# Succinate metabolism: a new therapeutic target for myocardial reperfusion injury

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**Abstract**

Myocardial ischemia/reperfusion (IR) injury is a major cause of death worldwide and remains a disease for which current clinical therapies are strikingly deficient. While the production of mitochondrial reactive oxygen species (ROS) is a critical driver of tissue damage upon reperfusion, the precise mechanisms underlying ROS production have remained elusive. More recently it has been demonstrated that a specific metabolic mechanism occurs during ischemia that underlies elevated ROS at reperfusion, suggesting a unifying model as to why so many different compounds have been found to be cardioprotective against IR injury. This review will discuss the role of the citric acid cycle intermediate succinate in IR pathology focussing on the mechanism by which this metabolite accumulates during ischemia and how it can drive ROS production at complex I via reverse electron transport (RET). We will then examine the potential for manipulating succinate accumulation and metabolism during IR injury in order to protect the heart against IR damage and discuss targets for novel therapeutics designed to reduce reperfusion injury in patients.

**Introduction**

Acute myocardial infarction (AMI) occurs when the complete occlusion of a coronary artery, for example due to rupture of an unstable atherosclerotic plaque, results in a region of myocardial ischemia. AMI is a major cause of death worldwide and is the primary cause of chronic heart failure (CHF). Despite a marked improvement in outcomes post-AMI in recent decades, due largely to the introduction of early reperfusion therapies, currently one quarter of patients will die or develop heart failure within one year (1). Irreversible myocardial injury progresses with increasing duration of ischemia, therefore the rapid restoration of blood flow to the ischemic area, via primary percutaneous coronary intervention (PPCI) or thrombolysis, is essential so as to salvage viable myocardium. Reperfusion itself however can paradoxically induce cardiomyocyte death independent of the ischemic episode by a process known as reperfusion injury (2). Indeed, experimental studies have shown that interventions applied at reperfusion can achieve an approximate reduction of 50% in final infarct size (3). Decreasing reperfusion injury is therefore a key target in the battle to preserve cardiac function in AMI patients. Despite this clear need, there is currently no effective therapy for the prevention of reperfusion injury available, and the translation of drugs from experimental studies to clinical trials has been disappointing (4–6). In this review we will discuss the role of mitochondrial reactive oxygen species (ROS) in the pathology of ischemia/reperfusion (IR) injury and how the citric acid cycle (CAC) intermediate, succinate, is emerging as a key driver of ROS production at reperfusion. From this we will go on to highlight the cardioprotective potential of intervening in succinate accumulation and metabolism in an attempt to identify novel targets for future therapies against reperfusion injury.

**Ischemia/reperfusion injury: reactive oxygen species and complex I**

An extraordinary amount of research has been carried out in order to understand the mechanisms by which the myocardium is damaged during IR injury, (3,7,8). In a normoxic heart 60-90% of the acetyl-CoA supplied to the CAC originates from the β-oxidation of fatty acids, with a smaller contribution from glycolysis and lactate oxidation (9). During acute ischemia, the lack of oxygen results in a switch to anaerobic glycolysis resulting in the build-up of lactate and a decrease in intracellular pH (10,11). ATP depletion and acidosis drive cytosolic $Na^+$ accumulation via the sodium/hydrogen exchanger (NHE) and as a consequence excess $Na^+$ is extruded, in exchange for calcium ($Ca^{2+}$), through the reverse action of the plasma membrane $Na^+/Ca^{2+}$, exchanger (NCX) (12). During ischemia, the usual $Ca^{2+}$ uptake by the sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) is prevented due to the decline in ATP resulting in cytosolic $Ca^{2+}$ overload (13,14). Furthermore, there is an accumulation of metabolic end-products, including hypoxanthine, xanthine and succinate (15–17). Reperfusion and subsequent re-oxygenation of the cell results in the rapid restoration of the mitochondrial membrane potential ($\Delta\psi_m$) providing a large driving force for $Ca^{2+}$ uptake into mitochondria via the mitochondrial calcium uniporter (MCU) (18). Reperfusion also leads to a large burst of ROS as oxygen reacts with leaked electrons to form superoxide (19). Within minutes of reperfusion, intracellular pH returns to normal due to the extrusion of protons by the NHE and the inhibitory effect on the mitochondrial permeability transition pore (mPTP) is released (11,20). Stimulated by the rise in mitochondrial ROS and $Ca^{2+}$, opening of the mPTP is induced resulting in a complete collapse of $\Delta\psi_m$, cytochrome *c* release and the activation of necrotic and apoptotic signalling cascades leading to cardiomyocyte death (21,22). Beyond triggering the onset of mPTP formation, excessive ROS can also induce tissue dysfunction directly through the peroxidation of lipids, oxidation of DNA and activation of matrix metalloproteinases (23,24) (Fig. 1). IR injury is therefore a highly complex process and one for which many aspects have yet to be fully characterised. Even so, it is clear that the large burst of ROS produced at reperfusion is incompatible with cell survival and is a critical factor in the pathogenesis of IR injury. ROS consequently constitute a potentially powerful pharmacological target in protecting the myocardium against infarction and antioxidant therapy, which inhibits or delays oxidative damage, is especially appealing. The development of effective antioxidants has however proven difficult, with blanket strategies, such as vitamin C and E, proving ineffective at improving clinical outcome (5). These disappointing results may in part be due to the precise molecular mechanisms responsible for ROS production remaining elusive with numerous sites having been implicated (25–27). The mitochondrial electron transport chain

has been recognized as a significant source of cellular ROS with complexes I (28–30) and III (25,31) identified as the predominant sites of superoxide production. The physiological relevance of ROS produced by complex III to *in vivo* scenarios remains uncertain given its requirement for conditions in which the ubisemiquinone radical is stable at the $Q_o$ site, most often achieved through its artificial inhibition by antimycin A (32). Moreover the complex III inhibitor myxothiazol, which inhibits ROS generation from the complex, was found to not decrease IR injury implying its limited involvement in the detrimental ROS burst at reperfusion (33). Interestingly however, complex III generated ROS has been implicated in the physiological signalling that occurs during volatile anesthestic and hypoxic pre-conditioning (33,34). Therefore in the context of IR, while a contribution to ROS by complex III should not be dismissed, under physiological conditions its involvement in ROS-mediated damage at reperfusion is thought to be lower than that of complex I (32,35). Indeed there is now an extensive body of work pointing towards mitochondrial complex I as a chief source of ROS at reperfusion with the inhibition of complex I during IR found to protect against IR injury (30,36). Complex I is the primary point of electron entry into the respiratory chain and is responsible for the oxidation of NADH and the extrusion of protons out of the mitochondrial matrix. In the context of IR, complex I undergoes an active/de-active transition in which during ischemia it converts to a 'de-active' state before being rapidly re-activated upon reperfusion (37,38). Importantly it has been shown that the inhibition of this re-activation, particularly through the reversible *S*-nitrosation of key cysteines, prevents ROS production and protects the myocardium against infarction (39–42) and long-term dysfunction (43). These findings suggest that in contrast to a largely unregulated response, a specific metabolic process occurs during ischemia priming the heart for ROS production through complex I at reperfusion which will be discussed in further detail below (17).

**Succinate accumulation is a metabolic signature of ischemia**

The heart is highly energy demanding and capable of utilising a variety of substrates, including free fatty acids, glucose, lactate and amino acids, for the production of ATP. The heart is also highly dependent on the constant delivery of oxygen, and disruption of this process, for example during ischemia or hypoxia, causes profound disturbances in myocardial metabolism (15,16,44). During ischemia, the lack of blood flow results in the loss of ATP, lactate accumulation and the build-up of metabolites including those within the CAC, purine nucleotide degradation pathways and those involved in fatty acid and amino acid metabolism (15,45,46). Despite metabolic adaptations to both myocardial hypoxia and ischemia being studied at length, the potential link to the generation of deleterious ROS during IR injury has been for the most part overlooked. Comparative metabolomics recently revealed that across multiple tissues three metabolites demonstrated conserved

accumulation during ischemia (17). Of these, two were components of the purine degradation pathway, hypoxanthine and xanthine. While both hypoxanthine and xanthine contribute to xanthine oxidase-derived generation of hydrogen peroxide, they interact with xanthine oxidase at the plasma membrane and do not contribute to mitochondrial ROS production (47). The third metabolite and sole mitochondrial component to show significant accumulation was the CAC intermediate and complex II substrate, succinate (17). Elucidation of the phenomenon of succinate accumulation is certainly not new. Hochachka *et al.* first demonstrated elevations in succinate during anaerobosis in their work on diving mammals in 1975 (48,49). Since then, ischemic succinate build-up has been observed in hypoxic rabbit papillary muscles (15), hypoxic isolated rat cardiomyocytes (50) and in the isolated mouse heart (51). Succinate accumulation can therefore be considered a universal signature of ischemia and an attractive candidate for a potential electron source for ROS production at reperfusion. The exact role of ischemic succinate however and the physiological basis behind its striking accumulation remains to be fully elucidated. Original propositions as to its function included forming part of an extra-glycolytic source for energy in situations of low nutrient and oxygen availability, therefore increasing tolerance to long-term anaerobosis (49). Wiesner *et al.* hypothesised that the production of succinate improved cytosolic redox state and thus was somehow beneficial during hypoxia (52). Indeed, improvements in cardiac function during hypoxia have been reported in isolated rat hearts perfused with potential precursors of succinate formation (53,54). In recent years the role of succinate has also expanded well beyond its action within the CAC and the electron transport chain with new roles discovered in inflammation (55) and GPCR stress-signalling (56). How these functions relate to the accumulation of succinate in anaerobic conditions remains to be investigated. Importantly, succinate can also drive the highest rate of mitochondrial ROS production in isolated mitochondria (32,35,57) in a process known as reverse electron transport (RET), and this will be discussed in detail later in the review.

**Succinate is rapidly oxidised at reperfusion**

As the burst of mitochondrial ROS production occurs within the first few minutes of reperfusion (19,42), it follows that ischemic metabolites fuelling ROS production should also be oxidised rapidly over a similar time-frame. Consistent with this hypothesis, succinate is abruptly lost from the tissue upon reperfusion, returning to pre-ischemic levels after only a few minutes (17,51). This is likely due to the rapid oxidation of succinate to fumarate by complex II in the mitochondrial matrix making succinate a highly attractive candidate for driving reperfusion-mediated ROS production. It should be noted that succinate in the mitochondria rapidly equilibrates with that in the cytosol via the mitochondrial dicarboxylate carrier (DIC) and may also be lost from the cell as a result of cell membrane disruption (58).

Consequently loss of a proportion of this metabolite through leakage upon reperfusion cannot be discounted (16). Despite this, it remains likely that a significant proportion of ischemic succinate is available to supply complex II-mediated oxidation upon reperfusion and recent work in which the inhibition of complex II slowed myocardial succinate loss at reperfusion now supports this (59).

**Ischemic succinate is produced by the reverse action of complex II**

In mammalian tissues, succinate is usually generated by the CAC via the oxidation of carbons from both glucose and fatty acids as a result of glycolysis and β-oxidation. Succinate can however also be produced from several mitochondrial reactions originating from amino acids (49). The first involves the ready conversion of glutamate to α-ketoglutarate (α-KG) by transamination. α-KG is then converted to succinate via succinyl-CoA, generating energy as GTP through standard operation of the CAC (15). This anaplerotic reaction not only acts to maintain levels of CAC intermediates but has been suggested to contribute significantly to total anaerobic maintenance of the mitochondrial membrane potential, although this remains contentious (60,61). The conversion of α-KG to succinyl-CoA is however strongly unfavourable due to the high $NADH/NAD^+$ ratio that occurs during ischemia and previous work has found no evidence of the conversion of α-KG to succinyl-CoA in the hypoxic isolated rat heart (50). Therefore while some contribution from α-KG to ischemic succinate cannot be entirely ruled out, it is likely to be less than that via other pathways. The other reaction involves the 'fumarate reductase' system. This system is composed of complex I and the reverse activity of complex II where succinate acts as the electron acceptor from reduced Coenzyme Q ($CoQH_2$). This reverse action of complex II has been proposed to enable proton pumping by complex I even in the absence of oxygen and the maintenance, to some extent, of the proton electrochemical potential gradient needed for ATP synthesis (62). Moreover, the fumarate required as substrate for this reaction can be generated from pathways such as the malate-aspartate shuttle (MAS) and purine nucleotide cycle (PNC). Indeed work by our group and collaborators recently demonstrated that these two key pathways did contribute to ischemic succinate formation in both the isolated mouse heart and *in vivo* mouse model of IR injury (17). By inhibiting each pathway selectively during ischemia, by means of the inhibitors 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) and aminooxyacetate (AOA) respectively, we significantly attenuated ischemic succinate levels *in vivo* (17). Moreover treating mice during ischemia with dimethyl malonate, a cell-permeable form of the complex II inhibitor malonate, ischemic succinate accumulation was similarly prevented (17). Interestingly these results suggest that $CoQH_2$, generated by complex I, is oxidised by complex II acting in reverse with fumarate acting as an electron acceptor resulting in the build-up of succinate.

**Succinate drives ROS production at reperfusion through RET at complex I**

At reperfusion, the large burst of mitochondrial superoxide that occurs appears to originate largely from mitochondrial complex I (30,42,63). While there are a number of ROS sources that may contribute to IR injury, including NADPH oxidases and xanthine oxidase, activation of these processes is thought to occur later in pathology and are thus not a focus of this review.

Complex I can produce superoxide via two potential mechanisms. The first occurs in the presence of a high matrix NADH/$NAD^+$ ratio in which a reduced flavin mononucleotide site reacts with oxygen to produce superoxide. This process occurs during conventional forward electron transport and is promoted by the complex I Coenzyme Q (CoQ) site inhibitor rotenone (32). Given that rotenone has been shown to be protective against oxidative damage during IR (36,63), it is unlikely that superoxide produced via this mechanism contributes significantly to reperfusion-induced ROS production. The second mechanism occurs via RET in which a highly reduced CoQ pool in conjunction with a maximal $\Delta\psi_m$ and low rate of ATP synthesis forces electrons from the reduced CoQ pool back through complex I (32,57). Notably this phenomenon has been observed in isolated mitochondria respiring on high concentrations of succinate and is associated with the greatest rate of mitochondrial ROS production known to occur (32). Despite RET being observed in brain, liver and heart mitochondria (64,65), it has generally been assumed to be solely an *in vitro* phenomenon of unknown physiological significance with the concentration of succinate in tissues being significantly lower than what is commonly used in *in vitro* experiments to evoke RET-mediated ROS (5-10 mM). However, recent work has shown that conditions at reperfusion are in fact sufficient to support RET with evidence of increased levels of ischaemic succinate and accelerated re-polarization of $\Delta\psi_m$ at reperfusion (17). In support of this mitochondrial ROS was tracked in a primary isolated rat cardiomyocyte model of simulated IR using the fluorescent probe dihydroethidium (DHE) (17). Upon reperfusion, DHE was rapidly oxidised consistent with increased superoxide production following the re-introduction of oxygenated buffer. Inhibiting complex II during ischemia with dimethyl malonate reduced reperfusion-mediated DHE oxidation. In contrast, the addition of dimethyl succinate, a cell-permeant derivative of succinate, to cardiomyocytes to artificially increase ischemic succinate levels significantly enhanced DHE oxidation at reperfusion. Critically, the selective inhibition of complex I with rotenone or MitoSNO abolished both endogenous and exogenous succinate-driven ROS production. These results are in accordance with previous work in which complex II inhibitors, including (dimethyl) malonate, diazoxide and atpenin A5 (AA5), have all been shown to reduce ROS production *in vitro* when administered prior to ischemia (17,66,67). Inhibiting succinate accumulation *in vivo* with dimethyl malonate

similarly abolished mitochondrial ROS production, as determined by the mass spectrometry ROS probe MitoB, and superoxide-mediated oxidative damage at reperfusion (17). These data therefore indicate that ischemic succinate levels control the extent of reperfusion ROS through complex I during IR injury both *in vitro* and *in vivo*.

**A unifying theory of ROS production upon reperfusion**

The recently defined metabolic transitions that occur within mitochondria during AMI offer a potential solution to the long sought mechanism for ROS production during IR injury. Readers are directed to a recent review in which this mechanism is described in more detail (68). During ischemia there is a build-up of succinate, as fumarate is converted to succinate via reverse action of complex II. The ATP/ADP ratio progressively decreases and accumulated AMP is further metabolised within the PNC as well as degraded to hypoxanthine and xanthine. Upon reperfusion the re-introduction of oxygen results in the rapid oxidation of the huge pool of electrons stored as succinate at complex II, resulting in a near maximal $\Delta\psi_m$ via complexes III and IV and a highly reduced CoQ pool (68). Adenine nucleotides depleted during ischemia and restoration to normoxic levels can take a significant amount of time (69,70). As a result, ATP synthesis is compromised at reperfusion and the reduced CoQ pool is maintained due to complex III being unable to consume all of the electrons supplied to the pool by succinate oxidation. The excess electrons are therefore forced back through complex I resulting in a large burst of ROS (Fig. 1). In conjunction with $Ca^{2+}$ overload, ROS triggers the opening of the mPTP and activation of the cells apoptotic machinery resulting in cardiomyocyte death. Inhibiting succinate build-up during ischemia with dimethyl malonate prevents ROS production from complex I by RET (Fig. 2). This unifying theory provides a possible explanation for why such a wide array of compounds, including complex I and II inhibitors and uncouplers, are cardioprotective and offers a potentially novel therapeutic target to help reduce infarct size during IR.

**Preventing succinate accumulation or oxidation as a therapeutic target for cardioprotection**

Ischemic succinate levels appear to control the extent of ROS at reperfusion. This suggests that manipulation of the pathways that increase succinate during ischemia, as well as those that oxidise it at reperfusion, should affect the degree of IR injury. In agreement with this the reversible inhibition of complex II with dimethyl malonate during ischemia and the attenuation of succinate accumulation significantly reduced infarct size *in vivo* (17). Indeed malonate, as well has other complex II inhibitors, have been previously shown to reduce ROS generation in isolated mitochondria (67,71) with AA5 protecting against IR injury in the isolated rat heart when given prior to ischemia (72). A recent study has also demonstrated

dimethyl malonate to be cardioprotective in the isolated mouse heart when infused prior to global ischemia (73). The exact mechanism by which this class of respiratory inhibitors protects against tissue damage however remains somewhat contentious with conflicting evidence with regards to the significance of the proposed mitochondrial ATP-sensitive potassium channel ($mK_{ATP}$) (74,75). The $mK_{ATP}$ has been implicated as a critical factor in ischaemic preconditioning-mediated cardioprotection (76). $mK_{ATP}$ has however also been functionally linked to complex II with significant pharmacological overlap demonstrated between the two mitochondrial components. Activators of $mK_{ATP}$, such as diazoxide, have been shown to inhibit complex II activity (77,78) while inhibitors of complex II, including malonate, can similarly activate $mK_{ATP}$ (75). Low concentrations of diazoxide, sufficient to activate mitochondrial potassium flux, however have no discernible effect on complex II activity and $mK_{ATP}$ specific actions can be inhibited by the $mK_{ATP}$ blocker, 5-hydroxydecanoate (79). Moreover, recent work supports a role for the renal outer medullary potassium channel (ROMK) as a pore-forming subunit for the $mK_{ATP}$ channel (80). Therefore, while the precise molecular composition of $mK_{ATP}$ remains to be fully elucidated and there are clear pharmacological parallels between the channel and complex II, it is likely to be distinct molecular entity. Whether dimethyl malonate is affecting $mK_{ATP}$ function during IR injury however remains unclear. Given that the restoration of succinate levels exogenously abolished dimethyl malonate induced cardioprotection, data do indicate that protection resulted solely from the blunting of succinate accumulation (17). The potential for off-target effects unrelated to the inhibition of RET however, cannot be entirely ruled out.

While inhibiting succinate accumulation during ischemia may be highly useful in situations of known ischemia, including elective surgery and organ transplantation, it is not clinically appropriate during an AMI where patients arrive at hospital with succinate already accumulated in the ischemic tissue. The usefulness of malonate *in vivo* as a chronic prophylactic treatment may also be limited by its effect on other organ systems with prolonged administration leading to striatal lesions that mimic Huntington's disease (81). It is therefore essential to determine if dimethyl malonate is equally effective at ameliorating cardiac injury when used later in IR, such as just prior to reperfusion. Succinate accumulation during ischemia only becomes pathological upon its rapid oxidation at reperfusion in which it drives RET-mediated ROS production through complex I. By suppressing succinate oxidation at the point of reperfusion through complex II inhibition and allowing a 'gradual wake-up' of mitochondrial metabolism, compounds including dimethyl malonate could be potentially valuable cardioprotectants. Support for this has recently been demonstrated in the isolated mouse heart when the administration of malonate at reperfusion only reduced infarct size and improved ventricular function (59). Moreover the authors directly attributed cardioprotection to the inhibition of succinate re-oxidation at

reperfusion and the prevention of ROS production (59). Malonate is therefore a potentially valuable tool for preserving mitochondrial function in a variety of settings and the model outlined here provides an avenue for the development of novel interventions against the generation of excessive mitochondrial ROS in a range of pathologies in which IR injury is implicated.

**Other potential therapeutic targets**

While the production and metabolism of ischaemic succinate is an important therapeutic target when targeting RET-mediated ROS, it is by no means the only target that should be considered. Downstream of succinate oxidation the inhibition of the transition of complex I to its active state at reperfusion will also prevent ROS production and has been shown frequently to protect against IR injury (28,41,42). Furthermore a critical condition required for the occurrence of RET is the generation of a near-maximal $\Delta\psi_m$ by the activity of complexes III and IV in combination with limited flux through the ATP synthase. Therapies that manipulate any of the numerous components involved could potentially have a beneficial effect by preventing RET induced ROS production. These would include the prevention of rapid $\Delta\psi_m$ repolarisation using inhibitors of complexes III and IV, such as myxothiazol (33) and hydrogen sulphide (82), dissipation of $\Delta\psi_m$ by mitochondrial uncouplers (83), and compounds that preserve or increase ADP content.

**Future Perspectives**

Despite considerable progress in treating AMI, there is a clear need for a novel secondary approach that can be applied in conjunction with current reperfusion therapy to protect the myocardium from infarction and achieve the full potential benefits of myocardial reperfusion. Succinate-mediated ROS production is emerging as a leading candidate for intervention during IR injury. Whether the inhibition of succinate-mediated ROS plays a significant role in other established cardioprotective mechanisms such as ischemic pre- and post-conditioning remains to be determined. The most important study however that remains to be carried out is to determine whether succinate accumulates in clinical settings of IR.

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**Conflicts of Interest**

ETC, CF, MPM and TK have filed patents in the area of therapies designed to prevent mitochondrial ROS production during cardiac IR injury.

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## Figures

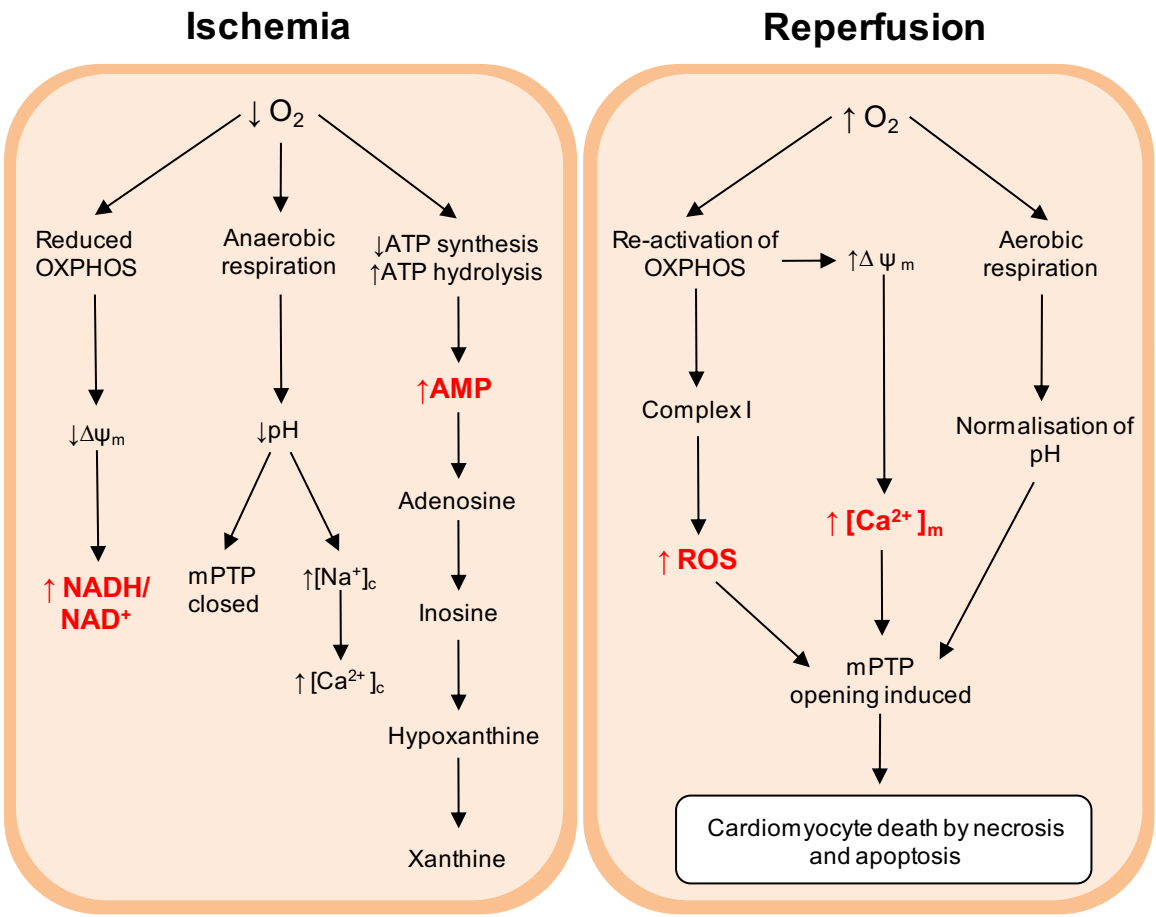


**Figure 1. Schematic illustrating the main components of ischemia/reperfusion injury.** During myocardial ischemia, the lack of oxygen causes a switch to anaerobic respiration, resulting in the production of lactate and a drop in intracellular pH. This disrupts ion haemostasis resulting in $Na^+$ and $Ca^{2+}$ overload. The low pH also prevents the opening of the mPTP. Oxidative phosphorylation is inhibited and NADH/$NAD^+$ ratio increases. ATP stores are depleted as ATP is hydrolysed to AMP by ATP synthase in order to maintain $\Delta\psi_m$. During reperfusion, the electron transport chain is re-activated and restored resulting in the normalisation of intracellular pH and $\Delta\psi_m$ and a large influx of $Ca^{2+}$ into the mitochondrion. Complex I is rapidly reactivated resulting in a large burst of ROS. Opening of the mPTP is induced resulting in the collapse of the $\Delta\psi_m$, the triggering of apoptosis and cardiomyocyte death. OXPHOS = oxidative phosphorylation. $\Delta\psi_m$ = mitochondrial membrane potential.

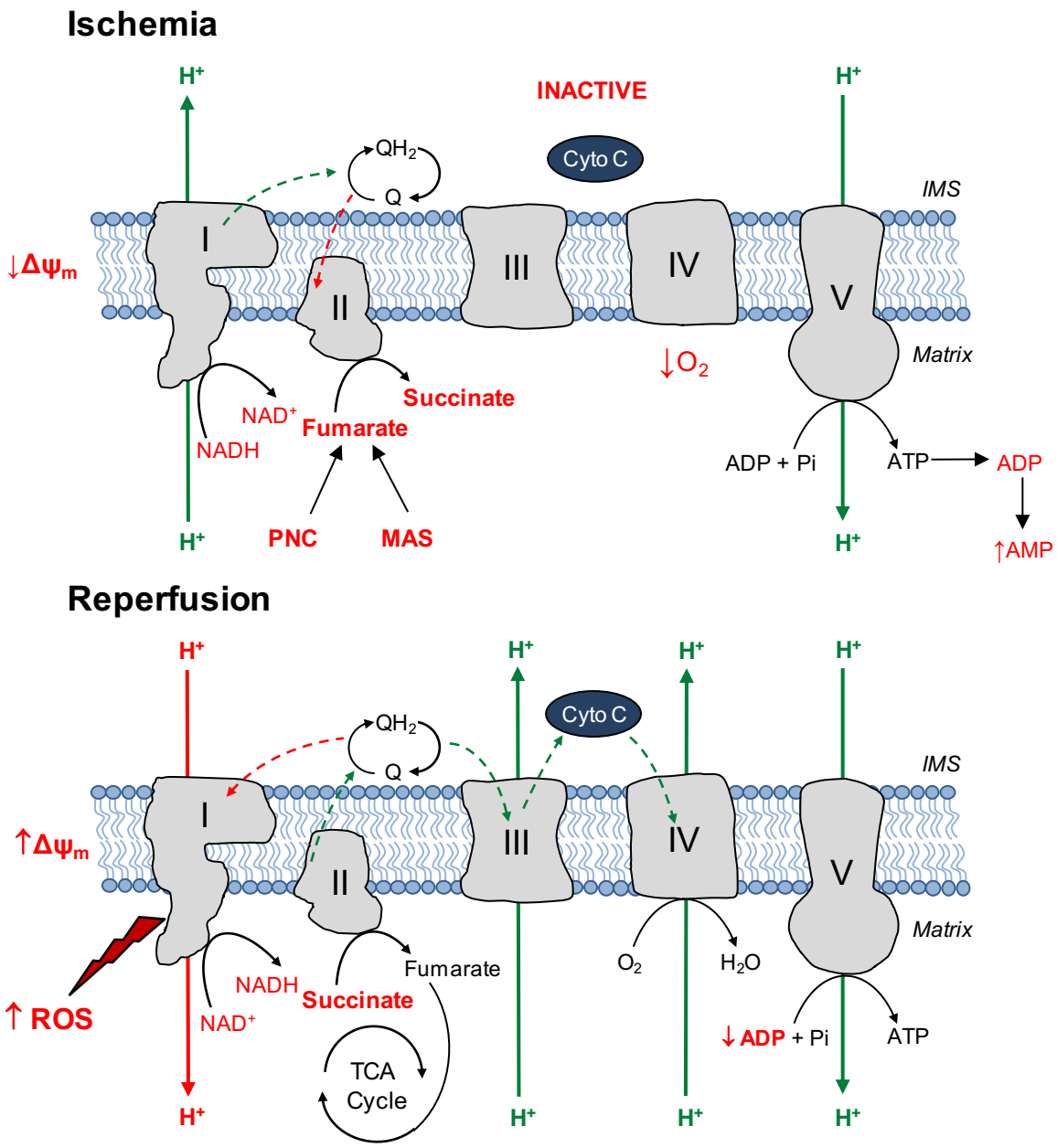


**Figure 2. ROS production by succinate-driven reverse electron transport during ischemia/reperfusion in the heart.** During ischemia, the purine nucleotide cycle (PNC) and malate aspartate shuttle (MAS) supply fumarate to complex II. Complex II acts in reverse by using $CoQH_2$ produced by complex I to reduce fumarate to succinate. ATP is hydrolysed to AMP due to insufficient ATP production. At reperfusion, oxygen is restored and the excess succinate is rapidly metabolised by complex II in its forward direction. A delay in the regeneration of ADP from AMP limits flux through ATP synthase, complex III and complex IV. This prevents complex III from using the ubiquinol generated by complex II as the membrane potential increases. Electrons are therefore forced back through complex I such that it runs in reverse generating large amounts of superoxide. Cyto C = cytochrome c. IMS = intermembrane space.