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In the eye of the storm: mitochondrial damage during heart and brain ischaemia

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We review research investigating mitochondrial damage during heart and brain ischaemia, focusing on the mechanisms and consequences of ischaemia-induced and/or reperfusion-induced: (a) inhibition of mitochondrial respiratory complex I; (b) release of cytochrome *c* from mitochondria; (c) changes to mitochondrial phospholipids; and (d) nitric oxide inhibition of mitochondria. Heart ischaemia causes inhibition of cytochrome oxidase and complex I, release of cytochrome *c*, and induction of permeability transition and hydrolysis and oxidation of mitochondrial phospholipids, but some of the mechanisms are unclear. Brain ischaemia causes inhibition of complexes I and IV, but other effects are less clear.

Introduction

Ischaemic heart disease and ischaemic brain disease are the first and second most common causes of death in the world [1]. Ischaemic injury is fast, multidimensional, and lethal, and at the centre of this pathological storm are mitochondria. Mitochondrial dysfunction is considered to be one of the main mechanisms involved in ischaemic injury in heart, brain, liver, kidney, and other organs and tissues. However, the causal relationships between various events occurring during ischaemic insult are not completely understood. Mitochondria themselves are affected by changes in the levels of oxygen, oxidants and Ca^{2+} during ischaemia or reperfusion. On the other hand, alterations in mitochondrial functions contribute to ischaemic or reperfusion pathology via loss of ATP production and Ca^{2+} transport and increased reactive oxygen species (ROS) production. Mitochondria may also directly initiate cell death by permeability transition or release of factors inducing apoptosis: cytochrome *c*, apoptosis-inducing factor (AIF), and endonuclease G.

In recent years, many reviews have been published on mitochondrial involvement in ischaemia-induced myocardial or cerebral dysfunction. In the present review, we focus on four key aspects of mitochondrial alterations during heart and brain ischaemia that have been relatively less discussed in recent reviews but remain controversial. These are: (a) inhibition of respiratory complex I and its pathological or protective roles; (b) inhibition of cytochrome *c* oxidase (COX) by

Abbreviations
AIF, apoptosis-inducing factor; COX, cytochrome *c* oxidase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

nitric oxide (NO) in competition with oxygen; (c) changes in mitochondrial lipids; and (d) mechanisms of cytochrome *c* release from mitochondria during ischaemia and/or reperfusion. Understanding these mechanisms may have important implications for designing mitochondria-targeted therapies against ischaemic injury during myocardial infarction and stroke.

Overview of ischaemia

The basic events of ischaemic pathology are well known. Ischaemia means insufficient blood flow to tissue, resulting in insufficient oxygen and glucose to maintain ATP levels. Within seconds of ATP levels falling below threshold, the heart stops contracting and the brain loses consciousness. This loss of tissue function may protect heart and brain viability by preserving limited ATP for cell functions required for cell survival. However, loss of heart contractility obviously causes global ischaemia and irreversible damage to the brain and body unless the blood supply is restored quickly. Insufficient cellular ATP means that the sodium and calcium pumps cannot extrude these ions from the cell, so that, if the ions continue to enter, then the cell swells, and loses its membrane potential and sodium-linked transport, and the cytosolic Ca^{2+} level starts to rise (potentially exacerbated by the loss of mitochondrial Ca^{2+} uptake). Continued swelling can kill the cell by rupturing the plasma membrane. Elevated Ca^{2+} can potentially kill the cell via activation of proteases, phospholipases, apoptosis, myocyte hypercontracture, or mitochondrial permeability transition (MPT). In the brain, these events are exacerbated by the ischaemic release from cells of the neurotransmitter glutamate and the activation of glutamate receptors, which increase neuronal Na^{+} and Ca^{2+} levels. Reperfusion of ischaemic tissue can prevent cell death by returning oxygen and glucose, but it can also trigger irreversible damage by enabling Ca^{2+} uptake into mitochondria and a burst of ROS production from the oxygen. Mitochondria isolated after ischaemia are found to be damaged, and this damage can occur during the ischaemic phase and/or the reperfusion phase.

Hypoxia, NO, and ROS

Factors thought to mediate mitochondrial damage during ischaemia–reperfusion include: hypoxia, NO, ROS, Ca^{2+}, and fatty acids. NO is produced by three NO synthase (NOS) isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS) are regulated by Ca^{2+} and phosphorylation, and are constitutively expressed in heart, endothelium, and some neurons, whereas inducible NOS (iNOS) is only expressed in inflammatory conditions, but, once expressed, produces high, sustained levels of NO [2–4]. ROS are compounds derived from oxygen that react uncatalysed with other molecules, and include superoxide, hydrogen peroxide, and the hydroxyl radical. Reactive nitrogen species (RNS) are compounds derived from NO that react uncatalysed with other molecules, and include peroxynitrite, NO_2, and S-nitrosothiols. The main sources of ROS are the NADPH oxidases, uncoupled NOSs, endoplasmic reticulum, peroxisomes, and mitochondria [5]. Superoxide (from, for example, mitochondria or NADPH oxidase) can react at the diffusion-limited rate with NO to produce peroxynitrite ($ONOO^{-}$), which is a strong oxidant of, for example, protein thiols. S-nitrosothiols, such as *S*-nitrosoglutathione, can transfer an NO^{+} group to other thiols, such as protein cysteine residues, and may thereby change protein function, or the reverse reaction may occur to remove the NO^{+} group. However, many S-nitrosothiols auto-oxidize, leaving an oxidized thiol.

A central, initiating event of ischaemic damage is inhibition of mitochondrial COX (complex IV) owing to insufficient levels of its substrate, oxygen. The apparent K_m of COX for oxygen is ~ 0.5 μm in normal conditions, which means that oxygen levels need to fall by one or two orders of magnitude before mitochondrial respiration is limited by oxygen [6]. However, NO inhibits COX in competition with oxygen, so NO can dramatically increase the apparent K_m of respiration for oxygen [7,8]. For example, 60 nm NO increases the apparent K_m of respiration for oxygen to 30 μm (the median level of oxygen found in the normal brain). Thus, NO could potentially cause dramatic sensitization to hypoxia. However, it should be noted that the NOSs (nNOS, eNOS, and iNOS) generally have apparent K_m values for oxygen in the range 15–30 μm [9], so that, during hypoxia, they will be strongly inhibited. On the other hand, if the NOSs are highly expressed or highly activated, or are located in a higher-oxygen microenvironment, then enough NO may still be produced to affect the oxygen affinity of COX. Also, during hypoxia, NO may be produced from nitrite by haemoglobin (and possibly myoglobin) without oxygen [10]. We found that inflammation-induced iNOS expression in the isolated rat aorta [11] and glial–neuronal cocultures [12] strongly sensitized to hypoxia-induced cell death, apparently via NO inhibition of COX. iNOS is expressed in glial cells in many neurological and neurodegenerative conditions [13,14]. iNOS is expressed in the heart during heart

failure [15,16]. In such inflammatory conditions, NO from iNOS might sensitize to hypoxic damage. However, again, it should be noted that low levels of NO, via stimulation of soluble guanylate cyclase, can protect cells by vasodilatation, by block of apoptosis, and by inhibition of MPT. For example, we have shown that NO can protect the ischaemic heart by inhibition of permeability transition [17]. Additionally, it has been suggested that NO inhibition of respiration during hypoxia may protect the heart by promoting myocardial 'hibernation' [18].

In neuronal cultures, we found that NO from nNOS sensitized to hypoxic neuronal death, apparently via NO inhibition of COX [19]. nNOS is structurally and functionally coupled to the glutamate *N*-methyl-D-aspartate receptor, which is activated during ischaemia, resulting in NO production that may potentially inhibit neuronal respiration [20,21]. Glutamate can induce the death of some neurons via stimulation of nNOS [22,23]. Genetic knockout of nNOS or iNOS protects against ischaemic brain damage, whereas knockout of eNOS is detrimental, presumably because the latter mediates vasodilatation [24].

Whereas NO can inhibit COX acutely, potently, and reversibly, the NO derivatives peroxynitrite and S-nitrosothiols can inactivate complex I and other mitochondrial components by oxidation or S-nitrosation [25]. Complex I is one of the main sources of mitochondrial ROS, and inhibition of complex I by peroxynitrite and S-nitrosothiols stimulates this ROS production [25–28].

RNS, ROS and Ca^{2+} can also activate the MPT pore (MPTP), which is a pore in the inner mitochondrial membrane that is freely permeable to all small molecules, thereby causing swelling of the mitochondria and depletion of cell ATP [29–31]. MPT can occur during heart and brain ischaemia and/or reperfusion, and inhibitors of MPT (in particular cyclosporin A) can block ischaemic–reperfusion damage in both organs [31,32]. Induction of MPT can damage mitochondria through the resultant release of cytochrome *c* (if swelling is sufficient to rupture the outer membrane), NADH and other components from the matrix space.

Which components of oxidative phosphorylation are affected by ischaemia?

Data concerning the effects of ischaemia–reperfusion on the mitochondrial oxidative phosphorylation system have been accumulating for approximately five decades [33,34].

To produce ATP, mitochondria need respiratory substrates and oxygen, the supply of which stops during ischaemia, thus blocking ATP synthesis. In these conditions, the mitochondrial ATP synthase reverses direction and starts working as an ATPase, hydrolysing glycolytic ATP [35], and thus accelerates the depletion of cellular ATP. The mitochondrial ATPase activity is regulated by an ATPase inhibitor protein, initiation factor 1 [36,37]. Initiation factor 1 contents and properties are different in different animal species and different tissue mitochondria, and therefore determine the rate of ATP exhaustion. For example, ATP in the dog myocardium falls to 5% of the control level after a 1-h ligation of the coronary artery [38,39], but ATP depletion may be slower during total ischaemia *in vitro* [40]. Importantly, the total content of adenine nucleotides also decreases [39,40]. Even after 15 min of occlusion of the coronary artery followed by reperfusion, the levels of heart ATP and total adenine nucleotides are not restored after 1 h [41] or even after 4 days of postischaemic reperfusion of the heart [42]. It was thought [33] that depletion of high-energy phosphates is (directly or indirectly) related to the development of irreversible injury, which proceeds to necrosis despite restitution of coronary flow. In the dog heart, necrosis occurs after 20–60 min of severe ischaemia, when ATP decreases to $< 10\%$ of the normal level [33]. In the brain, ischaemia for 5–15 min can lead to the death of sensitive neuronal populations, mediated by excitotoxicity [43]. Potential causes of irreversibility are: damage to the sarcolemma, decreases in high-energy phosphates, loss of cellular ionic gradients, Ca^{2+} overload, and activation of phospholipases and various proteases [33].

Measurements of mitochondrial respiration in state 3 with NAD^{+}-dependent substrates and succinate (complex II substrate), and of COX activity, showed large and variable changes when mitochondria were isolated from ischaemic heart (published in 31 papers; for review, see [35]). For example, 1 h of ligation of the dog coronary artery was reported to inhibit state 3 respiration by between 16% and 98% (eight papers; see [35]). Surprisingly, decreases in state 3 respiration rate of 40% and 50% were observed after just 5 or 10 min of ischaemia [44,45]. In most, but not all, cases, the decrease in state 3 respiration was stronger with NAD^{+}-dependent substrates than with succinate, whereas COX activity was generally unchanged, with rare exceptions (see [46]).

In the heart, fatty acids are the main respiratory substrates. During heart ischaemia, decreases in carnitine palmitoyltransferase activity and in palmitate but not hexanoate oxidation were found [44,47]. Attempts

to restore palmitate oxidation by addition of cytochrome *c* or malate were ineffective, suggesting damage to the upper part of the respiratory chain or β-oxidation system. We found that the respiratory inhibition caused by ischaemia was similar on ADP-stimulated and uncoupler-stimulated mitochondrial respiration, using several different respiratory substrates [35], in agreement with other studies [48], with the exception of one [49]. These findings suggest that the decrease in mitochondrial state 3 respiration during ischaemia (at least in some models of ischaemia/conditions) is probably attributable to failure of respiratory substrate oxidation but not to failure of adenine nucleotide transport or ATP synthesis.

This conclusion was supported by studies demonstrating that, during relatively short periods of ischaemia (20–30 min), complex I activity gradually decreases in parallel with the decrease in mitochondrial respiration with NAD^+-dependent respiration [48,50]. The activity of complex III was found also to decrease, but more gradually, and that of complex V decreased most rapidly (by 40% after 5 min of global ischaemia), whereas the activities of complexes II and IV were unchanged [50,51]. Total ligation of the dog heart left anterior descending coronary artery for 30 min and 60 min decreased the state 3 respiration rate (~ 80%) and complex I activity (~ 70%), and these did not recover on reperfusion [51]. A similar decrease in complex I activity was observed in rats after 20–25 min of global no-flow ischaemia [52]. In contrast, other investigators did not observe inhibition of complex I or other complexes during 40 min of hypoxic perfusion [53] or 30 min of coronary artery occlusion in rats [48], although inhibition of state 3 and uncoupled respiration was found. Lesnefsky *et al.* [54] also reported that 45 min of global no-flow ischaemia in isolated, perfused rabbit hearts caused state 3 respiration with glutamate to decrease without an effect on the activities of NADH–cytochrome *c* oxidoreductase (complexes I and III), succinate–cytochrome *c* reductase (complexes II and III), and COX. As this study found reduced levels of cytochrome *c* in ischaemic mitochondria, it is possible that the ischaemia-induced decrease in respiration could be a result loss of cytochrome *c* from mitochondria.

In the brain, global ischaemia for more than a few minutes causes similar inhibition of mitochondrial state 3 and uncoupled respiration with both NAD^+-dependent and FAD-dependent substrates [55], suggesting that the mitochondrial respiratory chain is inhibited rather than the phosphorylating subsystem. This initial decline in respiration almost fully recovers during 10–30 min of recirculation [56,57]. However, delayed suppression of mitochondrial respiratory capacity is observed after prolonged reperfusion in certain regions of the brain containing neurons that are selectively vulnerable to ischaemic–reperfusion insult [58,59]. This inhibition is thought to be related to inactivation or degradation of the pyruvate dehydrogenase complex [60–62]. Similar changes in mitochondrial respiratory capacity (inhibition by 45–60% of state 3 and uncoupled respiration with pyruvate or succinate), but over longer (~ 2 h) periods of ischaemia, are observed during regional, focal ischaemia induced by middle cerebral artery occlusion in rats [63,64]; these are also reversible during the first hours of reperfusion, but then secondary deterioration is seen after 2–4 h of reperfusion [55].

Ischaemic injury to the brain is thought to be at least partly mediated by overactivation of glutamate *N*-methyl-D-aspartate receptors, causing a form of neuronal death called excitotoxicity. The mechanisms of excitotoxicity are still unclear, but involve Ca^{2+} overload of the neurons, ROS production, and mitochondrial dysfunction [65]. Excitotoxic neuronal damage results in translocation of AIF from mitochondria to the nucleus, where it induces neuronal death via activation of DNases. AIF translocation during excitotoxicity and brain ischaemia is mediated by Ca^{2+}-activated calpain cleavage of mitochondrial AIF [66,67].

In conclusion, it is generally accepted that ischaemia in the heart, brain or other organs leads to progressive injury to the mitochondrial oxidative phosphorylation system, particularly the respiratory chain. In the heart, short periods of ischaemia rapidly induce inhibition of complex I and ATP synthase [50,68]. These are considered to be reversible events, as mitochondrial functions usually recover with restoration of blood flow [69]. If ischaemia continues, irreversible inhibition of complex IV occurs, along with the release of cytochrome *c* from mitochondria [50,69]. Similarly, during early brain ischaemia, the activities of complexes I, II and III of the mitochondrial respiratory chain are suppressed, but can be restored by reperfusion [58,70,71], whereas the activity of COX becomes inhibited only after long periods of reperfusion, suggesting that this inhibition may be caused by oxidative damage.

Changes in the phospholipid composition of mitochondrial membranes during ischaemia

Decreased ATP levels during tissue ischaemia–reperfusion disturb cellular ionic gradients and increase the Ca^{2+} concentration, which may cause activation of

numerous phospholipases, leading to degradation of membrane phospholipids [72,73]. Changes in the membrane phospholipid composition concomitant with free fatty acid accumulation may contribute to alteration of mitochondrial inner membrane integrity, and may lead to uncoupling, inhibition of mitochondrial respiratory enzymes and oxidative phosphorylation during ischaemia–reperfusion. For instance, Spencer *et al.* [74] showed that removal of just 1% of mitochondrial phospholipids with phospholipase A decreased ATP and ADP translocation in isolated rat liver mitochondria by 25–50%.

In an experimental model of heart ischaemia in dogs, the total mitochondrial phospholipid content was found to decrease after 15 min of coronary artery ligation followed by 5 min of reperfusion [75], and the cardiolipin content decreased after 10–20 min of coronary artery ligation [76] or 3 h of ischaemia [77]. We found significant decreases in the contents of phosphatidylethanolamine, phosphatidylcholine, cardiolipin and total phospholipids in rabbit hearts after 0.5 h and 1 h of total ischaemia, and after 1 h and 4 h of permanent coronary artery occlusion [35]. In mitochondria isolated from heart after 0.5–2 h of total ischaemia *in vitro*, the cardiolipin and total phospholipid contents were decreased [35]. Lesnefsky *et al.* [78] reported that heart ischaemia caused a specific decrease in mitochondrial cardiolipin content, whereas phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol levels were unchanged. This may have important implications, as many mitochondrial proteins bind to cardiolipin and may depend on it for activity, including various substrate carriers, NADH–ubiquinone reductase, cytochrome bc_1 complex, COX, ATP synthase, ADP/ATP carrier, phosphate transporter, and creatine phosphokinase [79,80].

As expected from the ischaemia-induced breakdown of phospholipids, accumulation of unesterified fatty acids in the ischaemic tissues and mitochondria was also observed. It was more pronounced during total ischaemia *in vitro* (1.8–4.8-fold increase after 0.5–2 h) than with permanent ligation of the coronary artery (1.5-fold and 1.6-fold after 1 h and 4 h) [35]. In our experiments, the greatest accumulation seen was of linoleic and arachidonic acids (an approximately eightfold increase after 2 h of total ischaemia) [35]. Similar increases have been found in open-chest dogs with the coronary artery occluded for 2 h [81]. Further degradation of membrane phosholipids and concomitant accumulation of fatty acids, including arachidonic acid, was observed during reperfusion of previously ischaemic hearts [82]. Large (6.4-fold), rapid (within 1 min of ischaemia) and specific accumulation of arachidonic acid was also detected in the mouse brain during ischaemia induced by decapitation [83]. It is important to note that Cocco *et al.* [84] found that addition of arachidonic acid to bovine heart mitochondria caused: (a) uncoupling of state 4 respiration; (b) a decrease in respiration of mitochondrial particles resulting from selective inhibition of complexes I and III; and (c) a dramatic increase in H_2O_2 production by mitochondria respiring on either pyruvate plus malate or succinate. A 50% increase in state 4 respiration and a 50% inhibition of uncoupled respiration was found with just 1–3 μM arachidonate; the uncoupling effect of palmitate was similar but less potent [84]. Albumin reversed the arachidonate-induced inhibition of mitochondrial respiration.

We have found that mitochondria isolated from rat heart after 30 min of ischaemia are partly uncoupled, and this uncoupling is reversed by the addition of albumin [85,86], indicating that the uncoupling is mediated by the accumulation of free fatty acids in mitochondria during ischaemia. This early uncoupling may contribute to ATP depletion during ischaemia (by promoting reversal of ATP synthase) and cause oxygen and ATP insufficiency during reperfusion.

Arachidonate also causes swelling and cytochrome *c* release from Ca^{2+}-loaded rat heart mitochondria [87]. However, the mitochondrial swelling and Ca^{2+} release induced by arachidonate and other unsaturated fatty acids in Ca^{2+}-loaded heart mitochondria appears not to be mediated by permeability transition (cyclosporin A-insensitive), but rather by the ATP/ADP translocator [88], in contrast to liver mitochondria, where arachidonate induces classic MPT. This mechanism of cytochrome *c* release might occur during heart ischaemia–reperfusion, as both Ca^{2+} and arachidonate are elevated in mitochondria in this situation.

Degradation of mitochondrial phospholipids also occurs during brain ischaemia. For instance, several studies using common carotid artery occlusion models have shown that, during the first 30 min of ischaemia, activation of phospholipase A_2 causes decreases in the mitochondrial contents of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin [89–91]. These changes were reversible during reperfusion, but recovery did not occur after an extended 60-min period of ischaemia. Changes in phospholipid content were accompanied by a decrease in mitochondrial state 3 respiration, and inhibition of COX and ATP synthase, which are known to depend on cardiolipin content and fatty acid composition [89,91].

Thus, breakdown of mitochondrial membrane phospholipids and accumulation of free fatty acids may increase the permeability of the mitochondrial inner

membrane to ions, and decrease mitochondrial membrane potential and ATP production (Fig. 1). However, mild uncoupling may be beneficial for cells, because it may decrease ROS production, as has been suggested by Skulachev [92].

Complex I suppression during ischaemia and its role in pathology

Complex I (NADH–ubiquinone oxidoreductase) is a large enzyme that couples electron transfer to the pumping of protons across the mitochondrial inner membrane (reviewed in [93,94]). Complex I is also a major site of mitochondrial ROS production, and is thought to be involved in MPT [95–98]. Complex I consists of at least 45 subunits and redox centres: flavin mononucleotide and eight iron–sulfur centres. The amount of complex I in the mitochondrial inner membrane is less than that of other complexes of the respiratory chain [99,100], so the respiration rate might be more sensitive to inhibition of complex I than to inhibition of other, more abundant respiratory chain complexes (although other parameters also determine rate limitation).

Inhibition of complex I activity is one of the earliest ischaemia-induced alterations in mitochondrial function, but the causes of this inhibition are not well understood. Possible factors that may mediate complex I inactivation during ischaemia or reperfusion are summarized in Fig. 2.

In early work, ischaemia-induced inhibition of complex I was suggested to be caused by elevated concentrations of Ca^{2+}, on the basis of several findings. Heart perfusion with Ruthenium Red (an inhibitor of Ca^{2+} uptake into mitochondria) prevented ischaemia–reperfusion-induced inhibition of complex I activity [53], implicating Ca^{2+} in the inhibition of complex I. Also, when isolated heart mitochondria were exposed to high Ca^{2+} levels, they became uncoupled and inhibited at complex I, a change similar to that observed in postischaemic mitochondria [101]. Ca^{2+}-induced

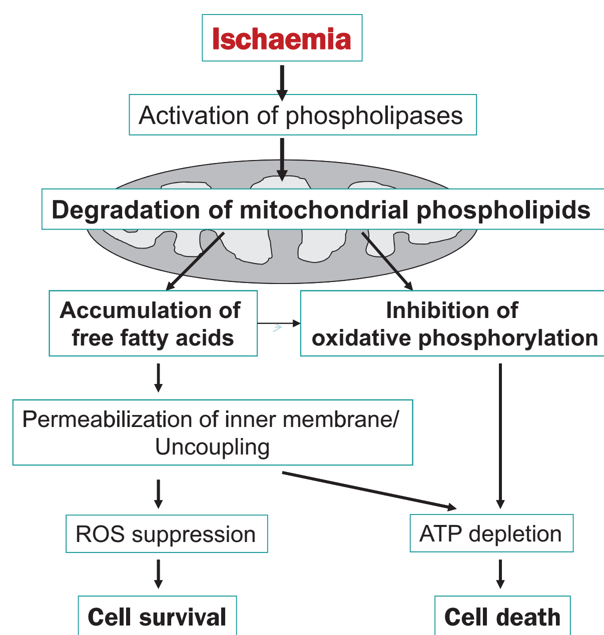

Fig. 1. Ischaemia-induced degradation of mitochondrial phospholipids and its consequences. Ischaemia causes activation of various phospholipases that act on mitochondrial membrane phospholipids. As a result, the phospholipid content decreases and free fatty acids accumulate in mitochondria. Degradation of mitochondrial phospholipids may cause inhibition of certain enzymes of the oxidative phosphorylation system, leading to further ATP depletion on reperfusion and cell death resulting from lack of energy. Increased concentrations of free fatty acids may cause the mitochondrial inner membrane to become pearmeable to ions (uncoupling) or, in the case of arachidonic acid, may induce mitochondrial swelling and loss of cytochrome *c*, leading to ATP depletion and cell death. Alternatively, mild uncoupling induced by free fatty acids may suppress ROS production at reperfusion, promoting cell survival.

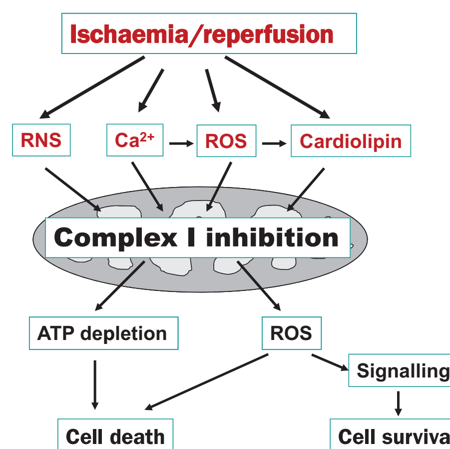

Fig. 2. Causes and consequences of ischaemia–reperfusion-induced inhibition of complex I of the mitochondrial respiratory chain. Ischaemia or reperfusion induces inhibition of complex I, which may caused by elevated concentrations of RNS, Ca^{2+}, or ROS, or changes in the phospholipid composition of the mitochondrial inner membrane, particularly regarding cardiolipin. The effect of elevated concentrations of Ca^{2+} may be indirect, being mediated by ROS, which may cause oxidative damage to cardiolipin. Complex I inhibition may lead to ATP depletion-mediated cell death, owing to the suppression of oxidative phosphorylation. Complex I inhibition may also cause generation of ROS by the respiratory chain, which may lead to cell death or, if ROS levels are not too high, may trigger intracellular signalling, activating cell survival pathways.

complex I inhibition might result from: (a) a direct effect of Ca^{2+} on complex I; (b) Ca^{2+}-induced permeability transition affecting complex I or loss of matrix NAD^+/NADH; or (c) Ca^{2+}-induced ROS inhibiting complex I. Ca^{2+}-induced inhibition of complex I in isolated mitochondria was shown to be prevented in the presence of antioxidants and superoxide dismutase, but not catalase, and could be reversed by dithiothreitol, suggesting that it was mediated by superoxide oxidizing cysteine residues in the proteins [102]. Interestingly, it was reported that Ca^{2+} suppressed superoxide production by complex III in heart submitochondrial particles, and this effect correlated with complex I inactivation [103]. Subsequently, it was suggested that Ca^{2+}-induced inactivation of complex I may represent a means of limiting superoxide production by a compromised electron transport chain [103].

Ischaemia–reperfusion-induced loss of function may also be related to direct oxidation of critical residues within complex I. Indeed, three tryptophans located on the 51-kDa and 39-kDa subunits of complex I were found to be oxidized in human and bovine hearts [104], suggesting that they are particularly vulnerable to oxidative damage. The activity of complex I is also decreased upon exposure of mitochondria to ROS [105], S-nitrosothiols, or peroxynitrite [25–27]. As mentioned above, peroxynitrite may nitrate tyrosines whereas S-nitrosothiols may cause S-nitrosation of cysteines in peptides of complex I, leading to its inactivation, which may be reversed by light or in the presence of glutathione or other thiols [25,27]. Increases in the nitrotyrosine levels of mitochondrial proteins were detected after 8 min of mouse heart ischaemia and prior to complex I inactivation, although the nitrated proteins were not identified [106]. S-nitrosation of the 75-kDa subunit of complex I during ischaemic preconditioning of the heart has been also reported [107]. However, a causal relationship between S-nitrosation/nitration of particular subunits and the loss of respiratory function of complex I during ischaemia–reperfusion has not yet been determined. Also, there is no direct evidence that ROS/RNS-induced effects are related to oxidation/nitrosation of critical residues or to oxidation of cardiolipin bound to complex I. In line with the latter, it has been shown that the decrease in complex I activity during ischaemia–reperfusion correlates with the cardiolipin content in heart mitochondria [108]. On the other hand, the functional importance of cardiolipin bound to complex I has not yet been conclusively demonstrated.

Another important question is whether the ischaemia-induced inactivation of complex I is pathological or protective. At first glance, inhibition of complex I activity leading to suppression of mitochondrial respiration during ischaemia–reperfusion may appear to be pathological. However, there is accumulating evidence that inhibition of complex I prior to ischaemia or at the beginning of reperfusion may be cardioprotective [109–111]. Various chemical compounds and medicines, such as amobarbital, ranolazine [112], volatile anaesthetics, and diazoxide [110], as well as S-nitrosating NO donors, such as *S*-nitrosoglutathione, *S*-nitroso-2-mercaptopropionyl-glycine [113], and mitochondria-targeted *S*-nitrosothiol [114], have been found to protect hearts against ischaemia–reperfusion injury by a mechanism thought to involve inhibition of complex I. It has been reported that mitochondrial proteins, particularly the 75-kDa subunit of complex I, are endogenously S-nitrosated in ischaemic preconditioning of the heart [107], which also suggests that reversible S-nitrosation and inhibition of complex I may be an innate cardioprotective mechanism. How inhibition of the mitochondrial respiratory chain may exert protection during ischaemia or reperfusion is unclear at present. A possible explanation is that complex I inhibition during ischaemia limits the Ca^{2+} overload and ROS production during reperfusion [113]. A similar mechanism may underlie the cardioprotective benefits of slow reperfusion or postconditioning. However, complex I inhibition does not normally inhibit ROS production unless electron transfer is occurring in the reversed direction from succinate to NAD^+, or the inhibition occurs at the NADH-binding site. Succinate may accumulate during ischaemia, and power ROS production during reperfusion. An alternative explanation might be that inhibition of complex I during ischaemia may stimulate ROS generation, which may trigger signalling cascades leading to cell survival on reoxygenation [115,116].

Does ischaemia induce release of cytochrome *c* from mitochondria?

Substantial evidence, obtained with direct and indirect assays, has accumulated over three decades that heart ischaemia (without reperfusion) causes rapid cytochrome *c* release from mitochondria. In the earliest studies, this was demonstrated by the so-called cytochrome *c* test, in which exogenous cytochrome *c* is added to mitochondria, and the change in respiration rate is measured. As cytochrome *c* cannot cross the intact mitochondrial outer membrane, stimulation of respiration after addition of cytochrome *c* is interpreted as an indication of damage to the mitochondrial outer membrane and a lack of endogenous

cytochrome *c*. With this methodology, it has been shown that addition of cytochrome *c* to mitochondria isolated from hearts after 30 min of ischaemia can restore the state 3 respiration rate to the preischaemic rate with the substrates glutamate plus malate [117] and succinate [35]. Direct measurements of mitochondrial and cytosolic cytochrome *c* have shown that cytochrome *c* is not released after 15 min of ischaemia but is progressively lost from mitochondria to the cytosol after 20–60 min of heart ischaemia [85]. The loss of cytochrome *c* during ischaemia is not an artefact of the mitochondrial isolation procedure, as it is observed in permeabilized cardiac fibres [85,118,119], which are considered to contain intact mitochondria [120]. Chen and Lesnevsky [69,121] found that subsarcolemmal mitochondria were more sensitive to ischaemia than interfibrillar mitochondria: cytochrome *c* was lost from subsarcolemmal mitochondria after 30 min of ischaemia, whereas interfibrillar mitochondria remained intact. Interestingly, these investigators found that blockade of the mitochondrial respiratory chain at complex I or complex III preserved the mitochondrial content of cytochrome *c* during ischaemia, whereas inhibition of COX had no effect on ischaemia-induced loss of cytochrome *c* from subsarcolemmal mitochondria. In another study, Lesnevsky *et al.* found that, in perfused rabbit hearts, loss of mitochondrial cardiolipin and cytochrome *c* occurred after 45 min of ischaemia, resulting in respiratory dysfunction of mitochondria, and reperfusion did not lead to any additional damage [69]. It was concluded that loss of cardiolipin and cytochrome *c* during ischaemia causes persistent mitochondrial dysfunction during the early reperfusion period.

Ischaemia-induced cytochrome *c* release from mitochondria into the cytosol has been also been reported in other organs, such as rat liver, where translocation of cytochrome *c* was detected 15 min after clamping of the blood vessels, and reached maximal levels after 90 min of ischaemia [122].

During transient cerebral ischaemia (90 min of occlusion of the middle cerebral artery) in rats, the release of cytochrome *c* from mitochondria into the cytosol was observed, at the earliest, after 4 h of reperfusion [123], and was associated with signs of neuronal apoptosis 24 h later. Similar results were obtained in other studies, where the appearance of cytochrome *c* in the cytosol was detected in neurons of the core and penumbra of the lesion after 60 min of ischaemia followed by 6 h of reperfusion [124]. In another study, cytochrome *c* release was observed after 20 min of ischaemia induced by occlusion of both common carotid arteries followed by 30 min of reperfusion [125]. Whether loss of cytochrome *c* occurs in brain during the ischaemic period is less clear, although, in a model of focal permanent ischaemia in mice, cytochrome *c* release was observed after 30 min of middle cerebral artery occlusion, and progressed further during 24 h of ischaemia [126]. Generally, in mice, the release of cytochrome *c* from mitochondria is seen at earlier times of ischaemia or ischaemia–reperfusion than in other species. However, the fraction of released cytochrome *c* is usually relatively small as compared with that remaining in the mitochondria [55]. In our experiments on cerebral ischaemia *in vivo* in piglets, inhibition of mitochondrial respiration with complex I substrates is observed, at the earliest, after 3 h of ischaemia, but it is not reversed in the presence of exogenous cytochrome *c*, suggesting that mitochondrial cytochrome *c* is not lost from mitochondria during this period of ischaemia (unpublished data). It is likely that brain mitochondria are more resistant to ischaemia-induced release of cytochrome *c* from mitochondria, and the release may be a relatively late event as compared with the time at which suppression of mitochondrial respiratory chain activity occurs.

Although there was evidence in the literature indicating that cytochrome *c* loss from mitochondria is induced by ischaemia, for some time this was questioned or neglected, owing to observations that reperfusion after the ischaemic period greatly promotes cardiac injury and cell death [127]. This notion was supported by studies from several groups demonstrating that the main event in ischaemic heart damage is opening of MPTPs, which leads to permeabilization of mitochondrial membranes. MPT is thought to occur during reperfusion rather than during ischaemia [32,119,128,129], potentially causing cytochrome *c* release. Consistent with this, many investigators have reported the release of cytochrome *c* from mitochondria at various times after ischaemia–reperfusion: in perfused rabbit hearts, after 30 min of ischaemia plus 15 min of reperfusion [130]; in cultured rat cardiomyocytes, after 3 h of anoxia plus 2 h of reoxygenation [131]; and in isolated chick cardiomycytes, after 60 min of ischaemia plus 5 min of reperfusion [132]. Thus, the idea arose that the apparent cytochrome *c* release during ischaemia was an artefact of reperfusion of the ischaemic heart during the isolation of mitochondria. However, there has recently been some progress in resolving this issue, as the group of Halestrap published data reassessing their previous findings, and confirmed that mitochondrial cytochrome *c* is released after 30 min of heart ischaemia without reperfusion [119].

Mechanisms of ischaemia-induced cytochrome *c* release from mitochondria

The release of cytochrome *c* from mitochondria requires dissociation of the protein from the outer surface of mitochondrial inner membrane, followed by translocation through the outer membrane owing to its rupture or via specific pores. Although the precise mechanisms by which cytochrome *c* is released from mitochondria during ischaemia are not fully understood, at least three pathways have been discussed in the literature (Fig. 3). First, loss of cytochrome *c* from mitochondria may be caused by opening of MPTPs, which causes mitochondrial swelling and rupture of the mitochondrial outer membrane. In line with this, we found that ischaemia-induced loss of cytochrome *c* from mitochondria was prevented when hearts were pre-perfused with an inhibitor of MPTPs – cyclosporin A [118]. However, using similar conditions, Pasdois *et al*. [119] found that cyclosporin A could not prevent ischaemia-induced cytochrome *c* release. We do not know the reason for this discrepancy. Pasdois *et al.* [119] suggested that ischaemia-induced cytochrome *c* release might result from the measured ischaemia-induced loss of hexokinase II and Bcl-xL from mitochondria, possibly owing to calpain truncation of Bcl-XL to a form that can pass through the outer

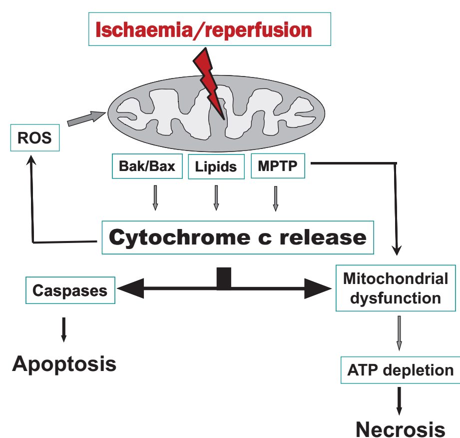

Fig. 3. Mechanisms and consequences of ischaemia-induced cytochrome *c* release from mitochondria. During ischaemia, mitochondrial cytochrome *c* may be released into the cytosol by mechanisms involving Bcl-2 proteins, lipids, or MPTP opening. In the cytosol, cytochrome *c* can participate in the activation of caspases, leading to apoptosis. Loss of cytochrome *c* from mitochondria may result in increased production of ROS, which, in turn, may lead to further oxidative damage to mitochondria. Loss of cytochrome *c* from mitochondria may result in mitochondrial dysfunction and ATP depletion, causing necrosis.

membrane. However, this suggestion remains speculative at present.

Another potential pathway of cytochrome *c* release from mitochondria involves the formation of large channels in the mitochondrial outer membrane via insertion of proapoptotic BH3 domain-containing proteins such as Bax, Bid, or Bad. It was shown that 20 min of simulated ischaemia induced p38 mitogen-activated protein kinase-dependent Bax translocation from the cytosol to mitochondria, and Bax translocation increased with longer periods of ischaemia [133,134]. This study also showed that Bax activation and translocation to mitochondria (preceding the release of cytochrome *c*) may be initiated during the ischaemic period itself, perhaps causing depletion of cellular energy resources and subsequent activation of AMP-dependent protein kinases [133]. Similarly, in another study using simulated ischaemia–reperfusion of isolated rat adult cardiomyocytes, it was demonstrated that 90 min of anoxia induced adenylyl cyclase-dependent and protein kinase A-dependent phosphorylation and translocation of cytosolic Bax to mitochondria, resulting in later cytochrome *c* release from mitochondria during simulated reperfusion [135]. According to this study, translocation of Bax was not sufficient to cause release of cytochrome *c*. Additional factors, such as oxidative stress during reperfusion, were needed to cause release. In the *in vivo* model of coronary artery occlusion/reperfusion of rat heart, translocation and tight association of Bax with mitochondria was detected only after 30 min of reperfusion (which followed 30 min of ischaemia), and was accompanied by massive release of cytochrome *c* from mitochondria [136]. Interestingly, a small but statistically significant increase in the cytosolic cytochrome *c* level was detected in this study even after 30 min of ischaemia itself, and was further promoted by reperfusion. This raises doubt about the involvement of Bax in ischaemia-induced cytochrome *c* release. Indeed, other investigators showed that cytochrome *c* loss during ischaemia was not associated with changes in the mitochondrial contents of Bax, Bad, Bak, or Bid, although a decrease in the content of the mitochondrial antiapoptotic protein Bcl-XL during ischaemia has been detected [119,137]. On the other hand, there is some evidence that MPT and Bax may be related, and may cooperate in providing a means for cytochrome *c* release from mitochondria, at least in certain models of apoptosis [138,139].

A further potential mechanism of cytochrome *c* release from mitochondria during ischaemia may be related to changes in the phospholipid composition of mitochondrial outer membranes, in particular

cardiolipin. Cardiolipin is present only in mitochondria, and is mainly located in the mitochondrial inner membrane, but it has also been found to be present in the outer membrane [140], where it may interact with Bcl-2-homologous proteins. Within the intermembrane space of mitochondria, cytochrome *c* is normally almost all reversibly bound to the cardiolipin of the inner membrane [141,142]. Cardiolipin depletion or oxidation causes dissociation of cytochrome *c* from the mitochondrial inner membrane, which is a requirement for cytochrome *c* release from mitochondria [143]. It has been shown that 45 min of ischaemia decreases the cardiolipin content of subsarcolemmal but not interfibrillar mitochondria of isolated rabbit heart without any further changes during reperfusion [69]. The cytochrome *c* content decreased in parallel with cardiolipin levels in subsarcolemmal mitochondria [69]. However, this study also revealed that cytochrome *c* was lost from interfibrillar mitochondria during ischaemia without any detectable changes in cardiolipin content, which may suggest that ischaemia leads to some loss of cytochrome *c* independently of cardiolipin depletion [69].

Kagan [144] has recently suggested that cytochrome *c* may form complexes with cardiolipin that catalyse H_2O_2-dependent cardiolipin peroxidation. Cardiolipin peroxidation may affect interactions of Bax and Bid with mitochondrial membranes, and in this way may be involved in mitochondrial outer membrane permeabilization [145]. Whether such a mechanism may operate during ischaemia is unclear.

Another potential mechanism is for saturated free fatty acids, which accumulate in ischaemic heart, to form complexes with elevated Ca^{2+}, leading to mitochondrial outer membrane permeabilization owing to the formation of (cyclosporin-insensitive) pores [146]. However, there is no experimental evidence yet for such a mechanism in ischaemic conditions.

What are the consequences of cytochrome *c* release during ischaemia? (a) We have found that cytochrome *c* release during heart ischaemia can trigger caspase activation, which itself can trigger further cytochrome *c* release. Thus, during prolonged ischaemia or reperfusion, part of the cytochrome *c* release can be prevented in the presence of caspase inhibitors [118]. Cytochrome *c* release via caspase activation can trigger apoptosis, which may contribute to heart damage during ischaemia or reperfusion. (b) Cytochrome *c* release during ischaemia is the main cause of respiratory inhibition (which can be reversed by adding back cytochrome *c*), which may block contractile function during reperfusion. (c) Cytochrome *c* release during ischaemia promotes mitochondrial ROS production during reperfusion, because it causes a reduction in the activity of of complexes I and III, and because mitochondrial cytochrome *c* normally oxidizes superoxide back to oxygen [119].

Conclusions

The data obtained over a half-century of intensive research on mitochondrial functions during ischaemia–reperfusion allow us to conclude that ischaemia itself has a detrimental effect on oxidative phosphorylation, particularly by inhibiting the mitochondrial respiratory chain. The primary effects of ischaemia seem to be exerted on mitochondrial complex I, which is suppressed reversibly at the beginning and irreversibly during prolonged ischaemia or reperfusion. Another early mitochondrial event during ischaemia that may cause irreversible damage to mitochondria is the release of cytochrome *c* from mitochondria into the cytosol. However, the mechanism(s) and consequences of ischaemia-induced complex I inhibition and cytochrome *c* release are still unclear, and require further research. Understanding these mechanisms may be important in developing better strategies for cardioprotection and neuroprotection. A promising current approach is ischaemic or pharmacological preconditioning and post-conditioning, the target of which is thought to be MPT. However, if MPT occurs only during reperfusion, but cytochrome *c* is lost during ischaemia, then additional strategies to prevent cytochrome *c*-mediated caspase activation and energy depletion resulting from suppression of oxidative phosphorylation in cytochrome *c*-deficient mitochondria may need to be applied together with inhibition of MPT.

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