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## **A Unifying Mechanism for Mitochondrial Superoxide Production During Ischemia-Reperfusion Injury**

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Ischemia-reperfusion (IR) injury occurs when blood supply to an organ is disrupted - ischemia – and then restored – reperfusion, leading to a burst of reactive oxygen species (ROS) from mitochondria. It has been tacitly assumed that ROS production during IR is a non-specific consequence of oxygen interacting with dysfunctional mitochondria upon reperfusion. Recently, this view has changed suggesting that ROS production during IR occurs by a defined mechanism. Here we survey the metabolic factors underlying IR injury, and propose a unifying mechanism for its causes that makes sense of the huge amount of disparate data in this area and provides testable hypotheses and new directions for therapies.

## **Introduction**

Ischemia-reperfusion (IR) injury occurs when the blood supply to a tissue is blocked for minutes to hours (ischemia), and then restored (reperfusion) (Eltzschig and Eckle, 2011; Hausenloy and Yellon, 2013). This pervasive pathological mechanism underlies a range of disorders, including heart attack and stroke, where the prevention of blood flow to the tissue, for example by a blood clot, is followed by reperfusion when the blockage is removed mechanically or pharmacologically (Pan et al., 2007; Yellon and Hausenloy, 2007). Similar situations affect many other tissues, for example during organ transplantation (Jaeschke and Woolbright, 2012), elective surgery (Hausenloy et al., 2012) or hypovolemic shock (Gutierrez et al., 2004). Consequently, there has been an enduring interest in understanding the nature of IR injury and in developing therapies to prevent or ameliorate its effects (Bernardi et al., 2015; Eltzschig and Eckle, 2011; Hausenloy and Yellon, 2013; Murphy and Steenbergen, 2008b; Yellon and Hausenloy, 2007).

The current consensus is that a period of ischemia primes the tissue for subsequent damage upon reperfusion, with the duration of ischemia required for reperfusion injury varying with tissue (Burwell et al., 2009; Jaeschke and Woolbright, 2012; Murphy and Steenbergen, 2008b; Pan et al., 2007). Ischemic cells will eventually die if blood flow is not restored, but it is during reperfusion itself that most IR damage is initiated. Thus paradoxically, the essential therapeutic intervention to treat ischemia - reperfusion – drives tissue pathophysiology (Murphy and Steenbergen, 2008b). The first few minutes of reperfusion are critical, because what happens then initiates long-term tissue damage and dysfunction (Eltzschig and Eckle, 2011). Early studies suggested a role for mitochondrial activity early in IR injury (Ganote et al., 1976). The first damaging event upon reperfusion is a burst of reactive oxygen species (ROS) production from mitochondria (Loor et al., 2011; Zweier et al., 1987). Mitochondrial ROS not only drive acute damage but also initiate the pathology that develops over the minutes, days and weeks following reperfusion (Eltzschig and Eckle, 2011). The initial burst of ROS production upon reperfusion directly causes oxidative damage to mitochondria, thereby disrupting ATP production (Murphy and Steenbergen, 2008b). In conjunction with dysregulation of calcium levels, this elevated ROS can also lead to induction of the

mitochondrial permeability transition (Bernardi et al., 2015; Burwell et al., 2009; Di Lisa and Bernardi, 2015; Halestrap, 2010). Together, this mitochondrial disruption leads to necrotic and apoptotic cell death following reperfusion (Loor et al., 2011; Murphy and Steenbergen, 2008b). ROS-mediated mitochondrial damage will also release damage-associated molecular pattern molecules (DAMPs), such as mitochondrial DNA, which can initiate the sterile inflammatory response (Arslan et al., 2011; Chen and Nunez, 2010; Kawaguchi et al., 2011). This activation of the innate immune system causes inflammation that contributes to IR injury and can continue for days after the initial damage (Arslan et al., 2011). Therefore, during the weeks and months following the initial IR injury there will be formation of fibrotic scar tissue to replace the dead cells along with extensive tissue remodeling (Frangogiannis, 2012). The end result is often an organ that is structurally altered and functions poorly, frequently leading to persistent long-term pathology such as chronic heart disease (White et al., 1987).

The causes of mitochondrial ROS production upon reperfusion are still unclear; the tacit assumption has been that the underlying mechanisms are imprecise and complicated. This view is consistent with the bewildering range of interventions that decrease IR injury (Burwell et al., 2009; Kornfeld et al., 2015; Murphy et al., 2012; Murphy and Steenbergen, 2008a, b). However, recent findings suggest instead that there is a common pathway for the initial burst of mitochondrial ROS production that underlies IR injury. Here we present a model that can rationalize the vast array of data in this area. To do this we first survey the literature on mitochondrial ROS production during IR and then propose a unifying hypothesis for how reperfusion injury occurs. Better understanding of this process should make it possible to develop therapies that limit this early event in IR injury, and thereby blunt the subsequent pathological cascade.

### **Mitochondrial superoxide formation upon reperfusion of ischemic tissue initiates ischemia-reperfusion (IR) injury**

There is consensus that a major damaging event upon reperfusion of ischemic tissue is a burst of ROS production. This was first proposed by Hess and Manson (Hess and Manson, 1984), demonstrated by Zweier and colleagues (Zweier et al., 1987), and confirmed by many others since (e.g. Murphy and Steenbergen, 2008b). This ROS

production arises as a burst of superoxide from mitochondria upon reperfusion, as has been corroborated in a range of tissues and types of IR injury (Burwell et al., 2009; Crack and Taylor, 2005; Jaeschke and Woolbright, 2012; Padanilam, 2003). Superoxide itself can cause damage, but also acts through the formation of hydrogen peroxide and other damaging reactive species (Murphy, 2009). There are a number of other sources of superoxide outside the mitochondrion that may also contribute to IR injury, for example xanthine oxidase (Linas et al., 1990) and NADPH oxidases (NOX) (Braunersreuther et al., 2013). However, activation of these processes seems to occur after the initial burst of mitochondrial ROS and their role in the proximal cascade of reperfusion pathology is unclear, suggesting that non-mitochondrial ROS contributes to secondary tissue damage and subsequent inflammation (Abramov et al., 2007). So, although these non-mitochondrial ROS sources are important contributors to the progression of the pathology in the hours that follow reperfusion, the role of mitochondrial ROS upon initiation of reperfusion seems to be critical and is our focus.

Initial work on mitochondrial ROS production during reperfusion used electron spin resonance in conjunction with spin traps to identify a flux of radical species in the first minutes of reperfusion (Arroyo et al., 1987; Bolli et al., 1989; Garlick et al., 1987; Zweier et al., 1987). More recently, fluorescence methods to detect reactive species in the outer layer of the heart (Pasdois et al., 2008), and mass spectrometric tools for the assessment of changes in mitochondrial hydrogen peroxide levels *in vivo* (Cocheme et al., 2011), have further demonstrated mitochondrial ROS production in the first few minutes of myocardial reperfusion (Chouchani et al., 2013; Chouchani et al., 2014). While most work has been on IR injury in the heart, similar findings have been found in studies in many other organs, including the brain (Kontos et al., 1992; Nelson et al., 1992), liver (Mukhopadhyay et al., 2012), and kidney (Dare et al., 2015). Together these data provide a strong body of evidence that reperfusion of ischemic tissue drives a burst of superoxide production in mitochondria.

Preventing the damage caused by this ROS production is a promising therapeutic strategy against IR injury, although many other therapeutic interventions can act later in the pathological cascade. Therefore, our working assumption is that the production of superoxide by mitochondria over the first few minutes of reperfusion is the essential

trigger in pathological IR injury.

### **Is complex I the major source of mitochondrial superoxide during reperfusion?**

During ischemia the respiratory chain, redox active enzymes, and electron carrier pools such as NADH and CoenzymeQ (CoQ) become maximally reduced. In parallel, mitochondria become progressively compromised due to factors such as ATP depletion, lack of ion homeostasis, calcium overload, and changes in pH (Burwell et al., 2009). Consequently, when ischemic tissue is reperfused with oxygenated blood, the tacit assumption has been that inappropriately reduced and damaged mitochondrial components will spill electrons onto oxygen from numerous sites to form superoxide (Hausenloy and Yellon, 2013; Murphy and Steenbergen, 2008b). While this model of non-specific superoxide production at multiple sites within mitochondria has appeal, there is now a large amount of evidence that points to respiratory complex I as the major source of mitochondrial superoxide upon reperfusion. Complex I is the entry point for electrons from NADH into the mitochondrial respiratory chain (Figure 1A), and is well established as a major source of mitochondrial superoxide *in vitro* (Murphy, 2009). Direct evidence for complex I as the major source of superoxide upon reperfusion comes from the fact that its selective pharmacological inhibition protects from reperfusion injury and ROS production (Chen et al., 2006a, b; Lesnefsky et al., 2004; Niatsetskaya et al., 2012).

While this evidence is consistent with complex I being the main site of mitochondrial superoxide production upon reperfusion, it does not eliminate the possibility that other sites, such as complex III, also contribute. However, a major reason for supporting a dominant role for complex I in superoxide production during reperfusion is that the conditions that favor complex I superoxide production are present during IR injury, in contrast to other potential sites of superoxide. Therefore, we next consider how complex I can produce superoxide during IR injury.

### **Reverse electron transport (RET) at complex I generates superoxide upon reperfusion**

Complex I produces superoxide under two conditions (Hinkle et al., 1967; Murphy, 2009; St-Pierre et al., 2002). The first occurs during conventional forward electron transport and arises when the flavin mononucleotide (FMN) that accepts electrons from NADH becomes reduced (Cadenas et al., 1977) (Figure 1A). This occurs, for example, when complex I is inhibited by the CoenzymeQ (CoQ)-site inhibitor rotenone, or when respiration rate is decreased by mitochondrial damage (Murphy, 2009; Pryde and Hirst, 2011). However, complex I inhibitors such as rotenone decrease ROS and oxidative damage during IR, and protect against reperfusion injury (Chen et al., 2006a, b; Lesnefsky et al., 2004), indicating that superoxide production associated with forward electron transfer at complex I is unlikely to contribute to IR injury. The other mode of superoxide production by complex I is that associated with reverse electron transport (RET) (Adam-Vizi and Chinopoulos, 2006; Chance, 1961; Chance and Hollunger, 1961; Kudin et al., 2004). RET at complex I has been known since the 1960s (Chance, 1961; Chance and Hollunger, 1961), but has generally been assumed to be an *in vitro* curiosity of uncertain physiological significance (Murphy, 2009). However, as delineated below, RET at complex I seems likely to be the main source of superoxide upon reperfusion of ischemic tissue.

RET occurs when electrons are forced backwards through complex I (Figure 1B), in contrast to conventional forward transport (Figure 1A). In forward operation two electrons transfer from NADH to reduce CoQ to CoQH<sub>2</sub>, and the redox energy difference drives the pumping of four protons across the mitochondrial inner membrane to maintain the protonmotive force ( $\Delta p$ ) that drives ATP synthesis (Figure 1A). For forward operation the redox driving force ( $\Delta E_h$ ) that pushes two electrons from NADH to CoQ has to be greater than the energy required to pump four protons across the inner membrane against the protonmotive force ( $\Delta p$ ):  $2\Delta E_h > 4\Delta p$  (Figure 1A). However, as the overall reaction is not displaced far from thermodynamic equilibrium the direction of electron transport can be reversed if the  $\Delta p$  is high and/or the CoQ pool is reduced so that  $4\Delta p > 2\Delta E_h$  (Figure 1B) (Murphy, 2009). If these conditions are met electrons flow backwards through complex I and onto the FMN from where they can reduce NAD<sup>+</sup> to NADH and also drive superoxide formation (Murphy, 2009).

Both the FMN and the CoQ reduction sites of complex I have been proposed as sites of superoxide production from complex I during RET (Lambert et al., 2008; Pryde and Hirst, 2011). We favor the FMN site, because from current complex I structures it is difficult to see how oxygen could access the CoQ site and there generate a negatively-charged superoxide molecule that could migrate in the low dielectric of the bilayer and also diffuse against the membrane potential to the matrix (Vinothkumar et al., 2014; Zickermann et al., 2015). An important additional point is that superoxide production during RET still occurs even when there is no reduction of  $\text{NAD}^+$  to NADH. Numerous experiments have shown extensive superoxide production from complex I with no net reduction of  $\text{NAD}^+$  in the presence of a highly reduced  $\text{NAD}^+/\text{NADH}$  pool (e.g Hirst et al., 2008). Superoxide production occurs during RET, despite no net  $\text{NAD}^+$  reduction, because as electrons build up on FMN they can then either reduce  $\text{NAD}^+$  or drive reduction of oxygen to superoxide (Pryde and Hirst, 2011) (Figure 1B). This mechanism enables complex I to continue to produce superoxide when the FMN site is at equilibrium with the NADH and CoQ pools, and the  $\Delta p$  (Figure 1B). Consequently under most conditions when RET drives superoxide formation, there is net flow of electrons from the CoQ pool to  $\text{NAD}^+$  while the redox state of the FMN is poised to support superoxide production (Figure 1B).

For reperfusion injury the most important aspect of RET is that it produces far more superoxide than any other mitochondrial process (Murphy, 2009). Furthermore, superoxide production by complex I during RET only requires a high  $\Delta p$  and a reduced CoQ pool, both of which occur under physiological conditions (Figure 1C), in contrast to ROS production following non-physiological interventions, such as respiratory complex inhibition (Turrens et al., 1985). Therefore, RET stands out as a physiologically plausible mechanism for superoxide production *in vivo*. Consistent with RET at complex I driving IR injury, many inhibitors of complex I are protective against IR injury, including rotenone (Chen et al., 2006a; Lesnefsky et al., 2004), amobarbitol (Chen et al., 2006b), MitoSNO (Chouchani et al., 2013) and metformin (Cahova et al., 2015; Calvert et al., 2008). The next question to consider is whether conditions that support RET – a high  $\Delta p$  from proton pumping by complexes III and IV and a reduced CoQ pool - arise upon reperfusion injury (Figure 1C).

### **A high protonmotive force during reperfusion may support RET**

Reperfusion will lead to  $\Delta p$  being rapidly restored as the reintroduction of oxygen drives proton pumping by complexes III and IV (Figure 1C). This observation is supported by evidence that isolated mitochondria and cells reestablish a high  $\Delta p$  within seconds of reperfusion (Chouchani et al., 2014; Kim et al., 2012; Korge et al., 2008). It is technically more challenging to assess changes in  $\Delta p$  in tissue slices, isolated perfused organs or *in vivo*, but the available data are consistent with a very rapid rise in  $\Delta p$  upon reperfusion (Venable et al., 2013). For isolated mitochondria a near-maximal  $\Delta p$  is required to drive RET (Chance and Hollunger, 1961), which is achieved by preventing ATP synthesis and by minimizing proton leak by sequestering free fatty acids (St-Pierre et al., 2002). This suggests that for RET to occur upon reperfusion of ischemic tissue, ATP synthesis and other mechanisms of  $\Delta p$  dissipation must be inhibited. ATP synthesis following prolonged ischemia is compromised by depletion of the intracellular adenine nucleotide pools (Grover et al., 2004; Harrison et al., 1998; Kinugasa et al., 2003). This occurs because as the ATP/ADP ratio progressively decreases during ischemia the accumulated AMP is deamidated and degraded to generate inosine and hypoxanthine, or further metabolized by the purine nucleotide cycle (Harrison et al., 1998; Kinugasa et al., 2003). Adenine nucleotide levels can take over 45 minutes to be restored to normoxic levels following reperfusion (Kinugasa et al., 2003; Lindsay et al., 1990), therefore  $\Delta p$  is likely to be high enough to sustain RET upon reperfusion. Consistent with this view, dissipation of  $\Delta p$  with uncouplers such as 2,4-dinitrophenol (DNP) and FCCP decrease IR injury and mitochondrial ROS production during early reperfusion (Hoerter et al., 2004; Korde et al., 2005).

### **Accumulation of succinate during ischemia maintains a highly reduced CoQ pool that supports RET and $\Delta p$ during reperfusion**

For RET from complex I to occur during IR the CoQ pool must be maintained highly reduced, so as to provide electrons to generate a near maximal  $\Delta p$  by complexes III and IV, while also donating electrons for reverse transport through complex I to the FMN site

(Figures 1B & 1C). To supply electrons continually to the CoQ pool during reperfusion, a store of reducing equivalents must be built up during ischemia. Supporting this possibility, IR injury requires a sufficiently long period of ischemia in order to produce mitochondrial ROS upon reperfusion: this varies with species and tissue but 20-25 minutes of ischemia is required for significant IR injury in the mouse heart (Hasche et al., 1995; Reimer et al., 1977; Wang et al., 2001). Ischemia could alter the abundance of many mitochondrial metabolites that can potentially act as electron stores during ischemia and CoQ reductants upon reperfusion (Chouchani et al., 2014; Hochachka and Mustafa, 1972; Hochachka et al., 1975). Recently our group set out to find metabolites that accumulated during ischemia in a range of tissues that underwent mitochondrial IR injury. Only three metabolites accumulated consistently in a wide range of tissues susceptible to IR injury (Chouchani et al., 2014). Two of these, hypoxanthine and xanthine, were well established to occur during ischemia and arose due to the breakdown of purine nucleotides by the purine nucleotide cycle (PNC) in response to ischemic AMP accumulation (Hagberg et al., 1987; Imai et al., 1964). The other metabolite, succinate, was a common ischemic signature across metabolically diverse tissues and it accumulated to a far greater extent than any other mitochondrial metabolite (Chouchani et al., 2014). Many previous studies had also shown that succinate increased during ischemia or anoxia (e.g. Hochachka and Mustafa, 1972; Hochachka et al., 1975), but this was seen as a general response to inhibition of the Krebs cycle. In contrast, these recent findings indicate that selective accumulation of succinate occurred due to reversal of succinate dehydrogenase (SDH), driven by accumulated ischemic NADH passing electrons through complex I on to the CoQ pool, which then reduces fumarate to drive selective succinate accumulation (Figure 2). There may also be a contribution to maintaining reduction of the CoQ pool by other pathways such as electron transfer flavoprotein (ETF) (St-Pierre et al., 2002),  $\alpha$ -glycerophosphate dehydrogenase (Tretter et al., 2007), dihydroorotate dehydrogenase (Hey-Mogensen et al., 2014), and the hydrogen sulfide CoQ oxidoreductase (Libiad et al., 2014).

The fumarate that accepts electrons from CoQH<sub>2</sub> at SDH probably arises by a number of pathways that are activated during ischemia. One is the degradation of the AMP that accumulates during ischemia by the PNC to generate adenylosuccinate, which

is in turn broken down to fumarate by adenylosuccinate lyase (Figure 2). That this provides the fumarate that goes on to form succinate during ischemia is supported by the prevention of succinate accumulation with AICAR (Chouchani et al., 2014), a known inhibitor of adenylosuccinate lyase (Stone et al., 1993). As there is no specific fumarate transporter in the mitochondrial inner membrane, its movement to the mitochondrial matrix most likely occurs indirectly. Mitochondrial fumarate uptake probably follows its conversion to malate by cytosolic fumarate hydratase (Adam et al., 2013; Fiermonte et al., 1998), followed by exchange for succinate by the DIC (Fiermonte et al., 1998). In the matrix the malate is then converted back to fumarate by mitochondrial fumarate hydratase for subsequent reduction by SDH to succinate (Figure 2). There is also the possibility that transamination of aspartate to oxaloacetate contributes to ischemic succinate. The oxaloacetate will be reduced to malate, catalyzed by cytosolic malate dehydrogenase and driven by the ischemic accumulation of cytosolic NADH. This malate can then exchange for succinate via the DIC and generate fumarate as an SDH substrate as above. A role for aspartate transamination in succinate accumulation during ischemia is supported by the decrease of ischemic succinate by the transaminase inhibitor aminooxyacetate (Chouchani et al., 2014). Aspartate can in principle lead to fumarate via both the transamination and the PNC pathways (Figure 2) and consistent with this pre-loading the heart with  $^{13}\text{C}$  aspartate prior to ischemia leads to increased  $^{13}\text{C}$ -incorporation into succinate (Chouchani et al., 2014). However, much more remains to be explored about how the supply of fumarate to SDH is brought about, but current evidence suggests that the metabolism of the NADH and AMP accumulated during ischemia will be central. The above model indicates that cytosolic malate exchanges for matrix succinate, thus succinate will accumulate in the cytosol. This process is facilitated because succinate is an excellent substrate for the mitochondrial DIC and is exchanged for malate as shown in Figure 2. Therefore the net flux during ischemia will be to convert fumarate to succinate, which will rapidly distribute throughout the cell (Chouchani et al., 2014). The several-fold, selective accumulation of succinate suggests that this metabolite cannot be further processed during ischemia (Chouchani et al., 2014). Indeed, the conversion of succinate to succinyl-CoA is thermodynamically unfavorable under these conditions because succinyl-CoA synthetase reverse operation requires GTP and CoA, both of which are

depleted in ischemia (Figure 2). More broadly, these findings suggest that succinate accumulation is a key metabolic signature of ischemia. Consistent with a role in ischemic-signaling, succinate in the cytosol, probably in conjunction with elevated ROS (Guzy et al., 2008), can stabilize the hypoxia-inducible factors (HIFs) by inhibiting their HIF- $\alpha$  prolyl hydroxylases-mediated degradation, thus leading to activation of a pseudohypoxic response (Selak et al., 2005; Tannahill et al., 2013) (Figure 2). In addition, extracellular succinate can activate the G protein-coupled receptor GPR91, which may be a relevant sensor responding to the succinate released into the extracellular space by cell death or lysis following ischemia (He et al., 2004). In summary, it is clear that succinate can be accumulated in great amounts as a terminal electron sink during ischemia, thereby acting as a potential electron store that can be used to drive superoxide formation from complex I by RET during reperfusion.

### **Oxidation of succinate during reperfusion**

The finding of succinate accumulation during ischemia was particularly intriguing in the context of ROS production during IR injury, as it has long been known that the most effective way of generating superoxide in isolated mitochondria is through RET driven by succinate oxidation (Boveris et al., 1972; Murphy, 2009; St-Pierre et al., 2002). *In vitro* succinate is rapidly taken up by mitochondria through the DIC and oxidized by SDH reducing the CoQ pool. This reduction of the CoQ pool drives proton pumping by complexes III and IV, leading to a large  $\Delta p$ , as long as there is limiting ADP and thus low  $\Delta p$  consumption for ATP synthesis. Under these conditions there is also extensive RET at complex I, that leads to superoxide and subsequent hydrogen peroxide formation in mitochondria. *In vivo*, the succinate that accumulates over time in ischemic tissues is very rapidly oxidized upon reperfusion back to baseline levels within 5 minutes (Ashrafian et al., 2012; Chouchani et al., 2014). This prompt clearance of succinate is likely due its rapid oxidation in the mitochondrial matrix, sustained by the uptake of cytosolic succinate via DIC in exchange for malate. This oxidation is required for RET driven ROS and IR injury, as was shown by manipulations that prevent the accumulation of succinate during ischemia (Chouchani et al., 2014). In this scenario accumulated succinate acts as

an electron sink during ischemia, which is then used to drive ROS by RET at complex I upon reperfusion.

### **The complex I active/deactive transition and reversible thiol modification in the prevention of IR injury**

As we propose that superoxide generation by RET at complex I upon reperfusion is the major source of IR injury, it is of particular relevance that complex I RET is dramatically altered by a structural change known as the active/deactive transition (Babot et al., 2014; Galkin et al., 2009). Our understanding of the active/deactive transition of complex I arose from the pioneering work of Vinogradov and colleagues (Babot et al., 2014; Kotlyar and Vinogradov, 1990; Vinogradov, 1998). When complex I is not oxidizing NADH and pumping protons across the mitochondrial inner membrane it gradually converts to a “deactive” state, which is characterized by a conformational transition (Kotlyar and Vinogradov, 1990; Vinogradov, 1998) (Figure 3A). The physiological role of the active/deactive transition is unclear, but in the context of IR injury the most interesting aspect is that complex I readily undergoes deactivation during ischemia with a half-life for deactivation of about 10-12 minutes (Ciano et al., 2013; Galkin et al., 2009; Gorenkova et al., 2013). Upon reperfusion complex I is rapidly reactivated and can support superoxide production by RET (Gorenkova et al., 2013) (Figure 3A).

The active/deactive transition of complex I provides an appealing explanation for many therapeutic interventions that decrease IR injury. Deactive complex I exposes a critical cysteine residue (Cys39 in the ND3 subunit in mammals) that is occluded in active complex I (Chouchani et al., 2013; Galkin et al., 2008; Gorenkova et al., 2013; Zickermann et al., 2015) (Figure 3B). Importantly, covalent modification of Cys39 locks complex I in the deactive state preventing RET (Chouchani et al., 2013; Galkin et al., 2008). Reversible modification of this cysteine residue by thiol reactive agents also temporarily locks complex I in the deactive state *in vivo*, thereby preventing ROS production by RET (Burwell et al., 2006; Chouchani et al., 2013; Galkin and Moncada, 2007; Shiva et al., 2007). In this case the modification is reversed by endogenous thiol reduction mechanisms such as the mitochondrial glutathione and thioredoxin systems following the first minutes of reperfusion with a half-life of ~ 5 minutes (Chouchani et

al., 2013). The overall effect is that electron flow through complex I is temporarily prevented upon reperfusion, thereby blocking superoxide production by RET during the crucial first few minutes of reperfusion. As the modification is reversible, complex I returns to full activity a few minutes after reperfusion, by which time the succinate accumulated during ischemia will have been oxidized. Indeed, this modification was demonstrated using the mitochondria-targeted S-nitrosothiol MitoSNO, and underlies the protective action of S-nitrosating agents against IR injury (Burwell et al., 2006; Chouchani et al., 2013; Methner et al., 2014; Prime et al., 2009).

More generally, similar reversible inhibition of complex I superoxide production by RET may explain a number of therapeutic interventions that decrease IR injury. Compounds can exert their effects by acting on Cys39 of the ND3 subunit of complex I when it is exposed during ischemia. These modifications include S-nitrosation by nitric oxide metabolites such as S-nitrosothiols (Kohr et al., 2011; Nadochiy et al., 2007, 2009) and sodium nitrite (Chouchani et al., 2013; Shiva et al., 2007), S-sulfenylation by hydrogen sulfide (Elrod et al., 2007), oxidation to a sulfenic acid and/or to glutathione mixed disulfide in response to oxidative stressors such as hydrogen peroxide (Gorenkova et al., 2013; Yaguchi et al., 2003), ischemic preconditioning (Chouchani et al., 2013; Kohr et al., 2011) and post-conditioning (Tong et al., 2014) (Figure 3C). These modifications could then be reversed by reductants such as the mitochondrial glutathione and thioredoxin systems (Chouchani et al., 2013). These manipulations of complex I are likely to be clinically useful because they act through reversible post-translational modification of deactive complex I and have no impact on the active complex (Chouchani et al., 2013; Galkin et al., 2008; Galkin and Moncada, 2007).

There are considerable uncertainties about the proportion of the complex I pool that undergoes the deactive transition *in vivo*. Furthermore, the way in which these changes in complex I activity link to their incorporation into supercomplexes and the extent to which this may alter the rates of interconversion and superoxide production are currently unclear. Even so, it is likely that a diverse range of therapeutic interventions act by inducing reversible modification of Cys39 on the ND3 subunit of complex I that is exposed following ischemia and thus prevents the burst of ROS due to RET upon reperfusion.

### **A unifying mechanism for mitochondrial superoxide production during IR injury**

The work discussed leads us to propose a unified model of IR injury. Defined metabolic transitions during ischemia re-route electrons to succinate, which acts as an electron store in the absence of oxygen (Figure 2). Upon reperfusion, succinate fuels RET due to the high  $\Delta p$  in the first minutes following ischemia, providing the initiating burst of superoxide that leads on to IR injury (Figure 1B & C). Many established therapeutic interventions act to decrease the accumulation of succinate during ischemia, its oxidation upon reperfusion or the generation of a large  $\Delta p$  upon reperfusion. Furthermore, many protective agents act by inhibiting RET by complex I, either by direct inhibition of the enzyme, or by temporarily locking complex I in the deactive state by reversible modification of Cys39. Many other potential therapies may act downstream of the burst of ROS, such as antioxidants that minimize the oxidative damage, compounds that block induction of the permeability transition pore and drugs that decrease the inflammatory response initiated by damaged mitochondria. This model helps rationalize the bewildering array of interventions that affect IR injury and provides a context for development of new therapies and to explore how other interventions such as ischemic preconditioning and cooling may exert their protective effects.

### **Outlook**

The unifying mechanism for mitochondrial ROS production during IR injury that we propose raises intriguing issues. One pertinent question is why mitochondria are set up to produce large amounts of superoxide by RET at complex I. It seems probable that complex I RET has a physiological role, perhaps as a way for mitochondria to rapidly communicate their status by a retrograde redox signal to the rest of the cell. RET at complex I enables mitochondria to combine the effects of two processes that are central to their function: the electron supply to the respiratory chain, sensed via the reduction potential of the CoQ pool, and the ATP demand, which is indicated by the magnitude of  $\Delta p$ . Thereby mitochondrial RET would be accelerated when mitochondria are inactive and over-supplied with electrons and minimized when mitochondria are active. The superoxide would be converted to hydrogen peroxide that can diffuse to the cytosol

acting as a redox signal by modifying redox sensitive protein thiols. In IR injury this signaling pathway is over-stimulated, leading to oxidative damage.

The model we present provides a unifying framework for an array of data that provides a number of predictions that can be tested in future experiments while also suggesting approaches for the future development of targeted therapies against the generation of mitochondrial ROS in IR and related pathologies.

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## Figure legends

### **Figure 1. Superoxide production by complex I during reperfusion injury**

(A) Operation of complex I in the forward direction oxidizing NADH in order to generate a protonmotive force ( $\Delta p$ ) to be used to synthesize ATP. For forward electron transport to occur the difference in reduction potential between the  $\text{NAD}^+/\text{NADH}$  and the Coenzyme Q (CoQ) pool across complex I ( $\Delta E_h$ ) has to be sufficient to pump protons across the mitochondrial inner membrane against the  $\Delta p$ . As 4 protons are pumped for every 2 electrons that pass through complex I,  $2\Delta E_h > 4\Delta p$  is the requirement for the forward reaction to occur. The red arrow in complex I indicates forward electron transport. SDH, succinate dehydrogenase.

(B) Reverse electron transport (RET) by complex I. When the  $\Delta p$  is large and/or the  $\Delta E_h$  across complex I is low such that  $4\Delta p > 2\Delta E_h$ , electrons can be driven backwards from the CoQ pool onto the flavin mononucleotide (FMN) of complex I, reducing the FMN which can donate a pair of electrons to  $\text{NAD}^+$  to form NADH, or pass one electron to oxygen to generate superoxide. The red arrow in complex I indicates reverse electron transport.

(C) The factors that favor RET at complex I during reperfusion. The condition to be met for RET to occur is that  $4\Delta p > 2\Delta E_h$ . The rapid oxidation of the succinate that accumulates during ischemia favors reduction of the CoQ pool thereby maintaining a large  $\Delta E_h$ . The reduced CoQ pool also favors proton pumping by complexes III and IV helping maintain a large  $\Delta p$  upon reperfusion. In addition, the degradation of adenine nucleotides during ischemia limits ADP availability upon reperfusion that would otherwise diminish  $\Delta p$  by stimulating ATP synthesis.

### **Figure 2. The pathways of succinate accumulation during ischemia and oxidation during ischemia-reperfusion injury**

(A) Mechanisms of accumulation of succinate during ischemia. During ischemia succinate dehydrogenase (SDH) operates in reverse, reducing fumarate to succinate. This occurs due to the increased reduction of the CoQ pool, which arises in response to the build up of NADH during ischemia that transfers electrons to the CoQ pool via complex I. Fumarate arises from the degradation of the AMP that accumulates during ischemia by the purine nucleotide cycle. This fumarate can then be hydrolyzed to malate by cytosolic fumarate hydratase. In parallel, deamination of aspartate to oxaloacetate transfer electrons from NADH in the cytosol to form malate. The malate in the cytosol is then exchanged for mitochondrial succinate by the action of the dicarboxylate carrier in the mitochondrial inner membrane. Once formed in the matrix, the succinate cannot go on to form succinyl CoA due to the lack of Coenzyme A (CoA) and GTP during ischemia. The succinate in the cytosol, probably in conjunction with elevated ROS (Guzy et al., 2008), can also activate the hypoxia inducible factor  $\alpha$  pathway. AOA, aminooxyacetate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; SDH, succinate dehydrogenase.

**Figure 3. The complex I active/deactive transition during IR injury**

(A) Schematic of complex I in the active and deactive states. During ischemia the complex adopts the deactive conformation that leads to cysteine 39 (red; bovine nomenclature) on the ND3 subunit (yellow) becoming exposed to the solvent. Upon reperfusion complex I is rapidly reactivated and then produces a burst of superoxide by RET. When exposed to the solvent Cys 39 can be reversibly modified (indicated as S-X) and can then be reactivated by mitochondrial matrix reductants such as the glutathione or thioredoxin systems. This slows the reactivation of complex I upon reperfusion consequently by the time complex I is fully active the succinate that drives RET has been oxidized and there is less superoxide production upon reperfusion.

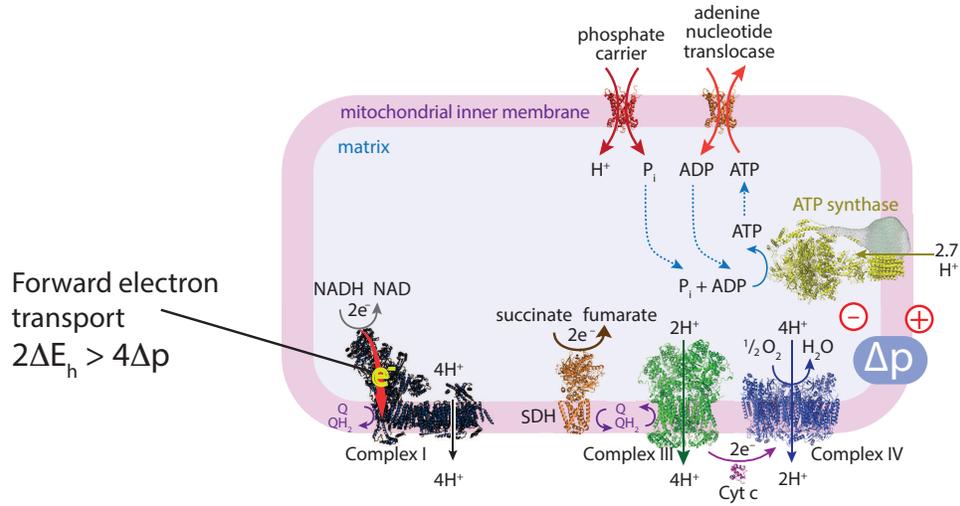
(B) The inset shows the structure of the homologous cysteine residue in the structure of deactive complex I from *Yarrowia lipolytica* (Zickermann et al., 2015) in the deactive state showing the cysteine equivalent to Cys 39 in mammals.

(C) The Cys 39 of the ND3 subunit of complex I that is exposed in the deactive complex I can react with a number of agents that modify the cysteine and thereby hold complex I

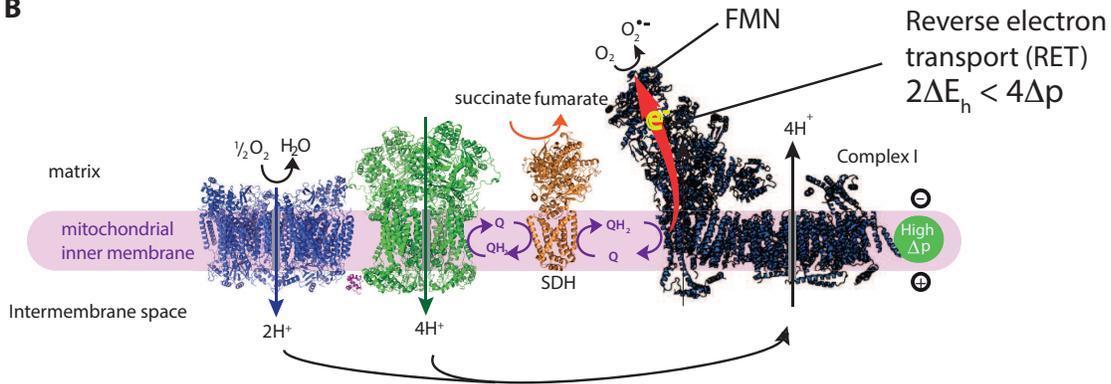
in the deactive state. These modifications can be reversible (in red) such that complex I can be restored to full activity, or irreversible modifications that render complex I permanently inactive (in green).

Figure 1

A



B



C

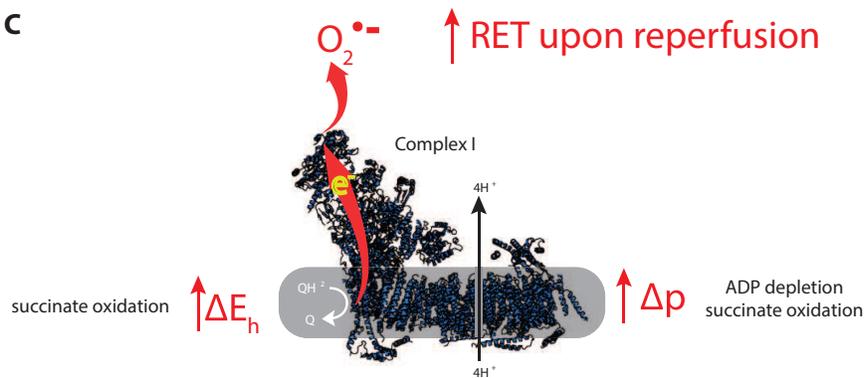
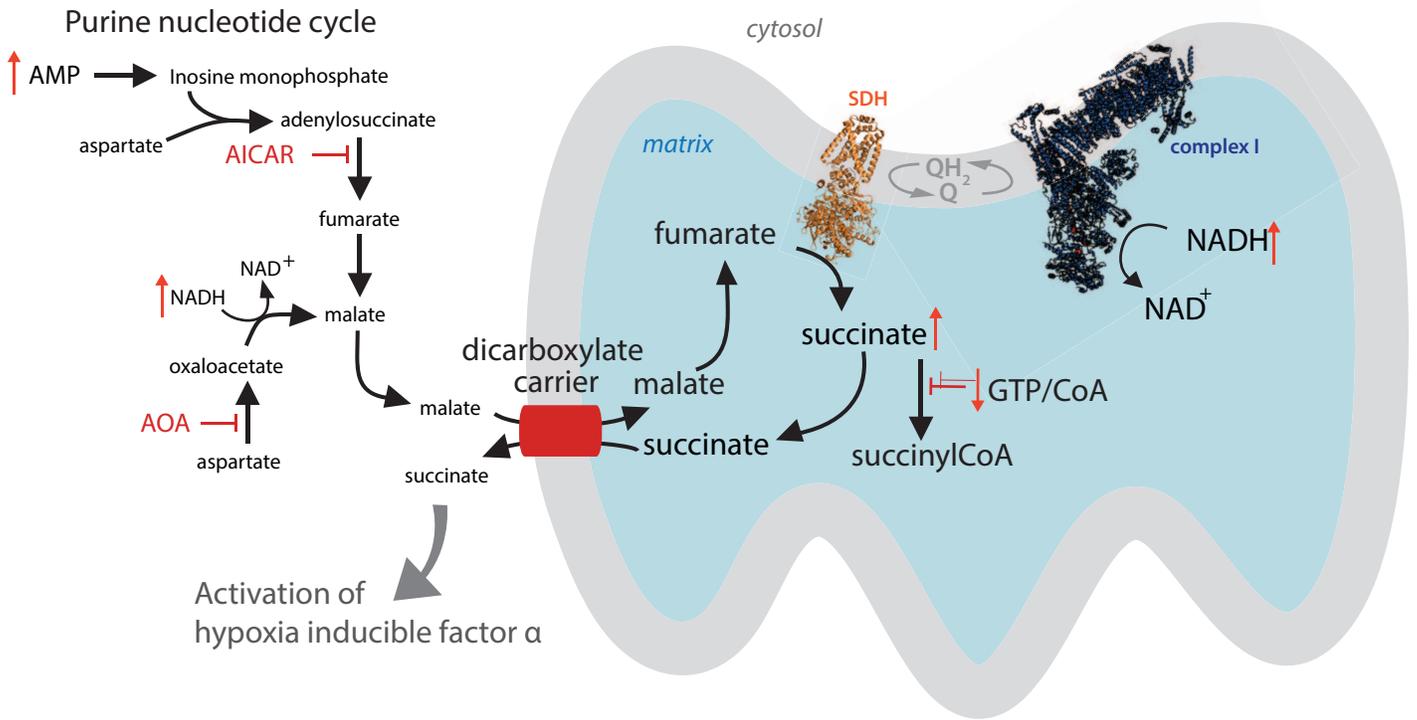
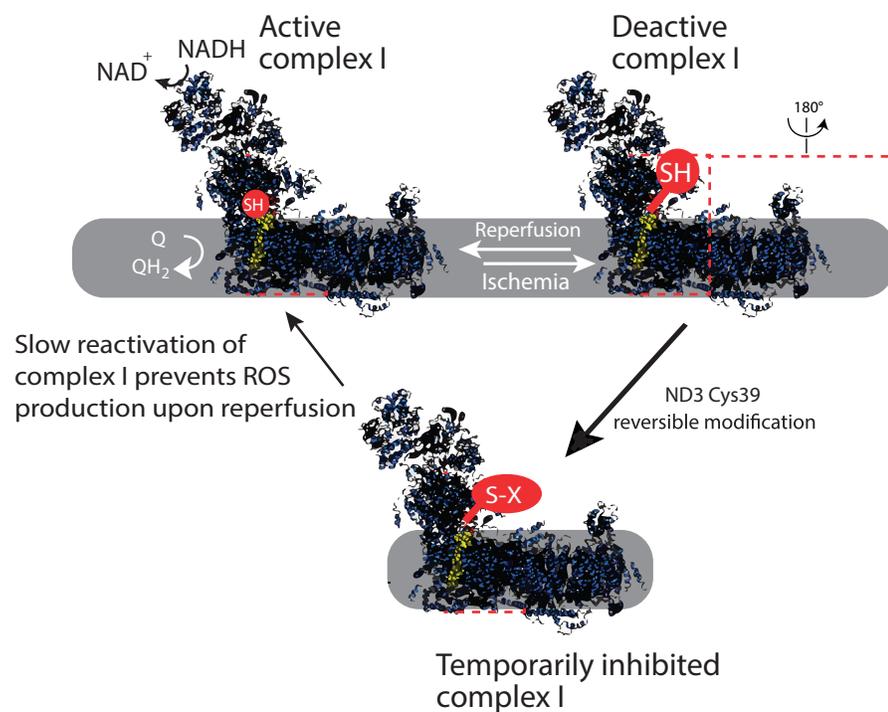


Figure 2

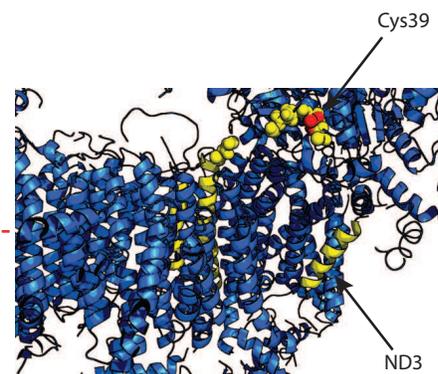


**Figure 3**

**A**



**B**



**C**

