Published in final edited form as:

J Mol Biol. 2018 October 19; 430(21): 3873–3891. doi:10.1016/j.jmb.2018.03.025.

Use the protonmotive force: mitochondrial uncoupling and reactive oxygen species

Brandon J. Berry^a, Adam J. Trewin^b, Andrea M. Amitrano^{c,d}, Minsoo Kim^{a,c,d}, and Andrew P. Wojtovich^{a,b,*}

^aUniversity of Rochester Medical Center, Department of Pharmacology and Physiology, 575 Elmwood Ave., Rochester NY, 14642. Box 711/604. United States of America. Phone: 585-275-4613

^bUniversity of Rochester Medical Center, Department of Anesthesiology and Perioperative Medicine, 575 Elmwood Ave., Rochester NY, 14642 Box 711/604. United States of America. Phone: 585-275-4613

^cUniversity of Rochester Medical Center, Department of Pathology, 601 Elmwood Ave., Rochester NY, 14642 Box 609. United States of America. Office: (585) 276-3917 Fax: (585) 273-2452

^dUniversity of Rochester Medical Center, Department of Microbiology and Immunology, 601 Elmwood Ave., Rochester NY, 14642 Box 609. United States of America. Office: (585) 276-3917 Fax: (585) 273-2452

Abstract

Mitochondrial respiration results in an electrochemical proton gradient, or protonmotive force (pmf), across the mitochondrial inner membrane (IM). The pmf is a form of potential energy consisting of charge (ψ_m) and chemical (pH) components, that together drive ATP production. In a process called uncoupling, proton leak into the mitochondrial matrix independent of ATP production dissipates the pmf and energy is lost as heat. Other events can directly dissipate the pmf independent of ATP production as well, such as chemical exposure or mechanisms involving regulated mitochondrial membrane electrolyte transport. Uncoupling has defined roles in metabolic plasticity and can be linked through signal transduction to physiologic events. In the latter case, the pmf impacts mitochondrial reactive oxygen species (ROS) production. Though capable of molecular damage, ROS also have signaling properties that depend on the timing, location, and quantity of their production. In this review we provide a general overview of mitochondrial ROS production, mechanisms of uncoupling, and how these work in tandem to affect physiology and pathologies, including: obesity, cardiovascular disease, and immunity. Overall, we highlight that isolated bioenergetic models—mitochondria and cells—only partially recapitulate the complex link between the pmf and ROS signaling that occurs *in vivo*.

*Corresponding author: andrew_wojtovich@urmc.rochester.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical Abstract

Keywords

mitochondria; proton leak; T cells; ischemia reperfusion injury; brown adipose tissue

Introduction

In 1961 Peter Mitchell proposed the chemiosmotic theory of mitochondrial energy production, which, while controversial at the time, is universally accepted today [1]. The chemiosmotic theory explained how establishment of the proton electrochemical gradient known as the protonmotive force (pmf) in mitochondria couples substrate oxidation by the electron transport chain (ETC) to the production of ATP, the energy currency in most cells. The discovery that the mitochondrial ATP synthase uses the pmf to catalyze phosphorylation of ADP confirmed the theory, and in 1978 Mitchell was awarded the Nobel Prize for Chemistry. Understanding the mechanisms of proton pumping by the ETC and subsequent ATP synthesis via the consumption of the pmf has linked mitochondrial biology to important biomedical problems. Notably, this has revealed that in addition to making ATP, mitochondria function to affect metabolism, substrate and ion uptake, calcium signaling, macromolecule synthesis, proteostasis and redox signaling in cells [2–5]. Importantly, each of these processes are driven by the pmf.

The ETC creates the pmf by pumping protons from the mitochondrial matrix to the intermembrane space (IMS), a process driven by the oxidation of reducing equivalents from metabolic substrates. The pmf across the mitochondrial inner membrane (IM) manifests as a charge gradient (ψ_m) and a chemical gradient (pH). The two components of the pmf are linked in that protons are positively charged, giving rise to the ψ_m component, and chemical separation across the IM gives rise to the pH component. The pH component has a smaller contribution to the pmf than the ψ_m , as the potential energy of charge separation is greater than that of the chemical separation [6–8]. The free energy available from electron transfer in the ETC drives proton translocation across the IM. When the pmf is high, the ETC slows as it must pump protons against a stronger electrochemical force, where

the energy of substrate oxidation is insufficient to move protons up the gradient [9]. Conversely, if the pmf is diminished, oxygen consumption increases as ETC activity increases to maintain the pmf (Fig. 1). Put concisely, flux through the ETC is driven by disequilibrium in free energies of electron transfer and proton translocation [10].

Electrons enter the ETC from various sites, including complexes I and II, and are transferred to complex III by ubiquinol (coenzyme Q, or Q). Additionally, electrons can be transferred via Q to complex III through dihydroorotate dehydrogenase (DHODH), mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), mitochondrial choline dehydrogenase (ChDH), sulphide:quinone oxidoreductase (SQOR) and the electron transfer flavoprotein (ETF) during fatty acid oxidation [11, 12]. Complex III passes electrons from Q to cytochrome c, which transfers them to complex IV, where reduction of molecular oxygen to water occurs. Complexes I, III and IV pump protons to the IMS during these electron transfer events. Electron transport is coupled to ATP production at the ATP synthase via the pmf. As protons flow down the electrochemical gradient into the matrix through a channel in the ATP synthase F_o domain, a rotation of the ATP synthase F_1 domain drives the phosphorylation of ADP to ATP. Protons can also reenter the matrix without going through the ATP synthase, however, this is an energy dissipating process that can be either regulated or unregulated. This leak of protons dissipates the pmf and may result in loss of ATP production, *uncoupling* ETC activity from phosphorylation. However, the ATP synthase has a maximal rate at which the pmf can increase yet ATP production rate does not change. Under these conditions the pmf can be mildly decreased without sacrificing ATP production [10, 13, 14].

Organisms have innate inefficiencies in energy transduction that involve futile cycles that dissipate energy as heat. Proton leak is one such futile cycle and was proposed to account for 20–50% of minimum metabolic function, or basal cellular respiration rate [15, 16]. Proton leak is regulated, however, and contributions to different respiratory states is likely dynamic [17]. Proton leak can be induced as well, increasing respiration beyond the basal rate [18]. Movement of any positively charged species into the matrix, such as potassium or calcium ions, will dissipate the ψ_m component of the pmf if uncompensated by movement of other charges. Therefore, proton leak across the IM is not the only mechanism of dissipating the pmf. Uncompensated cation movement into mitochondria can dissipate ψ_m , while proton leak/uncoupling dissipates both ψ_m and pH.

Fluctuations in coupling between ETC activity and ATP production can lead to electron leak from the ETC. Unpaired electrons can react with oxygen to form reactive oxygen species (ROS). Throughout, we use ROS as an umbrella term to refer to various oxidants produced by mitochondria. Often, the particular species that causes a biologic output is not fully characterized. The primary form of ROS produced by the ETC is superoxide, which results from a one electron reduction of oxygen. Superoxide can be dismutated to form hydrogen peroxide and the hydroxyl radical can subsequently be formed through the Fenton reaction. Additionally, superoxide can react with nitric oxide, resulting in the formation of peroxynitrite, a reactive nitrogen species (RNS) [19, 20]. These ROS have different chemistries, which influence their reactivity with different cellular targets and consequently, their potential signaling capacities. For example, the hydroxyl radical is highly reactive and

can initiate irreversible oxidative damage to cellular proteins, lipids and DNA. On the other hand, under physiologic conditions hydrogen peroxide can participate in reversible cellular signaling [21]. Therefore, ROS and RNS production can influence each other, and here we will consider inducible forms of uncoupling and pmf dissipation, and how they relate to ROS production.

Mitochondrial reactive oxygen species production

ROS are derived from oxygen and have historically been equated with oxidative stress and molecular damage [22, 23]. More recently, however, ROS are recognized as signaling molecules, with beneficial or detrimental effects contextually dependent on their timing and quantity of production [24, 25]. Mitochondria are not the only cellular source of ROS [26], but many sites in respiring mitochondria produce ROS in cells [27–29]. There are several electron transfer points in the ETC where electrons can exit the chain onto oxygen resulting in ROS before they reach complex IV [30]. Additionally, the redox state of metabolic reducing equivalents such as NADH or Q can impact ROS formation at non-ETC sites. Both consumption or production of NADH and the subsequent effect on electron shuttling through Q affect the respiratory state and are, therefore, pmf dependent. For example, glycerol-3-phosphate dehydrogenases [31], branched-chain 2-oxoacid dehydrogenase [32], and dihydroorotate dehydrogenases [33] can make ROS in a respiratory state-dependent manner [19, 30, 34]. Similarly, enzymes in the tricarboxylic acid cycle make ROS, such as pyruvate dehydrogenase [35] and α -ketoglutarate dehydrogenase [36, 37]. For discussion of uncoupling and ROS we will focus on the ROS generated from electrons flowing through the ETC, however, in-depth reviews of general mitochondrial ROS production can be found elsewhere [34, 37–39].

Mitochondrial ROS production is sensitive to metabolic substrate availability [40], oxygen availability [41], pathologic state [42], and the pmf (both ψ_m and pH [6]); these factors are interrelated and may influence ROS production context dependently, which complicates interpreting how ROS production will be affected under different conditions, but also diversifies their potential signaling outputs. For example, sites of ROS production in the ETC are differentially sensitive to the pmf [30, 43]. In general, however, higher pmf is associated with greater ROS formation [44, 45], while a decrease in pmf (or “mild uncoupling”) can greatly reduce ROS production [6, 46] (Fig. 1). There is a window where mild pmf dissipation can be used to decrease ROS production without impacting ATP production if ATP synthase is functioning maximally, as discussed above. Changes in pmf may regulate ROS production by affecting the rate of electron flow through the ETC.

When the pmf is high, electron flow rate is low, so the ETC is reduced for a longer period; electrons spend more time at reductive centers in the ETC. When electrons are in the ETC for a longer time, the probability of an electron reducing oxygen to ROS before it reaches complex IV is increased. ROS are also produced during low ETC activity when electron flux is inhibited by specific ETC complex inhibitors [37]. Dissipating the pmf accelerates the ETC, which may result in less time for electrons to reduce oxygen to ROS. Moreover, oxygen consumption is increased, thereby limiting oxygen availability for ROS formation [47, 48]. In general, it is widely accepted that increasing electron flux through the ETC

decreases the probability of unpaired electron leakage to form ROS [41, 49]. However, there is potential for ROS formation at high rates of ETC activity as well.

Accelerated ETC activity increases the volume of electrons flowing, which could increase the probability of ROS production [40]. Experimentally, ROS formation at high ETC rates can be observed when ROS scavenging pathways are overwhelmed [50], but under physiologic conditions these pathways maintain ROS homeostasis [40]. Further, it has been recently shown that ROS levels in cells are more dependent upon rates of mitochondrial ROS production than they are to cellular ROS scavenging rates [51]. Nevertheless, the capability of ROS production at both low and high rates of ETC activity highlights the complex nature of ROS production under different conditions. Recently, simultaneous measurement of oxygen consumption, ψ_m and ROS production showed that at low ψ_m and high ETC activity, ROS levels were low, but a transient ROS increase occurred when switching to a state of low ETC activity [50]. In summary, at both low ETC flux (high pmf) and high ETC flux (low pmf) there is potential for ROS production, but under most circumstances, increasing ETC flux decreases ROS (Fig. 1).

Complex I

A major source of mitochondrial ROS is complex I, where electrons from NADH reduce Q under normal conditions. ROS can be produced from different sites in complex I, especially when the NADH/NAD⁺ ratio is high in cells [37, 52]. A major site of ROS production at complex I is from the flavin mononucleotide cofactor [53]. ROS are also produced by the flavin in complex I under the condition of reverse electron transport (RET), when the pmf is elevated, such as during the pathology of ischemia reperfusion [54, 55]. A high pmf not only slows electron flux through complex I, but can also reverse it. Complex I reversal is driven by a highly reduced Q pool which can occur through over-reduction by complex II [42]. The pH component of the pmf is the main driving force of RET, more so than the ψ_m component [56]. When the pH is large resulting in an alkalinized matrix, ROS formation by RET is favored [6, 53]. Therefore, ROS production by RET is very sensitive to proton leak decreasing the pH [37, 43]. In terms of ψ_m , when transitioning from high to low ETC activities (low to high ψ_m) a transient production of ROS occurs at complex I [50].

Complex III

ROS production from complex III is also sensitive to the pmf, explained by a slowdown of electron transfer when the pmf is high (Fig. 1). Complex III catalyzes a Q cycle where electrons are transferred from Q to cytochrome c and protons from Q are pumped to the IMS in a series of reactions. As a part of the cycle, a Q radical (semiquinone) is transiently produced. Slow electron transfer results in a slower Q cycle, which results in more time for semiquinone to reduce oxygen to ROS [38, 45]. Therefore, this ROS production is dependent on kinetics and redox status during the Q cycle. [38]. Thus, increasing respiration to increase electron transfer rate to oxidize Q and semiquinone more rapidly may decrease the probability of ROS production [56]. Conversely, highly reduced Q when the pH component of the pmf is high leads to higher ROS production from complex III [30]. In line with this, alkaline matrix pH (which can be the result of a high pH) stabilizes semiquinone

radical formation [57]. Taken together, acceleration of the ETC and decreasing pH by uncoupling serves to decrease ROS production at complex III.

Mitochondrial uncoupling systems and ROS

There are different mechanisms of pmf dissipation that affect ROS production from the ETC. These mechanisms can dissipate one or both components of the pmf, in either controlled or uncontrolled ways, that can differently affect ROS production (Fig. 2). Uncoupling through proton leak can be measured precisely by simultaneously monitoring ψ_m and oxygen consumption to correlate changes in the pmf with changes in ETC activity [15, 58]. Often, however, only ψ_m or oxygen consumption is measured as an approximation of uncoupling. Studies measuring only one component of the pmf or oxygen consumption as a proxy of uncoupling should be carefully interpreted.

Protonophores

Small molecule protonophores, commonly referred to as chemical uncouplers, function by directly binding and transporting protons across the IM to equilibrium [59] while freely diffusing (Fig. 2a). Uncouplers were used before widespread adoption of Mitchell's chemiosmotic theory [60], and their effects on increasing respiration without ATP production were described [61]. Peter Mitchell used uncouplers to provide compelling evidence in support of his chemiosmotic theory that led to its acceptance [62]. Some widely used protonophores include carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and 2,4-dinitrophenol (DNP). There are many other small molecule protonophores that have been used to uncouple mitochondria for various research and clinical purposes as well [63–67]. For instance, mild uncoupling via controlled-release protonophores with oral bioavailability were recently shown to be beneficial in animal models of metabolic disease [68, 69].

Uncouplers are used as a pharmacologic approach to understand the link between uncoupling and ROS production both *in vitro* and *in vivo*. Uncouplers can be used to decrease both components of the pmf and decrease ROS production [64, 70]. Mice treated with long-term low dose DNP had extended lifespans and decreased oxidative stress [64]. Counterintuitively, low concentrations of FCCP (5–100 nM) can increase detection of ROS in some models [40, 71, 72], suggesting a more complex relationship between mild uncoupling and ROS production. Additionally, some uncouplers seem to work through association with the adenine nucleotide translocase (ANT) [73], which can endogenously uncouple, as discussed below.

It is worth noting that uncouplers as a pharmacologic approach lack spatial and temporal control. Once an uncoupler is added it cannot be precisely targeted in a sample or removed from it. Another caveat of uncouplers is that there is no selectivity for mitochondrial membranes. Above micromolar concentrations FCCP can depolarize the plasma membrane potential as well as the pmf [74]. Recently, photoactivatable uncouplers have been developed and used to confer spatial control over pharmacologic uncoupling using light [75, 76]. Mitochondria-targeted, light-activatable DNP (MitoPhotoDNP) accumulates into the matrix driven by the pmf via a lipophilic cation moiety, and upon light exposure DNP is released,

resulting in uncoupling. Light activation results in loss of mitochondria-targeting, therefore loss of spatial control could occur over time. However, the uncoupling effect of DNP did not spread to nearby mitochondria in cardiomyocytes treated with MitoPhotoDNP, suggesting that spatial control was maintained [76].

Uncoupling proteins

Uncoupling can be endogenously regulated by IM uncoupling proteins (UCPs) (Fig. 2b) [77]. UCPs are evolutionarily conserved transmembrane transport proteins [78]. *In vitro* evidence suggests that UCP proteins require fatty acids to uncouple, and significant uncoupling requires a sufficiently high pmf [79, 80]. *In vitro* electrophysiologic evidence provided a mechanism for UCP1, where free (unesterified) fatty acids act as a substrate and allow proton shuttling across the IM [81]. Research is active on molecular mechanisms for UCPs other than UCP1, and the proteins are described to varying extents [82].

UCPs can be activated by ROS through post-translational modification [39, 83]. Therefore, ROS production could be attenuated by decreasing the pmf through ROS activated UCP activity, setting up a negative feedback loop for the regulation of ROS production [84]. Though the relevance of ROS attenuation by UCPs is still not fully understood *in vivo* [85–87], UCP regulation of ROS production is widely accepted based on physiologic evidence [88–90]. In general, inhibition of UCP proton leak leads to mitochondrial ROS signaling, and UCP activation leads to decreased redox signaling [90]. Additionally, gene expression in response to oxidative stress can upregulate UCP3 [91]. It should be noted, however, that upregulation of UCPs at mRNA and protein levels does not always result in increased proton leak [92].

Another mitochondrial transport protein, the ANT, mediates proton leak under basal conditions and is inducible *in vitro* [93, 94]. The ANT normally exchanges ADP in the IMS for ATP in the matrix, a process that consumes the pmf in itself [95]. Many other carriers use the pmf for endogenous transport function like the ANT, such as the inorganic phosphate (Pi) carrier [96] and the glutamate/aspartate exchanger (both can decrease the pmf) [97]. Further, ANT can uncouple directly. Like UCPs, ANT is thought to facilitate uncoupling activity at high ψ_m [98] in the presence of fatty acids [99].

Cation flux

Since ψ_m is the major component of the pmf, there is a large driving force for cation (e.g. K^+ , Ca^{2+}) accumulation in the mitochondrial matrix in addition to protons [100]. It follows that any of these positive charges moving from the IMS to the matrix will decrease the pmf. The most abundant cation in the cytosol is K^+ . While at approximately equal concentrations in the cytosol and matrix, the ψ_m would allow an approximate 1000-fold K^+ accumulation into the matrix if ion flux was unregulated [101, 102]. Despite the low permeability of the IM to ions, K^+ can leak into the matrix. K^+ is followed by water and Pi, resulting in matrix swelling. The K^+ /proton exchanger (KHE) transports K^+ out of the matrix at the expense of the pmf [103]. Therefore, mitochondrial uptake of K^+ can impact ψ_m and pH, both of which can influence ROS generation [103].

K^+ ionophores, such as valinomycin, will allow K^+ to accumulate according to ψ_m (Fig. 2c). Titrations of valinomycin demonstrate a biphasic impact of K^+ fluxes on ROS production in respiring mitochondria [104, 105]. Low concentrations (<1 nM) allow K^+ uptake, however, small changes in ψ_m are often undetectable and are compensated by increased ETC activity [104–106]. Under conditions of low valinomycin-induced K^+ flux, ROS production was increased [104, 105]. Conversely, high concentrations of valinomycin (>1 nM) can decrease both ψ_m and ROS production [104, 105, 107]. In response to large K^+ fluxes, the pmf is redistributed from ψ_m to pH . The increased pH is reflected by matrix alkalization if not compensated by electroneutral P_i uptake, which cotransports P_i and a proton into the matrix.

As discussed above, mitochondrial ROS production depends on the pH component of the pmf, such that matrix alkalization is associated with increased ROS generation, especially at complex I [6, 57, 108]. The introduction of P_i to isolated mitochondria to decrease the pmf via the pH component lowers ROS production [6]. Overall, submaximal matrix K^+ influx (increased respiration without measurable impact on ψ_m) can enhance ROS production while maximal influx can decrease ψ_m and decrease ROS [104]. It is worth noting that large cation fluxes can also be self-limiting, as matrix cation accumulation decreases ψ_m , the driving force.

In addition to ionophores, mitochondrial K^+ channels are an inducible system to modulate the pmf. The mitochondrial inner membrane has numerous K^+ channels that allow K^+ flux into the matrix when activated [109]. K^+ channels are diversely regulated and widely implicated in many signaling paradigms [110–112]. Changes in K^+ levels in mitochondria are implicated in the maintenance of mitochondrial matrix volume and modulation of mitochondrial function.

The number of channels and their K^+ conductance capabilities vary greatly between cell and channel types. The mitochondrial ATP-sensitive channel is one of the most widely studied K^+ channels due to its putative role in protecting against ischemia reperfusion injury, yet its identity and precise role remain controversial [103]. The role of mitochondrial ATP-sensitive K^+ channels in ROS production is also unclear, since activation of the channel can either increase or decrease ROS levels [103, 104, 113]. K^+ channel activity in heart mitochondria resulted in matrix alkalization due to limited P_i transport compensation [114], potentially increasing pH to contribute to ROS production. However, reports also demonstrate that the activation of the channel can decrease ROS production. In brain mitochondria, which have higher channel activity or expression, ROS production was decreased in a K^+ -dependent manner [113, 115]. This decrease in ROS was present in heart and liver mitochondria, and persisted in the presence of excess P_i to minimize matrix alkalization (pH) [113]. The impact of K^+ fluxes may be the result of alterations in the contribution of ψ_m and pH to the pmf. The ability to compensate for changes in pH depends on availability of counter ions.

Moreover, while ATP-sensitive K^+ channel activity is thought to have minimal impact on ψ_m [116–120], the activities of other K^+ channels are larger than that of ATP-sensitive K^+ channels, and may have distinct impacts on the pmf and ROS production [104, 109, 121,

122]. The ATP- sensitive K^+ channel is also redox regulated and is activated by ROS [111, 123, 124]. However, identities of redox-sensitive residues or roles the channel plays in ROS generation remain unclear.

Redox slip and pmf dissipation

Under specific circumstances, ROS production itself can be an energy-dissipating system. ROS made at complex III can donate electrons to cytochrome c, taking the place of Q [125, 126]. Therefore, proton pumping is bypassed; protons from Q are not moved to the IMS as a result of electron transfer. Cytochrome c then functions as normal and shuttles electrons to complex IV. ROS can be used in this way as an electron donor for respiration and ATP generation [127]. This process is one example of redox slip, and can indirectly dissipate the pmf because proton pumping by complex III is bypassed. Fewer protons translocated results in a lower pmf compared to the condition where no ROS is used at complex III [84]. However, relative contribution of the redox slip mechanism to overall levels of uncoupling at high pmf is thought to be negligible [128].

Mitochondrial permeability transition pore

The permeability transition pore (PTP) is a large opening in the IM, the precise identity of which remains under investigation [129–132]. The PTP can be opened under pathologic conditions involving ROS and calcium entry into mitochondria, leading to collapse of pmf and to cell death [133, 134]. This loss of pmf lacks dynamic range and is not reversible. There is some evidence that decreased ψ_m , independent of calcium ion concentration, may be a cause as well as a consequence of PTP opening [135]. PTP opening may occur mainly through a decrease in the ψ_m component, since acidic conditions (dissipated pH) inhibits PTP opening [135]. In general, the PTP plays a role in changes of the pmf in response to pathology, including high levels of ROS. There is some indication, however, that transient PTP opening, or “flickering,” occurs under non-pathologic conditions to regulate mitochondrial pH, calcium ion concentration and pmf [136, 137].

Uncoupling, ROS, and signaling

Mitochondria are central hubs for sensing certain types of mild to moderate stress, and signal to initiate appropriate cellular responses. Since mitochondrial pmf and/or ROS production are interdependent [50], perturbations to either can induce retrograde stress signaling cascades in a process termed mitohormesis [24, 138]. For instance, modulation of mitochondrial ROS production may activate redox sensitive transcription factors such as nuclear factor (erythroid-like 2) related factor (NRF2), a master regulator of the cellular antioxidant response [139, 140]. Under basal conditions, NRF2 is bound to cytosol-localized Kelch-like ECH-associated protein1 (KEAP1) and Cullin 3, marking it for ubiquitin mediated proteolysis. During oxidative stress, cysteine residues on KEAP1 are oxidized causing conformational change and dissociation from NRF2. This allows NRF2 to evade proteolytic degradation and translocate to the nucleus where it can interact with MAF proteins, leading to DNA binding at antioxidant response elements [141]. This promotes transcription of numerous target genes which encode phase-II detoxification enzymes (i.e. antioxidant enzymes) that confer stress resistance against subsequent oxidative stress [139,

140]. Recent work has also identified NRF2 as a regulator of uncoupling via UCP3 expression. Notably, NRF2 RNAi blocked the upregulation of UCP3 in response to exogenous ROS, and UCP3 RNAi prevented increased mitochondrial uncoupled respiration in response to oxidative stress in cells [91]. Moreover, 4-hydroxynoneal (4HNE), an oxidized lipid, induced similar effects as ROS on NRF2 mediated UCP3 expression [142].

The interplay between the pmf and ROS underlies the sensing of mitochondrial proteostasis. The mitochondrial unfolded protein response (UPR^{mt}) is activated by an imbalance in the stoichiometry of mitochondrial:nuclear encoded proteins, as well as misfolded or damaged proteins, and leads to a retrograde signal to upregulate proteases and chaperones [143]. The UPR^{mt} is best described in the nematode *Caenorhabditis elegans*, where activating transcription factor associated with stress 1 (ATFS-1) is imported into the mitochondria and is used as a surrogate to monitor mitochondrial health [144]. When mitochondrial import of ATFS-1 is blocked, a nuclear localization sequence causes it to be trafficked to the nucleus where it triggers activation of a profile of stress-response genes that help reestablish mitochondrial homeostasis. Activating transcription factor 5 (ATF5) has been suggested to perform this function in mammals [144, 145], and in both worms and mammals activation of the UPR^{mt} requires ROS production [146]. Since protein import into mitochondria requires sufficient pmf [147], the ability of both ATFS-1/ATF-5 and their target gene products to enter mitochondria should require a pmf. While uncoupling alone is not required to induce the UPR^{mt} [145], instead, FCCP induces activating transcription factor 4 (ATF4) in mammalian cells [148], which is linked to the integrated stress response (ISR), a complementary pathway to the ATF5-mediated UPR^{mt} pathway [145, 149].

When the UPR^{mt} or ISR cannot overcome mitochondrial stress, mitophagy can be activated, which is an essential quality control process to target damaged or depolarized portions of mitochondria for removal and degradation. Receptor mediated mitophagy occurs when receptors on the outer mitochondrial membrane are phosphorylated and bind microtubule-associated protein 1A/1B-light chain 3 (LC3) to initiate autophagosome formation. Ubiquitin mediated mitophagy (mitophagy through PTEN-induced putative kinase (PINK) and parkin) occurs when outer mitochondrial membrane proteins are ubiquitinated, leading to adapter protein binding and subsequent LC77 mediated autophagosome formation. The autophagosome then fuses with a lysosome, which engulfs and digests the target mitochondrion. Uncoupling with FCCP is a commonly used pharmacologic approach to experimentally activate mitophagy [150], and pmf dissipation induces PINK/Parkin mediated mitophagy [151]. However, a recent report showed that mitophagy was independent of ψ_m and related to matrix acidification (loss of pH) instead [152].

Nevertheless, *in vivo*, another mechanism may exist for uncoupling-induced mitophagy via 5' AMP activated protein kinase (AMPK) [153]. Increased AMPK activity resulting from increased bioenergetic flux during exercise (a similar effect on AMPK as uncoupling) led to phosphorylation of ULK1 and initiation of mitophagy [154]. In addition, ROS can trigger mitophagy in a PINK/Parkin dependent manner [155, 156], as well as a recently identified mechanism of oxidative activation of p62/sequestosome1 [157].

Finally, uncoupling with FCCP induces mitochondrial biogenesis signaling via peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC1 α) [158]. This increased mitochondrial respiratory function could presumably modify ROS production characteristics [159]. Taken together, these redox-sensitive mitochondrial stress signaling pathways allow for a tightly regulated feedback loop between mitochondrial ROS formation and uncoupling (Fig. 3). Understanding the mechanistic link between uncoupling and ROS stress resistance signaling could impact therapeutic approaches for three avenues of research that represent important public health issues: obesity, cardiovascular disease, and immunity.

Brown adipose tissue

Obesity manifests as the accumulation of excessive body fat via lipid accumulation, primarily in white adipose tissue, which has low metabolic activity [160]. On the other hand, brown adipose tissue (BAT) has greater metabolic activity which is used for non-shivering thermogenesis, due to greater abundance of mitochondria with high levels of UCP1 expression [161]. UCP1 is extensively studied in BAT in the context of non-shivering thermogenesis and ROS signaling (discussed in detail elsewhere [162]). Activation of BAT thermogenesis by cold exposure through UCP1 may require mitochondrial ROS, as it can be reversed upon application of a mitochondria-targeted antioxidant [163]. This suggests that ROS induces UCP1 activity, and may decrease the pmf, and thereby pmf sensitive ROS production. However, greater temporal resolution in the measurement of ROS levels would help to fully understand the interplay of ROS levels and UCP1 activity in this model. Nonetheless, proton leak respiration rate was decreased in antioxidant treated primary adipocytes, supporting the idea that UCP1 is ROS activated. It remains unclear whether uncoupling on its own is sufficient for thermogenesis in BAT, or if further ROS signaling interacts with unidentified targets.

Different sources of ROS in mitochondria could be responsible for activating UCPs in brown fat models. In terms of obesity, specific upregulation of complex III and UCP1 occurs in BAT of obese mice [164]. ROS levels were increased in obese mice, observed using a fluorescent ROS detector, dihydrodichlorofluorescein diacetate (DCF). Although DCF has concerning caveats (see Perspectives section below, and references [165, 166]), ROS were also indirectly measured by quantifying lipid oxidation products, and by antioxidant gene responses. In general, ROS detection in obesity models suffers from a lack of standardization [167]. Basal respiration was increased in the obese mice, measured in isolated BAT, supporting increased proton leak by UCP1 [164]. These results show that even though uncoupling seems to be increased, ROS levels are also increased. This may be explained by aberrant ROS production activating UCP1 proton leak, where the leak is not sufficient to decrease ROS production. Alternatively, ROS could originate from non-mitochondrial sources, and therefore not be pmf sensitive. Additional studies with greater temporal resolution are necessary to discern the order and impact of the signaling events.

The possibility of browning other adipocyte types to combat obesity by activating fat burning is an actively researched topic. Beige adipocytes are found within white adipose tissue and can be induced by cold, which increases their UCP1 expression, like BAT. Although BAT represents a small fraction of adipocytes in adult humans (< 5%), especially

in obesity [168], BAT is also thought to function as an important endocrine tissue [169]. Therefore, strategies to induce beige adipocyte browning may have therapeutic potential, and depend on regulation of UCP1. It was recently shown that UCP1 can be activated to induce thermogenesis and is upregulated at both mRNA and protein levels by physiologically relevant cold exposure [170]. However, cold-induced thermogenesis is due to UCP1 in BAT alone, not beige adipocytes, and it is proposed by the authors that physiologic context must be carefully considered when studying browning models [170]. Notably, beige adipocytes were recently shown to have a different, UCP1-independent thermogenesis mechanism that is ATP-dependent, therefore not mediated by uncoupling [171]. Furthermore, brown and beige adipocytes have distinctly different developmental lineages, possibly explaining the different thermogenic mechanisms [172]. However, cold can activate UCPs other than UCP1 in different cell types, such as UCP4 in neurons [173]. The link between pmf sensitive ROS and browning is still unclear.

IR injury

The pmf and ROS production are involved in many cardiovascular pathologies, and here we will focus on ischemia reperfusion (IR) injury, the pathologic phenomenon that underlies the etiology of heart attack and stroke. Ischemia occurs when tissue experiences loss of blood flow and decreased oxygen and metabolic substrates. IR injury involves mitochondrial ionic imbalance, cell death signaling, and ROS generation upon reperfusion of blood. Specifically, succinate accumulation drives IR damage from ROS generated at complex I by RET [42, 174].

Interventions known to protect against IR injury involve mitochondria. Ischemic preconditioning is an intervention where brief bouts of tissue ischemia can protect against future pathologic ischemic insults. ROS triggers ischemic preconditioning, and antioxidants prevent it [175, 176]. Preconditioning upregulates redox-sensitive protective signaling responses to attenuate subsequent damage caused by overproduction of ROS during IR injury [175]. Stabilization of ψ_m in preconditioning models can result in lower ROS formation and protection against IR injury [177]. In summary, ROS are required for preconditioning signaling, but ROS damage is alleviated downstream of that signaling.

In UCP3 knockout hearts, ischemic preconditioning loses its protective effect, suggesting a role for uncoupling in preconditioning [178]. Pharmacologic uncoupling with FCCP protects UCP knockout hearts [178], supporting the role for uncoupling in preconditioning. Further, overexpression of UCP1 in the heart is sufficient for protection against IR injury in mice [179]. Therefore, mild uncoupling, whether through UCP activity or low concentration protonophores, protects against IR injury [178, 180–185], potentially through attenuation of ROS production by RET at reperfusion [186]. However, mild uncoupling protection by protonophores can be dependent on elevated ROS levels, as demonstrated with FCCP [71, 72]. This contrasts the notion of uncouplers generally decreasing ROS production. Similarly, FCCP induces vasorelaxation in arterial smooth muscle cells with a concomitant increase of mitochondrial ROS [187]. FCCP-induced vasorelaxation also resulted in AMPK activation [187], which is a major metabolic regulator that protects against IR injury [188, 189].

Additionally, AMPK activity is implicated in mediating the protective effects of mild uncoupling in models of IR injury [153].

ROS often mediate protective pathways in IR injury that involve uncoupling, but the precise details are still under investigation. For instance, ROS-dependent protection by mild uncoupling with protonophores is not dependent on K^+ channels, which also protect against IR injury [72, 190]. Therefore, protection through mild uncoupling and K^+ channels may be mediated through separate or convergent pathways. The mechanism of protection by K^+ channels is thought to involve volume regulation, attenuation of ROS production and decreased ψ_m at reperfusion [190]. ROS acts as a trigger for K^+ channel opening and protection, while antioxidants reverse the effect [105, 191–193]. Decreased ROS production mediated by the ATP-sensitive K^+ channel is also thought to lead to protection [113, 121, 124].

The PTP also plays a role in IR injury, where mitochondrial pmf depletion leads to calcium regulated cell death [194]. It is also suggested that after pmf collapse by PTP opening, ROS can still be made from damaged complex III [133]. There is evidence that transient PTP opening, or flickering, mediates protection that requires ROS [195]. Similarly, protection by the protonophore DNP was reversed by both inhibition of PTP activity and by antioxidants. This indicates PTP opening and ROS are required for protection by DNP [195]. These examples demonstrate a link between ROS signaling and mild decreases in the pmf, but the specific signaling targets and level of pmf variation remain unclear.

Recent evidence using pharmacologic techniques to separate the contributions of ψ_m and pH demonstrated that pH controls oxidation post IR [196]. ROS production was directly measured by electron paramagnetic resonance spin trapping, and contributions from ψ_m and pH showed decreased pH during IR results in oxidative stress, perhaps due to damaged ETC components [196]. Increased pH drives RET ROS production during IR [6, 174], but in this case, decreased pH resulted in oxidative damage in mitochondria post IR [196]. This inconsistency demonstrates how *in vivo* ROS pathology is more complicated than *in vitro* models suggest independently. Indeed, mice with a heterozygous knockout of a complex I subunit are resistant to IR injury, however, they showed increased cytosolic, but not mitochondrial, ROS levels [197], highlighting the potential role of different sites of ROS generation and removal in IR injury.

T cell function

T cells are lymphocytes produced in the thymus that play a central role in immunity. T cell metabolism is dynamic and plastic, and during development, T cells switch between different metabolic states that are best suited to their function [198]. Naïve T cells await activation to become effector T cells that rapidly proliferate in response to antigen. The metabolic switch in T cells occurs after T cell receptor (TCR) stimulation, and is referred to as T cell activation. After activation, T cell metabolism is still plastic and can respond to various nutrient availabilities in microenvironments.

Mitochondrial ROS play an important role in T cell function and development [199]. When a subunit of complex III was knocked down, there was decreased mitochondrial ROS, and

both CD4⁺ (“helper”) and CD8⁺ (“killer”) T cells were unable to be activated [199]. ROS produced by complex III can activate the nuclear factor of activated T cells (NFAT) to lead to activation, observed both *in vitro* and *in vivo* [199]. CD4⁺ T cells without functional complex III did not lead to activation because NFAT did not bind its target gene promoter after TCR stimulation. T cells with complex III knocked down could proliferate normally with no effect on cell viability, glycolysis, mitochondrial number, or mitochondrial membrane potential. Therefore, T cells without complex III ROS production can still meet bioenergetic demands, but are unable to become activated and undergo antigen-specific expansion due to a lack of signaling from mitochondrial ROS [199].

The role of the pmf in T cell activity is multifaceted, and many contrasting metabolic and functional differences result from modulation of ψ_m in distinct populations of T cells. When T cells are activated intracellular calcium increases [200] and more calcium can enter mitochondria. Increased mitochondrial calcium may result in TCA cycle enhancement and potentially increased pmf, explaining elevated ROS. In support of this notion, FCCP treatment inhibits activation [199]. Further, low ψ_m T cells have higher levels of fatty acid oxidation, increased ability to respire maximally, and reduced glycolysis, which is the metabolic phenotype of memory T cells [201]. Low ψ_m T cells are also able to self-renew and have lower levels of oxidative stress. Additionally, features of low ψ_m allow increased persistence, or ability of T cells to function longer. Persistence is elevated during immune responses in cancer models in T cells with lower ψ_m , but there is also a higher possibility for autoimmunity [202]. T cells with high ψ_m can be classified as effector T cells that have higher levels of both aerobic glycolysis and cytokine production, but lower persistence [201].

The magnitude of the ψ_m has a significant impact on both gene expression and metabolism in T cells. Hence, T cells with varying magnitudes of ψ_m have different effector phenotypes as well as survival capability [202]. TCR stimulation results in a transient increase in ψ_m . The elevated ψ_m promotes generation of ROS and increases the probability of apoptosis after T cell activation [203]. Patients with systemic lupus erythematosus (SLE) have abnormal T cell necrosis after activation, which likely increases inflammation. There is also ATP depletion and glutathione deficiency which can contribute to ROS-induced cell death [203]. Similarly, abnormal ψ_m and metabolism in T cells contributes to autoimmunity in type 1 diabetic patients [204]. Patients with type 1 diabetes often have increased ψ_m in all T cell subsets. These T cells also have increased mitochondrial ROS production and decreased intracellular ATP concentrations after activation [204]. Increased ψ_m did not correlate with disease duration or severity, or T cell activation condition, therefore high ψ_m appears to be an intrinsic defect in T cells in patients with type I diabetes [204]. The inability of T cells to alter the pmf to adapt to different conditions leads to disease.

A metabolic shift occurs during T cell activation that is associated with high ψ_m and increased ROS [199], which supports observations of metabolic shifts *in vivo* [50]. Further, after T cell activation, there is a significant increase in UCP2 protein expression. UCP2 likely has an important role in the metabolic switch and cell proliferation that follows T cell

activation. The mechanism for how UCP2 may be beneficial in activated T cells is unclear, but it is possible that UCP2 keeps the pmf lower to decrease ROS production [205].

The above examples of T cell physiology demonstrate yet another avenue of pmf sensitive mitochondrial ROS production. There are instances, however, where mitochondrial function in T cells can affect physiology independent of the pmf and ROS. For example, there is recent interest in optimizing T cell metabolism for adoptive T cell immunotherapies [201], a process where modified T cells are transferred and used to combat disease. One way to improve the effectiveness of these therapies is to promote mitochondrial fusion. Mitochondrial fusion increased both function and spare respiratory capacity of T cells while there was no significant change in ψ_m or ROS production [206]. A change in ψ_m or ROS production was not required for a shift in metabolic activity in this case. Redox signaling is dynamic and complex in T cells, and is impacted by ROS from sources other than mitochondria (details are discussed elsewhere [207]). A better understanding of the regulation of the pmf and ROS production will be important to advance this field of research.

Perspectives

A single model of pmf regulated ROS production to explain broad principles is often insufficient. The relationship between the pmf and ROS is dependent on ETC flux (Fig. 1), contributions from both ψ_m and pH (Fig. 2), and complex signaling regulation (Fig. 3). In general, ROS production seems to affect the pmf in a negative feedback loop. However, context-dependent molecular details of the link between uncoupling and ROS production could provide novel insight to advance the study and application of uncoupling and ROS signaling (Fig. 4). Clarification of these molecular details should be independently performed in each specific model to yield applicable results, as biologic context varies greatly.

One barrier to understanding the pmf and redox signaling is the use of antioxidant treatment to entirely abolish ROS outputs. While on one hand high levels of ROS can be damaging, on the other hand high levels of antioxidants can lead to reductive stress [24, 208, 209]. Antioxidant therapies have been largely unsuccessful, and in some cases antioxidants caused damage [210]. Particularly in cardiovascular disease models, global antioxidant treatment can reverse protective signaling triggered by ROS [71, 72]. Further, antioxidant supplementation in humans reverses beneficial adaptations from exercise [211, 212], demonstrating the importance of ROS signaling *in vivo*. Directly combating reductive stress requires intact oxidative quality control [213]. Excessive antioxidant activity can overwhelm these mechanisms, leading to cellular dysfunction.

Another barrier to mechanistic insight is wide misuse or misinterpretation of non-specific ROS indicators. For example, DCF is often used as an ROS level indicator, but DCF signal is not specific for ROS, is more indicative of iron or cytochrome c, and can even self-oxidize, artificially amplifying signals [165, 166]. Dihydroethidium (DHE) or its mitochondria-targeted derivatives are commonly used to detect superoxide by observing changes in fluorescence. However, to quantitatively use DHE, the superoxide-specific

oxidation product, 2-hydroxyethidium, must be separated from other non-specific oxidation products using high-performance liquid chromatography (HPLC) [214]. In addition to non-specific reactions, DHE intercalates DNA, which increases the fluorescence of oxidation products and may increase toxicity. DCF and DHE serve as examples for how qualitative observations can often be used inappropriately as quantitative measures in detecting ROS. However, selective mitochondria-targeted ROS sensors coupled with HPLC or mass spectrometry are addressing these caveats to improve ROS detection *in vivo* [215, 216].

Mitochondria-targeted ROS indicators or antioxidants used in conjunction with uncouplers must be carefully interpreted as well, as accumulation of such positively charged reagents depends on the pmf. Therefore, uncoupling can disrupt equilibrium of reagent accumulation in mitochondria and yield over- or underestimated results. Another barrier is the nature of direct pmf measurements and outputs. The contributions of ψ_m and pH or the impact of respiratory states remain unclear *in vivo*, as current knowledge comes from isolated systems that may have different conditions, such as ion availabilities [113]. As discussed in the cation flux section, counter ion availability can affect both ψ_m and pH, leading to differences in ROS production or even in simple mitochondrial function between models. Additionally, it should be noted that most fluorescent measurements of the pmf truly only measure the ψ_m component [217] and are difficult to normalize, further complicating interpretations. Changes in mitochondrial mass, morphology or location can impact fluorescent ψ_m probes and are often not considered [217]. Like ROS detection, measurement of ψ_m *in vivo* is often limited to qualitative observations. Finally, mild uncoupling to attenuate ROS has been questioned [85, 86, 218], as physiologic conditions are often difficult to apply when studying the phenomenon.

Novel technologies to directly uncouple mitochondria to study signaling will be instrumental in the progression of the field [219]. The complex interactions between ETC kinetics, ATP availability, metabolic and pathologic states of cells underlie the link between the pmf and ROS signaling, and each may differ in different contexts. This calls for model-specific study design and interpretation of data. Therefore, we propose that future investigation into the mitochondrial pmf and ROS should utilize physiologic models and whole organism approaches for translation to biomedical applications.

Acknowledgments

Work in the laboratory of APW is supported by a grant from National Institutes of Health (R01 NS092558), and BB is supported by an Institutional Ruth L. Kirschstein National Research Service Award (NIH T32 GM068411). We thank Paul Brookes and Keith Nehrke (University of Rochester Medical Center) for valuable discussion and critical review of the manuscript.

Abbreviations used

pmf	protonmotive force
IM	inner mitochondrial membrane
ROS	reactive oxygen species
ETC	electron transport chain

IMS	intermembrane space
Q	ubiquinol/ubiquinone
RNS	reactive nitrogen species
RET	reverse electron transport
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
CCCP	carbonyl cyanide <i>m</i> -chlorophenyl hydrazine
DNP	2,4-dinitrophenol
ANT	adenine nucleotide translocase
MitoPhotoDNP	mitochondria-targeted light-activatable DNP
UCP	uncoupling protein
K⁺	potassium ion
Pi	inorganic phosphate
KHE	K ⁺ /proton exchanger
PTP	permeability transition pore
NRF2	nuclear factor (erythroid-like 2) related factor
KEAP1	Kelch-like ECH-associated protein1
4HNE	4-hydroxynoneal
UPR^{mt}	mitochondrial unfolded protein response
ATFS-1	activating transcription factor associated with stress 1
ATF	activating transcription factor
ISR	integrated stress response
LC3	microtubule-associated protein 1A/1B-light chain 3
PINK	PTEN-induced putative kinase
AMPK	5' AMP activated protein kinase
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
BAT	brown adipose tissue
DCF	dihydrodichlorofluorescein diacetate
IR	ischemia reperfusion

TCR	T cell receptor
NFAT	nuclear factor of activated T cells
SLE	systemic lupus erythematosus
DHE	dihydroethidium
HPLC	high-performance liquid chromatography

References

1. Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*. 1961; 191:144–8. [PubMed: 13771349]
2. Meisner H, Palmieri F, Quagliariello E. Effect of cations and protons on the kinetics of substrate uptake in rat liver mitochondria. *Biochemistry*. 1972; 11:949–55. [PubMed: 5013818]
3. Nicholls DG. Mitochondria and calcium signaling. *Cell Calcium*. 2005; 38:311–7. [PubMed: 16087232]
4. Fox TD. Mitochondrial protein synthesis, import, and assembly. *Genetics*. 2012; 192:1203–34. [PubMed: 23212899]
5. Olsen RK, Cornelius N, Gregersen N. Redox signalling and mitochondrial stress responses; lessons from inborn errors of metabolism. *J Inher Metab Dis*. 2015; 38:703–19. [PubMed: 26025548]
6. Lambert AJ, Brand MD. Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem J*. 2004; 382:511–7. [PubMed: 15175007]
7. Nicholls DG. The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. *Eur J Biochem*. 1974; 50:305–15. [PubMed: 4452361]
8. Nicholls DG. Hamster brown-adipose-tissue mitochondria. The control of respiration and the proton electrochemical potential gradient by possible physiological effectors of the proton conductance of the inner membrane. *Eur J Biochem*. 1974; 49:573–83. [PubMed: 4442425]
9. Lodish H. *Molecular cell biology*. New York: Freeman - MacMillan; 2016.
10. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *Biochem J*. 2011; 435:297–312. [PubMed: 21726199]
11. Watmough NJ, Frerman FE. The electron transfer flavoprotein: ubiquinone oxidoreductases. *Biochim Biophys Acta*. 2010; 1797:1910–6. [PubMed: 20937244]
12. Lenaz G, Genova ML. Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. *Antioxid Redox Signal*. 2010; 12:961–1008. [PubMed: 19739941]
13. Kaim G, Dimroth P. ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. *EMBO J*. 1999; 18:4118–27. [PubMed: 10428951]
14. Lee I, Bender E, Kadenbach B. Control of mitochondrial membrane potential and ROS formation by reversible phosphorylation of cytochrome c oxidase. *Mol Cell Biochem*. 2002; 234–235:63–70.
15. Nobes CD, Brown GC, Olive PN, Brand MD. Non-ohmic proton conductance of the mitochondrial inner membrane in hepatocytes. *J Biol Chem*. 1990; 265:12903–9. [PubMed: 2376579]
16. Brand MD, Brindle KM, Buckingham JA, Harper JA, Rolfe DF, Stuart JA. The significance and mechanism of mitochondrial proton conductance. *Int J Obes Relat Metab Disord*. 1999; 23(Suppl 6):S4–11. [PubMed: 10454114]
17. Korzeniewski B. Contribution of proton leak to oxygen consumption in skeletal muscle during intense exercise is very low despite large contribution at rest. *PLoS One*. 2017; 12:e0185991. [PubMed: 29045413]
18. Nicholls DG. Hamster brown-adipose-tissue mitochondria. Purine nucleotide control of the ion conductance of the inner membrane, the nature of the nucleotide binding site. *Eur J Biochem*. 1976; 62:223–8. [PubMed: 1253787]

19. Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxid Med Cell Longev*. 2016; 2016:1245049. [PubMed: 27478531]
20. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol*. 1996; 271:C1424–37. [PubMed: 8944624]
21. Marinho HS, Real C, Cyrne L, Soares H, Antunes F. Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol*. 2014; 2:535–62. [PubMed: 24634836]
22. Longo VD, Liou LL, Valentine JS, Gralla EB. Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch Biochem Biophys*. 1999; 365:131–42. [PubMed: 10222047]
23. Papa S, Skulachev VP. Reactive oxygen species, mitochondria, apoptosis and aging. *Mol Cell Biochem*. 1997; 174:305–19. [PubMed: 9309704]
24. Van Raamsdonk JM, Hekimi S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc Natl Acad Sci U S A*. 2012; 109:5785–90. [PubMed: 22451939]
25. D’Autreaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol*. 2007; 8:813–24. [PubMed: 17848967]
26. Brown DI, Griendling KK. Nox proteins in signal transduction. *Free Radic Biol Med*. 2009; 47:1239–53. [PubMed: 19628035]
27. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev*. 1979; 59:527–605. [PubMed: 37532]
28. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)*. 2005; 70:200–14. [PubMed: 15807660]
29. Loschen G, Flohe L, Chance B. Respiratory chain linked H₂O₂ production in pigeon heart mitochondria. *FEBS Lett*. 1971; 18:261–4. [PubMed: 11946135]
30. Wong HS, Dighe PA, Mezera V, Monternier PA, Brand MD. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. *J Biol Chem*. 2017; 292:16804–9. [PubMed: 28842493]
31. Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL, Brand MD. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biol*. 2013; 1:304–12. [PubMed: 24024165]
32. Quinlan CL, Goncalves RL, Hey-Mogensen M, Yadava N, Bunik VI, Brand MD. The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I. *J Biol Chem*. 2014; 289:8312–25. [PubMed: 24515115]
33. Hey-Mogensen M, Goncalves RL, Orr AL, Brand MD. Production of superoxide/H₂O₂ by dihydroorotate dehydrogenase in rat skeletal muscle mitochondria. *Free Radic Biol Med*. 2014; 72:149–55. [PubMed: 24746616]
34. Brand MD. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radic Biol Med*. 2016; 100:14–31. [PubMed: 27085844]
35. Hagopian K, Chen Y, Simmons D, Dimer K, Soo Hoo R, Bentley T, McDonald RB, et al. Caloric restriction influences hydrogen peroxide generation in mitochondrial sub-populations from mouse liver. *J Bioenerg Biomembr*. 2011; 43:227–36. [PubMed: 21505800]
36. Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, et al. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. *J Neurosci*. 2004; 24:7779–88. [PubMed: 15356189]
37. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009; 417:1–13. [PubMed: 19061483]
38. Drose S, Brandt U. Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Adv Exp Med Biol*. 2012; 748:145–69. [PubMed: 22729857]
39. Mailloux RJ, Harper ME. Mitochondrial proticity and ROS signaling: lessons from the uncoupling proteins. *Trends Endocrinol Metab*. 2012; 23:451–8. [PubMed: 22591987]
40. Aon MA, Cortassa S, O’Rourke B. Redox-optimized ROS balance: a unifying hypothesis. *Biochim Biophys Acta*. 2010; 1797:865–77. [PubMed: 20175987]
41. Hoffman DL, Brookes PS. Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions. *J Biol Chem*. 2009; 284:16236–45. [PubMed: 19366681]

42. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014; 515:431–5. [PubMed: 25383517]
43. Miwa S, Brand MD. Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem Soc Trans*. 2003; 31:1300–1. [PubMed: 14641047]
44. Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett*. 1997; 416:15–8. [PubMed: 9369223]
45. Rottenberg H, Covian R, Trumpower BL. Membrane potential greatly enhances superoxide generation by the cytochrome bc₁ complex reconstituted into phospholipid vesicles. *J Biol Chem*. 2009; 284:19203–10. [PubMed: 19478336]
46. Korshunov SS, Korkina OV, Ruuge EK, Skulachev VP, Starkov AA. Fatty acids as natural uncouplers preventing generation of O₂^{•-} and H₂O₂ by mitochondria in the resting state. *FEBS Lett*. 1998; 435:215–8. [PubMed: 9762912]
47. Massari S, Bosel A, Wrigglesworth JM. The variation of K_m for oxygen of cytochrome oxidase with turnover under de-energized and energized conditions. *Biochem Soc Trans*. 1996; 24:464S. [PubMed: 8879008]
48. Gnaiger E, Lassnig B, Kuznetsov A, Rieger G, Margreiter R. Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *J Exp Biol*. 1998; 201:1129–39. [PubMed: 9510525]
49. Tahara EB, Navarete FD, Kowaltowski AJ. Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. *Free Radic Biol Med*. 2009; 46:1283–97. [PubMed: 19245829]
50. Sharaf MS, Stevens D, Kamunde C. Mitochondrial transition ROS spike (mTRS) results from coordinated activities of complex I and nicotinamide nucleotide transhydrogenase. *Biochim Biophys Acta*. 2017; 1858:955–65.
51. Munro D, Treberg JR. A radical shift in perspective: mitochondria as regulators of reactive oxygen species. *J Exp Biol*. 2017; 220:1170–80. [PubMed: 28356365]
52. Kussmaul L, Hirst J. The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc Natl Acad Sci U S A*. 2006; 103:7607–12. [PubMed: 16682634]
53. Bazil JN, Pannala VR, Dash RK, Beard DA. Determining the origins of superoxide and hydrogen peroxide in the mammalian NADH:ubiquinone oxidoreductase. *Free Radic Biol Med*. 2014; 77:121–9. [PubMed: 25236739]
54. Scialo F, Fernandez-Ayala DJ, Sanz A. Role of Mitochondrial Reverse Electron Transport in ROS Signaling: Potential Roles in Health and Disease. *Front Physiol*. 2017; 8:428. [PubMed: 28701960]
55. Stepanova A, Kahl A, Konrad C, Ten V, Starkov AS, Galkin A. Reverse electron transfer results in a loss of flavin from mitochondrial complex I: Potential mechanism for brain ischemia reperfusion injury. *J Cereb Blood Flow Metab*. 2017; 37:3649–58. [PubMed: 28914132]
56. Speijer D. Being right on Q: shaping eukaryotic evolution. *Biochem J*. 2016; 473:4103–27. [PubMed: 27834740]
57. Ohnishi T, Trumpower BL. Differential effects of antimycin on ubisemiquinone bound in different environments in isolated succinate. cytochrome c reductase complex. *J Biol Chem*. 1980; 255:3278–84. [PubMed: 6245075]
58. Brand MD, Chien LF, Ainscow EK, Rolfe DF, Porter RK. The causes and functions of mitochondrial proton leak. *Biochim Biophys Acta*. 1994; 1187:132–9. [PubMed: 8075107]
59. Benz R, McLaughlin S. The molecular mechanism of action of the proton ionophore FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone). *Biophys J*. 1983; 41:381–98. [PubMed: 6838976]
60. Goldsby RA, Heytler PG. Uncoupling of Oxidative Phosphorylation by Carbonyl Cyanide Phenylhydrazones. II. Effects of Carbonyl Cyanide M-Chlorophenylhydrazone on Mitochondrial Respiration. *Biochemistry*. 1963; 2:1142–7. [PubMed: 14087375]

61. Heytler PG, Prichard WW. A new class of uncoupling agents--carbonyl cyanide phenylhydrazones. *Biochem Biophys Res Commun.* 1962; 7:272–5. [PubMed: 13907155]
62. Mitchell P, Moyle J. Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Eur J Biochem.* 1969; 7:471–84. [PubMed: 5776240]
63. Childress ES, Alexopoulos SJ, Hoehn KL, Santos WL. Small Molecule Mitochondrial Uncouplers and Their Therapeutic Potential. *J Med Chem.* 2017
64. Caldeira da Silva CC, Cerqueira FM, Barbosa LF, Medeiros MH, Kowaltowski AJ. Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging Cell.* 2008; 7:552–60. [PubMed: 18505478]
65. Goldhof M, Xiao C, Chanturiya T, Jou W, Gavrilova O, Reitman ML. The chemical uncoupler 2,4-dinitrophenol (DNP) protects against diet-induced obesity and improves energy homeostasis in mice at thermoneutrality. *J Biol Chem.* 2014; 289:19341–50. [PubMed: 24872412]
66. Perry RJ, Kim T, Zhang XM, Lee HY, Pesta D, Popov VB, et al. Reversal of hypertriglyceridemia, fatty liver disease, and insulin resistance by a liver-targeted mitochondrial uncoupler. *Cell Metab.* 2013; 18:740–8. [PubMed: 24206666]
67. Khailova LS, Silachev DN, Rokitskaya TI, Avetisyan AV, Lyamsaev KG, Severina II, et al. A short-chain alkyl derivative of Rhodamine 19 acts as a mild uncoupler of mitochondria and a neuroprotector. *Biochim Biophys Acta.* 2014; 1837:1739–47. [PubMed: 25038514]
68. Perry RJ, Zhang D, Zhang XM, Boyer JL, Shulman GI. Controlled-release mitochondrial protonophore reverses diabetes and steatohepatitis in rats. *Science.* 2015; 347:1253–6. [PubMed: 25721504]
69. Abulizi A, Perry RJ, Camporez JPG, Jurczak MJ, Petersen KF, Aspichueta P, et al. A controlled-release mitochondrial protonophore reverses hypertriglyceridemia, nonalcoholic steatohepatitis, and diabetes in lipodystrophic mice. *FASEB J.* 2017; 31:2916–24. [PubMed: 28330852]
70. Starkov AA, Fiskum G. Regulation of brain mitochondrial H₂O₂ production by membrane potential and NAD(P)H redox state. *J Neurochem.* 2003; 86:1101–7. [PubMed: 12911618]
71. Brennan JP, Berry RG, Baghai M, Duchon MR, Shattock MJ. FCCP is cardioprotective at concentrations that cause mitochondrial oxidation without detectable depolarisation. *Cardiovasc Res.* 2006; 72:322–30. [PubMed: 16979603]
72. Brennan JP, Southworth R, Medina RA, Davidson SM, Duchon MR, Shattock MJ. Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation. *Cardiovasc Res.* 2006; 72:313–21. [PubMed: 16950237]
73. Lou PH, Hansen BS, Olsen PH, Tullin S, Murphy MP, Brand MD. Mitochondrial uncouplers with an extraordinary dynamic range. *Biochem J.* 2007; 407:129–40. [PubMed: 17608618]
74. Nicholls DG. Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. *J Biol Chem.* 2006; 281:14864–74. [PubMed: 16551630]
75. Chalmers S, Caldwell ST, Quin C, Prime TA, James AM, Cairns AG, et al. Selective uncoupling of individual mitochondria within a cell using a mitochondria-targeted photoactivated protonophore. *J Am Chem Soc.* 2012; 134:758–61. [PubMed: 22239373]
76. Glancy B, Hartnell LM, Combs CA, Fenmou A, Sun J, Murphy E, et al. Power Grid Protection of the Muscle Mitochondrial Reticulum. *Cell Rep.* 2017; 19:487–96. [PubMed: 28423313]
77. Parker N, Crichton PG, Vidal-Puig AJ, Brand MD. Uncoupling protein-1 (UCP1) contributes to the basal proton conductance of brown adipose tissue mitochondria. *J Bioenerg Biomembr.* 2009; 41:335–42. [PubMed: 19705265]
78. Walker JE, Runswick MJ. The mitochondrial transport protein superfamily. *J Bioenerg Biomembr.* 1993; 25:435–46. [PubMed: 8132484]
79. Rupprecht A, Sokolenko EA, Beck V, Ninnemann O, Jaburek M, Trimbuch T, et al. Role of the transmembrane potential in the membrane proton leak. *Biophys J.* 2010; 98:1503–11. [PubMed: 20409469]
80. Nicholls DG. The effective proton conductance of the inner membrane of mitochondria from brown adipose tissue. Dependency on proton electrochemical potential gradient. *Eur J Biochem.* 1977; 77:349–56. [PubMed: 19250]

81. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell*. 2012; 151:400–13. [PubMed: 23063128]
82. Ledesma A, de Lacoba MG, Rial E. The mitochondrial uncoupling proteins. *Genome Biol*. 2002; 3:REVIEWS3015. [PubMed: 12537581]
83. Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, et al. Superoxide activates mitochondrial uncoupling proteins. *Nature*. 2002; 415:96–9. [PubMed: 11780125]
84. Brookes PS. Mitochondrial H(+) leak and ROS generation: an odd couple. *Free Radic Biol Med*. 2005; 38:12–23. [PubMed: 15589367]
85. Cannon B, Shabalina IG, Kramarova TV, Petrovic N, Nedergaard J. Uncoupling proteins: a role in protection against reactive oxygen species--or not? *Biochim Biophys Acta*. 2006; 1757:449–58. [PubMed: 16806053]
86. Shabalina IG, Nedergaard J. Mitochondrial ('mild') uncoupling and ROS production: physiologically relevant or not? *Biochem Soc Trans*. 2011; 39:1305–9. [PubMed: 21936806]
87. Bouillaud F. UCP2, not a physiologically relevant uncoupler but a glucose sparing switch impacting ROS production and glucose sensing. *Biochim Biophys Acta*. 2009; 1787:377–83. [PubMed: 19413946]
88. Mailloux RJ, Harper ME. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radic Biol Med*. 2011; 51:1106–15. [PubMed: 21762777]
89. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, et al. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic Biol Med*. 2004; 37:755–67. [PubMed: 15304252]
90. Jezek P, Holendova B, Garlid KD, Jaburek M. Mitochondrial uncoupling proteins: subtle regulators of cellular redox signaling. *Antioxid Redox Signal*. 2018
91. Anedda A, Lopez-Bernardo E, Acosta-Iborra B, Saadeh Suleiman M, Landazuri MO, Cadenas S. The transcription factor Nrf2 promotes survival by enhancing the expression of uncoupling protein 3 under conditions of oxidative stress. *Free Radic Biol Med*. 2013; 61:395–407. [PubMed: 23597505]
92. Cadenas S, Buckingham JA, Samec S, Seydoux J, Din N, Dulloo AG, et al. UCP2 and UCP3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged. *FEBS Lett*. 1999; 462:257–60. [PubMed: 10622707]
93. Azzu V, Brand MD. The on-off switches of the mitochondrial uncoupling proteins. *Trends Biochem Sci*. 2010; 35:298–307. [PubMed: 20006514]
94. Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, et al. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem J*. 2005; 392:353–62. [PubMed: 16076285]
95. Duszynski J, Bogucka K, Letko G, Kuster U, Kunz W, Wojtczak L. Relationship between the energy cost of ATP transport and ATP synthesis in mitochondria. *Biochim Biophys Acta*. 1981; 637:217–23. [PubMed: 7295709]
96. Kramer R. Structural and functional aspects of the phosphate carrier from mitochondria. *Kidney Int*. 1996; 49:947–52. [PubMed: 8691742]
97. Hoek JB, Njogu RM. The role of glutamate transport in the regulation of the pathway of proline oxidation in rat liver mitochondria. *J Biol Chem*. 1980; 255:8711–8. [PubMed: 7410389]
98. Azzu V, Parker N, Brand MD. High membrane potential promotes alkenal-induced mitochondrial uncoupling and influences adenine nucleotide translocase conformation. *Biochem J*. 2008; 413:323–32. [PubMed: 18426390]
99. Samartsev VN, Smirnov AV, Zeldi IP, Markova OV, Mokhova EN, Skulachev VP. Involvement of aspartate/glutamate antiporter in fatty acid-induced uncoupling of liver mitochondria. *Biochim Biophys Acta*. 1997; 1319:251–7. [PubMed: 9131047]
100. Garlid KD, Beavis AD, Ratkje SK. On the nature of ion leaks in energy-transducing membranes. *Biochim Biophys Acta*. 1989; 976:109–20. [PubMed: 2675980]
101. Kowaltowski AJ, Cosso RG, Campos CB, Fiskum G. Effect of Bcl-2 overexpression on mitochondrial structure and function. *J Biol Chem*. 2002; 277:42802–7. [PubMed: 12207028]
102. Akerman KE, Wikstrom MK. Safranin as a probe of the mitochondrial membrane potential. *FEBS Lett*. 1976; 68:191–7. [PubMed: 976474]

103. Garlid KD, Halestrap AP. The mitochondrial K(ATP) channel--fact or fiction? *J Mol Cell Cardiol.* 2012; 52:578–83. [PubMed: 22240339]
104. Heinen A, Camara AK, Aldakkak M, Rhodes SS, Riess ML, Stowe DF. Mitochondrial Ca²⁺-induced K⁺ influx increases respiration and enhances ROS production while maintaining membrane potential. *Am J Physiol Cell Physiol.* 2007; 292:C148–56. [PubMed: 16870831]
105. Andrukhiv A, Costa AD, West IC, Garlid KD. Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. *Am J Physiol Heart Circ Physiol.* 2006; 291:H2067–74. [PubMed: 16798828]
106. Brookes PS, Rolfe DF, Brand MD. The proton permeability of liposomes made from mitochondrial inner membrane phospholipids: comparison with isolated mitochondria. *J Membr Biol.* 1997; 155:167–74. [PubMed: 9049110]
107. Morota S, Piel S, Hansson MJ. Respiratory uncoupling by increased H(+) or K(+) flux is beneficial for heart mitochondrial turnover of reactive oxygen species but not for permeability transition. *BMC Cell Biol.* 2013; 14:40. [PubMed: 24053891]
108. Selivanov VA, Zeak JA, Roca J, Cascante M, Trucco M, Votyakova TV. The role of external and matrix pH in mitochondrial reactive oxygen species generation. *J Biol Chem.* 2008; 283:29292–300. [PubMed: 18687689]
109. Szabo I, Zoratti M. Mitochondrial channels: ion fluxes and more. *Physiol Rev.* 2014; 94:519–608. [PubMed: 24692355]
110. Wojtovich AP, Smith CO, Haynes CM, Nehrke KW, Brookes PS. Physiological consequences of complex II inhibition for aging, disease, and the mKATP channel. *Biochim Biophys Acta.* 2013; 1827:598–611. [PubMed: 23291191]
111. Queliconi BB, Wojtovich AP, Nadtochiy SM, Kowaltowski AJ, Brookes PS. Redox regulation of the mitochondrial K(ATP) channel in cardioprotection. *Biochim Biophys Acta.* 2011; 1813:1309–15. [PubMed: 21094666]
112. Wojtovich AP, Nadtochiy SM, Brookes PS, Nehrke K. Ischemic preconditioning: the role of mitochondria and aging. *Exp Gerontol.* 2012; 47:1–7. [PubMed: 22100642]
113. Ferranti R, da Silva MM, Kowaltowski AJ. Mitochondrial ATP-sensitive K⁺ channel opening decreases reactive oxygen species generation. *FEBS Lett.* 2003; 536:51–5. [PubMed: 12586337]
114. Costa AD, Quinlan CL, Andrukhiv A, West IC, Jaburek M, Garlid KD. The direct physiological effects of mitoK(ATP) opening on heart mitochondria. *Am J Physiol Heart Circ Physiol.* 2006; 290:H406–15. [PubMed: 16143645]
115. Akopova OV, Kolchinskaya LI, Nosar VI, Bouryi VA, Man'kovskaya IN, Sagach VF. Effect of Valinomycin-Induced Potassium Influx on the Formation of Reactive Oxygen Species in the Rat Brain Mitochondria. *Neurophysiology+.* 2013; 45:388–95.
116. Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD. Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. *Am J Physiol Heart Circ Physiol.* 2001; 280:H649–57. [PubMed: 11158963]
117. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K⁺ channels modulate cardiac mitochondrial function. *Am J Physiol.* 1998; 275:H1567–76. [PubMed: 9815062]
118. Holmuhamedov EL, Wang L, Terzic A. ATP-sensitive K⁺ channel openers prevent Ca²⁺ overload in rat cardiac mitochondria. *J Physiol.* 1999; 519(Pt 2):347–60. [PubMed: 10457054]
119. Bajgar R, Seetharaman S, Kowaltowski AJ, Garlid KD, Paucek P. Identification and properties of a novel intracellular (mitochondrial) ATP-sensitive potassium channel in brain. *J Biol Chem.* 2001; 276:33369–74. [PubMed: 11441006]
120. Cancherini DV, Trabuco LG, Reboucas NA, Kowaltowski AJ. ATP-sensitive K⁺ channels in renal mitochondria. *Am J Physiol Renal Physiol.* 2003; 285:F1291–6. [PubMed: 12952853]
121. Kulawiak B, Kudin AP, Szewczyk A, Kunz WS. BK channel openers inhibit ROS production of isolated rat brain mitochondria. *Exp Neurol.* 2008; 212:543–7. [PubMed: 18572168]
122. Yang M, Camara AKS, Aldakkak M, Kwok WM, Stowe DF. Identity and function of a cardiac mitochondrial small conductance Ca(2+)-activated K(+) channel splice variant. *Biochim Biophys Acta.* 2017; 1858:442–58.

123. Costa AD, Garlid KD. Intramitochondrial signaling: interactions among mitoKATP, PKCepsilon, ROS, and MPT. *Am J Physiol Heart Circ Physiol.* 2008; 295:H874–82. [PubMed: 18586884]
124. Facundo HT, de Paula JG, Kowaltowski AJ. Mitochondrial ATP-sensitive K⁺ channels are redox-sensitive pathways that control reactive oxygen species production. *Free Radic Biol Med.* 2007; 42:1039–48. [PubMed: 17349931]
125. Pereverzev MO, Vygodina TV, Konstantinov AA, Skulachev VP. Cytochrome c, an ideal antioxidant. *Biochem Soc Trans.* 2003; 31:1312–5. [PubMed: 14641051]
126. Guidot DM, Repine JE, Kitlowski AD, Flores SC, Nelson SK, Wright RM, et al. Mitochondrial respiration scavenges extramitochondrial superoxide anion via a nonenzymatic mechanism. *J Clin Invest.* 1995; 96:1131–6. [PubMed: 7635949]
127. Mailer K. Superoxide radical as electron donor for oxidative phosphorylation of ADP. *Biochem Biophys Res Commun.* 1990; 170:59–64. [PubMed: 2164811]
128. Brand MD, Chien LF, Diolet P. Experimental discrimination between proton leak and redox slip during mitochondrial electron transport. *Biochem J.* 1994; 297(Pt 1):27–9. [PubMed: 8280106]
129. Bernardi P, Di Lisa F. The mitochondrial permeability transition pore: molecular nature and role as a target in cardioprotection. *J Mol Cell Cardiol.* 2015; 78:100–6. [PubMed: 25268651]
130. Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, et al. The mitochondrial permeability transition from in vitro artifact to disease target. *FEBS J.* 2006; 273:2077–99. [PubMed: 16649987]
131. Beutner G, Alavian KN, Jonas EA, Porter GA Jr. The Mitochondrial Permeability Transition Pore and ATP Synthase. *Handb Exp Pharmacol.* 2017; 240:21–46. [PubMed: 27590224]
132. He J, Carroll J, Ding S, Fearnley IM, Walker JE. Permeability transition in human mitochondria persists in the absence of peripheral stalk subunits of ATP synthase. *Proc Natl Acad Sci U S A.* 2017; 114:9086–91. [PubMed: 28784775]
133. Korge P, John SA, Calmettes G, Weiss JN. Reactive oxygen species production induced by pore opening in cardiac mitochondria: The role of complex II. *J Biol Chem.* 2017; 292:9896–905. [PubMed: 28450394]
134. Kowaltowski AJ, Castilho RF, Vercesi AE. Mitochondrial permeability transition and oxidative stress. *FEBS Lett.* 2001; 495:12–5. [PubMed: 11322939]
135. Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem.* 1992; 267:8834–9. [PubMed: 1374381]
136. Brenner C, Moulin M. Physiological roles of the permeability transition pore. *Circ Res.* 2012; 111:1237–47. [PubMed: 23065346]
137. Wong R, Steenbergen C, Murphy E. Mitochondrial permeability transition pore and calcium handling. *Methods Mol Biol.* 2012; 810:235–42. [PubMed: 22057571]
138. Ristow M, Schmeisser S. Extending life span by increasing oxidative stress. *Free Radic Biol Med.* 2011; 51:327–36. [PubMed: 21619928]
139. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun.* 1997; 236:313–22. [PubMed: 9240432]
140. Nguyen T, Huang HC, Pickett CB. Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J Biol Chem.* 2000; 275:15466–73. [PubMed: 10747902]
141. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* 1999; 13:76–86. [PubMed: 9887101]
142. Lopez-Bernardo E, Anedda A, Sanchez-Perez P, Acosta-Iborra B, Cadenas S. 4-Hydroxynonenal induces Nrf2-mediated UCP3 upregulation in mouse cardiomyocytes. *Free Radic Biol Med.* 2015; 88:427–38. [PubMed: 25843654]
143. Martinus RD, Garth GP, Webster TL, Cartwright P, Naylor DJ, Hoj PB, et al. Selective induction of mitochondrial chaperones in response to loss of the mitochondrial genome. *Eur J Biochem.* 1996; 240:98–103. [PubMed: 8797841]

144. Nargund AM, Pellegrino MW, Fiorese CJ, Baker BM, Haynes CM. Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science*. 2012; 337:587–90. [PubMed: 22700657]
145. Fiorese CJ, Schulz AM, Lin YF, Rosin N, Pellegrino MW, Haynes CM. The Transcription Factor ATF5 Mediates a Mammalian Mitochondrial UPR. *Curr Biol*. 2016; 26:2037–43. [PubMed: 27426517]
146. Runkel ED, Liu S, Baumeister R, Schulze E. Surveillance-activated defenses block the ROS-induced mitochondrial unfolded protein response. *PLoS Genet*. 2013; 9:e1003346. [PubMed: 23516373]
147. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. *Annu Rev Biochem*. 2007; 76:723–49. [PubMed: 17263664]
148. Quiros PM, Prado MA, Zamboni N, D'Amico D, Williams RW, Finley D, et al. Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress response in mammals. *J Cell Biol*. 2017; 216:2027–45. [PubMed: 28566324]
149. Arnould T, Michel S, Renard P. Mitochondria Retrograde Signaling and the UPR mt: Where Are We in Mammals? *Int J Mol Sci*. 2015; 16:18224–51. [PubMed: 26258774]
150. Georgakopoulos ND, Wells G, Campanella M. The pharmacological regulation of cellular mitophagy. *Nat Chem Biol*. 2017; 13:136–46. [PubMed: 28103219]
151. Okatsu K, Oka T, Iguchi M, Imamura K, Kosako H, Tani N, et al. PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. *Nat Commun*. 2012; 3:1016. [PubMed: 22910362]
152. Berezhnov AV, Soutar MP, Fedotova EI, Frolova MS, Plun-Favreau H, Zinchenko VP, et al. Intracellular pH Modulates Autophagy and Mitophagy. *J Biol Chem*. 2016; 291:8701–8. [PubMed: 26893374]
153. Weisova P, Anilkumar U, Ryan C, Concannon CG, Prehn JH, Ward MW. 'Mild mitochondrial uncoupling' induced protection against neuronal excitotoxicity requires AMPK activity. *Biochim Biophys Acta*. 2012; 1817:744–53. [PubMed: 22336583]
154. Laker RC, Drake JC, Wilson RJ, Lira VA, Lewellen BM, Ryall KA, et al. Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. *Nat Commun*. 2017; 8:548. [PubMed: 28916822]
155. Xiao B, Goh JY, Xiao L, Xian H, Lim KL, Liou YC. Reactive oxygen species trigger Parkin/PINK1 pathway-dependent mitophagy by inducing mitochondrial recruitment of Parkin. *J Biol Chem*. 2017; 292:16697–708. [PubMed: 28848050]
156. Sakellariou GK, Pearson T, Lightfoot AP, Nye GA, Wells N, Giakoumaki II, et al. Mitochondrial ROS regulate oxidative damage and mitophagy but not age-related muscle fiber atrophy. *Sci Rep*. 2016; 6:33944. [PubMed: 27681159]
157. Carroll B, Otten EG, Manni D, Stefanatos R, Menzies FM, Smith GR, et al. Oxidation of SQSTM1/p62 mediates the link between redox state and protein homeostasis. *Nat Commun*. 2018; 9:256. [PubMed: 29343728]
158. Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, Spiegelman BM. A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. *Proc Natl Acad Sci U S A*. 2007; 104:7933–8. [PubMed: 17470778]
159. Drose S, Brandt U, Wittig I. Mitochondrial respiratory chain complexes as sources and targets of thiol-based redox-regulation. *Biochim Biophys Acta*. 2014; 1844:1344–54. [PubMed: 24561273]
160. Heymsfield SB, Wadden TA. Mechanisms, Pathophysiology, and Management of Obesity. *N Engl J Med*. 2017; 376:254–66. [PubMed: 28099824]
161. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. *Physiol Rev*. 1984; 64:1–64. [PubMed: 6320232]
162. Chouchani ET, Kazak L, Spiegelman BM. Mitochondrial reactive oxygen species and adipose tissue thermogenesis: Bridging physiology and mechanisms. *J Biol Chem*. 2017; 292:16810–6. [PubMed: 28842500]
163. Chouchani ET, Kazak L, Jedrychowski MP, Lu GZ, Erickson BK, Szpyt J, et al. Corrigendum: Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1. *Nature*. 2016; 536:360.

164. Alcalá M, Calderon-Dominguez M, Bustos E, Ramos P, Casals N, Serra D, et al. Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose tissue from obese mice. *Sci Rep*. 2017; 7:16082. [PubMed: 29167565]
165. Karlsson M, Kurz T, Brunk UT, Nilsson SE, Frennsson CI. What does the commonly used DCF test for oxidative stress really show? *Biochem J*. 2010; 428:183–90. [PubMed: 20331437]
166. Forman HJ, Augusto O, Brigelius-Flohe R, Dennery PA, Kalyanaraman B, Ischiropoulos H, et al. Even free radicals should follow some rules: a guide to free radical research terminology and methodology. *Free Radic Biol Med*. 2015; 78:233–5. [PubMed: 25462642]
167. McMurray F, Patten DA, Harper ME. Reactive Oxygen Species and Oxidative Stress in Obesity-Recent Findings and Empirical Approaches. *Obesity (Silver Spring)*. 2016; 24:2301–10. [PubMed: 27804267]
168. Leitner BP, Huang S, Brychta RJ, Duckworth CJ, Baskin AS, McGehee S, et al. Mapping of human brown adipose tissue in lean and obese young men. *Proc Natl Acad Sci U S A*. 2017; 114:8649–54. [PubMed: 28739898]
169. Kajimura S, Spiegelman BM, Seale P. Brown and Beige Fat: Physiological Roles beyond Heat Generation. *Cell Metab*. 2015; 22:546–59. [PubMed: 26445512]
170. Kalinovich AV, de Jong JM, Cannon B, Nedergaard J. UCP1 in adipose tissues: two steps to full browning. *Biochimie*. 2017; 134:127–37. [PubMed: 28109720]
171. Ikeda K, Kang Q, Yoneshiro T, Camporez JP, Maki H, Homma M, et al. UCP1-independent signaling involving SERCA2b-mediated calcium cycling regulates beige fat thermogenesis and systemic glucose homeostasis. *Nat Med*. 2017; 23:1454–65. [PubMed: 29131158]
172. Wang W, Seale P. Control of brown and beige fat development. *Nat Rev Mol Cell Biol*. 2016; 17:691–702. [PubMed: 27552974]
173. Liu D, Chan SL, de Souza-Pinto NC, Slevin JR, Wersto RP, Zhan M, et al. Mitochondrial UCP4 mediates an adaptive shift in energy metabolism and increases the resistance of neurons to metabolic and oxidative stress. *Neuromolecular Med*. 2006; 8:389–414. [PubMed: 16775390]
174. Chouchani ET, Pell VR, James AM, Work LM, Saeb-Parsy K, Frezza C, et al. A Unifying Mechanism for Mitochondrial Superoxide Production during Ischemia-Reperfusion Injury. *Cell Metab*. 2016; 23:254–63. [PubMed: 26777689]
175. Otani H. Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. *Antioxid Redox Signal*. 2004; 6:449–69. [PubMed: 15025947]
176. Puisieux F, Deplanque D, Bulckaen H, Maboudou P, Gele P, Lhermitte M, et al. Brain ischemic preconditioning is abolished by antioxidant drugs but does not up-regulate superoxide dismutase and glutathione peroxidase. *Brain Res*. 2004; 1027:30–7. [PubMed: 15494154]
177. Quarrie R, Lee DS, Steinbaugh G, Cramer B, Erdahl W, Pfeiffer DR, et al. Ischemic preconditioning preserves mitochondrial membrane potential and limits reactive oxygen species production. *J Surg Res*. 2012; 178:8–17. [PubMed: 22763215]
178. Ozcan C, Palmeri M, Horvath TL, Russell KS, Russell RR 3rd. Role of uncoupling protein 3 in ischemia-reperfusion injury, arrhythmias, and preconditioning. *Am J Physiol Heart Circ Physiol*. 2013; 304:H1192–200. [PubMed: 23457013]
179. Hoerter J, Gonzalez-Barroso MD, Couplan E, Mateo P, Gelly C, Cassard-Doulcier AM, et al. Mitochondrial uncoupling protein 1 expressed in the heart of transgenic mice protects against ischemic-reperfusion damage. *Circulation*. 2004; 110:528–33. [PubMed: 15262832]
180. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol*. 2015; 6:524–51. [PubMed: 26484802]
181. Sack MN. Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance. *Cardiovasc Res*. 2006; 72:210–9. [PubMed: 16914124]
182. Ganote C. Effects of CCCP-induced mitochondrial uncoupling and cyclosporin A on cell volume, cell injury and preconditioning protection of isolated rabbit cardiomyocytes. *Journal of Molecular and Cellular Cardiology*. 2003; 35:749–59. [PubMed: 12818565]
183. Korde AS, Pettigrew LC, Craddock SD, Maragos WF. The mitochondrial uncoupler 2,4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia. *J Neurochem*. 2005; 94:1676–84. [PubMed: 16045446]

184. Minners J, van den Bos EJ, Yellon DM, Schwalb H, Opie LH, Sack MN. Dinitrophenol, cyclosporin A, and trimetazidine modulate preconditioning in the isolated rat heart: support for a mitochondrial role in cardioprotection. *Cardiovasc Res.* 2000; 47:68–73. [PubMed: 10869531]
185. Rodrigo GC, Lawrence CL, Standen NB. Dinitrophenol pretreatment of rat ventricular myocytes protects against damage by metabolic inhibition and reperfusion. *J Mol Cell Cardiol.* 2002; 34:555–69. [PubMed: 12056859]
186. Dost T, Cohen MV, Downey JM. Redox signaling triggers protection during the reperfusion rather than the ischemic phase of preconditioning. *Basic Res Cardiol.* 2008; 103:378–84. [PubMed: 18347834]
187. Zhang YQ, Shen X, Xiao XL, Liu MY, Li SL, Yan J, et al. Mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone induces vasorelaxation without involving KATP channel activation in smooth muscle cells of arteries. *Br J Pharmacol.* 2016; 173:3145–58. [PubMed: 27534899]
188. Kim AS, Miller EJ, Wright TM, Li J, Qi D, Atsina K, et al. A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. *J Mol Cell Cardiol.* 2011; 51:24–32. [PubMed: 21402077]
189. Qi D, Young LH. AMPK: energy sensor and survival mechanism in the ischemic heart. *Trends Endocrinol Metab.* 2015; 26:422–9. [PubMed: 26160707]
190. Smith CO, Nehrke K, Brookes PS. The Slo(w) path to identifying the mitochondrial channels responsible for ischemic protection. *Biochem J.* 2017; 474:2067–94. [PubMed: 28600454]
191. Krenz M, Oldenburg O, Wimpee H, Cohen MV, Garlid KD, Critz SD, et al. Opening of ATP-sensitive potassium channels causes generation of free radicals in vascular smooth muscle cells. *Basic Res Cardiol.* 2002; 97:365–73. [PubMed: 12200636]
192. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem.* 1998; 273:18092–8. [PubMed: 9660766]
193. Cohen MV, Yang XM, Liu GS, Heusch G, Downey JM. Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals and opening mitochondrial K(ATP) channels. *Circ Res.* 2001; 89:273–8. [PubMed: 11485978]
194. Rasola A, Bernardi P. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. *Cell Calcium.* 2011; 50:222–33. [PubMed: 21601280]
195. Hausenloy D, Wynne A, Duchon M, Yellon D. Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation.* 2004; 109:1714–7. [PubMed: 15066952]
196. Kang PT, Chen CL, Lin P, Chilian WM, Chen YR. Impairment of pH gradient and membrane potential mediates redox dysfunction in the mitochondria of the post-ischemic heart. *Basic Res Cardiol.* 2017; 112:36. [PubMed: 28508960]
197. Hu H, Nan J, Sun Y, Zhu D, Xiao C, Wang Y, et al. Electron leak from NDUFA13 within mitochondrial complex I attenuates ischemia-reperfusion injury via dimerized STAT3. *Proc Natl Acad Sci U S A.* 2017; 114:11908–13. [PubMed: 29078279]
198. Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. *Immunity.* 2013; 38:633–43. [PubMed: 23601682]
199. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity.* 2013; 38:225–36. [PubMed: 23415911]
200. Tamas P, Hawley SA, Clarke RG, Mustard KJ, Green K, Hardie DG, et al. Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca²⁺ in T lymphocytes. *J Exp Med.* 2006; 203:1665–70. [PubMed: 16818670]
201. Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. *J Exp Med.* 2015; 212:1345–60. [PubMed: 26261266]
202. Sukumar M, Liu J, Mehta GU, Patel SJ, Roychoudhuri R, Crompton JG, et al. Mitochondrial Membrane Potential Identifies Cells with Enhanced Stemness for Cellular Therapy. *Cell Metab.* 2016; 23:63–76. [PubMed: 26674251]

203. Perl A, Gergely P Jr, Banki K. Mitochondrial dysfunction in T cells of patients with systemic lupus erythematosus. *Int Rev Immunol.* 2004; 23:293–313. [PubMed: 15204090]
204. Chen J, Chernatynskaya AV, Li JW, Kimbrell MR, Cassidy RJ, Perry DJ, et al. T cells display mitochondria hyperpolarization in human type 1 diabetes. *Sci Rep.* 2017; 7:10835. [PubMed: 28883439]
205. Rupprecht A, Brauer AU, Smorodchenko A, Goyn J, Hilse KE, Shabalina IG, et al. Quantification of uncoupling protein 2 reveals its main expression in immune cells and selective up-regulation during T-cell proliferation. *PLoS One.* 2012; 7:e41406. [PubMed: 22870219]
206. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, et al. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. *Cell.* 2016; 166:63–76. [PubMed: 27293185]
207. Kesarwani P, Murali AK, Al-Khami AA, Mehrotra S. Redox regulation of T-cell function: from molecular mechanisms to significance in human health and disease. *Antioxid Redox Signal.* 2013; 18:1497–534. [PubMed: 22938635]
208. Alleman RJ, Katunga LA, Nelson MA, Brown DA, Anderson EJ. The “Goldilocks Zone” from a redox perspective-Adaptive vs. deleterious responses to oxidative stress in striated muscle. *Front Physiol.* 2014; 5:358. [PubMed: 25278906]
209. Villanueva C, Kross RD. Antioxidant-induced stress. *Int J Mol Sci.* 2012; 13:2091–109. [PubMed: 22408440]
210. Steinhubl SR. Why have antioxidants failed in clinical trials? *Am J Cardiol.* 2008; 101:14D–9D.
211. Ristow M, Zarse K, Oberbach A, Klötting N, Birringer M, Kiehnopf M, et al. Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A.* 2009; 106:8665–70. [PubMed: 19433800]
212. Trewin AJ, Lundell LS, Perry BD, Patil KV, Chibalin AV, Levinger I, et al. Effect of N-acetylcysteine infusion on exercise-induced modulation of insulin sensitivity and signaling pathways in human skeletal muscle. *Am J Physiol Endocrinol Metab.* 2015; 309:E388–97. [PubMed: 26105008]
213. Maity S, Rajkumar A, Matai L, Bhat A, Ghosh A, Agam G, et al. Oxidative Homeostasis Regulates the Response to Reductive Endoplasmic Reticulum Stress through Translation Control. *Cell Rep.* 2016; 16:851–65. [PubMed: 27373166]
214. Kalyanaraman B, Cheng G, Hardy M, Ouari O, Bennett B, Zielonka J. Teaching the basics of reactive oxygen species and their relevance to cancer biology: Mitochondrial reactive oxygen species detection, redox signaling, and targeted therapies. *Redox Biol.* 2018; 15:347–62. [PubMed: 29306792]
215. Shchepinova MM, Cairns AG, Prime TA, Logan A, James AM, Hall AR, et al. MitoNeoD: A Mitochondria-Targeted Superoxide Probe. *Cell Chem Biol.* 2017; 24:1285–98e12. [PubMed: 28890317]
216. Cocheme HM, Quin C, McQuaker SJ, Cabreiro F, Logan A, Prime TA, et al. Measurement of H₂O₂ within living *Drosophila* during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. *Cell Metab.* 2011; 13:340–50. [PubMed: 21356523]
217. Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques.* 2011; 50:98–115. [PubMed: 21486251]
218. Tretter L, Adam-Vizi V. Uncoupling is without an effect on the production of reactive oxygen species by in situ synaptic mitochondria. *J Neurochem.* 2007; 103:1864–71. [PubMed: 17854347]
219. Tkatch T, Greotti E, Baranauskas G, Pendin D, Roy S, Nita LI, et al. Optogenetic control of mitochondrial metabolism and Ca²⁺ signaling by mitochondria-targeted opsins. *Proc Natl Acad Sci U S A.* 2017; 114:E5167–E76. [PubMed: 28611221]

Highlights

- Mitochondria use a protonmotive force (pmf) for energy transduction.
- The pmf and reactive oxygen species (ROS) production are linked.
- Dissipating the pmf, or uncoupling, can decrease ROS production.
- Uncoupling can affect ROS signaling and diverse physiologic outputs.

Figure 1.

Electron transport chain activity can affect reactive oxygen species production. Electron transport results in proton translocation (not depicted) across the mitochondrial inner membrane (IM) establishing the protonmotive force (pmf) represented by + and -. The magnitude of the pmf is represented by the size of the signs on either side of the IM. (a) Efficient flux through the electron transport chain (ETC) results in a basal amount of reactive oxygen species (ROS) production under basal pmf. (b) Decreased flux through the ETC due to high pmf results in higher than basal ROS production. Electron flux is slower, increasing the chance of reduction of oxygen to ROS. A high pmf slows electron transport by increasing the energy required for proton pumping by the ETC. ETC complex inhibitors can also slow electron transport and induce ROS production (not pictured).

Figure 2.

General mechanisms of inducible dissipation of the protonmotive force. (a) The protonophore FCCP is depicted to outline pharmacologic depletion of the pmf, dissipating both ψ_m and pH. (b) Mitochondria contain uncoupling proteins (UCP) in the inner membrane (IM). UCPs facilitate proton leak, affecting both ψ_m and pH components of the protonmotive force (pmf). (c) Cation movement can be induced using the ionophore valinomycin (depicted) or by the activation of mitochondrial cation channels. Potassium (K⁺) influx directly affects only the ψ_m component of the pmf as positively charged ions such as K⁺ can flow down the pmf but only decrease the major ψ_m contribution. However, downstream compensation by exchangers (e.g. the KHE or phosphate carrier) can affect the pH as well. The principles of uncoupling outlined here represent distinct physiologic and nonphysiologic avenues of decreasing the pmf, highlighting that there are mechanisms to affect both components of the pmf separately or together.

Figure 3.

Stress signaling regulated by the pmf and ROS. ROS signaling paradigms center around mitochondria and can impact the pmf. Nuclear factor (erythroid-like 2) related factor (NRF2) is activated by ROS and leads to upregulation of UCP3 activity (along with antioxidant defenses), decreasing the pmf, potentially regulating ROS production. The mitochondrial unfolded protein response (UPR^{mt}) is activated by ROS and impacts proteostasis and cellular health downstream of activating transcription factors (ATFs). Localized collapse of pmf activates mitophagy through PINK and parkin proteins, stimulating mitochondrial turnover, preserving the pmf by degrading depolarized mitochondria. Changes in metabolic flux and pmf can activate the metabolic sensor 5' AMP activated protein kinase (AMPK) to activate mitophagy. These signaling networks integrate the pmf and ROS production to maintain homeostasis. The interactions shown do not represent every interaction between the outlined pathways, but highlight how the pmf and ROS coordinate them.

Figure 4.

The pmf and ROS production affect diverse physiologic outputs. (a) Mitochondrial ROS elevation coincides with brown fat activation of thermogenesis and UCP activity. Modulation of mitochondrial ROS production and uncoupling to affect browning of other fat tissues is unclear. (b) Increases in ROS are involved in both damage and protection in ischemia reperfusion (IR) injury. Mild uncoupling protects against IR damage through an unknown mechanism that may require ROS. (c) T cell activation requires elevated ROS levels. UCP2 expression is upregulated with T cell activation, potentially to prevent ROS overproduction. Optimizing the pmf could affect T cell phenotypes and novel immunotherapies. These three physiologic examples of interplay between the pmf and ROS production highlight the complexity of the interaction, and the necessity for careful experimental design and interpretation.