Mitochondrial reprogramming through cardiac oxygen sensors in ischaemic heart disease

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Under hypoxic conditions, mitochondria can represent a threat to the cell because of their capacity to generate toxic reactive oxygen species (ROS). However, cardiomyocytes are equipped with an oxygen-sensing pathway that involves prolyl hydroxylase oxygen sensors and hypoxia-inducible factors (HIFs), which induces a tightly regulated programme to keep ischaemic mitochondrial activity under control. The aim of this review is to provide an update on the pathways leading to mitochondrial reprogramming, which occurs in the myocardium during ischaemia, with particular emphasis on those induced by HIF activation. We start by studying the mechanisms of mitochondrial damage during ischaemia and upon reperfusion, highlighting the importance of the formation of the mitochondrial permeability transition pore during reperfusion and its consequences for cardiomyocyte survival. Next, we analyse hypoxia-induced metabolic reprogramming through HIF and its important consequences for mitochondrial bioenergetics, as well as the phenomenon known as the hibernating myocardium. Subsequently, we examine the mechanisms underlying ischaemic preconditioning, focusing, in particular, on those that involve the HIF pathway, such as adenosine signalling, sub-lethal ROS generation, and nitric oxide production. Finally, the role of the mitochondrial uncoupling proteins in ischaemia tolerance is discussed.

Keywords Hypoxia-inducible factor • Ischaemia • Mitochondria • Preconditioning • Prolyl hydroxylase

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1. Introduction

Ischaemic heart disease represents a heterogeneous group of pathological conditions characterized by insufficient perfusion of the heart. Mitochondria are essential organelles in any tissue, but when oxygen availability is limited they can become detrimental to the performance of the diseased heart. Moreover, during reperfusion, mitochondria overproduce reactive oxygen species (ROS) that may damage cardiomyocytes. It was recently revealed, however, that cardiomyocytes can activate an endogenous oxygen-sensitive transcriptional programme that modulates mitochondrial activity when the myocardium is challenged by a low oxygen supply in areas of acute or chronic ischaemia. This biological response is controlled by prolyl hydroxylases (PHDs) that act as oxygen sensors, and by hypoxia-inducible factors (HIFs). This system regulates the expression of numerous genes that induce a metabolic reprogramming, which ultimately makes mitochondria harmless but less active. In the present review, we will discuss the beneficial and detrimental consequences of this programme in both acute and chronic ischaemia. In addition, the HIF pathway has also been implicated in the endogenous cardioprotective phenomenon of ischaemic preconditioning (IPC), although the signalling transduction pathways involved in this process are not completely understood. Along with adenosine synthesis and PI3K/Akt activation, the endogenous gas nitric oxide (NO) plays a key role in IPC. Moreover, the mitochondrial uncoupling proteins (UCPs) UCP2 and UCP3 attenuate ROS production and ROS-related cellular damage, and their activation during IPC decreases ROS production upon reperfusion and hence increases tolerance to ischaemia–reperfusion (IR) damage.

2. IR damage

Increased ROS production, initiated during ischaemia and exacerbated upon reperfusion, coupled with increased cellular [Ca\(^{2+}\)], is thought to be the main cause of reperfusion injury. The electron transport chain (ETC) is the main source of ROS, and it is generally believed that the superoxide anion (O\(_2^-\)) is mainly produced at complexes I and III. Thus, mitochondria are both producers of ROS and targets of ROS and calcium damage.

2.1 Damage occurring during ischaemia

During ischaemia, increased glycolysis causes lactic acid accumulation and hence a decrease in the intracellular pH. In an attempt to revert...
the drop in pH, the Na\(^{+}/H^{+}\) antiporter is activated, which leads to a rise in intracellular [Na\(^{+}\)] as the Na\(^{+}/K^{+}\) ATPase becomes inhibited by the decline in ATP concentration. As a consequence of the increased [Na\(^{+}\)], the intracellular [Ca\(^{2+}\)] concentration also increases, because the Na\(^{+}/Ca^{2+}\) antiporter, which usually pumps Ca\(^{2+}\) out of the cell, is inhibited or reversed.

The production of ROS is also a feature of ischaemia. During ischaemia, ROS are generated in the myocardium.\(^{11,12}\) This occurs particularly during low-flow ischaemia, when residual oxygen still reaches the tissue. ROS are formed at complexes I and III of the ETC, and mainly through the action of xanthine oxidase on the xanthine formed by the degradation of adenosine.\(^{1,10,13,14}\) After a prolonged period of ischaemia, the inner mitochondrial membrane potential in cardiomyocytes is reduced.\(^{11}\) The ATP synthase reverses direction to maintain the membrane potential by ATP hydrolysis. However, elevated [Ca\(^{2+}\)] and levels of ROS, combined with ATP depletion, provoke a gradual loss of cellular integrity as ATP-dependent repair processes become inoperative.\(^{8,10,15}\) Recovery or necrosis of the tissue on reperfusion depends on the length of the ischaemic period. Thus, after a short period of ischaemia, mitochondria might be able to generate ATP and the mild tissue damage can be repaired. If the ischaemic period has been too long, however, recovery is impossible and reperfusion causes further damage to the heart.

### 2.2 Damage occurring during reperfusion

There is increasing evidence that mitochondrial dysfunction plays a central role in mediating necrosis and apoptosis during reperfusion injury. Reperfusion is associated with a burst of ROS production,\(^{16}\) although the source of these ROS is unclear. Most ROS are probably formed by complexes I and III of the respiratory chain.\(^{1,10,14}\) The superoxide anion (O\(_{2}^{−}\)) is produced from the transfer of an electron from the partially reduced ubiquinone to oxygen, and it is then reduced to hydrogen peroxide (H\(_{2}\)O\(_{2}\)) by superoxide dismutase. This H\(_{2}\)O\(_{2}\) is then removed by glutathione peroxidase or catalase, or it can react via Fenton chemistry with ferrous ions and other transition metals to form the highly reactive hydroxyl radical (·OH). ROS have direct effects on different respiratory chain components, such as complexes I and III. Moreover, ROS can cause thiol oxidation and inhibition of the ATP synthase and adenine nucleotide translocase (ANT). They can also cause peroxidation of the unsaturated fatty acids in membrane phospholipids, especially cardiolipin at the inner mitochondrial membrane, which leads to further inhibition of respiratory chain activity.\(^{17,18}\) As a consequence of lipid peroxidation, reactive aldehydes such as 4-hydroxynonenal are released and can modify membrane proteins.\(^{19}\) The combined effects of ROS and elevated [Ca\(^{2+}\)] lead to the opening of the mitochondrial permeability transition pore (mPTP) that plays a critical role in reperfusion damage.\(^{2,3,8,10,20}\)

#### 2.2.1 The mitochondrial permeability transition pore

The mitochondrial inner membrane is impermeable to most metabolites and ions. This allows a membrane potential and pH gradient to be established, which are essential for oxidative phosphorylation and ATP synthesis. In certain conditions, when the calcium matrix concentrations increase, and especially when this is accompanied by low adenine nucleotide and high phosphate concentrations, as well as under situations of oxidative stress, the non-specific mPTP opens in the inner mitochondrial membrane (reviewed in reference 21). The opening of the mPTP facilitates the free passage of molecules of <1.5 kDa, including protons, into the mitochondria. This uncouples oxidative phosphorylation and results in the collapse of the mitochondrial membrane potential, ATP depletion (glycolytic ATP is hydrolysed by the reverse operation of the ATP synthase), and necrotic cell death. Another consequence of mPTP opening is that small-molecular-weight molecules equilibrate across the inner membrane with the subsequent disruption of the metabolic gradients between the mitochondria and cytosol. This phenomenon also causes mitochondrial swelling, and as the matrix expands, it exerts pressure on the outer membrane that eventually bursts, releasing cytochrome c and other pro-apoptotic proteins that may initiate apoptotic cell death.

The properties of the pore are well defined, although the identity of the membrane components remains controversial. Several proteins have been implicated in the structure or regulation of mPTP. Over the past 20 years, three proteins have been accepted as key structural components of the mPTP: the ANT in the inner membrane, cyclophilin-D (CyP-D) in the matrix, and the voltage-dependent anion channel (VDAC) in the outer membrane. However, recent studies have eliminated VDAC as an essential component of mPTP, and ANT has been attributed a regulatory role. Moreover, the mitochondrial phosphate carrier (PiC) appears to play a crucial role in mPTP formation. Several other proteins have been proposed as components of the mPTP, although the evidence for their involvement is scant. All these components act as potential targets for cardioprotection.

The damage suffered by the heart at reperfusion after a period of ischaemia may be produced by pore opening. This was first suggested in 1987\(^{22,23}\) and was later demonstrated in isolated cardiomyocytes,\(^{24,25}\) as well as by using Langendorff-perfused hearts.\(^{26,27}\) Accordingly, IR-induced cell death is diminished in CyP-D knockout mice in vivo.\(^{28}\) Likewise, preventing pore opening with the potent inhibitor cyclosporine A (CsA), CsA analogues, or the CyP-D inhibitor sanglifehrin A (SFA) provides protection against perfusion injury.\(^{29-31}\) Both CsA and SFA reduce the size of infarct in hearts subjected to coronary artery occlusion and re-opening, a protocol that mimics the treatment of coronary thrombosis. However, the most powerful inhibitor of mPTP opening and the best protector of the heart is pyruvate.\(^{32,33}\) The effects of pyruvate are mediated by its ability to scavenge free radicals, to maintain a lower intracellular pH at the beginning of reperfusion, and to serve as a fuel for ATP synthesis.

#### 2.2.2 Reperfusion-induced cardiomyocyte hypercontracture

During the first minutes of reperfusion that follow myocardial ischaemia, cell death occurs by necrosis. Post-reperfusion infarcts are formed by areas of contraction band necrosis composed of hypercontracted cardiomyocytes.\(^{34,35}\) Several studies have shown that transient blockage of contraction at the onset of reperfusion prevents cell death,\(^{36-40}\) indicating the causal influence of hypercontracture in cell death during reperfusion. Although all the mechanisms involved are not fully understood, contractile activation induced by the resumption of ATP synthesis in the presence of high cytosolic [Ca\(^{2+}\)] is the main factor involved in reperfusion-induced hypercontracture.\(^{41-43}\) Moreover, hypercontracture can be propagated to adjacent cardiomyocytes by the passage of Na\(^{+}\) through gap junctions, and through secondary entry of Ca\(^{2+}\) via reverse Na\(^{+}/Ca^{2+}\) exchange.\(^{44}\) In addition, mPTP opening may induce hypercontracture in Ca\(^{2+}\)-overloaded cardiomyocytes, provided a sufficient number of non-permeabilized mitochondria are present to sustain ATP synthesis.\(^{45}\)
2.2.3 Protection of the heart by postconditioning

Ischaemic postconditioning consists of brief periods of ischaemia alternating with brief periods of re-flow applied during reperfusion after a prolonged ischaemia. This protocol dramatically reduces infarct size\(^{46,47}\), to be effective, however, postconditioning must be applied at the onset of reperfusion. The mechanisms underlying the protection offered by postconditioning have recently been reviewed.\(^{48}\) Briefly, they include the formation and release of several autacoids and cytokines, the maintenance of acidosis during early reperfusion, the activation of protein kinases, and the preservation of mitochondrial function by attenuating the opening of the mPTP. Experimental evidence for the last mentioned mechanism comes from studies showing that when administered at the time of reperfusion, CsA could limit infarct size in the isolated rat heart.\(^{49}\) Moreover, the Ca\(^{2+}\) load required to open the mPTP in mitochondria isolated from the myocardial risk region of postconditioned hearts was higher than that of control hearts.\(^{50}\) Indeed, the specific inhibitor of the mPTP, NIM811, and IPC (see below) both limited infarct size to a similar extent. Although promising, the use of these cardioprotective therapies in humans first requires an improvement in clinical outcome to be demonstrated in a large number of patients.

3. Hypoxia-induced metabolic reprogramming

3.1 Cellular response to hypoxia: the HIF pathway

A decrease in oxygen concentration has a significant effect on gene transcription due to the activation of HIF, a transcription factor that activates the expression of a number of genes involved in the adaptation of tissues to hypoxia.\(^{51}\) HIF is an α/β-heterodimer of two proteins, both of which contain basic helix–loop–helix PAS domains. The expression of HIF-β (also known as aryl hydrocarbon receptor nuclear translocator) is not influenced by oxygen levels, and HIF-β is constitutively expressed in all cell types. In contrast, the accumulation of HIF-α subunits is oxygen sensitive. Three α isoforms have been defined in humans (HIF-1α, HIF-2α, and HIF-3α), each encoded by a distinct gene locus. These proteins are substrates of a family of proline hydroxylases that catalyse the hydroxylation of specific residues in HIF-α subunits (Pro402 and Pro564 in human HIF-1α) utilizing oxygen, ferrous iron (Fe\(^{2+}\)), and 2-oxoglutarate (α-ketoglutarate). These residues are located within oxygen-dependent degradation domains.\(^{52,53}\) Hydroxylation of an asparagine residue also occurs (Asp803 in human HIF-1α) due to the activity of FIH (factor inhibiting HIF), which prevents transcriptional activity by inhibiting the interaction with the transcriptional co-activator CBP/p300.

Prolyl hydroxylation enables HIF-α to interact with the von Hippel–Lindau tumour suppressor (pVHL), the recognition component of a ubiquitin E3 ligase that targets HIF-α subunits for proteasome degradation. Thus, in the presence of sufficient oxygen, the HIF system is inactivated by prolyl hydroxylation and subsequent proteolysis. In addition, HIF-driven transcription is impaired by FIH. In hypoxic conditions, oxygenase activity is reduced, leading to HIF-α accumulation and its dimerization with HIF-β, as well as the recruitment of the CBP/p300 co-activator and the induction of the transcription of specific targets (for review, see reference \(^{54}\)). These target genes contain a core DNA sequence (G/ACGTG) in hypoxia response elements to which HIF binds.

In humans, prolyl hydroxylation is catalysed by three oxygenases: PHD1 (also called EglN2), PHD2 (also called EglN1), and PHD3 (also called EglN3).\(^{55–57}\) A decrease in environmental pO\(_2\) inhibits hydroxylase activity leading to the activation of the HIF pathway. Prolyl hydroxylase activity is very sensitive to changes in oxygen availability in vitro because of its relatively high K\(_m\) for oxygen.\(^{56,58}\) The fact that an oxygen-dependent enzyme can control HIF stability provides a simple explanation for how changes in oxygen concentration are translated into changes in gene expression. Moreover, PHD activity has also been shown to be inhibited during hypoxia by mitochondrial ROS formation in response to low-oxygen tension,\(^{59}\) although the exact mechanisms involved remain elusive.\(^{60–62}\)

3.2 Metabolic reprogramming through the HIF pathway

Individual cells adapt to oxygen deprivation by reprogramming their metabolism in part through metabolic alterations induced by the PHD/HIF system (Figure 1).

The processes stimulated by HIFs include:

- expression of the glucose transporters GLUT1 and GLUT4;
- expression of glycolytic enzymes;
- expression of lactate dehydrogenase A;
- expression of pyruvate dehydrogenase kinase (PDK);
- the COX4-1 to COX4-2 subunit switch; and
- mitochondrial autophagy.

Diverting pyruvate away from the mitochondria is an important alteration induced by hypoxia. This is achieved by the PHD/HIF-mediated activation of the PDK gene, which encodes the pyruvate dehydrogenase (PDH) kinase isoenzymes 1,\(^{63,64}\) 3,\(^{65}\) and 4.\(^{66}\) PDK phosphorylates and inhibits PDH, the enzyme that converts pyruvate into acetyl coenzyme A (AcCoA) for entry into the mitochondrial citric acid cycle, which generates reducing equivalents that are donated to the ETC. Reduced delivery of substrate to the mitochondria for oxidative phosphorylation results in reduced ATP synthesis. Simultaneously, there is an increase in glucose uptake via glucose transporters and increased conversion of glucose to lactate by the activity of glycolytic enzymes and lactate dehydrogenase A, all of which are encoded by HIF-1 target genes.\(^{67–70}\)

The induction of PDK expression inhibits the oxidative metabolism of AcCoA derived from glucose, although it does not affect the oxidative metabolism of AcCoA derived from fatty acids. However, some studies have reported the active destruction of mitochondria by mitochondrial autophagy during hypoxia.\(^{71}\) Indeed, mouse embryo fibroblasts (MEFs) cultured in 1% oxygen reduce their mitochondrial mass by ~75% over 48 h through autophagy. This process is initiated by the HIF-1-dependent expression of BNIP3, a mitochondrial protein that competes with Beclin-1 for binding to Bcl2, displacing Beclin-1, which triggers autophagy.\(^{71}\) It has also been proposed, however, that hypoxia-induced mitochondrial autophagy is independent of HIF-1 and BNIP3.\(^{72}\)

Another adaptation to reduced oxygen levels is a subunit switch that occurs in complex IV, whereby the COX4-1 regulatory subunit is replaced by the COX4-2 isoform. This event is mediated by HIF-1, which activates the transcription of the genes encoding COX4-2 and LON, a mitochondrial protease required for the...
The aim of this subunit switch is to optimize the efficiency of electron transport and minimize $O_2^\cdot_2$ production in hypoxic conditions. Indeed, a CcO subunit switch in response to hypoxia also occurs in the yeast *Saccharomyces cerevisiae*, although this is achieved by a completely different mechanism since this organism does not have an HIF-1 homologue. The parallel regulation of CcO activity in yeast and human cells indicates that oxygen-dependent regulation of mitochondrial respiration is an ancient process and is likely to be shared by all eukaryotic organisms.

These metabolic responses to hypoxia have an adaptive significance in the context of protection against excessive ROS production. It has long been known that mitochondrial ROS production increases under hyperoxic conditions. As discussed above, however, acute hypoxia also leads to increased mitochondrial ROS production, and this is thought to be required to inhibit PHD activity. There is a reduction in ROS when wild-type MEFs are exposed to hypoxia for 48–72 h, whereas ROS levels are markedly increased in HIF-1α-deficient MEFs under such conditions, causing cell death. Survival improves by the overexpression of PDK1 or BNIP3, or in the presence of free-radical scavengers.

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**3.3 Chronic hypoxia: the hibernating heart**

Heart failure and underlying coronary artery disease are characterized by the presence of chronic ischaemic areas in which cardiomyocytes remain viable but hypocontractile. This condition is known as ‘myocardial hibernation’ and it refers to the sustained reduction of contractile function in hypoperfused but viable myocardium, which recovers completely upon reperfusion. Cardiomyocytes located in poorly perfused regions induce this cellular state in an attempt to match metabolism to the chronically reduced oxygen supply, as well as to prepare cardiac tissue to avoid the oxidative damage associated with the eventual restoration of blood flow. Several studies have shown that the molecular mechanisms of chronic ischaemia involved in the protection against subsequent reperfusion damage differ from those described above, which are associated with reperfusion followed by acute ischaemia. Chronic ischaemia has been associated with the downregulation of proteins involved in mitochondrial oxidative metabolism, as well as with the upregulation of genes involved in the unfolded protein response and an increase in ‘self-cannibalism’ called autophagy. Recent evidence suggests a role for the PHD and HIF system in the execution of these responses in chronic hibernating myocardium. Indeed, in a mouse model of reduced vascular endothelial growth factor expression, the heart suffers from vessel pruning, cardiac hibernation, and subsequent cessation of contractile function, mimicking the events observed in chronic ischaemic heart.
4. Ischaemic preconditioning

IPC is a very effective way of protecting the heart from reperfusion injury. This involves one or more short non-lethal cycles of IR that protect the heart against a subsequent prolonged period of ischaemia. This procedure induces two phases of protection: an immediate effect that lasts 2–3 h, and a delayed sustained effect evident 24–48 h later. Since the seminal study demonstrating the protection of the myocardium from IR injury by IPC,87 the search for the mechanisms involved has identified several key mediators, including adenosine, sub-lethal ROS, and NO. It seems clear that IPC protects the heart by reducing oxidative stress during ischaemia and reperfusion, and that this leads to less mPTP opening. However, the signalling pathways involved in mediating these effects are still to be elucidated.85

4.1 IPC through the HIF pathway

In contrast to chronic ischaemia, it is well known that the acute oxygen fluctuations that take place during IPC induce tolerance to IR challenge. Cardiac-delayed IPC leads to the upregulation of HIF-1α or some of its downstream genes, suggesting an essential role for the PHD/HIF system in cardiac IPC.91–93 Indeed, recent studies have shown that IPC-induced protection against myocardial ischaemia is lost in partially deficient HIF-1α mice, as well as after intraventricular infusion of siRNA against HIF-1α.92,94 Conversely, intraventricular infusion of siRNA against PHD2 only (not PHD1 or PHD3) leads to HIF-1α activation and myocardial IPC, which in turn induces cardiac tolerance to severe IR challenge.92,94 Moreover, pharmacological inhibition of PHDs or FiH induces myocardial preconditioning in vivo.95,96 In terms of the HIF-1-dependent genes involved in cardiac preconditioning, recent reports unveiled new genes that affect cardiac signalling pathways that counteract reperfusion-induced oxidative damage (Figure 2).

4.1.1 Adenosine signalling

Hearts subjected to IPC (or silencing of the PHD2 sensor) upregulate key molecules in adenosine signalling through HIF-1α, such as the ecto-5’-nucleotidase CD73 that generates adenosine, and the A2B adenosine receptor (A2BAR). Importantly, intraventricular infusion of siRNA HIF-1α, as well as of CD73 and A2BAR, abrogates IPC-induced cardioprotection.92,97,98 Notably, cardioprotection induced by DMOG, a pharmacological PHD inhibitor, is completely lost in CD73- and A2BAR-deficient mice, which suggests a critical role for the adenosine pathway in the PHD/HIF-induced cardiac IPC.92

How does adenosine ultimately produce IPC? Phosphatidylinositol-3-kinase (PI3-kinase) activation is involved in IPC.99 Thus, its pharmacological inhibition with wortmannin or LY 294002 attenuates the cardioprotective effect of IPC.100 PI3-kinase can activate several targets via phospholipid-dependent kinases, one of which is PKB (also known as Akt). Thus, PI3-kinase activation results in rapid phosphorylation and activation of Akt.101 Among the intracellular events triggered by adenosine is Akt activation,102,103 which confers cardioprotection. Akt is a serine–threonine kinase, the activation of which prevents the opening of the mPTP, an essential event associated with reperfusion-induced cardiomyocyte death.104–107

4.1.2 Generation of sub-lethal ROS via HIF-1

Studies performed in heterozygous HIF-1α-deficient mice revealed that HIF mediates IPC through the generation of low (sub-lethal) amounts of mitochondrial ROS that act as an intracellular signal.94,108,109 However, the molecular mechanism triggered by HIF that leads to sub-lethal ROS accumulation remains unknown, although PTEN (a protein/lipid phosphatase that negatively regulates the PI3K/ Akt pathway) is a target of ROS. PTEN oxidation and the subsequent inactivation by ROS enhance Akt phosphorylation, which promotes cardioprotection by reducing mPTP opening (see above).110,111 Therefore, HIF contributes to the overactivation of Akt, not only through adenosine signalling (see above) but also by PTEN inactivation. Indeed, these recent insights into oxygen sensors and the role of HIFs in IPC point to Akt activation as a meeting point for different HIF-dependent pathways that ultimately confer cardiac IPC. Nevertheless, it is still conceivable that other mechanisms co-operate with the intracellular pathways discussed above.
4.1.3 Cardioprotective effects of nitric oxide in IR

The endogenous gas NO has been implicated in cardiac protection by IPC, and indeed, several reports show the ability of NO donors to protect the myocardium against IR injury. Cardiac IPC upregulates the inducible isoform of NO synthase (iNOS), and iNOS deficiency abolishes the infarct-sparing effect of IPC. Interestingly, HIF-1 regulates iNOS, and studies in endothelial NO synthase (eNOS) knockout mice revealed that infarct size was significantly augmented following IR, consistent with eNOS-derived NO acting as an endogenous cardioprotective agent. Conversely, transgenic mice expressing significantly more eNOS than their littermates presented a significantly smaller infarct area following IR. It is thought that NO generated by eNOS triggers multiple signalling pathways that lead to the upregulation of a number of proteins, including iNOS. Thus, NO could be a trigger and a mediator of IPC.

In terms of the mechanisms by which NO protects the heart during IR injury, NO is a known activator of soluble guanylate cyclase, which stimulates the production of cGMP and activates PKG (protein kinase G). The addition of exogenous PKG and cGMP to isolated mitochondria results in the opening of mitoKATP channels located in the inner mitochondrial membrane. Hence, PKG is responsible for transmitting the cardioprotective signal from the cytosol to the mitochondria. Openers of the mitoKATP channel, such as diazoxide, prevent reoxygenation-induced hypercontracture of cardiomyocytes. Moreover, the opening of mitoKATP channels results in an influx of potassium that causes the mitochondria to swell, leading to the production of sub-lethal ROS. Exposure to sub-lethal oxygen radicals mimics IPC protection in the absence of ischaemia, suggesting that ROS are involved in IPC. Conversely, an ROS scavenger could block the protection from IPC, which suggests that ROS not only contribute to reperfusion injury but are also capable of acting as second messengers. An important target of redox signalling is PKC (protein kinase C), and activated PKC provides protection that persists during the prolonged period of ischaemia. Protection is ultimately achieved by inhibiting the formation of mPTP early in reperfusion through the activation of the survival kinases Akt and ERK (extracellular signal-regulated protein kinase). These survival kinases are thought to inhibit pore formation by phosphorylating GSK-3β (glycogen synthase kinase-3β), which reduces its activity. Interestingly, iNOS is phosphorylated and activated by Akt, and thus, modulation of the cGMP signalling pathway is a promising approach to prevent reperfusion injury.

The second mode of action involves the effects of NO on oxygen consumption, since NO is a major regulator of mitochondrial respiration. The cardioprotective effects of NO on the ETC are thought to occur by reversibly inhibiting electron entry into the ETC and through the generation of low levels of ROS that initiate signalling cascades. Reversible inhibition of proximal components of the ETC by NO results in a slow reintroduction of electrons into the ETC on reperfusion. Indeed, pharmacological inhibitors of complexes I and II are cardioprotective, and their response may be elicited by forcing the ETC to undergo a ‘gradual wake-up’ from ischaemia on reperfusion. This gradual wake-up would attenuate mitochondrial Ca2+ overload, ROS overproduction, and mPTP formation, and it
could also delay and/or reduce the magnitude of reperfusion-induced hypercontraction. However, a limited amount of ROS is needed to initiate preconditioning signalling pathways.\textsuperscript{128} Low ROS concentrations could be generated by ETC through interactions of NO with distal components of the ETC, such as complexes III and IV. NO acts as a competitive inhibitor of complex IV by competing for binding with molecular oxygen,\textsuperscript{129–131} and inhibition of CoQ by NO may activate superoxide generation by upstream complexes.\textsuperscript{132} However, further studies will be necessary to clarify the mechanisms by which NO triggers the generation of ROS needed to initiate preconditioning signalling pathways.

5. UCPs and ischaemia tolerance

The UCPs are mitochondrial inner membrane proteins that belong to the superfamily of mitochondrial anion carriers. Unlike UCP1, which is expressed in brown adipose tissue and that mediates adaptive thermogenesis by uncoupling oxidative phosphorylation, the physiological functions of UCP2 and UCP3, two UCP1 homologues present in the heart, remain unclear.\textsuperscript{133} Although initial studies explored the possible thermogenic role of these proteins, it is now generally accepted that they do not increase the basal proton conductance of the inner mitochondrial membrane, as UCP2 and UCP3 knockdown does not affect basal proton conductance in mouse mitochondria,\textsuperscript{19,134–137} even though they do catalyse an inducible proton conductance in the presence of activators, including O$_2^\cdot$ radicals\textsuperscript{138} and reactive alkenals.\textsuperscript{19,139}

It has been well established that there is a strong positive correlation between membrane potential and ROS production.\textsuperscript{139–141} Thus, a small decrease in membrane potential (‘mild-uncoupling’) was suggested to have a natural antioxidant effect.\textsuperscript{142} The activation of UCP2 or UCP3 might slightly lower the membrane potential, thereby attenuating mitochondrial ROS production and protecting against ROS-related cellular damage. Experimental evidence supporting this hypothesis has been expertly reviewed.\textsuperscript{143}

UCPs have been implicated in the protection against IR injury.\textsuperscript{144} UCP2 overexpression in rat neonatal cardiomyocytes does not have intrinsic uncoupling activity; rather, it confers tolerance to oxidative stress by diminishing mitochondrial Ca$^{2+}$ overload and reducing ROS generation.\textsuperscript{145} Ectopic expression of UCP1 in cardiac-derived H9c2 cells\textsuperscript{146} and in the murine heart\textsuperscript{147} reveals a cytoprotective effect of UCP-mediated uncoupling against IR injury. However, this finding contrasts with the reported resistance to cerebral ischaemia injury in UCP2 knockout mice,\textsuperscript{148} which have reduced (not increased) infarct sizes after cerebral IR injury. Additional evidence supporting a protective role of UCPs against IR injury comes from RNA interference studies in which UCP2 and UCP3 were partially depleted in cardiac-derived H9c2 cells.\textsuperscript{149} These studies demonstrated diminished tolerance to anoxic stress in association with increased ROS production, with UCP2 playing a more important role than UCP3 in promoting tolerance to anoxia and reoxygenation.

The mitochondria of cells that have been acutely preconditioned in situ are uncoupled, as reflected by their lower inner membrane potential, reduced ATP levels, and greater oxygen consumption when compared with mitochondria from non-preconditioned control cells.\textsuperscript{150}

The association of classic preconditioning (an acute adaptive phase evident within minutes to 2–4 h after IPC) and proton leak has been confirmed directly in mitochondria isolated from perfused rat hearts that were subjected to preconditioning.\textsuperscript{151} The proton leak in these mitochondria was greater than in control mitochondria, and this increased leakage was prevented by the UCP inhibitor GDP, or the ANT inhibitor carboxylatractylate, thereby implicating both these proteins in this phenomenon. In contrast, delayed preconditioned mitochondria do not exhibit increased proton leak; rather, they are more tolerant to anoxia-reperfusion insults than non-preconditioned mitochondria.\textsuperscript{152} The transcriptional activator, peroxisome-proliferator-activated receptor-γ co-activator-1α (PGC-1α), is upregulated as a component of the delayed preconditioning programme.\textsuperscript{152} PGC-1α is a powerful regulator of ROS metabolism,\textsuperscript{153} and its transient activation in skeletal muscle cells upregulates UCP2 and UCP3, together with increased proton leak.\textsuperscript{154} Hence, it is reasonable to speculate that UCPs are involved in the protection against ROS-induced damage observed in delayed preconditioning. Indeed, upregulation of UCP2 and UCP3 is evident in rat hearts subjected to delayed preconditioning.\textsuperscript{149} This upregulation is associated with increased ROS-induced proton leak that is inhibited by GDP, and with lowered ROS generation upon reperfusion after prolonged anaoxia.\textsuperscript{155} The role of UCP2 in increasing tolerance to IR injury after delayed IPC has also been shown in the brain.\textsuperscript{155,156} Together, these results indicate that UCP2 and UCP3 are induced in response to ischaemic stress as cytoprotective proteins with antioxidant capacity, and therefore these proteins are potential therapeutic targets for the management of ischaemic diseases.

6. Conclusions and perspectives

The key role of HIF-induced mitochondrial reprogramming in protecting tissues against ischaemic damage has raised great interest due to its clinical implications. Decreased mitochondrial activity helps to keep ROS generation under control in chronic hypoxic areas (as observed in PHD1-deficient mice), and both NO and sub-lethal amounts of ROS prevent mPTP opening during cardiac preconditioning. Therefore, therapeutic strategies that mimic the activation of oxygen-sensing pathways in reprogramming mitochondria but that do not compromise cardiac performance would clearly have clinical value to counteract cardiomyocyte damage in the context of chronic or acute ischaemic heart disease. Likewise, mitochondrial UCPs are upregulated during delayed IPC to confer cardiac ischaemia tolerance. Elucidating the signalling pathways involved in IPC would help to develop clinical interventions to counteract ischaemic damage.

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