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Cell Death Pathways in Acute I/R Injury

Abstract

The consequence of myocardial ischemia is energetic stress, while reperfusion is accompanied by abrupt ionic shifts and considerable oxidative stress. Cells die by apoptotic and necrotic pathways. After the acute injury, the healing myocardium is subject to biomechanical stress and inflammation, which can trigger a smaller but more sustained wave of cell death, as well as changes in the metabolic and functional characteristics of surviving cells. The goal of cardioprotection is to prevent cell death during the acute injury as well as to modulate the detrimental processes that ensue during remodeling. This review will focus on acute injury, and the central premise is that mitochondria are the key determinant of cardiomyocyte fate.

Keywords

Apoptosis; necrotic cell death; mitochondria; autophagy; cardioprotection

Mitochondria Integrate the Signals of I/R Injury and Cytoprotection

Research over the past 25 years has yielded a still-expanding list of factors that contribute to mitochondrial dysfunction, and an equally long list of factors that protect the mitochondria. Some factors appear on both lists, and the issues may include timing, magnitude, or context. The mitochondria integrate the diverse signals and respond by altering ATP production, calcium homeostasis, ROS production, and in dire cases, membrane permeability. As shown in Figure 1, the signals that destabilize mitochondria include reactive oxygen species (ROS), protein kinases such as PKC delta and JNK, calpain, and pro-apoptotic members of the Bcl-2 family (Bax/Bak and a BH3-only family member such as Bid, Bnip3, Nix, etc.)^{1, 2}. Calcium in combination with ROS can lower the threshold for activation of mitochondrial iPLA2, liberating arachidonic acid and plasmalogens which contribute to further mitochondrial damage³. In contrast, signals that help to stabilize mitochondrial function include protein kinase C epsilon, Akt or its downstream effectors, and pro-survival members of the Bcl-2 family². This concept of mitochondria as a global signal integrator for cellular homeostasis is based on findings that many different interventions can change the outcome, yet they are not necessarily interdependent. In the following sections, several of these pathways will be discussed in more detail since they have led to therapeutic interventions some of which have gone to clinical trial including the PTP blocker cyclosporine A, a peptide inhibitor of delta PKC (KAI 9803), and cariporide, a regulator of ion homeostasis.

Unhappy Mitochondria: Intracellular Troublemakers

Functional integrity of the cardiomyocytes requires an abundance of mitochondria capable of producing ATP and participating in calcium homeostasis. However, beyond merely lying down on the job, unhappy mitochondria can actively contribute to the demise of the cell through activation of apoptotic and necrotic pathways. Apoptosis is a form of programmed cell death characterized by activation of caspases, notably caspase 3. Activation of caspase 3

—the effector caspase—by caspase 9 requires assembly of a large multimeric complex comprising caspase 9, APAF1, and cytochrome *c*. Cytochrome *c* is released from mitochondria after the outer membrane is permeabilized with the help of Bax (or Bak) and a BH3-only protein such as Bid. Also residing in the intracellular space with cytochrome *c* are other pro-apoptotic factors such as SMAC/DIABLO, a factor that binds to IAPs (inhibitor of apoptosis proteins) that interfere with APAF1 activation. The intricacies of the apoptotic pathway and participation of mitochondria have been covered extensively in other reviews^{4, 5}. Mitochondria may trigger cell death through a second pathway involving the mitochondrial permeability transition pore (MPTP)⁶. This large-conductance pore is operationally defined, and its molecular identity remains elusive, although genetic studies have clearly demonstrated a requirement for cyclophilin D⁷. Opening of the pore results in rapid exchange of solutes up to 1.5 kDa in size, with redistribution of NADH to the cytosol and an influx of cytosolic water, causing matrix expansion. In extreme cases, matrix swelling is sufficient to unwrinkle the inner membrane; however, the outer membrane is less distensible and ruptures, releasing pro-apoptotic factors from the intermembrane space. The catastrophic mitochondrial swelling often leads to cell death before apoptosis can proceed, due to energetic collapse and uncontrolled enzymatic processes. Another troublesome mitochondrial phenomenon is ROS-induced ROS release. One unhappy mitochondrion can emit large quantities of ROS which will trigger like-minded behavior on the part of neighbors, propagating a wave of ROS and depolarizations; this unstable electrical behavior within the cell can contribute to electrical conduction defects across a segment of tissue, culminating with cardiac arrhythmias^{8, 9}. This propensity for one or a few damaged mitochondria to trigger an epidemic of mitochondrial mischief-making is the reason they are such an important target for intervention in cardioprotection.

Calcium Overload and Mitochondrial Damage

Based on our understanding of the factors that contribute to catastrophic MPTP opening (summarized in Figure 2), a number of interventions have been explored. During ischemia, intracellular acidosis develops as lactic acid from glycolysis builds up and as protons are released with ATP hydrolysis. At reperfusion, the Na⁺/H⁺ exchanger (NHE-1) exchanges intracellular protons for extracellular sodium ions. The increased intracellular sodium ions compete with calcium for extrusion via the Na⁺/Ca⁺⁺ exchanger (NCX), resulting in a buildup of intracellular calcium. Interventions which inhibit this process including acidic reperfusion or inhibition of NHE-1 with cariporide limits the calcium overload, and have been shown to protect the heart, presumably through benefits to mitochondria¹⁰. Mitochondria acquire calcium more directly through close interaction with specialized regions of sarcoplasmic reticulum (SR)¹¹; it remains to be shown whether disrupting those close interactions which are mediated in part through mitofusin 2 could attenuate mitochondrial calcium overload. However, the significance of mitochondrial calcium overload as a determinant of MPTP opening has been called into question by Kim et al.¹². Calcium uptake and mitochondrial dynamics may also be modulated by other calcium-sensitive regulatory proteins including protein kinases and phosphatases, hexokinase, Drp-1, and Bcl-2 family members¹³⁻¹⁵. Beyond direct effects on mitochondria, calcium may activate phospholipases and calpain, which in turn will cause mitochondrial damage. Inhibiting calpain can reduce infarct size^{16, 17}, presumably without altering calcium overload, suggesting that calcium overload is not nearly as problematic as the downstream events. Calpain has been implicated in the processing and release of the mitochondrial protein AIF which translocates to the nucleus to mediate DNA fragmentation¹⁸.

ROS, Complex I, and the MPTP

It is widely accepted that excessive ROS production is a consistent feature of myocardial ischemia/reperfusion injury, and that protective interventions are associated with diminished ROS production. However, the evidence that ROS scavengers are beneficial is less compelling, and a clinical trial with recombinant human superoxide dismutase failed to demonstrate efficacy despite promising results in small pilot studies¹⁹. A number of enzymatic sources of ROS exist, including Complex I, alpha-ketoglutarate dehydrogenase, NADPH oxidase, and a variety of other NAD(P)H-dependent dehydrogenases. Modestly encouraging results have emerged from efforts to inhibit ROS production from selected enzymes using agents such as amytal (complex I inhibitor), allopurinol (xanthine oxidase inhibitor), and apocynin (NADPH oxidase inhibitor).²⁰ Given the abundance of NAD(P)H-dependent enzymes that can generate ROS, it is surprising that inhibiting just a single enzyme can have a measurable effect on infarct size. A more global approach may be to prevent the buildup of NADH that occurs during ischemia; this may be one reason why pyruvate is beneficial, as it would support NADH oxidation, thus limiting availability of NADH for other ROS-generating dehydrogenases. Administration of a cell-permeable NADH dehydrogenase targeted to mitochondria provided more direct evidence that driving NADH oxidation could attenuate ROS production and limit infarct size in the isolated perfused rat heart²¹. Complex I and the ROS derived from it have been implicated in triggering the MPTP; this might be a problem in the setting of high levels of NADH and impaired electron flow through the distal subunits of Complex I, resulting in superoxide production from the FAD site²². In aggregate, these studies indicate that the MPTP is the central target of calcium overload, calpain, NADH, and ROS.

Strategies to Inhibit the MPTP

Although the molecular identity of the MPTP remains uncertain, a variety of drugs have been shown to modulate its activity, although genetic studies indicate that most of these must work indirectly. However, considerable work has shown that cyclosporine A is cardioprotective through its action to inhibit the MPTP, and genetic deletion of cyclophilin D (the target of cyclosporine A) results in a heart that is resistant to ischemia/reperfusion injury^{7, 23, 24}. Importantly, cyclosporine A administered at reperfusion was shown to reduce infarct size in some, but not all, large-animal models²⁵⁻²⁸, providing a compelling rationale for its use in a clinical trial for patients with acute myocardial infarction. In a randomized double-blinded clinical trial reported by Piot et al., cyclosporine A was shown to significantly decrease myocardial enzyme release²⁹. A subset of patients underwent cardiac magnetic resonance imaging, and the patients receiving cyclosporine A had smaller infarcts²⁹. Thus strategies aimed at preventing MPTP opening may have value in the clinical setting.

Mitochondrial Fission and Fusion

In all eukaryotic cell types studied to date, mitochondria behave as a reticulum which undergoes dynamic remodeling on a time-scale of minutes. Individual mitochondria undergo fusion and fission events so frequently that photoactivation of GFP in the matrix of a single mitochondrion within a cell will result in redistribution of the GFP to all mitochondria within 40 to 60 minutes^{30, 31}. This phenomenon has been described in a variety of cell lines and primary cells, and has been assumed to be universal. However, the rigid architecture of the cardiomyocytes, including a network of sarcoplasmic reticulum between mitochondria, has raised the possibility that cardiac mitochondria may undergo little to no fusion and fission, although the relevant proteins (Mfn1, Mfn2, Opa1, and Drp1) are all expressed in cardiomyocytes. A recent study³² appears to implicate fusion in cardioprotection, although

the evidence that fusion and fission events occur in cardiomyocytes is far from compelling. More work will be required to understand this dynamic process in the cardiomyocytes. However, it is attractive to entertain the notion, as fusion would alter the surface area to volume ratio, resulting in a higher calcium threshold before the MPTP would open. On the other hand, mitochondrial fission could be beneficial, because in other systems it has been shown to be a prerequisite for mitophagy³⁰, which is a key mechanism to eliminate damaged organelles.

Mitophagy in Cardioprotection

Efforts to understand the role of autophagy in cardiomyocytes have been accelerated by new tools, including several lines of knockout mice that are deficient in key factors required for autophagy (Atg5, Atg7) and transgenic mice that express fluorescent fusion proteins that allow imaging of autophagosomes in situ (GFP-LC3 and mCherry-LC3)^{33,34}. Early studies using Beclin1 heterozygous knockout mice may be misleading as Beclin1 plays a role in initiating autophagy but can also interfere with flux^{35,36}. Reduction of Beclin1 expression has been reported to accelerate flux³⁷, yet most studies have assumed that reduction of Beclin1 decreases the amount of autophagy. Autophagy is a mechanism for bulk removal of intracellular aggregates and organelles, and provides energy-generating substrates under conditions of nutrient limitation. Our own work has demonstrated a requirement for autophagy in cardioprotection by ischemic preconditioning and by various pharmacologic agents including sulfaphenazole and the adenosine A1 agonist CCPA³⁸⁻⁴⁰. Autophagy has been shown to occur with administration of diazoxide, ranolazine, UTP, rapamycin, hydrophobic statins, and chloramphenicol^{38,41-43}. The signal transduction pathway that activates autophagy has much in common with ischemic and pharmacologic preconditioning. Thus AMPK, PKC, ROS, and nitric oxide (NO) have been shown to be important to induction of autophagy as well as to cardioprotection^{40,44,45}. Mitochondrial depolarization (as might occur with opening of the mitochondrial ATP-sensitive potassium channel) is a trigger for selective removal of mitochondria, and an important element of cardioprotective autophagy appears to be the sequestration and degradation of damaged mitochondria^{46,47}. Mitochondrial turnover, which is a balance of mitophagy and biogenesis, represents an essential aspect of mitochondrial quality control, a homeostatic process that is critical to the integrity of long-lived cells such as neurons and cardiomyocytes. Thus removal of damaged mitochondria will be followed by biogenesis; the replacement mitochondria may differ with respect to protein composition, protein phosphorylation status, and oxidative modifications. At the present time, it is not known whether the differences in protein composition and post-translational modifications observed in mitochondria from preconditioned hearts⁴⁸ are due to modifications of pre-existing mitochondria or wholesale replacement of substantial numbers of mitochondria.

Translational Next Steps

The purpose of these interesting studies is to identify targets and drugs that can reduce the amount of injury associated with myocardial ischemia and reperfusion. Mitochondria are clearly a central target for cardioprotection. Strategies include stabilization of the mitochondria, particularly through inhibition of the MPTP with cyclosporine A, or through elimination of damaged mitochondria through selective mitophagy (Figure 3). Since mitophagy is initiated in part through mitochondrial depolarization⁴⁹, drugs such as cyclosporine A that inhibit the MPTP may antagonize the therapeutic effects of agents that stimulate mitophagy⁴⁹; this may be an important consideration in patients receiving statins (which induce autophagy) as well as cyclosporine A. Although it is important to understand the mechanisms by which these various agents elicit cardioprotection, there are other questions that must be addressed in order to select candidates for clinical development from

among the panoply of small molecules and interventions that have shown promise in the laboratory. Because positive results in rodents are not always replicated in large animal models (e.g. pigs), it is important to validate efficacy in two or possibly three species, as well as to validate the findings in independent labs. If protection is achieved by inhibiting the MPTP, inducing autophagy, or some other mechanism, how long does the effect need to be sustained? If protection is demonstrated in an acute model, durable benefit must be determined in chronic studies. Are all agents similar with respect to speed of onset, duration of effect, and potency? These questions of pharmacodynamics must be addressed before a drug can be selected for clinical advancement, yet these rather mundane questions are not hypothesis-driven questions that fit easily into the milieu of R01 grant proposals.

Reconsidering the Models

A key consideration in planning future studies is the need to use animal models that more closely resemble the human condition. Lean, young, isogenic male mice are a far cry from the studies that must be conducted in the human population. Before an agent can be considered for clinical advancement, it will need to demonstrate reproducible efficacy in both sexes, in aged animals, and in outbred species. Myocardial infarctions are more common and more severe in the presence of co-morbid conditions such as metabolic syndrome, diabetes, and advanced age. For that reason it is reasonable to recommend that a drug that fails to show benefit in animal models of metabolic syndrome, diabetes, or advanced age should not be developed further. Another consideration is the duration of ischemia. Laboratory models have been designed with an optimized duration of ischemia that is quite sensitive, permitting easy detection of a beneficial effect. Unfortunately, many of the drugs which passed this “easy” test subsequently failed to show efficacy in clinical trials. Animal studies involving longer ischemia times (as are typically the case in humans), incomplete reperfusion (which is to be expected in patients with atherosclerosis, microvascular disease, and no-reflow), and presence of co-morbid conditions might be better predictors of which drugs will succeed in the clinical setting. For this reason, efforts should be directed towards developing more clinically relevant models that will be better predictors of which drugs will succeed or fail.

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Figure 1.

Mitochondria integrate the signals of I/R injury and cytoprotection. Mitochondria respond to a variety of inputs, both good and bad, including ROS, phospholipases, kinases, phosphatases, Bcl-2 family members (both pro- and anti-apoptotic), Ca⁺⁺, and the pH of the cytosol and mitochondrial matrix.

Figure 2.

Ion homeostasis protects mitochondria. At reperfusion NHE-1 exchanges extracellular sodium for the excess hydrogen which accumulated during ischemia (cariporide inhibits NHE1); however, the increased intracellular sodium now causes the sodium-calcium exchanger (NCX) to bring in more calcium in order to eliminate the sodium (KB-R7943 inhibits NCX). Calpain proteolytically activates Bid to trigger apoptosis. Calcium uptake via the uniporter may contribute to triggering the MPTP. The mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) can open in response to protective signaling and cause mild depolarization, thereby limiting calcium uptake and opposing MPTP opening. Depolarization via mitoK_{ATP} may also trigger mitophagy. Mitochondrial stability is also modulated by fusion and fission, regulated by Mfn1, Mfn2, Drp1, Fis1, and Opa1. Closely appositioned SR is an important mechanism of calcium delivery to mitochondria, and its association is mediated by Mfn2 on SR and mitochondrial outer membrane. Kinases and phosphatases regulate many targets in the outer mitochondrial membrane.

Figure 3.

Strategies for mitoprotection. Reperfusion injury is largely due to mitochondrial dysfunction, which may be controlled by inhibiting the MPTP with cyclosporine A or by selective elimination of damaged mitochondria via autophagy.