thus, autocatalytic oxidation of flavin by O_2 gives rise to flavin radicals that react with $O_2^{\bullet-}$ to form H_2O_2 at a rate of ~10^8 M^{-1} s^{-1} [71]. Several studies have demonstrated that sites along the respiratory chain can form a mixture of $O_2^{\bullet-}$ and H_2O_2 [57, 72, 73], with H_2O_2 generated by two-electron transfer to O_2. Complex II's flavin site has been demonstrated to produce $O_2^{\bullet-}$ and H_2O_2 [57]. Whether each site along the chain can transfer one-electron or a mixture of one and two-electrons is not well established and is difficult to accurately determine especially in complex biological systems. Mitochondria contain glutathione peroxidases and peroxiredoxin 3 (Prdx3) which react with H_2O_2 at rates in the order of ~10^7 M^{-1} s^{-1} [74], and hence effectively remove H_2O_2 as long as these enzymes are recycled. H_2O_2 can be transported out of the mitochondrial matrix through aquaporins, a family of transmembrane proteins that transport neutral solutes across cell membranes. Aquaporins 8 and 9 (AQP8, AQP9) are located in the inner mitochondrial membrane [75, 76] and these have been demonstrated to facilitate H_2O_2 movement in mammalian cells [77, 78]. Consequently, mitochondria derived H_2O_2 can potentially participate in redox reactions outside the mitochondrial matrix. In the presence of reduced transition metals such as Fe^{2+}, H_2O_2 can give rise to hydroxyl radical ($^{\bullet}OH$) via Fenton chemistry [79]. $O_2^{\bullet-}$ can liberate iron from Fe-S clusters [50, 79], and maintain it in the reduced form and hence potentially propagate this process resulting in cycles of oxidant production.

Mitochondrial lipid peroxidation

Lipid peroxidation is a biologically important process that results in intermediate unstable oxidized lipid species and stable end products some of which act as bioactive lipid mediators. Phospholipids present in the inner and outer mitochondrial membranes and containing bisallylic hydrogen are targets for lipid peroxidation (for a detailed review of this process see [80]). Cardiolipin is of particular interest as it is exclusive to mitochondria and has a high content of unsaturated fatty acids with bisallylic hydrogen [81, 82]. In a series of studies, Kagan and co-workers reported that binding to cardiolipin affords

cytochrome *c* with peroxidase activity that results in cardiolipin oxidation [83], and that this process is essential for apoptosis [84], thereby linking mitochondrial oxidants, lipids and mitochondrial function.

Reactive nitrogen species

Nitric oxide ($^{\bullet}NO$) is a gaseous signaling molecule generated by nitric oxide synthases (NOS) and well known to facilitate a wide range of physiological processes. Two groups have reported the existence of a mitochondrial form of NOS [85, 86] and intramitochondrial formation of peroxynitrite ($ONOO^-$) formation [87]. While the existence of a mitochondrial NOS has remained under debate [88], the presence of $^{\bullet}NO$ in mitochondria can have important consequences regardless its site of generation. It is well established that low concentrations of $^{\bullet}NO$ cause rapid and reversible inhibition of cytochrome *c* oxidase [89, 90], such that $^{\bullet}NO$ is a potential physiological regulator of respiration. $O_2^{\bullet-}$ and $^{\bullet}NO$ combine rapidly to $ONOO^-$ [91], itself a strong oxidant and nitrating agent. $ONOO^-$ may act as an oxidant itself, isomerize to nitrate, or decompose to nitrogen dioxide radical ($^{\bullet}NO_2$) and $^{\bullet}OH$, all strong oxidants that can induce lipid peroxidation. $ONOO^-$ readily oxidizes sulfur groups in cysteine, glutathione (GSH) and methionine [92, 93], ascorbate [94], and the bases of DNA [95], $CoQH_2$ and NADH [96]. $ONOO^-$ has also been reported to inhibit respiratory complexes [97]. These reactions can lead to increased $O_2^{\bullet-}$ and H_2O_2 formation. $ONOO^-$ can also start the process of lipid peroxidation [98, 99] (**Figs. 1 and 2**).

Systems that regulate mitochondrial redox homeostasis

Antioxidant defense systems in mammalian mitochondria are a critical factor in setting the steady state levels of mitochondrial reactive species and the overall maintenance of mitochondrial redox homeostasis. Dedicated enzymatic systems are in place to remove oxidants generated in mitochondria (**Fig. 2**). Mn-SOD is responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2 and critical for optimal cell functioning, exemplified by the fact that Mn-SOD gene knockout mice die shortly after birth [100, 101]. H_2O_2 and other hydroperoxides can be removed by the actions of glutathione peroxidase 1 and 4 (GPx1, 4) and/or peroxiredoxin 3 (Prdx3). The relative abundance of Prdx and GPx is an important factor governing peroxide removal and signaling, with a study estimating that Prdx3 is approximately 30-times more abundant than Gpx1 in HeLa cells [102]. Both Gpx1 and Prdx3 have a high reactivity with H_2O_2 and function to keep endogenous concentrations of H_2O_2 in the sub-micromolar range [103], while GPx4 has a preference for lipid hydroperoxides. Unlike Prdx3 which is exclusively located in mitochondria, GPx1 and GPx4 are located across the cell compartments [104]. Despite similarity in their function, Prdx and GPx rely on different redox cycles. Prdx utilizes the thioredoxin system (**Fig. 2**) with a complete thioredoxin system found in mitochondria. As the name suggests GPx utilize a mitochondrial glutathione-based redox cycle (**Fig. 2**). Reduced glutathione (GSH) is a ubiquitous low-molecular weight thiol and is generally considered to provide the primary cellular redox buffer in eukaryotic systems. The appropriate functioning of these redox cycles is essential in maintaining redox

state and oxidants concentrations. Therefore, homeostatic mechanisms regulating the abundance and activity of each component are important mechanisms to control mitochondrial redox state.

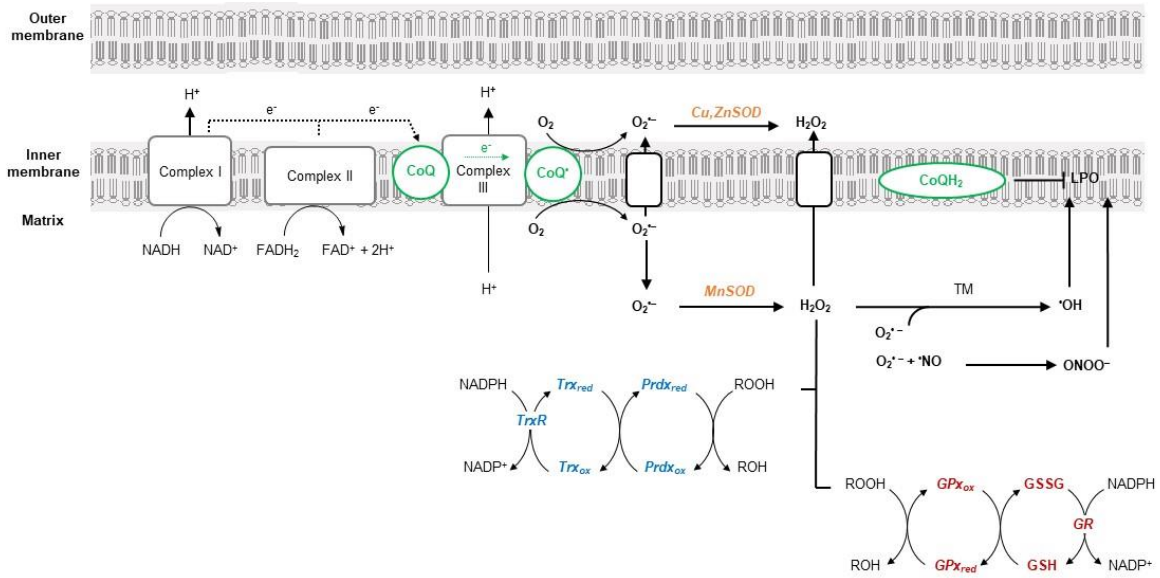

Fig. 2. Mitochondrial antioxidant defense systems. Superoxide radical anion ($O_2^{\bullet-}$) is generated through one-electron reduction of molecular oxygen. Once generated, $O_2^{\bullet-}$ can be released into either the matrix or inner membrane space. Mn-SOD (encoded by *Sod2*) catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and water (not shown) within the matrix, and there is evidence that Cu,Zn-SOD (encoded by *Sod1*) is localized to the intermembrane space. Within the matrix, H_2O_2 and other peroxide species (ROOH) including lipid hydroperoxides (LOOH) can be reduced into their respective hydroxides (ROH) by either the thioredoxin (Trx) or glutathione (GSH) systems. The thioredoxin redox cycle is comprised of thioredoxin reductase (TrxR), thioredoxins (Trx) and peroxiredoxins (Prdx). Similarly, the glutathione redox cycle is comprised of glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG) and glutathione peroxidases (GPx). Both the thioredoxin and glutathione redox cycles utilize NADPH as the ultimate reductant. LPO, lipid peroxidation; TM, transition metal.

The NADPH/$NADP^+$ redox couple is critical for the glutathione and thioredoxin systems. Intracellular reductive/anabolic systems often utilize NADPH as electron donating cofactor. Reduction of $NADP^+$ to NADPH is a two-electron reaction and has a highly reducing E_0' of -315 mV [105]. This makes NADPH an extremely efficient electron donor. In cells, the NADPH/$NADP^+$ ratio is approximately 100:1 [106], with the maintenance of NADPH as the dominant species favoring reductive reactions. To date, a mitochondrial transport system for mitochondrial uptake of NADPH has not been described. Rather mitochondria have their own distinct set of NADPH-producing reactions [107] comprised of (i) $NADP^+$ transhydrogenation by nicotinamide nucleotide transhydrogenase (NNT) using NADH as cofactor; (ii) conversion of glutamate to α-ketoglutarate by glutamate dehydrogenase 1 (GDH1); (iii) NADH phosphorylation by mitochondrial NAD kinase (NADK2); (iv) isocitrate dehydrogenase 2 (IDH2); (v) malic enzymes (ME2/3); and (vi) the mitochondrial folate cycle. Maintenance of NADPH concentrations is essential to allow the continued cycling of the glutathione and thioredoxin systems. Defects in mitochondrial NADPH synthesis, *e.g.*, through mutation in *NNT* (discussed in subsequent sections) significantly affect mitochondrial reactive oxygen species production redox state.

In addition to its role in mitochondrial energy production, $CoQH_2$ is the only endogenous lipid-soluble antioxidant in mammals. $CoQH_2$ is a highly effective antioxidant due to its superior hydrogen atom donor activity, together with its abundance in cellular membranes and the presence of enzymatic systems that maintain CoQ in the reduced, antioxidant-active form. By acting as a hydrogen donor, $CoQH_2$ prevents the propagation of lipid peroxidation, as well as protein and DNA oxidation [108]. The CoQ pool plays a key role in mitochondrial function and CoQ deficiency has been associated with a wide variety of pathologies [109], including insulin resistance. A recent study [44] has revealed a role for CoQ in insulin resistance, specifically that CoQ is a proximal driver of insulin resistance through its role in modulating mitochondrial $O_2^{\bullet-}$. This will be discussed below in more detail (see *Evidence for and against causal role of mitochondrial oxidants in IR*).

Mitochondrial redox signaling

It is now well understood that reactive species can act as important signaling molecules for both, maintenance of normal physiology as well as driving disease. Mitochondrially-derived species initiate redox signaling in a spatially restricted mode, including their generation and reaction with their targets. However, mitochondrially-derived reactive oxygen species may also signal outside of mitochondria to affect targets in other parts of the cell, and this is of considerable interest in the field of insulin resistance. Several common features underpin mitochondrial redox signaling. Foremost, Mn-SOD controls the conversion of $O_2^{\bullet-}$ to H_2O_2 on kinetic grounds, and thereby favors signaling by the two-electron oxidant H_2O_2. Indeed, little is known about direct redox signaling by $O_2^{\bullet-}$ so much focus has been on the comparatively more stable H_2O_2 as a signaling molecule. Two key factors modulate H_2O_2-mediated signal transduction: i) the combined effect of location, expression, and activities of H_2O_2-detoxifying enzymes that restricts or allows H_2O_2 to engage with target molecules; and ii) the mechanism by which-H_2O_2 induces signal transduction in target molecules. Several models have been proposed to explain how H_2O_2 signals, *i.e.,* the floodgate and redox-relay models [110-115]. These mechanisms are based on reaction of H_2O_2 with peroxiredoxins to allow signaling to occur in the presence of 'professional' detoxification enzymes. While better characterized for cytosolic redox systems, these mechanisms could also operate in mitochondria.

Mitochondrial H_2O_2 has the potential to affect cellular processes through signaling within the mitochondrial matrix (*e.g.*, UCP-1 redox state [116]). As H_2O_2 can be transported out of the mitochondrial matrix via aquaporins in the inner membrane, mitochondria-derived H_2O_2 can impact processes in the inner membrane space (*e.g.*, Lyn/Syk signaling cascades [117]), the cytosol (*e.g.*, via the OMA1-DELE1-HRI pathway [118, 119], or via the redox "bridge" (MIC60–Miro interaction) [120]), nucleus (*e.g.*, regulation of transcription factors like HIF1α [121]) or the endoplasmic reticulum [122]. However, a critical issue in this area of research is the ability to distinguish mitochondrial and mitochondria derived H_2O_2 from that derived from non-mitochondrial sources and/or other oxidants, as

these may have different roles. In this context, it is important to consider the appropriateness of the different methods and tools to assess mitochondrial oxidants and redox couples.

Assessing the role of mitochondrial oxidants and redox state in insulin resistance

Accurate assessment of mitochondrial oxidants and changes in redox state are key in demonstrating their role in the physiology of glucose metabolism and pathogenesis of insulin resistance, or any biological study. Two broad approaches can be utilized. First, the direct or indirect measurement of oxidant(s) that can provide important information on the nature of oxidant(s) involved and alterations in oxidant steady-state concentration(s). Second, modulation of mitochondrial redox state or oxidant production through pharmacological or genetic means to study the role of specific oxidant(s). The two approaches can be used in tandem. Despite a plethora of published methods, accurate and reliable measurement(s) of mitochondrial oxidants, especially H_2O_2, and their effects on biological systems remains challenging, and results need to be interpreted with caution and considering the methodological limitations. The following provides a brief overview of techniques, the basis of each method, their use and important considerations, together with references to relevant in-depth reviews. A focus will be on methods that have been applied to mitochondria in the context of insulin resistance.

Measurement of oxidants

Mitochondria targeted-hydroethidine for detection of $O_2^{\bullet-}$

Mitochondria-targeted hydroethidine (Mito-HE, more commonly known as Mito-SOX) is a popular, commercially available fluorogenic probe used to assess mitochondrial $O_2^{\bullet-}$ formation. The chemical basis of reactivity and use of HE-based probes including Mito-HE have been reviewed extensively [123], with detailed protocols for their use published [124]. The use of Mito-HE has provided considerable evidence for the involvement of mitochondrial $O_2^{\bullet-}$ in a range of physiological and pathological conditions. While used commonly in *ex vivo* and in cell culture, HE-based probes including Mito-HE can be administered to mice [125] allowing for *in vivo* measurement of reactive species. Upon reaction with $O_2^{\bullet-}$, Mito-HE forms a specific adduct, Mito-2-hydroxyethidium (2-OH-E^+) [126, 127]. Like with other redox probes, the challenge with using Mito-HE in experimental systems is ensuring the use of appropriate methods and controls to allow for accurate oxidant determination. An important issue relates to the fluorescent properties of different oxidation products. Mito-HE (like HE) also form less specific two-electron oxidation products, Mito-E^+ and Mito-E^+-Mito-E^+, via reaction with cytochrome *c* and other heme proteins [128-131]. Both Mito-2-OH-E^+ and Mito-E^+ exhibit overlapping fluorescence spectra [123] precluding their differentiation by fluorescent methods. Thus, Mito-HE-derived fluorescence does not specifically measure mitochondrial $O_2^{\bullet-}$ and reliance on this mode of detection can lead to erroneous conclusions, especially under conditions associated with apoptosis, when there is increased release of cytochrome *c*. Specific detection of Mito-2-OH-E^+ (and 2-OH-E^+) requires use of high-pressure liquid chromatography (HPLC) or liquid chromatography-tandem mass

spectrometry (LC-MS/MS) with several methods published [44, 45, 124]. Despite this, Mito-HE-derived fluorescence is still commonly attributed to $O_2^{\bullet-}$ formation. The specificity of fluorescent approaches can be improved with inclusion of controls, *e.g.*, the presence of SOD, in which a SOD-inhibitable signal would indicate $O_2^{\bullet-}$-dependent reaction of the probe. Finally, the amount of Mito-2-OH-E^+ formed is affected not only by $O_2^{\bullet-}$, but also by other processes, including Mito-HE uptake, consumption, availability and efficiency in the competition for $O_2^{\bullet-}$ with SOD [124]. These issues must be taken into consideration and controlled, when possible (*e.g.*, use of internal standards, 2-OH-E^+ normalization to total Mito-HE), though this is evidently more challenging in complex systems.

Mitochondrial H_2O_2 quantification by Amplex Red-derived fluorescence

Mitochondrial H_2O_2 can be quantified using peroxidase-catalyzed oxidation of fluorogenic probes, of which Amplex Red is used most. The assay is based on Amplex Red reacting with H_2O_2 in the presence of horseradish peroxidase (HRP) which stoichiometrically generates fluorescent resorufin. While the assay was developed originally for use in cell free systems, it has been coopted to measure H_2O_2 release from mitochondria, most commonly isolated mitochondria [132, 133]. It is assumed that the H_2O_2 detected is derived from $O_2^{\bullet-}$, and that H_2O_2 diffuses across the inner/outer mitochondrial membrane(s), as HRP cannot cross biological membranes. The method has also been applied to permeabilized isolated skeletal muscle fibers [134] treated with saponin/digitonin to selectively permeabilize sacrolemmal membranes while keeping mitochondrial membranes intact. Monitoring mitochondrial H_2O_2 'emission' (referred to hereafter as 'efflux') in this way can be done in the presence of electron transport chain substrates and inhibitors to identify site topology of oxidant generation, often in conjunction with respiration measurements [135, 136]. It is assumed that the cellular architecture of the myofibers and structural integrity of the mitochondria are maintained [137]. While popular, the 'Amplex red assay' assumes that internal consumption of H_2O_2 by matrix-based antioxidants (*e.g.*, Prdx3) is negligible, and it does not consider the role of aquaporins in facilitating diffusion of H_2O_2 across membranes [75, 76]. Also, Amplex Red can be oxidized by $ONOO^-$ [137, 138], and resorufin can undergo photosensitized reduction by NADH or Amplex Red itself, or oxidation by peroxidases or via redox cycling, that affect the estimation of H_2O_2. Therefore, it is important to validate results, *e.g.*, by testing that H_2O_2 generation is being measured (*e.g.*, sensitivity of the signal to catalase) and that the reaction is catalyzed by HRP (*e.g.*, sensitivity of the signal to azide). Even when considering these caveats and the widespread use of this assay, the H_2O_2 measured using this assay is unlikely to provide direct information on mitochondrial H_2O_2, and it cannot be used for absolute quantification.

Mitochondrial-targeted boronate-based probes for H_2O_2 and $ONOO^-$

Boronate-based probes such as monoboronated 2-methyl-4-methoxy Tokyo Green (Peroxy Green 1, PG1), monoboronated resorufin (Peroxy Crimson 1, PC1) [139] and their mitochondria-targeted versions have been described for H_2O_2 detection (reviewed in [140]). These probes react slowly but

stoichiometrically and selectively with H_2O_2 and can be used with detection modalities including fluorescence and mass spectrometry. Subsequent to the first mitochondria-targeted Peroxy Yellow 1 (MitoPY1, [141, 142]), the ratiometric probe, MitoB, in which the ratio of unreacted and reacted probe can be quantified by LC-MS/MS, has also been validated for use in mice [143]. While MitoPY1 and MitoB are structurally distinct, both probes can identify and track H_2O_2 within cells and tissue [141, 144, 145]. For non-ratiometric probes like mitoPY1, the signal is affected by probe uptake, whereas for non-ratiometric and ratiometric probes, quantification of absolute concentrations of H_2O_2 is challenging due to competing reactions. Another limitation with boronate-based probes is their reactivity with $ONOO^-$, so that controls such as catalase treatment are recommended to validate the presence of H_2O_2. In an attempt to overcome this issue, *o*-MitoPh$(OH)_2$ [146, 147] was developed in which reaction products specific for $ONOO^-$ and H_2O_2 are formed and can be detected by HPLC.

Other mitochondrial probes

Chemical probes for mitochondrial compounds such as hydrogen sulfide (H_2S) have also been developed. Mitochondria generate H_2S and H_2S signaling is intricately associated with redox processes. For example, H_2S can be metabolized in the mitochondrial inner membrane by CoQ and GSH. While numerous H_2S fluorescent probes have been developed [148], including mitochondria-targeted probes such as MitoA [149] and Mito-BT. MitoA is a ratiometric probe that can be used *in vivo*. Upon reaction with H_2S, it forms a specific product called MitoN, and both MitoA and MitoN can be determined by LC-MS/MS. Mito-BT [150] is a ratiometric fluorescent probe more suited to *in vitro* studies.

Genetically encoded redox sensors

The development of compartment-specific fluorescent and FRET-based probes have allowed for real-time, organelle -specific changes in redox state and oxidant production to be monitored in a dynamic and relatively non-invasive manner. The sensors can report redox/oxidant changes without cell disruption and can be highly specific for the redox couple in question, with redox potentials and both reduced and oxidized forms measured. Genetic sensors fall in two large categories, i) redox-sensitive fluorescent proteins, where the protein has been engineered to respond to redox changes, *e.g.,* roGFP or rxYFP; or ii) composite fluorescent sensors. The latter couple redox-sensitive fluorescent proteins to redox-responsive proteins such as glutaredoxins, thioredoxins, or peroxidases. Composite sensors ensure continuous equilibration against the redox couple in question, *e.g.*, glutaredoxin-linked probes equilibrate with the glutathione redox state. Sensors have been developed to measure oxidants such as H_2O_2, specific redox analytes such as NADH, NADPH and methionine sulfoxide, and redox couples such as 2GSH/GSSH and NADH/NAD^+. Many of these are available commercially facilitating their use. For an in-depth reviews on genetically encoded-redox probes see [151, 152].

Mitochondria-targeted versions of redox sensors are relatively common, with specific targeting achieved by the fusion of specific mitochondrial leader sequences to the protein, resulting in their highly selective expression in the mitochondrial matrix, or the mitochondrial inner membrane space (IMS)

[153-157]. The ability of these sensors to be targeted to multiple sub-cellular locations allows for information of redox dynamics in mitochondria versus other sub-cellular compartments and thus provides important information about the spatial regulation of redox processes. This is important in the context of disease, as the changes in mitochondrial redox dynamics may be distinct or reflect more widespread redox changes occurring in the cell. Most fluorescent redox probes function by changing their fluorescent properties in response to redox or oxidant changes, and this can be followed using fluorescent plate readers, microscopy or flow cytometry. Unlike many chemical probes, genetic sensors can be used in living cells, tissues and whole organisms [158]. However, genetic probes rely on efficient methods to introduce them into organisms, and this can be a challenge in certain experimental systems, *e.g.*, cells with low transfection potential, although techniques like adenoviral transfection can mitigate these [159]. Many but not all genetically encoded fluorescent sensors are ratiometric, such that issues like transfection efficiency do not present a limitation. While genetically encoded redox sensors can be used to estimate sub-cellular redox potentials (*e.g.*, E_{GSH}) these measurements can be complex, requiring stringent controls. Reporting relative changes in mitochondrial redox state or oxidant content is considerably simpler. Initially limited to cellular systems, advances in genetic technologies, cytosolic and mitochondrial transgenic redox biosensor models have been developed in small organisms such as such as *Caenorhabditis elegans* [160] and *Drosophila melanogaster* [161, 162]. More recently, mice expressing transgenic redox sensor have become available, albeit with limited cellular expression (see [158] for a review of *in vivo* use of genetically encoded fluorescent sensors). These mice can be used for semi-quantitative analyses of mitochondrial oxidants and tissue redox state. For example, *db/db* mice expressing mitochondria-targeted roGFP have been used to interrogate dynamic monitoring of redox changes in kidneys [163]. The analysis of redox biosensor responses *in vivo* at the tissue level is contingent on the use of suitable fluorescent imaging techniques (*e.g.*, confocal, two-photon or wide-field microscopy). Technical challenges remain when trying to image redox probes in internal organs *in situ* resulting so that cells or tissue isolated from transgenic mice are studied most. Redox histology, a method that preserves the *in vivo* redox state of genetic redox biosensors in tissue sections [164], has increased the scope of transgenic mice in studying cell- and organelle-specific redox changes.

Mitochondrial lipid oxidation and oxidized lipids

Oxidized lipids and changes to the lipid redox state affect mitochondrial processes such as respiration and apoptosis. One of the challenges in this area is the extensive heterogeneity of oxidized lipids. Fluorescent probes such as C11-BODIPY(581/891) have been used extensively for measuring lipid oxidation, although it remains unknown what this probe measures specifically, and it does not report changes in distinct sub-cellular compartments. A mitochondria-targeted derivative of C11-BODIPY(581/891), called MitoPerOx, has been developed [165]. Other examples include MitoCLox [166], a cardiolipin-specific fluorescent lipid oxidation probe, and Mito-BODIPY-TOH that targets the inner mitochondrial membrane and is sensitive to the lipid peroxyl radicals [167]. Fluorescent lipid

oxidation probes have the advantage that they can be used for live-cell imaging and can yield information about lipid oxidation in general. However, they cannot give detailed information on specific species of oxidized lipids, which is important for understanding biological effects. More specific assays are based on determination of specific oxidized lipids in mitochondria (*e.g.*, targeted lipid analyses). Determination of oxidized lipids is best achieved using mass spectrometry-based methods such as gas-chromatography mass spectrometry (GC-MS), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry [168], which allow simultaneous profiling of multiple lipid classes. These methods can be used on whole tissue or cells especially if looking for oxidation of mitochondria-specific lipids such as cardiolipins [81]. However, most lipid classes are distributed across sub-cellular membranes and identification of mitochondria-localized lipids can be challenging and require mitochondrial isolation. Kappler and colleagues undertook a systematic comparison of three common mitochondrial isolation procedures and observed significant differences in the lipid classes identified by each method [169]. As a result, the authors recommended isolation of mitochondria by ultracentrifugation for lipidomic analyses of cell culture and highlighted the need for controls to minimize issues arising from contamination.

From an insulin resistance perspective, the analysis of mitochondrial CoQ is of significant interest. As CoQ is present in all sub-cellular membranes, isolated mitochondria must be used to measure mitochondrial CoQ. Reduced and oxidized forms of CoQ can be analyzed by HPLC with electrochemical detection and by LC-MS/MS [44, 170]. However, accurate determination of the CoQ redox state is challenging due to oxidation of ubiquinol during isolation, extraction, and analysis. *para*-benzoquinone is used commonly to oxidize reduced CoQ in biological samples with total CoQ content then reported. This approach obviously does not allow the CoQ redox state to be determined. Analyses of CoQ redox state generally requires careful sample workup with samples collection, preparation and analyses all occurring under an atmosphere of argon [171-173].

Surrogate measures

Surrogate markers do not directly measure oxidants themselves but rather alterations in these markers can signal potential changes in mitochondrial oxidants or redox state. Surrogate markers can be a useful experimental starting point. If changes are observed, more detailed confirmatory analyses can be undertaken. **Table 1** outlines commonly used surrogates of mitochondrial oxidants, techniques involved, and advantages and disadvantages of each.

Manipulation of mitochondrial oxidants and redox state

a. Modulating mitochondrial redox state - Promotion of mitochondrial oxidant production or redox dysfunction

Many studies, in particular proof-of-concept studies, modulate mitochondrial redox systems as a strategy to interrogate its importance in the underlying pathogenesis of disease processes. Promotion of reactive oxygen species production or changes in redox state can be induced pharmacologically or

genetically. Inhibitors such as auranofin (thioredoxin reductase inhibitor), 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU; glutathione reductase inhibitor) or redox-cyclers (*e.g.*, paraquat) will affect mitochondria. However, their lack of specificity for mitochondria is problematic and specific effects on mitochondria are hard to ascertain. Mitochondria-targeted inhibitors (pharmacological or genetic) provide a better approach. **Table 2** outlines methods to promote mitochondrial oxidants in cell culture and animal models, and key considerations when using each.

b. Modulating mitochondrial redox state – mitochondria-targeted electron scavengers and antioxidants

Mitochondria-targeted antioxidants have been used to study the role of mitochondrial oxidants in insulin resistance. Such compounds have also been suggested at the basis for novel therapeutic strategies. Supportive of this concept, eye drops developed against dry eye syndrome, cataract and glaucoma containing SkQ1, a mitochondria-targeted plastaquinone, have passed clinical trials phase I-II in the USA [174], and are approved for use in Russia [175]. **Table 3** lists mitochondrial antioxidants that can be used in cell culture and small animal models and key considerations when using each.

A commonly used mitochondrial 'antioxidant' in biological studies is MitoQ which contains a TPP moiety joined via a 10-carbon alkyl chain to the redox-active, fully substituted benzoquinone moiety contained in CoQ [176]. MitoQ is reported to accumulate in mitochondria 100 to 1000-fold and the 10-carbon alkyl chain is thought to insert into the mitochondrial inner membrane [176]. MitoQ is structurally different to CoQ as it lacks the 30-50 carbon polyisoprenoid tail. Nevertheless, the quinone moiety of MitoQ can undergo redox changes. Complex II is the principal site of MitoQ reduction [177], likely due to the orientation of insertion of MitoQ in the inner membrane. By comparison, it is a poor substrate for complex I, complex III and electron-transferring flavoprotein:quinone oxidoreductase [177], and unable to function as an electron carrier in oxidative phosphorylation. Care must be taken in interpreting results and understanding how MitoQ may exert biological effects via changes to mitochondrial redox state, especially as it can enhance formation of $O_2^{\bullet-}$ rather than acting as an 'antioxidant' [178].

Emerging modalities

The development of techniques for the detection of oxidants in live animals includes positron electron transmission (PET) radiotracers based on ethidium [179, 180] for imaging $O_2^{\bullet-}$. While these probes require further study to characterize their selectivity, and kinetics and may have similar limitations to other ethidium-based probes, their development is nevertheless an exciting prospect in the way oxidants may be identified in complex systems.

Studying the role of mitochondrial oxidants and redox state in insulin resistance – merging two fields

A key question in the field of insulin resistance and Type 2 diabetes is the pathophysiological role of mitochondrial oxidants. While there is convincing *in vitro* and *in vivo* data for a role for oxidants in

insulin resistance, demonstrating causality is critical to link changes in specific mitochondrial oxidants or redox state to the induction of disease. This requires accurate and reliable measurement of mitochondrial oxidants in complex biological systems and proper data interpretation. The following sections outline some important considerations in this context.

First, the experimental system used will likely dictate the types of assessment techniques that can be practically employed. For insulin resistance studies, cell culture and small animal models are the mainstay of research, and each system will lend itself to various techniques. An example of this is high-throughput analyses, as mitochondria-based techniques are often challenging to scale up. However, the use of mitochondria-specific redox probes, including genetically encoded fluorescent sensors, has aided in this [147, 181], as they can be used without the need to isolate mitochondria. In terms of clinical research and *in vivo* human studies, most current laboratory techniques are not feasible, such that analysis of mitochondrial oxidants has been limited to the Amplex Red assay in isolated muscle biopsies, and *ex vivo* determination of steady state concentration of oxidants, proteins, lipids and metabolites.

It is equally important to consider how to best measure insulin resistance. **Tables 4-7** list several different methods used to designate tissues as insulin resistant. Here, we classify insulin resistance in muscle and adipose as a state of impaired insulin-stimulated glucose transport into these tissues/cell types. Therefore, methods that directly measure this process are best for assessing whether a tissue is insulin resistant or not. For example, while assessing insulin signaling using phospho-specific antibodies may provide evidence for altered insulin signaling, this does not always translate to impaired glucose uptake (see [23]), and relying on such measures may be misleading. Similarly, proxy measures (*e.g.*, HOMA-IR [182]) and routinely performed tests such as glucose tolerance (GTT) and insulin tolerance tests (ITT), do not provide a direct measure of muscle or adipose insulin sensitivity [183]. Hyperinsulinemic-euglycemic clamps are the gold standard technique for measuring whole-body insulin sensitivity in humans and rodents, and can be used in conjunction with tracers to provide estimates of endogenous glucose production and disposal (which occurs predominantly in muscle [184, 185]. The inclusion of additional glucose tracers also allows determination of adipose or muscle glucose uptake as a direct measure of tissue glucose uptake [185]; tracers can be included in ITTs as well [186]. Indeed, *ex vivo* analyses (e.g., [45] perhaps provide the most reductionist platform for assessing tissue responses, with the caveat that this removes the tissues from the whole body context.

Understanding the genetics of common mouse lines is also important when studying insulin resistance since different strains have markedly different responses to high fat diet feeling in terms of both increased adiposity and insulin resistance [187]. This is especially pertinent for studies into the relationship between mitochondrial redox and insulin resistance. While C57BL/6J mice are the most-widely used inbred strain, 6J mice contain a naturally occurring deletion in exons 7-11 in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene, one of the mitochondrial NADPH generation systems. The glucose-intolerant phenotype of the 6J strain was mapped to the deletion of the *Nnt* gene

[188, 189]. Reintroduced functional NNT rescued the impaired insulin secretion/glucose clearance phenotype of 6J mice [188], connecting NNT loss of function with glucose intolerance. Analysis of C57BL/6J mice compared to mice expressing NNT such as C57BL/6N mice, revealed that the *Nnt* mutation affects mitochondrial redox state and increased mitochondrial H_2O_2 release in liver [190, 191] and is essential for peroxide removal in skeletal muscle [67, 192]. Interestingly, a head-to-head comparison of high-fat induced metabolic responses in C57BL/6N and C57BL/6J mice revealed that both 6N and 6J mice present with impairments in glycemia manifest after just one-day of high-fat diet feeding despite several diet-induced measured of metabolic syndrome observed between genotypes [192]. While working in both 6J and 6N mouse backgrounds may not always be feasible, recognition of the impact the *Nnt* mutation has on studies and their interpretation is valuable.

Second, researchers also need to consider which reactive species they want to analyze. As outlined earlier, mitochondrial oxidants are made up of a range of species, so the experimental systems used are highly dependent on the reactive oxygen species in question. It is also important to consider how each experimental tool assesses oxidant status and following from this, what conclusions can be validly drawn. For example, are reactive oxygen species production or steady state concentrations being measured? Does the tool used measure a specific species or a range of species? Often researchers may want to begin with a more generic tools then follow this up with more sensitive and specific methods. A major issue especially in complex systems is that the output of many assays such as Amplex Red or Mito-HE collectively reflects oxidant generation, the activity of oxidant removal systems, potential competing side reactions, probe uptake and/or mitochondrial efflux of oxidants. As discussed earlier, the use of genetic and pharmacological tools to modulate mitochondrial oxidants and redox state are increasingly popular strategies in this field (see **Table 5-7** for examples of such studies). Most antioxidant probes interact with many mitochondrial processes, and the beneficial effect from antioxidant treatment may not implicate a specific oxidant species or redox process. We suggest that, if possible, the use of multiple strategies (induction and removal and measurement of reactive oxygen species) as an effective way to link mitochondrial reactive oxygen species more specifically with disease and possible treatment strategies.

Finally, experimental controls are critical when assessing reactive oxygen species in biological systems. There are many established factors that give rise to artifacts in redox assays (*e.g.*, elevated temperature, light, the presence of redox-active transition metal ions) and these can be limited using a dark room, anoxic environments, and inclusion of chelators like diethylenetriaminepentaacetic anhydride in buffers. On top of this, there are issues specific to the assessment of mitochondrial reactive oxygen species such as isolation procedures, an issue discussed earlier. If changes in mitochondrial reactive oxygen species are observed, analyzing parameters such as membrane potential and mitochondrial mass is recommended as changes in membrane potential can affect reactive oxygen species production and the uptake of TPP-based probes and increased/decreased reactive oxygen species concentration may merely reflect changes in mitochondrial mass. Additionally, excessive

accumulation of hydrophobic TPP compounds can disrupt mitochondrial function [177, 193], and it has been suggested that the some "antioxidant" effects of lipophilic cations may be due to mild disruption of proton motive force that lowers mitochondrial $O_2^{\bullet-}$ production by RET [62]. Therefore, controls with compounds with matched physicochemical properties are essential to correct for nonspecific effects in experiments using TPP-conjugated molecules.

Evidence for and against causal role of mitochondrial oxidants in IR

In the next sections, we will refer to studies that have investigated the role of mitochondrial reactive oxygen species in insulin resistance, with a focus on studies in skeletal muscle and adipose tissue. This evidence has generally appeared in three forms: 1) measures of increased reactive oxygen species in mitochondria under insulin resistant conditions using various, mostly indirect, methods; 2) interventions thought to lower mitochondrial reactive oxygen species improve insulin action; and 3) interventions that promote mitochondrial reactive oxygen species impair insulin responses. We have tabulated these studies; dividing them into the experimental systems used (human versus pre-clinical), techniques employed to measure/interrogate mitochondrial oxidants, and how the relationship between oxidants and insulin action were assessed (**Tables 4-7**). These tables comprise the key studies that explored the role of oxidants (mainly of mitochondrial origin) in insulin resistance.

Human studies

Only a relatively small number of studies have investigated mitochondrial reactive oxygen species and insulin resistance in human cohorts. Three of these studies used hyperinsulinemic-euglycemic clamps to determine insulin sensitivity [194-196], although only one included tracers to calculate glucose disposal rates into muscle [194]. The other studies used HOMA-IR to determine insulin sensitive or resistant status [42, 197]. In terms of redox measures, it is challenging to measure or modulate mitochondrial reactive oxygen species in humans and most studies in humans are limited to *ex vivo* analyses. This poses a significant limitation in trying to understand whether reactive oxygen species, and those in mitochondria, associate with human insulin resistance. In addition, accessing material from live subjects can be complicated. While blood and plasma can be collected readily, these can be less valuable when studying mitochondrial biology compared to systemic changes in blood glucose and utilization. Despite this, lipid oxidation markers such as plasma 8-F_2 isoprostane concentration suggest increased systemic 'oxidative stress' in insulin resistance [195, 198], even in response to relatively short (7-28 days) dietary interventions [195, 198]. These same studies also reported increased protein carbonyls in adipose [195] and muscle tissue [198]. However, neither of these measures provide evidence of reactive oxygen species from a specific subcellular site such as mitochondria. When considering data on mitochondrial reactive oxygen species, skeletal muscle is most studied, likely because biopsies can be obtained under local anesthesia. The only technique used to probe changes in mitochondrial reactive oxygen species specifically has been *ex vivo* mitochondrial H_2O_2 release as

assessed by Amplex Red fluorescence in skeletal muscle (**Table 4**). While these data support a correlation between *ex vivo* H_2O_2 release from mitochondria and insulin resistance, they do not provide information on the causal species (*e.g.*, $O_2^{\bullet-}$ versus H_2O_2) in insulin resistance.

From the studies presented in **Table 4**, there is both evidence supporting and contradicting a role for mitochondrial reactive oxygen species in insulin resistance in human skeletal muscle. For example, both Anderson [42] and Kane and colleagues [197] demonstrated increased mitochondrial H_2O_2 release in insulin resistant male and female subjects. In addition to baseline insulin resistance, a high fat meal or diet (for 5 days) in lean men was also sufficient to increase mitochondrial H_2O_2 release [42], suggesting that changes in mitochondrial reactive oxygen species are sensitive to changes in diet and may be a causal feature of insulin resistance. Contrasting these findings [194], Abdul-Ghani *et al.* observed no change in mitochondrial H_2O_2 release per ATP by skeletal muscle from insulin resistant humans. Conflicting results, differing methods to determine insulin resistance, and small subject numbers make is difficult to draw definitive conclusions from these studies using *ex vivo* measures. Rather, these studies highlight the complexity of studying the relationship between reactive oxygen species and insulin resistance in humans, including the importance of variables such as gender. For instance, progesterone levels were observed to modulate mitochondrial H_2O_2 release [197]. This builds on previous evidence suggesting an inhibitory action of progesterone on mitochondrial respiration, providing a potential link between sex hormones and the reduced insulin sensitivity reported during phases of the menstrual cycle (reviewed in [199]).

Induction of insulin resistance by seven-day bedrest was not sufficient to induce changes in mitochondrial H_2O_2 release in the presence of submaximal ADP concentrations (*i.e.*, concentrations likely to be found in resting muscle) [196]). Mitochondrial H_2O_2 release was increased in insulin resistant muscle when tested using complex I or II substrates with no exogenously supplied ADP, but this is not representative of the *in vivo* situation, and the implication of these data is not clear. Nevertheless, these data raise the possibility that mitochondrial reactive oxygen species may play a role in certain types of insulin resistance (*e.g.*, obesity-driven) not others (*e.g.*, bed rest driven).

Overall, there is no consensus view regarding a causal or associative role of mitochondrial reactive oxygen species in human insulin resistance and more studies are required to fully understand these issues *in vivo*. This is complicated by the likely sensitivity of mitochondria (and H_2O_2 release) to acute changes in nutritional, hormonal and exercise environments, all of which may be hard to control in human studies. One interesting addendum to this is that there is at most only a small effect of common genetic variants within nuclear-encoded mitochondrial genes [200] or oxidative phosphorylation genes [201] in insulin resistance risk in humans, although this may be due to most studies relying on surrogate measures of muscle/adipose insulin sensitivity.

Pre-clinical data

Preclinical models, including cultured cells and rodents, have allowed use of a wider range of techniques to measure mitochondrial oxidants and provide the strongest evidence for a role in insulin resistance. In **Tables 5-7**, we have separated the studies by tissue type to help understand the evidence for mitochondrial reactive oxygen species across metabolic tissue. The majority of these studies have reported increased reactive oxygen species in mice that are insulin resistant following high fat diet feeding. A significant issue is that, despite working in more tractable model systems, only a handful of redox techniques have been utilized by researchers, and in general the techniques used to date only provide indirect measures of reactive species. For example, most studies use mitochondrial H_2O_2 release to determine changes in mitochondrial H_2O_2 production. For these assays, maximal H_2O_2 release is reported [202-204] though some studies have use respiratory chain inhibitors to identify specific sites of H_2O_2 release along the respiratory chain [67]. Along the same lines, many studies use Mito-HE-derived fluorescence as a marker of mitochondrial $O_2^{\bullet-}$. As we have learned, these assays, especially in isolation, do not aid in understanding the exact species that may be involved in the pathogenesis of insulin resistance. A challenge for researchers is to use tools that selectively identify distinct oxidants. Keeping this in mind, we believe that data from preclinical studies provide a level of experimental support for an increase in mitochondrial reactive species during insulin resistance. However, more detailed analyses of specific species are without doubt required.

One line of evidence for a causal role for mitochondrial oxidants in adipose insulin resistance comes from experiments that have assessed insulin responses and reactive species in tissues early after transition from a normal to a high fat diet. Under these conditions, epididymal adipose tissue becomes insulin resistant within 5 to 7 days (as measured by decreased glucose uptake [44] and insulin signaling [205]) with concomitant increases Prdx3 dimerization [44] and mitochondrial H_2O_2 release. This contrasts other proposed drivers of insulin resistance, for example inflammation, which is detected later, after the onset of insulin resistance [206]. Although the extent of insulin resistance and Prdx3 dimerization was maintained to 14 days [44], longer dietary interventions (8 weeks) exacerbated mitochondrial H_2O_2 release in both inguinal and visceral adipose depots [207]. These data suggest that reactive species may increase in response to prolonged high fat feeding. Whether this correlates with worsening insulin resistance, as measured by insulin-stimulated glucose uptake, is not clear. Nevertheless, the observed rapid changes in reactive species parallels data in humans where even 7 day diet interventions arre sufficient to produce measurable effects on protein carbonylation [195]. Overall, detection of mitochondrial reactive species at a time that coincides with the occurrence of insulin resistance provides correlative support that mitochondrial reactive species play a causal role in insulin resistance.

Stronger evidence that reactive oxygen species play a casual role in insulin resistance comes from genetic approaches that modulate mitochondrial $O_2^{\bullet-}$ (*i.e.*, *Sod2*) [37, 208-210] or H_2O_2 (*i.e.*, mitochondrial-targeted catalase; mCAT) [202, 203, 205, 211]. We note that Smith *et al* (2021) [203] reported that mCAT overexpression improved glucose tolerance, but not glucose uptake into the

extensor digitorum longus (EDL) muscle, in a genetic model of increased β-oxidation [203]. These data highlight the importance of using direct measures of tissue insulin responses. Therefore, here we focus on studies that have measured muscle/myocyte and/or adipose/adipocyte insulin resistance by hyperinsulinemic-euglycemic clamp or tissue-specific glucose tracer uptake in mice, or glucose tracer uptake or GLUT4 responses in cultured cells. In these studies, overexpression of mCAT improved insulin sensitivity in a range of models of insulin resistance in mice including age [202] and high fat diet [42, 202, 212], and in cultured adipocytes including treatment with tumor necrosis factor-α (TNFα) or dexamethasone, and inhibition of CoQ synthesis [37, 44]. *Sod2* overexpression in cultured myotubes and adipocytes also improved insulin responses in cells rendered insulin resistant through incubation with chronic insulin, TNFα, dexamethasone, palmitate and antimycin A [37, 208]. Similarly, more acute interventions using pharmacological agents that target mitochondrial oxidants have also improved insulin sensitivity in C2C12 cells (SkQ [204]), 3T3-L1 adipocytes (MnTBAP [37, 208]), L6 myotubes (MitoTempol, MnTBAP, and MnTMPyP [208]) and adipose tissue (MnTBAP [208]). Conversely, small molecules that increase mitochondrial $O_2^{\bullet-}$ (*e.g.*, mito-paraquat or antimycin A) have been reported to induce insulin resistance [45, 208, 213], further supporting a role for mitochondrial $O_2^{\bullet-}$.

In contrast to *in vivo* data from mCAT transgenic mice, reports of a positive effect of *Sod2* overexpression in mice are limited to GTTs [43, 214], and assessment of insulin action using hyperinsulinemic-euglycemic clamps reported no effect of *Sod2* overexpression on glucose disposal (as a measure of muscle glucose uptake) in chow or high fat diet-fed mice [212]. Similarly, *Sod2* heterozygous mice exhibit impaired glucose tolerance [208], but have normal muscle and adipose tissue insulin-stimulated glucose uptake [209]. Therefore, despite positive data from cells expressing *Sod2*, data supporting a positive effect on insulin sensitivity in muscle and fat tissue *in vivo* are lacking. It is also important to note that Lee *et al* [211] present evidence that mCAT overexpression protection from high fat diet-induced insulin resistance may be mediated by changes in mitochondrial oxygen consumption and lipid accumulation in muscle, rather than by H_2O_2 scavenging directly. This highlights that there is likely substantial cross talk between distinct putative drivers of insulin resistance, and that there can be difficulties in attributing the effects in insulin sensitivity observed with long term mCAT or *Sod2* expression or gene knockout directly to changes in reactive oxygen species.

Together, the genetic and pharmacological data described here provide reasonably strong proof–of-principal evidence for a role for mitochondrial reactive oxygen species in the development of insulin resistance in muscle and adipose tissues (see **Fig. 3** for a schematic outlining how mitochondrial ROS may affect insulin signaling). We note that it is likely that this may occur in other metabolic tissues, including liver, pancreatic β-cells and the endothelium (reviewed in [215]), where redox mechanisms have been associated with insulin resistance and non-alcoholic fatty liver disease [216, 217], endothelial dysfunction ([218, 219]) and cytokine-mediated toxicity [220-223]. What can these data, in combination with the measures of reactive oxygen species, tell us about which species is the most detrimental for insulin resistance? Focusing on genetic data, both mCAT and *Sod2* overexpression seem to benefit

insulin responses, at least in cell culture models, despite having opposing effects on H_2O_2. However, data from clamp studies in mice that show the benefit of mCAT, but not of *Sod2* or double *Sod2* and mCAT overexpression [212], suggest that H_2O_2 is most likely to be the causal species *in vivo*. While we currently do not have appropriate tools available to measure H_2O_2 accurately and quantitatively in complex biological samples, a casual role of H_2O_2 is supported by genetic models of lowered mitochondrial antioxidant activity where mice lacking Prdx3 (lower H_2O_2 scavenging) are insulin resistant [224] (as measured by ITT) [208, 209]. This conclusion is somewhat consistent with data presented from methods to measure redox status described above, although these assays have in general not been performed in a way that can distinguish different reactive species. It is reasonable to suggest that given the reactivities of reactive oxygen species and the antioxidant systems in place, combinations of redox methodologies are required to start understanding which reactive oxygen species drive insulin resistance. Indeed, there is room for studies to adopt best practice methods from both the redox and insulin resistance fields to interrogate the role of reactive oxygen species in muscle and adipose insulin resistance. This includes using methods than can specifically measure distinct reactive species, preferably *in vivo*, in combination with methods that specifically measure muscle and/or adipose insulins sensitivity.

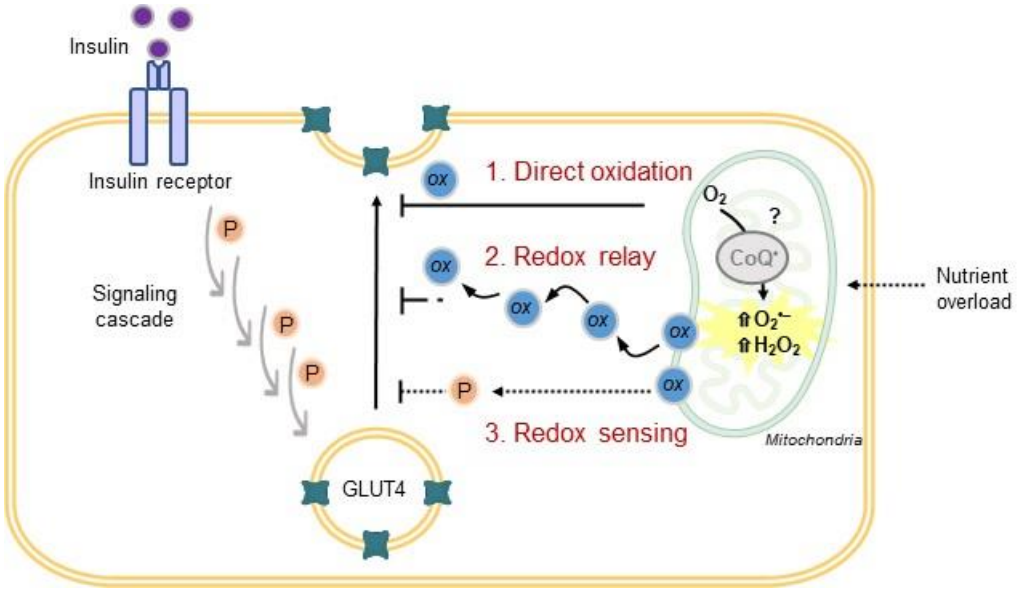

Fig. 3. Linking mitochondrial reactive oxygen species generation and insulin resistance. Nutrient overload (*e.g.*, high-fat diet feeding) leads to the generation of reactive species such as $O_2^{\bullet-}$ and H_2O_2 though the exact mechanism underpinning this is still unknown (denoted by ?). Data suggests that increased $O_2^{\bullet-}$ and H_2O_2 lead to changes in cytoplasmic insulin signaling, most likely through alterations in GLUT4 signaling and trafficking resulting in a blunted insulin response. This may be mediated through direct reaction of reactive oxygen species with GLUT4 (1), though it is likely that mitochondrial reactive species either initiate a complex redox signaling cascade (2) or initiate a redox sensing pathway that leads to changes, *e.g.*, post-translational modifications like phosphorylation (3) that promote changes in cytoplasmic insulin signaling. Abbreviations: $CoQ^{\bullet}$, semiquinone radical; H_2O_2, hydrogen peroxide; O_2, molecular oxygen; $O_2^{\bullet-}$, superoxide radical anion; ox, oxidation; P, phosphorylation.

An emerging role for subcellular CoQ content in linking mitochondrial reactive oxygen species and insulin resistance

Another outstanding question is what drives increased oxidants in insulin resistance. It may not be unexpected that a positive energy balance (*i.e.*, obesity) is associated with increased reactive species because higher $NADH/NAD^+$ or $FADH_2/FAD^+$ ratios under these conditions can promote reactive oxygen species formation via mechanisms such as RET, as discussed above [62]. Alternatively, the specific substrate oxidized in mitochondria may be important. Fatty acid oxidation is more prone to reactive oxygen species production than other carbon sources [225], providing a possible link between high fat intake and increased reactive oxygen species. While these are possibilities, there are lines of evidence that suggests that there are changes to mitochondria that support grater reactive oxygen species production/less detoxification in insulin resistance. For example, isolated mitochondria from insulin resistant tissues assayed in *ex vivo* exhibited greater H_2O_2 release (as measured by Amplex Red-derived fluorescence) in response to the same substrate conditions as mitochondria from more insulin sensitive tissues [42]. These data suggest that there are intrinsic changes to mitochondria themselves that make them more prone to generate reactive oxygen species. Recent studies have indicated that mitochondrial CoQ may play a role in reactive oxygen species production in adipose and muscle insulin resistance. As described above, CoQ plays a critical role in electron transfer from Complex I and II to Complex III. Studies in humans, mice and cultured cells revealed that insulin resistance was associated with decreased mitochondrial CoQ content [44]. Lowering mitochondrial CoQ was associated with greater Prdx3 dimerization, placing loss of CoQ in mitochondria upstream of increased H_2O_2 in mitochondria [44]. Indeed, restoration of mitochondrial CoQ, by providing high doses of CoQ via intraperitoneal injection, lowered Prdx3 dimerization and improved insulin sensitivity[44]. Intriguingly, the loss of CoQ was specific to mitochondria, implicating this sub-cellular pool of CoQ in insulin resistance. Until recently, the mechanisms controlling CoQ subcellular distribution remained largely obscure. However, Kemmerer and colleagues [226] identified two proteins in yeast, named Cqd1 and Cqd2 that appear to be critical for correct CoQ localization. It will be if interest to find out whether the mammalian homologues of these proteins exhibit altered expression in insulin resistant muscle and adipose tissues. More recently, the mitochondrial methylation capacity, assessed by the *S*-adenosylmethionine to *S*-adenosylhomocysteine (SAM-to-SAH) ratio, was identified as a major determinant of mitochondrial CoQ content. As with exogenous CoQ, experimental manipulation of the SAM-to-SAH in mitochondria to increase mitochondrial CoQ improved insulin sensitivity in adipocytes [227]. Together, these studies place the subcellular localization and mitochondrial content of CoQ, rather than 'whole cell' CoQ, as a determinant of muscle and adipose tissue mitochondrial reactive oxygen species and insulin sensitivity.

How loss of CoQ confers increased mitochondria reactive oxygen species and insulin resistance is a pertinent question. Given the role CoQ plays in electron transport and as a lipid antioxidant, it is feasible that either or both increased mitochondrial oxidants and/or mitochondrial lipid peroxidation may link loss of CoQ to insulin resistance. As mentioned above, specific peroxidation of mitochondrial

membrane lipids is largely understudied, especially in the context of insulin resistance. However, data on Prdx3 dimer/monomer ratios cells and adipose tissue suggest that decreased mitochondrial CoQ results in increased mitochondrial H_2O_2 [44]. This is overall supported by a range of studies reporting modest losses of CoQ inducing increases in mitochondrial reactive oxygen species [228-232] and that experimentally increasing mitochondrial CoQ decreases mitochondrial $O_2^{\bullet-}$ [227]. Although the precise mechanism for how CoQ depletion results in increased reactive oxygen species remains unknown, aberrant electron flux along the respiratory chain likely plays a central role. It has been previously proposed that high rates of electron flux from lipid metabolism (*e.g.*, high fat diet feeding) into the CoQ pool could increase $O_2^{\bullet-}$ production at Complex I or III due to decreased availability of oxidized CoQ to act as an electron acceptor, or RET [40]. However, no changes in CoQ redox state were observed [44]. How could more oxidants be produced in a situation where the $CoQH_2$/CoQ ratio remains unaltered, yet the pool of total CoQ is decreased? Recently it has been suggested that the lowering of the total CoQ pool could lower the threshold for electron leak and subsequent H_2O_2 production [7]. Alternatively, it may be related to the specifics of Complex II electron flux. Whereas electron transfer from complex I to CoQ is limited by the energetic constraints determined by the membrane potential, electron transfer from flavoproteins in complex II to CoQ is not. This means that electrons can be transferred to CoQ, provided oxidized CoQ is available to receive them. Therefore, decreasing the absolute amount of oxidized CoQ available to accept electrons at complex II may increase oxidant production (**Fig. 4**). However, given the central role of CoQ in mitochondrial electron transfer it remains likely that decreasing CoQ acts on several sites along the respiratory chain to increase oxidant production. This is supported by inhibition of complex II by thenoyltrifluoroacetone or malonate only partially rescuing Prdx3 dimerization [44].

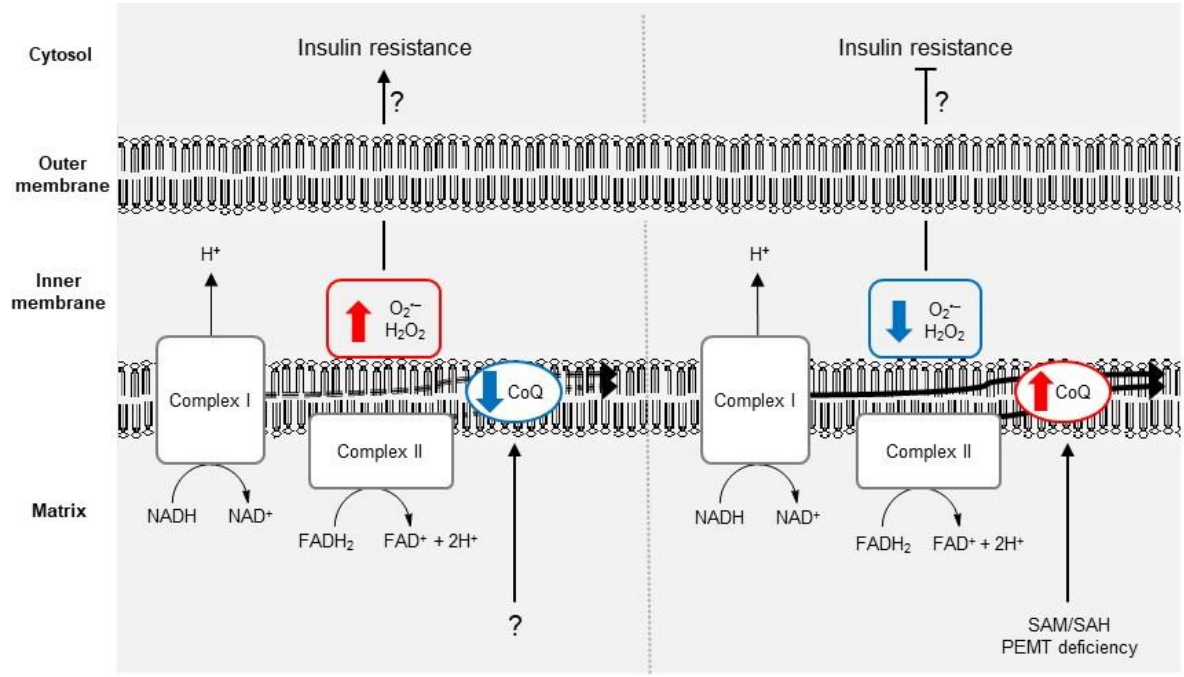

Fig. 4. CoQ links mitochondrial reactive species generation and insulin resistance. Recent evidence has demonstrated that decreased mitochondrial CoQ (left hand panel) leads to insulin resistance through the increased production of reactive oxygen species at Complex II [44]. While this process, including how CoQ decreases (denoted by ?), is not fully understood, altered electron flux through the respiratory chain appears to be a central element. How an increase in mitochondrial $O_2^{\bullet-}$ or H_2O_2 leads to insulin resistance also is currently unknown. Modulation of CoQ regulators such as phosphatidylethanolamine transferase (PEMT) or the mitochondrial *S*-adenosylmethionine to *S*-adenosylhomocysteine (SAM-to-SAH) ratio increases mitochondrial CoQ (right hand panel) leading to a decrease in $O_2^{\bullet-}$ or H_2O_2 and maintenance of insulin sensitivity [227].

Very little is known about the factors that regulate CoQ concentrations in cells. A recent study identified a previously unrecognized set of genetic regulators of CoQ content, *e.g.,* phosphatidylethanolamine methyltransferase (PEMT) [227]. PEMT deficiency increased mitochondrial CoQ through modulation of the mitochondrial SAM-SAH ratio and prevented insulin resistance. While PEMT is not a suitable therapeutic candidate as loss of PEMT results in non-alcoholic steatohepatitis [233], the study does provide proof-of-concept that modulation of endogenous CoQ regulators can increase mitochondrial CoQ, decrease mitochondrial reactive oxygen species production and ameliorate insulin resistance. Increased understanding of regulatory pathways could increase the potential new methods to modulate CoQ in experimental systems, which is a significant challenge as methods to alter CoQ *in vivo* are currently lacking. In addition, identification of what initiates CoQ depletion may be an interesting strategy to target the CoQ pathway in insulin resistance.

What about brown adipose tissue?

The data from adipose tissue discussed so far is from visceral or subcutaneous adipose depots. Brown adipose tissue (BAT) is highly vascularized and comprises adipocytes that are morphologically distinct from those in white adipose tissue. Brown adipocytes contain multiple small lipid droplets rather than one large lipid droplet, high numbers of mitochondria, and high expression of uncoupling protein-1 (UCP-1) in the inner mitochondrial membrane. In addition to classical brown adipocytes, brown-like cells termed 'beige' or 'brite' adipocytes, can form under certain circumstances in white depots, a process referred to as 'browning'. When UCP-1 is activated, the proton-motive force is uncoupled from ATP synthesis resulting in increased respiration and non-shivering thermogenesis, which is the primary role of BAT. Since brown and brown-like adipocytes can dissipate energy, they have garnered substantial interest as a potential target for treating obesity. This was accelerated by the fist description of functional BAT in human adults in 2009 [234-238]. Indeed, there is accumulating evidence that higher BAT activity promotes leanness and improves whole body glucose metabolism and insulin sensitivity likely through increased energy expenditure [239-242] (see [12] for a recent review).

There is significant evidence supporting a physiological role for mitochondrial reactive species, particularly mitochondrial $O_2^{\bullet-}$ in supporting UCP-1-dependent thermogenesis *in vivo* (reviewed in [243]). For example, mitochondrial $O_2^{\bullet-}$ and H_2O_2 were elevated during acute activation of BAT thermogenesis, MitoQ administration inhibited BAT-mediated thermogenic respiration [116], and adipose-specific deletion of *Sod2* elevated $O_2^{\bullet-}$ and increased thermogenesis without paralleled

increases in oxidative adducts [244]. However, the type and location of reactive oxygen species are likely crucial for proper activation of thermogenesis. For example, knockout of genes in NADPH production such as mitochondrial isocitrate dehydrogenase [245] or glucose-6-phosphate dehydrogenase [246], or of CD36 which is required for CoQ uptake into BAT [247], increased oxidants and led to defective thermogenesis.

BAT glucose uptake is responsive to both insulin and norepinephrine, which activate glucose transport primarily via GLUT4 and GLUT1, respectively [248]. BAT also exhibits insulin resistance in response to high fat diet feeding [249], but the specific role of mitochondrial reactive oxygen species in BAT insulin resistance has not been well studied. Interestingly, despite the apparent requirement for mitochondrial reactive species for thermogenesis, activated BAT retains insulin responses to sustain thermogenesis [250]. As such, understanding the relationship between mitochondrial reactive oxygen species, thermogenesis and insulin action in BAT may be informative in understanding the types and location of the reactive species that underlie insulin resistance in other adipose depots and muscle tissues.

Summary and future directions

How could one establish more firmly/directly the relationship between mitochondrial reactive oxygen species and insulin resistance?

There is strong experimental evidence from various models associating mitochondria-derived reactive oxygen species with insulin resistance *in vivo*. There is also reasonable evidence for a causal role for mitochondrial reactive oxygen species in insulin resistance, although more work is needed to establish this exact causal mechanism(s) (See **Fig. 5**). The questions remaining to be answered include the source, chemical nature and molecular target(s) of the oxidant(s), and how this 'signals' to the insulin-GLUT4 pathways in the cytoplasm of muscle and fat cells. Unlike the present situation in insulin resistance, such knowledge is available for other scenarios of redox signaling in pathophysiology. For example, in the setting of blood pressure regulation in inflammation, the chemical nature of the oxidant involved, how and where it is formed, its molecular target (down to the level of amino acid residue affected) and the ensuing mechanism by which the oxidized target protein modifies blood pressure have been reported recently [251]. This knowledge allowed modification and study of this redox pathway in a highly specific manner (*e.g.*, by mutating the amino acid residue in the target protein and studying specific protein modifications) *in vivo*. The current lack of such detailed information provides a substantial barrier to our understanding of the molecular basis of insulin resistance.

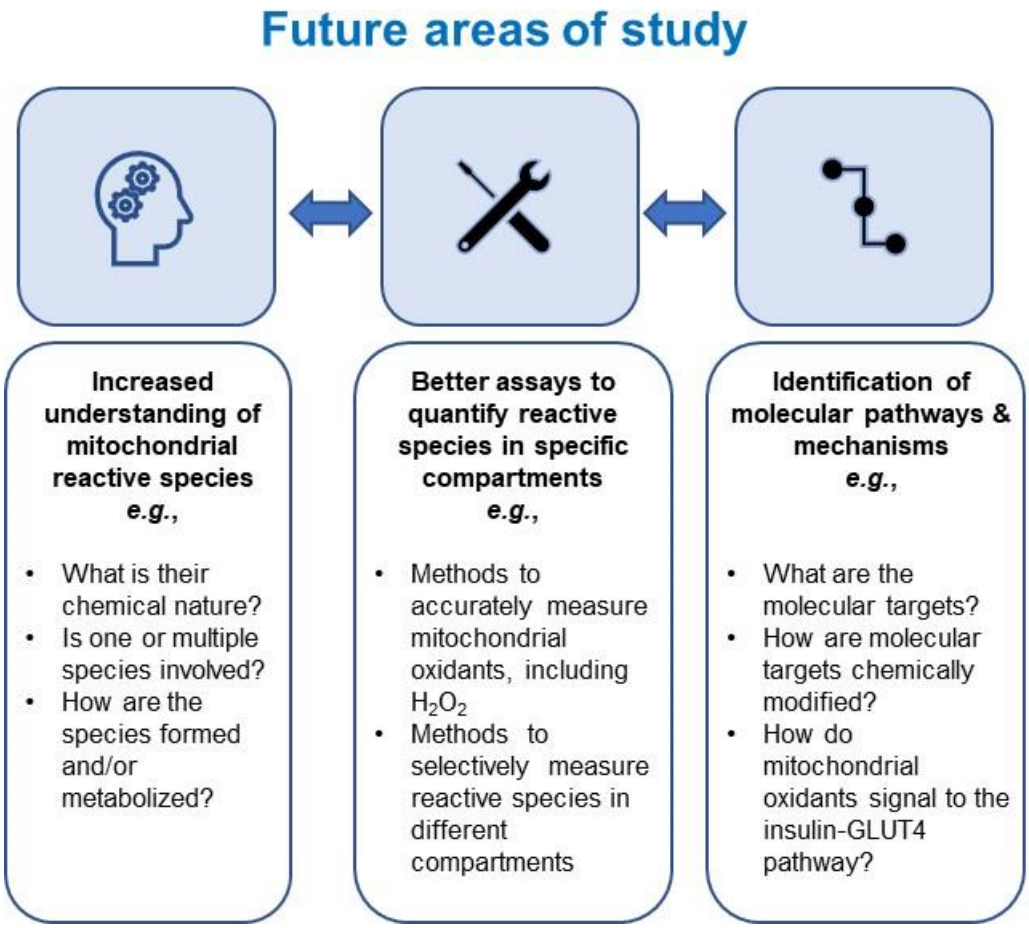

Fig. 5. Outstanding issue to be addressed to uncover the exact causal mechanisms between mitochondrial reactive oxygen species and insulin resistance.

To answer the initial questions about the exact causal reactive species, we suggest that new methods may be required to measure of species of interest, in specific cellular compartments, in models relevant to insulin resistance (*e.g.*, *in vivo*). This would be aided researchers in metabolism and redox biochemistry working in collaboration to develop new, practicable technologies that may facilitate stronger research outcomes. A major technological limitation remains the accurate determination of H_2O_2, especially in a compartmentalized manner. Current methodological challenges result in indirect techniques such as redox proteomics being the mainstay of attempts to attribute a specific function to H_2O_2 in the pathogenesis of insulin resistance. In a similar vein, significant contributor to the lack of clarity regarding the role mitochondrial reactive oxygen species play in insulin resistance is that current methods to measure these species have limited *in vivo* application. New strategies to image and monitor reactive oxygen species and redox modifications *in vivo* should help resolve some outstanding issues. Transgenic animals expressing sub-compartmental localized redox probes are an exciting tool that allow real time monitoring of redox state with sub-cellular resolution *in vivo*. These models may provide insight in the spatial and temporal generation of reactive oxygen species and potential differences in mitochondrial redox dynamics across models of insulin resistance but are yet to be used in pre-clinical insulin resistance studies. Other examples of new methods that could be adopted to study the relationship between reactive oxygen species and insulin resistance *in vivo* include positron electron

transmission (PET) or biomedical Overhauser Magnetic Resonance Imaging (OMRI). PET radiotracers based on ethidium have been synthesized for $O_2^{\bullet-}$ imaging *in vivo* [159, 160], and OMRI combined with a nitroxyl contrast agent has been used to study brain redox changes in models of Parkinson's disease [252]. Imaging mass spectrometry is another exciting analytical method with potential for use to study redox modifications. Imaging mass spectrometry allows the study of the topography of proteins and lipids, with secondary-ion imaging mass spectrometry capable of imaging at sub-cellular resolution. This technique has been used to visualize CoQ localization in the mouse brain [253] and imaging of cardiolipin molecular species in brain tissue [254] with direct imaging of oxidized lipids suggested as likely in the future [255]. While still in their infancy, the development of newer technologies that allow for imaging and monitoring of redox state represent exciting new avenues for the way reactive oxygen species are studies in complex systems in both physiology and disease. Adopting such techniques will be critical to revealing the true relationship between reactive oxygen species and insulin action in tissues *in vivo*.

Identifying the signaling pathway that links mitochondria to the insulin-GLUT4 pathway in the cytoplasm is critical to establishing how mitochondrial reactive oxygen cause insulin resistance (**Fig. 5**). For example, is this a direct pathway from reactive oxygen species to oxidation of a protein involved in GLUT4 traffic (*e.g.*, oxidation of GLUT4 [195])? Rather, are mitochondrial reactive oxygen species sensed and indirectly signaled to regulate GLUT4?, for example via protein phosphorylation [117] or other mitochondria-cytosol signaling pathways (*e.g.*, [118-120]). This concept is supported by the changes in Prdx3 redox state observed in response to mitochondrial CoQ content [44], which indicate altered H_2O_2 concentrations in the mitochondrial matrix. Whether this is also associated with H_2O_2 outside mitochondria is not known and, at face value, these data suggest the involvement of mitochondrial pathways in mediating insulin resistance. One possibility is that this involves opening of the mitochondrial permeability transition pore, supported by the protection from insulin resistance conferred by blocking this channel [256]. Regardless, there remains an ill-defined pathway that communicates from mitochondria to mediators of insulin action in the cytoplasm. We have two some clues as to the nature of this pathway and its target. First, the induction of reactive oxygen species in mitochondria can rapidly induce insulin resistance (*e.g.*, within 30-120 min), suggesting a post-translational mechanism [45, 208], rather than via previously reported transcriptional mechanisms [257], *e.g.*, Nrf2 [258] or the FOXO family [259]. Further, mito-paraquat induced insulin resistance was only observed if mito-paraquat treatment was administered prior to insulin stimulation [213]. This suggests that mitochondrial reactive oxygen species (or their effects) likely target insulin signaling or GLUT4 trafficking processes that are required for the initial GLUT4 response to insulin (*i.e.*, GLUT4 release from intracellular sequestration), rather than those required to maintain the insulin response. Overall, studies into how mitochondrial reactive oxygen species impinge on GLUT4 traffic are made more challenging by the lack of knowledge about how insulin signaling to GLUT4 translocation is impaired in insulin resistance, preventing targeted approaches. Therefore, unbiased approaches that can

map protein modifications such as oxidation and phosphorylation across the proteome may be more appropriate (see *e.g.*, [260]). A deeper understanding of the link between mitochondrial reactive oxygen species and insulin resistance may provide a framework and impetus to find the exact site of the lesion in the insulin-GLUT4 pathway that causes insulin resistance.

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Table 1. Surrogate measures for assessing mitochondrial oxidants and changes in mitochondrial redox state

Assay	Experimental technique	Readout	Notes
Prdx3 redox state	Redox Western blotting assessing the ratio of oxidized and reduced Prdx3	Changes in steady state mitochondrial peroxide concentrations	● Can be done in whole tissue/cells as Prdx3 (mitochondrial isoform) can be detected using specific antibodies
Protein carbonylation	Immunoblot using Oxyblot	Protein carbonylation content	● Commercially available assay system ● Isolated mitochondria must be used to make the assay specific to mitochondria ● Overall readout provided rather than changes in individual proteins
'Redox' gene expression	qPCR analyses of mitochondrial genes involved in oxidant production and redox homeostasis	Changes in gene expression	● Can be done on whole/cell tissues. mRNA changes do not necessarily translate to changes in protein expression or enzyme activity ● Not all genes of interest may be transcriptionally regulated ● Many genes can be analyzed simultaneously
'Redox' protein content	Analyses of mitochondrial proteins involved in oxidant production and redox homeostasis (*e.g.*, Western blotting, proteomics)	Changes in protein expression	● Can be done on whole/cell tissues. Protein changes do only always affect changes enzyme activity ● Mass spectrometry-based proteomics facilitates analyses of multiple changes in multiple proteins across cellular compartments
Aconitase inactivation	Biochemical assay measuring aconitase activity by spectroscopy [261]	Aconitase activity. Ratio of active and inactive aconitase	● Can be done on whole tissue or cells, as aconitase is localized to mitochondria ● Surrogate for $O_2^{\bullet-}$, as aconitase is susceptible to oxidative inactivation by $O_2^{\bullet-}$
Cytochrome *c* reduction	Biochemical assay following cytochrome *c* reduction at 550-540 nm [262, 263]	Reduction of ferricytochrome *c*	● Read out for $O_2^{\bullet-}$ production ● Acetylation of cytochrome c increases specificity for $O_2^{\bullet-}$

			• Addition of catalase to minimize reoxidation of reduced cytochrome *c* by H_2O_2 [264] • SOD controls recommended
SOD activity	Biochemical assay, commonly following loss of NADPH at 340 nm (reviewed in [265])	SOD activity	• Can be used on whole tissue or cells as isoforms of MnSOD can be easily distinguished using NaCN inactivation of Cu,ZnSOD
	Activity gels		• Can be used on whole tissue or cells as isoforms of SOD can be easily distinguished by SDS-PAGE migration patterns
Mitochondrial DNA (mtDNA) oxidation	qPCR assay of mtDNA damage [266]	mtDNA lesion frequency	• Whole tissue/cell DNA can be used as primers used are specific for mtDNA • Damage to mitochondrial and nuclear DNA can be assayed simultaneously • Not all forms of oxidative DNA damage are detected • Not possible to uncover the type of DNA lesion
Mitochondrial oxygen consumption	Oxygen consumption measured via polagraphy, SeaHorse or Oroboros respirometers	Oxygen consumption measured in various respiration states	• Can be done in a variety of samples and species (*e.g.*, whole cells, permeabilized cells/tissues, frozen tissue, isolated mitochondria) • Respiratory complex substrate-inhibitor systems can be used to probe selected components of the electron transport chain
Mitochondrial integrity	Fluorometric membrane potential measurements (*e.g.*, JC-1) or apoptosis assays	Changes in membrane potential or apoptosis	• Can occur independently of oxidant production

Table 2. Modulation of mitochondrial redox state by increasing oxidant production

Genetic inhibition mitochondrial protein expression	
Animal models with altered mitochondrial protein expression	● Allows *in vivo* study of the effect of the loss of mitochondrial enzymes in a tissue-specific manner. For mouse models see (*Prdx3*$^{-/-}$ [224], *Sod2*$^{+/-}$ [100, 101], succinate dehydrogenase mutations; [267]) ● Amenable to a range of interventions related to insulin resistance (*e.g.*, high-fat diet, streptozotocin treatment, exercise). ● Amenable to a range of insulin resistance related assays (glucose uptake, euglycemic clamp, metabolic cage) ● Effect of gene deletion may lead to upregulation of compensatory pathways which may obscure findings ● Animal models can be created relatively rapidly and selectively with CRISPR technology
Deletion of mitochondrial proteins in stable-cell lines	● Can be generated in a wide variety of cells ● Time-consuming to construct ● Effect of gene deletion may lead to upregulation of compensatory pathways which may obscure findings
Transient gene silencing of mitochondrial enzymes involved in oxidant scavenging	● Various genetic strategies can be used (small interfering RNA, antisense-oligonucleotides, short hairpin RNA, adenoviral delivery, etc.) ● Amenable to a range of interventions related to insulin resistance (*e.g.*, glucose uptake). ● Can be done in cells and animals relatively simply ● Appropriate scrambled/vector only controls should be used ● Effect of gene deletion may lead to upregulation of compensatory pathways which may obscure findings
Pharmacological inhibition of mitochondrial redox processes	
ETC inhibitors	● Complex I-III inhibitors [268], mitochondrial DNA transcription [269] ● Will affect respiration and this can confound interpretation of results
Mito-Paraquat (Mito-PQ)	● TPP cation conjugated to the redox cycler paraquat ● Commercially available ● Increases mitochondrial $O_2^{\bullet-}$ in isolated mitochondria and cells ● MitoPQ produces $O_2^{\bullet-}$ by redox cycling at the flavin site of complex I

	• Experimentally validated in isolated tissue and cultured cells [270] • Accumulation in mitochondria affected by mitochondrial membrane potential
Mito-1-chloro-2,4-dinitrobenzene	• TPP cation conjugated to 1-chloro-2,4-dinitrobenzene • Commercially available • Depletes mitochondrial GSH, catalyzed by glutathione *S*-transferases, and indirectly inhibits mitochondrial TrxR2 and Prdx3 [271] • Can have off-target effects such as inhibition iron-sulfur containing dehydrogenases • Accumulation in mitochondria affected by mitochondrial membrane potential

Table 3. Modulation of mitochondrial redox state using mitochondrially-targeted antioxidant strategies

Genetic inhibition mitochondrial protein expression	
Mouse model with ectopic expression of human catalase in mitochondria (MCAT) [272]	● Allows *in vivo* study of the effect of increased mitochondrial H_2O_2 scavenging ● Amenable to a range of interventions related to insulin resistance (*e.g.*, high-fat diet, streptozotocin treatment, exercise). ● Amenable to a range of *in vivo* assays related to insulin resistance (glucose uptake, euglycemic clamp, metabolic cage) ● Tissue-specific MCAT expression (for heart and skeletal muscle) ● Permanent gene overexpression (*i.e.*, stable cell lines) may lead to upregulation of compensatory pathways which may obscure findings ● Transgenic mice with peroxisome- and nucleus-targeted catalase can be used as controls [272]
Overexpression of mitochondrial antioxidant enzymes	● Various genetic strategies can be used (*e.g.*, plasmids, adenoviral delivery) ● Amenable to a range of interventions related to insulin resistance (*e.g.*, glucose uptake). ● Can be done in cells and animals relatively simply ● Appropriate scrambled/vector only controls should be used ● Permanent gene overexpression (*i.e.*, stable cell lines) may lead to upregulation of compensatory pathways which may obscure findings/confound interpretation of results
Pharmacological inhibition of mitochondrial redox processes	
MitoQ [176]	● Extensively used in biological systems ● Possibility exists that it acts as a pro-oxidant under some conditions ● Reduced MitoQ has the potential to react with several different oxidants, making conclusions on which oxidant(s) are involved difficult ● Accumulation in mitochondria affected by mitochondrial membrane potential
Inhibitors of ETC-derived $O_2^{\bullet-}/H_2O_2$	● Specific sites along the ETC can be targeted to attenuate oxidant production at Complex I and III [268, 273] ● Suppress oxidant generation during reverse electron transport ● Do not affect oxidative phosphorylation
MitoC [274]	● TPP cation conjugated to ascorbate

	● MitoC reacts with a range of reactive species, and is rapidly recycled to the antioxidant active form by the glutathione and thioredoxin systems ● Broad acting therefore it is difficult to identify specific biological process affected by MitoC ● Accumulation in mitochondria affected by mitochondrial membrane potential
SkQ1 [175]	● Plastoquinone conjugated to a "Skulachev ion" which promotes mitochondrial accumulation ● Can be administered *in vivo* for long periods of time without toxicity ● Eyedrops containing SkQ1 approved for use in Russia and passed Phase II trials in the USA ● Broad acting therefore it is difficult to identify specific biological process affected by SkQ1 treatment
Szeto-Schiller peptides [275] (*e.g.* SS-31)	● Water soluble and accumulates in inner mitochondrial membrane ● Interaction with cardiolipin ● Mitochondrial accumulation not dependent on mitochondrial membrane potential ● Evidence for decreasing H_2O_2 and inhibiting lipid oxidation *in vitro* ● Broad acting therefore it is difficult to identify specific biological process affected by peptide treatment
Mitochondrially-targeted vitamin E analogues	● Several probes developed, such as Mito-ChM [276], Mito-Vit E/TPPB [277] and Mito-VES [278], based on conjugation of vitamin E analogues to a TPP moiety ● Broad acting therefore it is difficult to identify specific biological process affected ● Possibility exists that it may act as a pro-oxidant under some conditions ● Accumulation in mitochondria affected by mitochondrial membrane potential
Mito-TEMPOL [279]	● Nitroxide (TEMPOL) conjugated to a TPP moiety ● Commercially available ● Mito-TEMPO and its reduced form (Mito-TEMPOL-H) are thought to exert antioxidant effects ● Can be administered *in vitro* and *in vivo* ● Broad acting therefore it is difficult to identify specific biological process affected by Mito-TEMPOL ● Accumulation in mitochondria affected by mitochondrial membrane potential

Table 4: Human studies investigating a link between mitochondrial reactive oxygen species and insulin resistance

Study	System(s) used	Tissue	Mitochondrial intervention	Mitochondrial assay(s)	Cellular/tissue assay(s)	Insulin Resistance assay(s)
[42]	● Human male cohort ○ Lean ○ Morbidly obese ● Rat ○ Chow ○ Lard ○ HFD ● GM mice	● Skeletal muscle	● mCAT (skeletal muscle and cardiac tissue) ● Mito-targeted antioxidant supplementation (SS31)	● Mito H_2O_2 release by Amplex Red fluorescence	● Total GSH/GSSG	● OGTT ● Hyperinsulinemic-euglycemic clamp ● Phospho-Akt
[194]	● Human male cohort ○ Lean healthy ○ Obese non-diabetic ○ Type 2 diabetic	● Skeletal muscle		● Mito H_2O_2 release by Amplex Red fluorescence		● Hyperinsulinemic-euglycemic clamp
[197]	● Human female non-menopausal cohort ○ Insulin sensitive ○ Insulin resistant	● Skeletal muscle		● Mito H_2O_2 release by Amplex Red fluorescence		● HOMA-IR
[196]	● Human male cohort ○ Healthy ○ 7 d bed rest	● Skeletal muscle		● Mito H_2O_2 release by Amplex Red fluorescence	● 4-HNE ● Total GSH/GSSG	● Hyperinsulinemic-euglycemic clamp

Abbreviations: 4-HNE, 4-hydroxynonenal; GM, genetically modified; GSH, reduced glutathione; GSSG, oxidized glutathione, HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; MCAT, mitochondria-targeted human catalase; mito, mitochondria; OGTT, oral glucose tolerance test.

Table 5: Pre-clinical studies investigating mitochondrial reactive oxygen species and insulin resistance in skeletal muscle

Study	System(s) used	Intervention	Assay(s)	Cellular/tissue assay(s)	Insulin resistance assay(s)

[202]	• GM mice
	  ○ MCAT
	  ○ Old vs young
		• Ectopic mCAT expression	• Mitochondrial H_2O_2 assessed by DCF-derived fluorescence in presence of horseradish peroxidase
			• Mitochondrial protein carbonylation
			• *Sod2* expression
				• Gpx1 expression
				• Protein carbonylation
					• 2DOG addition at end of hyper-insulinemic euglycemic clamp
					• Akt activity assay

[280]	• Rats
	  ○ Chow vs HFD
	  ○ Sedentary vs exercise
			• Mitochondrial H_2O_2 efflux assessed by Amplex Red-derived fluorescence
			• TnxRd2 content
				• qPCR of 'redox' genes
				• Total GSH/GSSG
				• 4-HNE modified adducts
					• HOMA-IR

[204]	• WT mice ○ Chow vs HFD • C2C12 muscle cells	• Treatment with mitochondria-targeted antioxidant (Skulachev ion)	• SOD2 enzyme assay • H_2O_2 efflux by isolated mitochondria assessed by Amplex Red-derived fluorescence	• Protein carbonylation blots • GPX1 assay	• 2DOG uptake in C2C12 cells • *i.p.* GTT • Phospho-Akt

[211]	• GM mice
	  ○ mCAT
	  ○ Chow vs HFD vs acute lipid infusion
	• Mouse derived embryonic fibroblasts (mCAT)
		• Ectopic mCAT expression	• H_2O_2 efflux by isolated mitochondria assessed by Amplex Red-derived fluorescence		• 2DOG addition at end of hyper-insulinemic euglycemic clamp
					• Phospho-Akt
					• PKCθ translocation

[203]	• GM mice
	  ○ MCK-PPARα
	  ○ Lipin-1 deficient mice
	  ○ mCAT Mice
		• Ectopic mCAT expression	• Mitochondrial H_2O_2 efflux assessed by Amplex Red-derived fluorescence
			• Prdx3 redox blot
				• Total GSH/GSSG
				• Prdx2 redox blot
					• *i.p.* GTT
					• *ex vivo* 2DOG skeletal muscle uptake

	• Phospho-Akt • Phospho-TBC1D4

Abbreviations: 2DOG, 2-deoxyglucose; DCF, dichlorofluorescein; GM, genetically modified; GSH, reduced glutathione; GSSG, oxidized glutathione; HFD, high-fat diet, 4-HNE, 4-hydroxynonenal; HOMA-IR, homeostatic model assessment of insulin resistance; *i.p.* GTT, intraperitoneal glucose tolerance test; MCAT, mitochondria-targeted human catalase; OGTT, oral glucose tolerance test; SOD2, manganese superoxide dismutase (Mn-SOD); TnxRd2, thioredoxin reductase 2.

Table 6: Pre-clinical studies investigating mitochondrial reactive oxygen species and insulin resistance in adipose tissue

Study	System(s) used	Intervention	Assay(s)	Cellular/tissue assay(s)	Insulin resistance assay(s)

[37]	• GM mice
	  ○ *Ob/Ob*
	• 3T3-L1 adipocytes
	  ○ TNFα
	  ○ Dexamethasone
		• MnTBAP treatment (SOD mimetic)
		• Ectopic mCAT expression
		• *SOD2* overexpression
				• Microarray for redox gene expression
				• DCF-derived fluorescence
				• Protein carbonylation
				• Overexpression of cytosolic catalase
				• Overexpression of SOD1
				• *N*-acetylcysteine treatment
					• 2DOG uptake
					• phospho-Akt
					• Subcutaneous GTT
					• Subcutaneous ITT

[208]	• GM mice
	  ○ *SOD2* overexpressing
	  ○ $SOD2^{+/-}$
	• 3T3-L1 adipocytes
	  ○ TNFα
	  ○ Chronic insulin
	  ○ Palmitate
	  ○ Dexamethasone
	• L6 myotubes overexpressing HA-GLUT4
		• *SOD2* overexpression
		• SOD mimetic treatment (mito-TEMPO, MnTMPyP and MnTBAP)
		• Antimycin A treatment
			• $O_2^{\bullet-}$ assessed by mitoSOX-derived fluorescence
			• Amplex Red-derived fluorescence in isolated mitochondria
					• Plasma membrane GLUT4 translocation (cell culture)
					• Phospho-Akt
					• GTT
					• 2DOG uptake into adipose tissue and muscle during GTT

[205]	• WT mice
	  ○ Chow vs HFD
	• GM mice
	  ○ mCAT
		• Ectopic mCAT expression	• Mitochondrial H_2O_2 efflux assessed by Amplex Red-derived fluorescence	• Catalase protein expression
				• SOD1 protein expression
				• 4-HNE
				• Protein carbonylation
					• *i.v.* ITT
					• HOMA-IR
					• Phospho-Akt

[281]	• Human visceral adipose
	  ○ Morbidly obese vs nonobese
	• Rat
	  ○ Control vs HFD
		• MitoQ administration		• 4-HNE
				• Total Prdx4
					• Phospho-IRS-1

	• 3T3-L1 adipocytes ○ TNFα				

[282]	• Primary human myotubes
	  ○ Oleate
	  ○ Palmitate
			• $O_2^{\bullet-}$ assessed by mitoSOX-derived fluorescence	• ER stress by PERK phosphorylation	• Insulin stimulated 2DOG uptake
					• Phospho-Akt
					• Phospho-TBC1D4

[45]	• WT mice
	  ○ Chow vs HFD
	• 3T3-L1 adipocytes
	  ○ TNFα
	  ○ Chronic insulin
	• L6 myotubes overexpressing HA-GLUT4
		• Mito-paraquat treatment	• Prdx2 and Prdx3 redox blot
			• $O_2^{\bullet-}$ assessed by mitoSOX using LC-MS/MS for specific determination of 2-hydroxy-mitoSOX
					• 2DOG uptake (cells)
					• Plasma membrane GLUT4 translocation (cells)
					• 2DOG uptake in tissue explants
					• Phospho-Akt
					• Phospho-TBC1D4

[213]	• 3T3-L1 adipocytes • Mature primary adipocytes	• Treatment with Mito-paraquat	• Mito-roGFP fluorescence • Prdx3 redox blot	• Prdx2 redox blot • Auranofin treatment	• 2DOG uptake • Plasma membrane GLUT4 translocation • Phospho-Akt • Phospho-TBC1D4
[207]	• WT mice ○ HFD vs LFD		• Mitochondrial H_2O_2 efflux assessed by Amplex Red-derived fluorescence induced by various mitochondrial substrates		• *i.p.* GTT • Insulin administration • Phospho-Akt

[283]	• GM mice
	  ○ BAMBI KO
	  ○ Adipose-specific BAMBI KO
	• Primary adipocytes
		• $O_2^{\bullet-}$ assessed by mitoSOX-derived fluorescence	• *N*-acetylcysteine supplementation
			• DCFH-DA-derived fluorescence
				• *i.p.* GTT
				• *i.p.* ITT
				• Phospho-Akt

Abbreviations: 2DOG, 2-deoxyglucose; DCF, dichlorofluorescein; DCFH-DA, dichloro-dihydro-fluorescein diacetate dichlorofluorescein; ER, endoplasmic reticulum; GM, genetically modified; GSH, reduced glutathione; GSSG, oxidized glutathione; GTT, glucose tolerance test; HFD, high-fat diet, HNE, hydroxynonenal; HOMA-IR, homeostatic model assessment of insulin resistance; *i.p.* GTT, intraperitoneal glucose tolerance test; *i.p.* ITT, intraperitoneal insulin tolerance test; *i.v.* ITT, intravenous insulin tolerance test; LC-MS/MS, liquid chromatography tandem mass spectrometry; LFD, low-fat diet; MCAT, mitochondrially targeted human catalase; OGTT, oral glucose tolerance test; Prdx, peroxiredoxin; SOD, superoxide dismutase; TNFα, tumor necrosis factor-α; TnxRd2, thioredoxin reductase 2.

Table 7: Pre-clinical studies investigating mitochondrial reactive oxygen species and insulin resistance in multiple tissues

Study	System(s) used	Intervention	Assay(s)	Cellular/tissue assay(s)	Insulin resistance assay(s)

[209]	• GM mice
	  ○ *Sod2*$^{+/-}$
	  ○ Chow vs HFD
	• Tissues
	  ○ Skeletal muscle
	  ○ Liver
	  ○ Islets
			• SOD2 protein quantification
			• SOD2 activity assay
			• Mitochondrial H_2O_2 efflux assessed by Amplex Red-derived fluorescence
				• DHE staining
				• TBARS
				• Protein carbonylation
				• Total GSH and GSSG
					• Hyperinsulinemic-euglycemic clamp with 2DOG administration at end of clamp
					• Hyperglycemic clamp
					• Phospho-Akt

[44]	• Human adipose tissue
	• WT mice
	  ○ Chow vs HFD
	• Tissues
	  ○ Liver
	  ○ Skeletal muscle
	  ○ Adipose
	• 3T3-L1 adipocytes
	  ○ TNFα
	  ○ Chronic insulin
	  ○ Dexamethasone
		• Treatment with Mito-paraquat
		• Mito-catalase overexpression
		• Inhibition of CoQ synthesis
			• Mitochondrial CoQ
			• *Coq7* and *Coq9* knockdown
			• Prdx3 redox blot
			• $O_2^{\bullet-}$ assessed by mitoSOX using LC-MS/MS
					• 2DOG uptake (cell culture)
					• Plasma membrane GLUT4 translocation (cell culture)
					• 2DOG uptake in tissue explants
					• *in vivo i.p.* GTT and ITT
					• *in vivo* 2DOG tracer uptake
					• Phospho-Akt
					• Phospho-TBC1D4

[210]	• WT mice
	  ○ Chow and HFD
	• GM mice
	  ○ *db/db* Mice
	  ○ Bardet-Biedl syndrome mouse model (BBS)
	• Tissues
	  ○ Liver
	  ○ Skeletal muscle
	  ○ Adipose
		• Liver-specific adenoviral overexpression of *SOD2*	• $O_2^{\bullet-}$ assessed by mitoSOX-derived fluorescence
			• SOD2 protein expression
				• Plasma F_2-isoprostanes
				• Plasma and liver GSH and GSSG
				• Plasma GSH, E_h
				• Protein glutathionylation
				• Protein cysteinylation
				• Amplex Red fluorescence
					• 2DOG uptake into brown and white adipose tissue, skeletal muscle, heart, and brain
					• 2DOG incorporation into liver and muscle glycogen
					• Hyperinsulinemic-euglycemic clamp

					• DHE-derived fluorescence • SOD1, Nrf2 and catalase protein expression • Liver catalase activity

Abbreviations: 2DOG, 2-deoxyglucose; CoQ, coenzyme Q; DHE, dihydroethidine; E_h, half-cell redox potential; GM, genetically modified; GSH, reduced glutathione; GSSG, oxidized glutathione; GTT, glucose tolerance test; HFD, high-fat diet; *i.p.* GTT, intraperitoneal glucose tolerance test; LC-MS/MS, liquid chromatography tandem mass spectrometry; Nrf2, nuclear factor-erythroid factor 2-related factor 2; Prdx, peroxiredoxin; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNFα, tumor necrosis factor-α.