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Review

Heart mitochondria: gates of life and death

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Mitochondria are important generators of energy, providing ATP through oxidative phosphorylation. However, mitochondria also monitor complex information from the environment and intracellular milieu, including the presence or absence of growth factors, oxygen, reactive oxygen species, and DNA damage. Mitochondria have been implicated in the loss of cells in various cardiac pathologies, including ischaemia/reperfusion injury, cardiomyopathy, and congestive heart failure. The release of factors such as cytochrome *c*, Smac, Omi/Htr2A, and AIF from mitochondria serves to activate a highly complex and regulated cell death program. Furthermore, mitochondrial calcium overload might trigger opening of the mitochondrial permeability transition pore, causing uncoupling of oxidative phosphorylation, swelling of the mitochondria due to influx of water, and rupture of the mitochondrial outer membrane. In this review, we discuss the role of mitochondria in the control of cell death in cardiac myocytes.

1. Introduction

Mitochondria play critical roles in both the life and death of cardiac myocytes. In healthy cells, their primary function is to meet the high energy demand of the beating heart by providing ATP through oxidative phosphorylation. Mitochondria occupy a large portion of each myocyte and are located between the myofibrils and just below the sarcolemma. The strategic positioning and abundance of mitochondria ensure a highly efficient localized ATP delivery system to support contraction, metabolism, and ion homeostasis.[1] However, mitochondria are also important regulators of cell death, responding to a wide variety of stress signals, including loss of growth factors, hypoxia, oxidative stress, and DNA damage. The switch to a cell death program can be mediated by opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane, causing collapse of the membrane potential and swelling of the mitochondria,[2] often culminating in necrotic cell death, or permeabilization of the mitochondrial outer membrane (MOM) with release of proapoptotic proteins such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor (AIF) to activate an energy-dependent apoptosis.[3] It is important to remember that both forms of cell death are highly regulated and activated by mitochondria.

During apoptosis, the cell activates a signalling cascade which leads to cell death without triggering an inflammatory response. In contrast, necrosis is characterized by swelling of the cell and disruption of the plasma membrane. The resulting release of the cell's content into the extracellular space triggers an inflammatory response which can cause further damage to surrounding cells.[4] Both processes have been implicated in loss of myocardial cells in pathologies such as ischaemia/reperfusion (I/R), cardiomyopathy, and congestive heart failure. This review discusses the mechanism(s) of mitochondrial dysfunction and how malfunctioning mitochondria might contribute to loss of cardiac myocytes.

2. Mitochondria and reactive oxygen species

Mitochondria are a major source of reactive oxygen species (ROS), which are a byproduct of mitochondrial electron transfer activity. As much as 0.2–2% of the molecular oxygen consumed by mitochondria during respiration is converted to superoxide primarily by Complexes I and III.[5–7] Molecular oxygen (O_2) is highly electrophilic and superoxide production occurs when O_2 captures an electron from Complex I[8] or from the ubisemiquinone located in Complex III.[9] At higher oxygen concentrations, such as during reperfusion, diminished availability of reduced co-factors of the respiratory chain will increase mitochondrial ROS formation. In addition, a reduction in Complex I activity was shown to result in elevated levels of superoxide.[10] The density of mitochondria in cardiac myocytes and the high rate of oxidative phosphorylation can result in generation of significant amounts of superoxide. Normally, superoxide is detoxified by the combined activity of the mitochondrial antioxidant enzymes manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase (GPx).[11,12] Superoxide anions are quickly dismuted to hydrogen peroxide by MnSOD, which is converted to water by catalase and GPx. Oxidative stress occurs when excess ROS are generated

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that cannot be adequately countered by the antioxidant systems. Mice deficient in MnSOD develop normally but die within the first 10 days of life with cardiomyopathy.[13] Moreover, an increase in superoxide caused by partial inhibition of SOD resulted in hypertrophy and apoptosis in isolated cardiac myocytes.[14] These studies demonstrate the importance of SOD in the regulation of superoxide formed as a byproduct of oxidative phosphorylation.

Production of ROS at levels that exceed the detoxification capacity results in oxidative modification of mitochondrial proteins, lipids, and mtDNA, culminating in mitochondrial dysfunction and cell death. Oxidative stress has been associated with loss of cells in heart failure, I/R injury, and doxorubicin-induced cardiomyopathy, whereas reducing ROS production has been shown to be cardioprotective. For instance, increased generation of ROS was associated with mitochondrial dysfunction in failing hearts after myocardial infarction where mitochondria exhibited increased lipid peroxidation, decreased mtDNA copy number, and a reduced oxidative capacity due to lower activity of electron transfer enzymes.[15] In addition, development of heart failure was shown to be associated with decreased antioxidant capacity and increased oxidative stress. For instance, SOD activity was decreased in an animal model of congestive heart failure,[16] and catalase and GPx activities were reduced by 50% after myocardial infarction, whereas lipid peroxidation in the heart was increased by 50%.[17] Moreover, transgenic mice overexpressing either MnSOD or the cytosolic Cu/ZnSOD had reduced infarct size compared to wild type,[18,19] and treatment with a ROS scavenger protected against hypoxia/reoxygenation and hydrogen peroxide treatment in isolated cardiac myocytes.[20] Doxorubicin, an anthracycline, is an effective anti-cancer drug known to cause severe cardiac toxicity. The toxicity is thought to be mediated through the generation of the ROS and impairment of mitochondrial function in cardiac myocytes. In support of this, antioxidants can protect isolated cardiac myocytes and intact hearts from anthracyline toxicity,[21–23] and transgenic mice overexpressing MnSOD had less mitochondrial damage at the ultrastructural level and reduced serum levels of creatine kinase and lactate dehydrogenase than wild type littermates after doxorubicin treatment.[24] Similarly, Xiong *et al*.[25] found that doxorubicin treatment led to uncoupling of electron transfer and oxidative phosphorylation in mitochondria and that mice with cardiac overexpression of Gpx1 were more resistant than wild type mice to acute cardiac toxicity of doxorubicin. These studies suggest that production of ROS and damage to mitochondria play an important role in doxorubicin-mediated cardiac toxicity.

3. Reactive oxygen species-induced reactive oxygen species release (RIRR)

ROS produced initially in mitochondria have been shown to act in a positive feedback, where mitochondria can respond to elevated ROS by increasing their own ROS production in a process known as ROS-induced ROS release (RIRR). Zorov *et al*.[26] initially discovered that ROS production in adult cardiac myocytes proceeded in two distinct phases; an initial slow increase in ROS production induced by photoactivation of mitochondrial dyes was followed by a second burst of ROS originating from the electron transport chain (ETC) occuring simultanously with dissipation of mitochondrial membrane potential ($\Delta\Psi_m$). Two different pathways of RIRR have been described. The first pathway of RIRR is dependent on the mPTP, where the initial enhanced ROS leads to opening of the mPTP and depolarization of mitochondria, yielding a short-lived burst of ROS originating from the mitochondrial ETC. Bongkrekic acid, an inhibitor of the mPTP, was shown to inhibit both $\Delta\Psi_m$ collapse and the second mitochondrial ROS burst.[26] More recently, a second pathway of RIRR was described by O'Rourke and colleagues. This type of RIRR occurs through a mechanism involving opening of the inner membrane anion channel (IMAC) which is regulated by the mitochondrial benzodiazepine receptor (mBzR). They found that increased ROS in the mitochondria triggered opening of the IMAC, resulting in a brief increase in ETC-derived ROS. The RIRR was inhibited by ligands of the mBzR, but not by mPTP inhibitors or Ca^{2+} overload, suggesting that the RIRR occurred through a separate pathway that was independent of the mPTP.[27]

Intracellular generation of mitochondrial ROS by both types of RIRR causes collapse of the $\Delta\Psi_m$ and destabilization of the action potential, and is emerging as an important mediator of I/R injury. Several independent studies have demonstrated that RIRR is linked to hypoxia/reoxygenation-activated pathways of cell death via the mPTP. Cyclosporine A (CsA), a classic inhibitor of the mPTP, and sanglifehrin A, a recently described specific inhibitor of the mPTP, reduced infarct size following I/R in rat hearts[28] and reduced the responsiveness of the mPTP to photo-triggering via confocal imaging of the cardiac myocyte.[29,30] In addition, 4'-chlorodiazepam, an antagonist of the mBzR, shortened action potential duration and prevented arrhythmias during reperfusion, whereas the high affinity agonist, FGIN-1-27, amplified myocardial dysfunction.[31] This suggests that the second pathway of RIRR participates in I/R-induced myocardial arrhythmias.

4. Mitochondria and calcium

Cardiac mitochondria also play an important role in regulating calcium homeostasis and can act as a sponge to buffer Ca^{2+}. Since elevated [Ca^{2+}] may be deleterious, it is necessary to maintain a physiologic Ca^{2+} concentration in the cytoplasm; much of the cellular Ca^{2+} is stored in the sarcoplasmic/endoplasmic reticulum (SR/ER). Mitochondria have been shown to be positioned near Ca^{2+}-release sites on the SR/ER and can capture a substantial amount of the released Ca^{2+}.[32] The ability of mitochondria to accumulate Ca^{2+} helps to prevent the level of calcium in the cytosol from becoming too high and to prevent SR/ER depletion by recycling Ca^{2+} to the SR/ER.[33] Mitochondrial calcium can be stored as calcium phosphate precipitates, permitting sequestration of surprisingly large amounts of Ca^{2+}.[34] Mitochondrial Ca^{2+} homeostasis also regulates energy metabolism to synchronize ATP generation with cell function. For instance, uptake of Ca^{2+} results in the activation of three dehydrogenases in the mitochondrial matrix.[35] This causes an increase in mitochondrial NADH/NAD^+ ratio which may result in increased energy available for mitochondrial functions. In addition, matrix Ca^{2+} has been suggested to activate ATP synthesis through a direct effect on F_0F_1-ATPase.[36] Mitochondrial matrix [Ca^{2+}] is controlled by a Ca^{2+} uniporter channel for influx and by a Na^+/Ca^{2+}

exchanger (NCX) for efflux.[37] The activity of NCX saturates as mitochondrial matrix [Ca^{2+}] increases, whereas the uniporter acts as a channel and is not saturated with increasing [Ca^{2+}]. As a result, the [Ca^{2+}] increases in the matrix and beyond a certain threshold, the mitochondria can no longer regulate matrix [Ca^{2+}], resulting in mitochondrial overload.[37] If the mitochondrial Ca^{2+} load is substantially increased, Ca^{2+} can promote opening of the mPTP thereby triggering cell death.[2] However, the role of Ca^{2+} as a mediator of mPTP opening in cardiac myocytes has recently come into question. Most experiments demonstrating mPTP opening in response to Ca^{2+} has been done *in vitro* using isolated mitochondria, but Juhaszova *et al.*[29] found that increased uptake of Ca^{2+} into mitochondria did not result in mPTP opening in isolated cardiac myocytes. Similarly, another study using a model of simulated I/R found that mPTP opening during reperfusion was not dependent on mitochondrial uptake of Ca^{2+} during ischaemia.[38] Clearly, the role of Ca^{2+} in mediating mPTP opening needs to be further investigated *in vivo*.

It is well established that myocardial I/R injury is accompanied by mitochondrial Ca^{2+} overload which contributes to mitochondrial dysfunction and cell death. During ischaemia, there is a drop in ATP/ADP and a build-up of lactic acid, with a consequent drop in intracellular pH. In an attempt to restore the pH, the cell utilizes the Na^+/H^+ exchanger, but this results in loading of the cell with Na^+. This Na^+ cannot be pumped out via the Na^+/K^+-ATPase due to compromised ATP levels. Consequently, the plasma membrane NCX which normally extrudes Ca^{2+} operates in reverse and the cell becomes loaded with Ca^{2+}.[39] Upon reperfusion, Ca^{2+} re-enters the mitochondria and consequently produces mitochondrial Ca^{2+} overload, which may lead to cell death. Strategies that limit mitochondrial Ca^{2+} accumulation have significant beneficial effects on cardiac function following I/R. For instance, treatment of hearts with ruthenium red (RR) or Ru360, inhibitors of the Ca^{2+} uniporter, improved functional recovery and reduced infarct size in hearts subjected to I/R.[40,41] In addition, isolated heart mitochondria were more resistant to Ca^{2+}-induced swelling in the presence of Ru360 or RR, suggesting that pore opening during Ca^{2+} overload is modulated by activity of the Ca^{2+} uniporter.[40] Other studies have demonstrated the beneficial effects of plasma membrane NCX inhibitors on myocardial I/R injury. For instance, KB-R7943 or SEA0400, two different inhibitors of the NCX, reduced infarct size and improved post-ischaemic recovery of function,[42,43] whereas enhancing expression of NCX in the heart increased susceptibility to I/R injury.[44]

5. Mitochondrial permeability transition pore (mPTP)

The mPTP is a voltage-dependent, high-conductance channel with a diameter of 3 nm allowing for rapid equilibration of ions and water, as well as passage of molecules up to 1.5 kDa.[45] The mPTP is regulated by cyclophilin D (CycD) in the inner mitochondrial membrane, and is important for mPTP function.[46] Various studies have implicated VDAC and ANT as elements of the mPTP, yet mitochondria from knockouts for multiple isoforms of VDAC or ANT are still able to demonstrate pore opening albeit with higher thresholds.[47,48] Other studies have revealed a role for Complex I in mPTP function.[49,50] Under normal conditions, the inner mitochondrial membrane is impermeable to all but a few ions and metabolites for which specific transport mechanisms are present. This tight regulation is necessary for mitochondria to maintain a pH gradient and membrane potential which drives oxidative phosphorylation. Opening of the pore causes collapse of the proton gradient and electrical potential across the inner mitochondrial membrane, leading to uncoupling of oxidative phosphorylation. The high colloid osmotic pressure in the matrix causes influx of water, and swelling of the matrix; whereas the inner mitochondrial membrane can expand through cristae remodelling, the outer mitochondrial membrane is unable to expand resulting in rupture and release of cytochrome *c* and other pro-apoptotic proteins into the cytosol.

Many studies have found that opening of the mPTP occurs during I/R. During ischaemia, depletion of adenine nucleotides and accumulation of inorganic phosphate (Pi) sensitizes the mPTP to opening. The mPTP is regulated by pH, and the low pH during ischaemia prevents opening of the pore. Upon reperfusion, the mitochondria resume respiration and generate a membrane potential, resulting in an increase in the production of ROS. Reperfusion also triggers rapid accumulation of Ca^{2+} within the mitochondria. Within a few minutes of reperfusion, the pH returns to normal and mPTP opening occurs. Halestrap *et al.*[51] demonstrated that mPTP opening did not take place during ischaemia but occurred approximately 2 min into reperfusion. Opening of the mPTP contributes to mitochondrial dysfunction in cardiac myocytes and inhibition of pore opening is cardioprotective. For instance, inhibitors of the mPTP protected against hypoxia/re-oxygenation-mediated cell death in isolated cardiac myocytes,[52,53] and reduced infarct size in the *ex vivo* model of I/R.[28,30,54] Treatments that reduce mPTP opening, such as decreasing oxidative stress with free radical scavengers, and maintaining a lower pH during reperfusion are also cardioprotective.[51] Moreover, CycD deficient mice, whose mPTP is more resistant to Ca^{2+}- and oxidative stress-induced opening, had reduced injury after myocardial I/R compared to wild-type litter mates.[46,55] Interestingly, cells lacking CycD were still sensitive to apoptotic stimuli, suggesting that mPTP opening is not required for induction of apoptosis via the mitochondrial pathway.[46] In contrast, cardiac mitochondria from transgenic mice overexpressing CycD in the heart displayed a lower threshold for mPTP opening *in vitro*, and hearts displayed increased signs of apoptosis, hypertrophy, and decreased cardiac function.[46] Moreover, the mPTP has been shown to contribute to loss of myocytes during progression to heart failure. For instance, transgenic mice overexpressing the alpha subunit of Gq develop cardiac hypertrophy and subsequent lethal cardiomyopathy.[56] Adams *et al.*[57] reported that overexpression of a constitutively active form of $Gq\alpha$ in isolated cardiac myocytes caused mitochondrial dysfunction and cell death, which was prevented in the presence of bongkrekic acid. These studies demonstrate that opening of the mPTP contributes to loss of cardiac myocytes in various cardiovascular diseases.

Reduced mPTP opening has also been suggested to play a role in pre- and post-conditioning of the heart brief, i.e. periods of ischaemia performed at the prior to ischaemia

or at onset of reperfusion, respectively. Pre-conditioning was shown to be associated with reduced mPTP opening in Langendorff-perfused hearts.[58] Studies using isolated cardiac myocytes and mitochondria have implicated the inhibition of the mPTP in both calcium- and diazoxide-mediated pre-conditioning.[53,59,60] Similarly, mPTP inhibition has been suggested to be responsible for the protection provided by post-conditioning,[61] which agrees with the study by Hausenloy *et al*.[59] who found that CsA treatment at the start of reperfusion reduced infarct size.

6. Mitochondrial mediators of cardiac protection

The mitochondrial ATP-sensitive K^+ (mK_{ATP}) channel plays a central role in mediating protection in ischaemic pre-conditioning (IPC). Pharmacological activation of this channel protects against I/R injury, whereas inhibition of the mK_{ATP} prevents the protective effects of IPC.[62] Opening of the mK_{ATP} channel has been reported to reduce mitochondrial Ca^{2+} uptake and to inhibit mPTP opening,[60,63] as well as to increase ROS production by the ETC.[64,65] Whereas excess levels of ROS triggers cell death, lower levels of ROS produced during brief periods of ischaemia and reperfusion have been shown to play a role in IPC.[66] The exact mechanism for opening of the mK_{ATP} during pre-conditioning is still under intense investigation. Recently, it was demonstrated that mK_{ATP} and PKCε directly interact in the mitochondrial inner membrane and that PKCε is required for the opening of the mK_{ATP}.[67] Previous studies have found that activation and translocation of PKCε to the mitochondria are important for pre-conditioning.[68–70] Gray *et al*.[71] demonstrated that treatment with a PKCε selective inhibitor abolished the protective effects of pre-conditioning. In addition, transgenic mice overexpressing PKCε in the heart had reduced infarct size after myocardial I/R,[72] and PCKε knockout mice did not retain the protective effects of pre-conditioning.[73] These studies suggest that PKCε mediates protection of mitochondria in the myocardium by activation of the mK_{ATP}.

Activation of the PI 3-kinase/Akt signalling pathway has also been demonstrated to provide cardiac protection against various stressors by preserving mitochondrial integrity and function. PI 3-kinase signalling has been shown to provide protection against I/R injury during pre-conditioning[74] and Akt activation has been shown to decrease apoptosis, as well as to reduce infarct size and to improve cardiac function after I/R.[75] Furthermore, adenovirally infecting mouse[76] or rat hearts[77] with constitutively active Akt gene constructs confers protection against I/R injury. Akt has been shown to protect mitochondrial integrity and inhibit cytochrome *c* release following an apoptotic stimulus.[78–80] However, the exact mechanism for Akt-mediated cardioprotection is still unclear and it appears that Akt acts on multiple target to provide its protective effects. For instance, preservation of mitochondrial integrity and function by Akt has been shown to be dependent on the presence of glucose and hexokinase. Akt was shown to preserve mitochondrial membrane potential in response to growth factor deprivation by increasing glucose transporter expression and glytolytic activity resulting in greater substrate availability for mitochondrial electron transport.[81] Akt also elevated mitochondrial hexokinase association and activity at the mitochondria.[78] In addition, Akt has been shown to provide protection by phosphorylation and inactivation of the BH3-only protein Bad[82] and prevention of Bax translocation to the mitochondria,[83] as well as induction of anti-apoptotic Bcl-2 proteins.[84,85] Akt has also been shown to phoshorylate and inactivate glycogen synthase kinase-3β (GSK-3β),[86] and inhibition of GSK-3β prevents opening of the mPTP.[29] GSK-3β is thought to contribute to I/R-mediated cell death,[87,88] and has been shown to be inhibited by pre-conditioning.[88,89]

7. Mitochondria and Bcl-2 family proteins

The Bcl-2 family proteins are important regulators of the mitochondrial apoptotic pathway. This family is composed of both pro- and anti-apoptotic proteins that share up to four conserved regions known as Bcl-2 homology (BH) domains. Anti-apoptotic members such as Bcl-2 and Bcl-X_L contain all four subtypes of BH domains, and promote cell survival by inhibiting the function of the pro-apoptotic Bcl-2 proteins. The pro-apoptotic members can be separated into two structurally distinct subfamilies. The 'multidomain' proteins (Bax and Bak) share three BH regions and lack the BH4 domain. They are structurally similar to the anti-apoptotic proteins.[90,91] In contrast, 'BH3-only' proteins which include Bid, Bnip3, Nix/Bnip3L, and Puma, share only the BH3 domain and are structurally diverse.[92] The BH3-only proteins function as death signal sensors in the cell and play a major role in transducing signals from the cytosol to the mitochondria. All BH3-only proteins initiate cell death through the activation of Bax and Bak, and studies using cells derived from knock-out mice lacking both Bax and Bak have demonstrated that Bax and Bak are essential for initiation of cell death through the mitochondrial pathway. In response to an apoptotic stimulus, BH3-only proteins bind to and neutralize the anti-apoptotic Bcl-2 proteins, thereby releasing Bax and Bak. Certain BH3-only proteins, such as tBid, can directly interact with the pro-apoptotic Bax and Bak to trigger apoptosis. In healthy cells, Bax is localized in the cytosol and upon a cell death signal, Bax rapidly translocates to the mitochondria, whereas Bak is already present in the mitochondria as an integral membrane protein. Although the precise mechanisms of membrane permeabilization are unclear, it has been proposed that it can result from a conformational change of Bax or Bak, their full insertion into mitochondrial membranes as homo-oligomerized multimers, and formation of giant protein-permeable pores.[93]

The Bcl-2 family proteins play a central role in regulating apoptosis in the heart. The anti-apoptotic Bcl-2 proteins provide protection of mitochondria by acting on multiple targets. For instance, Bcl-2 has been shown to prevent permeabilization of the outer mitochondrial membrane by inhibiting activation of Bax/Bak,[94] and to increase the calcium threshold for mPTP opening in heart mitochondria by blocking opening of the pore.[95] Moreover, transgenic mice overexpressing Bcl-2 in the heart had fewer apoptotic cells, reduced infarct size and improved recovery of cardiac function after I/R.[96–98] Mice null for desmin develop cardiomyopathy characterized by ultrastructural abnormalities in mitochondria and extensive cell death, but overexpression of Bcl-2 in these hearts corrected the mitochondrial

defects and improved cardiac function.[99] During ischaemia, electron transport and mitochondrial generation of ATP are inhibited, and the F_1F_0-ATPase runs in reverse, consuming glycolytically generated ATP.[100,101] Interestingly, transgenic mice overexpressing Bcl-2 in the heart had a decreased rate in decline of ATP during ischaemia as well as reduced acidification, suggesting that Bcl-2 provides protection by inhibiting consumption of glycolytically generated ATP by the F_1F_0-ATPase.[96] Moreover, elevated expression of Bcl-X_L by adenoviral gene transfer inhibited Bax translocation from the cytosol to the mitochondria, reduced cytochrome *c* release from mitochondria and decreased apoptosis after I/R.[102] Similarly, the BH4 domain of Bcl-X_L has been shown to be necessary and sufficient for protection against mitochondrial dysfunction and apoptosis and recently was shown to interact with truncated Bid.[103] Perfusion of hearts with this BH4 peptide linked to a protein transduction domain (TAT-BH4) reduced infarct size and creatine kinase release after I/R.[104] These studies demonstrate a cardioprotective role for the anti-apoptotic Bcl-2 proteins in preventing mitochondrial dysfunction and cell death in cardiac myocytes in response to various stressors.

Many of the pro-apoptotic Bcl-2 proteins have also been implicated in the pathogenesis of various cardiac diseases including myocardial hypertrophy, infarction, and heart failure. For instance, chronic hypoxia, stretch, and chronic pressure overload in rat hearts correlated with increased Bax and decreased Bcl-2 levels accompanied by mitochondrial dysfunction and cell death.[105,106] Moreover, Bax has been reported to be activated in cardiac cells in response to oxidative stress[107] and during ischaemia.[108] Hearts from Bax deficient mice had reduced mitochondrial damage and decreased infarct size after I/R compared to wild type, implicating Bax as a major player of mitochondrial dysfunction in I/R.[109] Among the BH3-only proteins, Bid, Puma, Bnip3, and Nix/Bnip3L have been implicated in cardiac myocyte death. For instance, Bid has been reported to be subjected to proteolytic cleavage during myocardial I/R. The activated truncated Bid (tBid) then translocates to the mitochondria causing activation of Bax/Bak and release of cytochrome *c* into the cytosol.[104,110,111] Puma was upregulated in cardiac myocytes in response to hypoxia/reoxygenation, whereas Puma knockout mice had reduced infarct size and improved cardiac function after I/R.[112] Moreover, Bnip3 and Nix/Bnip3L have been associated with mitochondrial dysfunction and cell death in the heart. Bnip3 has been shown to contribute to I/R injury via activation of Bax[113,114] and was found to be upregulated in failing hearts,[115] whereas Nix/Bnip3L has been implicated in cardiac hypertrophy and development of cardiomyopathy.[116]

8. Consequences of mitochondrial permeabilization

Permeabilization of the MOM and loss of $\Delta\Psi_m$ are universal features of cell death and are generally considered as the 'point of no return' in the events leading to cell death.[117] Once permeabilization has occurred, it leads to rapid cell death through a variety of independent and redundant mechanisms. These include caspase activation, irreversible metabolic changes, disruption of oxidative phosphorylation, and the release of caspase independent effectors such as apoptosis inducing factor (AIF).

The most well known protein released from mitochondria during apoptosis is cytochrome *c*. The function of cytochrome *c* in the mitochondria is to shuttle electrons from complexes III to IV in the respiratory chain. However, during apoptosis mitochondria release cytochrome *c* into the cytosol, where it associates with Apaf-1 along with dATP and caspase-9 to form the macromolecular complex known as the apoptosome.[118] The formation of this complex triggers activation of caspase-9 which in turn cleaves and activates the effector, caspase-3, culminating in cell death. Caspases are responsible for the proteolytic inactivation of crucial cellular targets, including mitochondria. For instance, caspases have been shown to enter the permeabilized outer membrane of mitochondria and cleave essential subunits in Complex I, disrupting mitochondrial respiration.[119] Interestingly, it has been shown that inhibition of caspases has no effect on loss of $\Delta\Psi_m$ and delays—but does not prevent—cell death induced by stimuli that activate the mitochondrial pathway.[120]

Other apoptotic factors released from the mitochondria are Smac/Diablo, Omi/Htr2A, AIF, and endonuclease G (EndoG). Inhibitors of apoptosis proteins (IAPs) are endogenous inhibitors of initiator caspase-9 and downstream effector caspases-3 and -7. Once released, Smac/Diablo and Omi/HtrA2 antagonize IAPs, thereby permitting activation of caspases.[121–123] Another mitochondrial protein that promotes cell death upon release into the cytosol is AIF. In the mitochondria, AIF is critical for optimal detoxification of ROS and the assembly and stabilization of Complex I.[124,125] Cardiac myocytes isolated from harlequin mutant mice that have downregulated expression of AIF were found to be more sensitive to hydrogen peroxide-induced cell death, and hearts displayed increased injury after I/R.[124] In addition, targeted disruption of AIF in the heart leads to severe defects in Complex I activity and development of dilated cardiomyopathy.[126] These studies demonstrate that AIF is essential for normal mitochondrial function. However, upon induction of apoptosis, release of AIF into the cytosol is followed by rapid translocation to the nucleus where it facilitates chromatin condensation and large-scale DNA fragmentation. For instance, Siu *et al.*[127] found AIF and Omi/Htr2A in the cytosol in an animal model of salt-induced heart failure. Another study reported that AIF was detected in the cytosolic and nuclear fractions of hearts subjected to I/R, and that IPC attenuated AIF release, implicating AIF as a contributor to myocardial cell death in I/R.[128] EndoG is a mitochondrion specific nuclease that also translocates to the nucleus during apoptosis, where it cleaves chromatin DNA into nucleosomal fragments.[129] Bahi *et al.*[130] found that ischaemia induced the release of EndoG from mitochondria and translocation to the nucleus in isolated neonatal myocytes, and that knock-down of EndoG by siRNA reduced DNA laddering suggesting that EndoG is an important executor of DNA degradation during ischaemia in cardiac myocytes. However, the importance of EndoG in cell death has recently been questioned. Two independent studies reported no obvious effect on cell death and DNA degradation in EndoG deficient mice. The mice are viable and there is no effect on mitochondrial DNA copy number or mutation rate.[131] Moreover, cells isolated from these mice show no difference

in susceptibility in response to various apoptotic stimuli.[132] However, these studies did not assess whether the hearts were less susceptible to injury in response to stress such as I/R or pressure overload. Clearly, more studies are required to elucidate the role of EndoG in apoptosis. In our laboratory, we found that some antibodies to EndoG cross-reacted strongly with creatine kinase (unpublished data), which may add to the confusion.

9. Mitochondrial fission and 'mitophagy'

Mitochondria are dynamic organelles that are constantly undergoing fission and fusion to adapt to changing conditions of the cell. Recently, several studies have reported that mitochondrial morphology changes during apoptosis, resulting in small round mitochondrial fragments.[133–135] For instance, Karbowski *et al.*[135] reported that Bax co-localized with Dynamin-Related Protein-1 (Drp-1), a protein involved in mitochondrial fission, at defined foci on the mitochondrial membrane at the onset of apoptosis. More importantly, a dominant negative of Drp-1 inhibited fragmentation and apoptosis, but not Bax translocation to the foci in response to staurosporine treatment. Drp-1 has also been reported to be required for optimal release of cytochrome *c* induced by the BH3-only protein Bik, presumably through remodelling of the cristae.[136] Moreover, the mitochondrial network in HL-1 cardiac myocytes was shown to undergo extensive fragmentation in response to simulated I/R or overexpression of Bnip3.[113,137] The Bnip3-mediated mitochondrial fragmentation correlated with accumulation of Bax and Drp-1 at distinct clusters on the mitochondrial fragments.[114] These studies suggest that the Bcl-2 proteins can mediate apoptosis through the mitochondrial fission pathway.

Mitochondrial dysfunction has also been reported to lead to upregulation of autophagy. Autophagy is a process important in cellular homeostasis and in removing excess or damaged organelles.[138] For instance, mitochondrial fragmentation correlated with upregulation of autophagy and sequestration of mitochondrial fragments in autophagosomes (mitophagy) in HL-1 myocytes overexpressing Bnip3.[113] Autophagy plays an important role in the cellular response to stress and has been shown to be upregulated in the myocardium in response to I/R.[137,139] Upregulation of autophagy in HL-1 myocytes was found to protect against simulated I/R cell death in HL-1 myocytes,[137] while enhanced autophagy during chronic myocardial ischaemia correlated with decreased apoptosis, suggesting that induction of autophagy is a protective response.[140] In contrast, Beclin-1 is a protein essential in the autophagic pathway and heterozygous knockout mice had reduced autophagy, smaller infarcts, and apoptosis after I/R compared to wild type,[139] suggesting that upregulation of autophagy in response to I/R may be detrimental. Clearly, further studies are required to elucidate the roles of mitochondrial fragmentation and autophagy in myocardial cell death.

10. Clinical perspective

Many pharmacological agents that directly or indirectly protect mitochondrial integrity in response to stress have been used in clinical trials for the treatment of various cardiovascular diseases. For instance, beta-blockers such as carvedilol have been shown to be very effective for the treatment of ischaemic heart disease and congestive heart failure. Carvedilol has been demonstrated to have additional effects independent of blocking the beta-adrenergic receptor and has been shown to directly protect against mitochondrial permeability transition by reducing oxidative stress.[141,142] Although most antioxidants protect against myocardial cell death in animal models, clinical trials have produced mixed results. For instance, large clinical trials using antioxidant vitamins have failed to show any clinical benefits on the heart.[143–145] In contrast, clinical studies have demonstrated that infusion of N-acetyl cysteine (NAC) duing thrombolysis correlated with a reduced infarct size and increased preservation of left ventricular function.[146,147] In addition, administration of NAC in combination with streptokinase reduced oxidative stress and improved left ventricular function in patients with acute myocardial infarction.[148]

Pharmacological openers of the mK_{ATP} channel were developed for the treatment of angina pectoris and hypertension even before this channel was identified as an important mediator of protection during IPC. Clinical studies have demonstrated that infusion of nicorandil, a hybrid K_{ATP} channel opener and nitrate, improved left ventricular function in patients with acute myocardial infarction.[149] Although pre-conditioning provides great protection against myocardial injury in animal models of I/R, its clinical application is limited since the coronary artery is already occluded in patients with acute myocardial infarction by the time they are admitted to the hospital. In contrast, post-conditioning offers greater clinical potential and can be applied to a number of clinical procedures such as cardiac transplantation and coronary angioplasty. The study by Staat *et al.*[150] demonstrated that patients subjected to four cycles of 1 min ischaemia and 1 min reperfusion immediately upon reperfusion during coronary angioplasty for acute myocardial infarction had reduced infarct size compared to control patients.

11. Conclusions

Mitochondria are critical to cardiac cell survival through their essential roles in energy production and calcium homeostasis, but they also function as central regulators of apoptotic and necrotic cell death (*Figure 1*). Bax/Bak-mediated permeabilization of the MOM leads to apoptosis, while opening of the mPTP leads to a caspase-independent (necrotic) form of cell death. In either case, failure of mitochondrial ATP production also contributes to cell death in these energy-intensive cells. To protect cardiac myocytes from death, it is important to preserve mitochondrial integrity. Loss of mitochondrial membrane integrity is generally considered a point of no return, as inhibiting the post-mitochondrial phase of apoptosis does not rescue the cardiomyocyte. Preservation of mitochondrial integrity is of utmost importance in the design of cardioprotective therapies.

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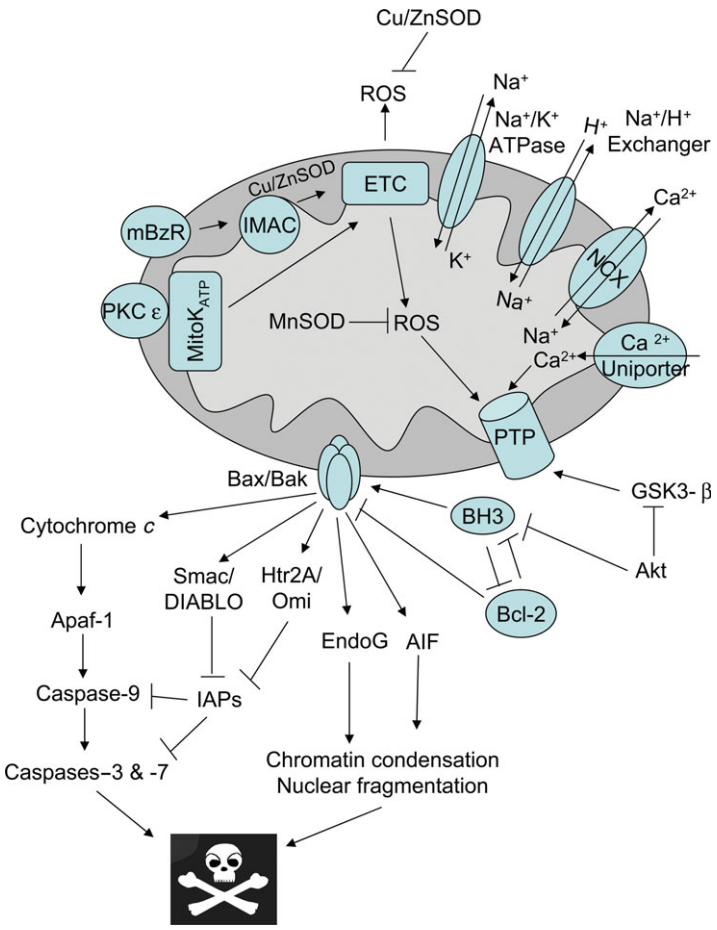

Figure 1 Mitochondria as a central integrator of multiple stress signals.

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References

1. Andrienko T, Kuznetsov AV, Kaambre T, Usson Y, Orosco A, Appaix F *et al*. Metabolic consequences of functional complexes of mitochondria, myofibrils and sarcoplasmic reticulum in muscle cells. *J Exp Biol* 2003; **206**:2059–2072.
2. Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans* 2006;**34**:232–237.
3. Gustafsson AB, Gottlieb RA. Mechanisms of apoptosis in the heart. *J Clin Immunol* 2003;**23**:447–459.
4. Searle J, Kerr JF, Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol Annu* 1982;**17**:229–259.
5. Staniek K, Nohl H. H(2)O(2) detection from intact mitochondria as a measure for one-electron reduction of dioxygen requires a non-invasive assay system. *Biochim Biophys Acta* 1999;**1413**:70–80.
6. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003;**552**:335–344.
7. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* 2003;**278**:36027–36031.
8. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 1980;**191**:421–427.
9. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 1985;**237**:408–414.
10. Koopman WJ, Verkaart S, Visch HJ, van Emst-de Vries SE, Nijtmans LG, Smeitink JA *et al*. Human NADH:ubiquinone oxidoreductase deficiency: radical changes in mitochondrial morphology? *Am J Physiol Cell Physiol* 2007;**293**:C22–29.
11. Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 2001;**31**: 1287–1312.
12. Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest* 2005;**115**:500–508.
13. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL *et al*. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995;**11**:376–381.
14. Siwik DA, Tzortzis JD, Pimental DR, Chang DL, Pagano PJ, Singh K *et al*. Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes in vitro. *Circ Res* 1999;**85**:147–153.
15. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K *et al*. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* 2001;**88**: 529–535.
16. Dhalla AK, Singal PK. Antioxidant changes in hypertrophied and failing guinea pig hearts. *Am J Physiol* 1994;**266**:H1280–H1285.
17. Hill MF, Singal PK. Right and left myocardial antioxidant responses during heart failure subsequent to myocardial infarction. *Circulation* 1997;**96**:2414–2420.
18. Chen EP, Bittner HB, Davis RD, Folz RJ, Van Trigt P. Extracellular superoxide dismutase transgene overexpression preserves postischemic myocardial function in isolated murine hearts. *Circulation* 1996;**94**: II412–II417.
19. Chen Z, Siu B, Ho YS, Vincent R, Chua CC, Hamdy RC *et al*. Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J Mol Cell Cardiol* 1998;**30**:2281–2289.
20. Yamawaki M, Sasaki N, Shimoyama M, Miake J, Ogino K, Igawa O *et al*. Protective effect of edaravone against hypoxia-reoxygenation injury in rabbit cardiomyocytes. *Br J Pharmacol* 2004;**142**:618–626.

21. Spallarossa P, Garibaldi S, Altieri P, Fabbi P, Manca V, Nasti S *et al*. Carvedilol prevents doxorubicin-induced free radical release and apoptosis in cardiomyocytes in vitro. *J Mol Cell Cardiol* 2004;**37**:837–846.
22. Kim DS, Kim HR, Woo ER, Kwon DY, Kim MS, Chae SW *et al*. Protective effect of calceolarioside on adriamycin-induced cardiomyocyte toxicity. *Eur J Pharmacol* 2006;**541**:24–32.
23. Oz E, Erbas D, Surucu HS, Duzgun E. Prevention of doxorubicin-induced cardiotoxicity by melatonin. *Mol Cell Biochem* 2006;**282**:31–37.
24. Yen HC, Oberley TD, Vichitbandha S, Ho YS, St Clair DK. The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J Clin Invest* 1996;**98**: 1253–1260.
25. Xiong Y, Liu X, Lee CP, Chua BH, Ho YS. Attenuation of doxorubicin-induced contractile and mitochondrial dysfunction in mouse heart by cellular glutathione peroxidase. *Free Radic Biol Med* 2006;**41**:46–55.
26. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med* 2000;**192**:1001–1014.
27. Aon MA, Cortassa S, Marban E, O'Rourke B. Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. *J Biol Chem* 2003; **278**:44735–44744.
28. Clarke SJ, McStay GP, Halestrap AP. Sanglifehrin A acts as a potent inhibitor of the mitochondrial permeability transition and reperfusion injury of the heart by binding to cyclophilin-D at a different site from cyclosporin A. *J Biol Chem* 2002;**277**:34793–34799.
29. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW *et al*. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 2004;**113**:1535–1549.
30. Hausenloy DJ, Duchen MR, Yellon DM. Inhibiting mitochondrial permeability transition pore opening at reperfusion protects against ischaemia-reperfusion injury. *Cardiovasc Res* 2003;**60**:617–625.
31. Akar FG, Aon MA, Tomaselli GF, O'Rourke B. The mitochondrial origin of postischemic arrhythmias. *J Clin Invest* 2005;**115**:3527–3535.
32. Rizzuto R, Brini M, Murgia M, Pozzan T. Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. *Science* 1993;**262**:744–747.
33. Arnaudeau S, Kelley WL, Walsh JV Jr, Demaurex N. Mitochondria recycle Ca(2+) to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions. *J Biol Chem* 2001;**276**: 29430–29439.
34. Chalmers S, Nicholls DG. The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. *J Biol Chem* 2003;**278**:19062–19070.
35. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 1990;**70**:391–425.
36. Balaban RS. Cardiac energy metabolism homeostasis: role of cytosolic calcium. *J Mol Cell Cardiol* 2002;**34**:1259–1271.
37. Gunter TE, Buntinas L, Sparagna G, Eliseev R, Gunter K. Mitochondrial calcium transport: mechanisms and functions. *Cell Calcium* 2000;**28**: 285–296.
38. Ruiz-Meana M, Garcia-Dorado D, Miro-Casas E, Abellan A, Soler-Soler J. Mitochondrial Ca2+ uptake during simulated ischemia does not affect permeability transition pore opening upon simulated reperfusion. *Cardiovasc Res* 2006;**71**:715–724.
39. Karmazyn M, Moffat MP. Role of Na+/H+ exchange in cardiac physiology and pathophysiology: mediation of myocardial reperfusion injury by the pH paradox. *Cardiovasc Res* 1993;**27**:915–924.
40. Zhang SZ, Gao Q, Cao CM, Bruce IC, Xia Q. Involvement of the mitochondrial calcium uniporter in cardioprotection by ischemic preconditioning. *Life Sci* 2006;**78**:738–745.
41. Garcia-Rivas Gde J, Carvajal K, Correa F, Zazueta C. Ru360, a specific mitochondrial calcium uptake inhibitor, improves cardiac post-ischaemic functional recovery in rats in vivo. *Br J Pharmacol* 2006; **149**:829–837.
42. Magee WP, Deshmukh G, Deninno MP, Sutt JC, Chapman JG, Tracey WR. Differing cardioprotective efficacy of the Na+/Ca2+ exchanger inhibitors SEA0400 and KB-R7943. *Am J Physiol Heart Circ Physiol* 2003;**284**: H903–H910.
43. Wang J, Zhang Z, Hu Y, Hou X, Cui Q, Zang Y *et al*. SEA0400, a novel Na(+)/Ca(2+) exchanger inhibitor, reduces calcium overload induced by ischemia and reperfusion in mouse ventricular myocytes. *Physiol Res* 2007;**56**:17–23.
44. Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E. Overexpression of the cardiac Na+/Ca2+ exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. *Circ Res* 1998;**83**:1215–1223.
45. Zoratti M, Szabo I. The mitochondrial permeability transition. *Biochim Biophys Acta* 1995;**1241**:139–176.
46. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA *et al*. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 2005;**434**:658–662.
47. Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 2007;**9**:550–555.
48. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP *et al*. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* 2004;**427**:461–465.
49. Batandier C, Leverve X, Fontaine E. Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. *J Biol Chem* 2004;**279**: 17197–17204.
50. Fontaine E, Eriksson O, Ichas F, Bernardi P. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex i. *J Biol Chem* 1998;**273**:12662–12668.
51. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion—a target for cardioprotection. *Cardiovasc Res* 2004;**61**:372–385.
52. Nazareth W, Yafei N, Crompton M. Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. *J Mol Cell Cardiol* 1991;**23**:1351–1354.
53. Xu M, Wang Y, Hirai K, Ayub A, Ashraf M. Calcium preconditioning inhibits mitochondrial permeability transition and apoptosis. *Am J Physiol Heart Circ Physiol* 2001;**280**:H899–H908.
54. Griffiths EJ, Halestrap AP. Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. *J Mol Cell Cardiol* 1993;**25**:1461–1469.
55. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H *et al*. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005; **434**:652–658.
56. Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB *et al*. Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure, 2nd ed. *Proc Natl Acad Sci USA* 1998;**95**:10140–10145.
57. Adams JW, Pagel AL, Means CK, Oksenberg D, Armstrong RC, Brown JH. Cardiomyocyte apoptosis induced by Galphaq signaling is mediated by permeability transition pore formation and activation of the mitochondrial death pathway. *Circ Res* 2000;**87**:1180–1187.
58. Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. *J Physiol* 2003;**549**: 513–524.
59. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res* 2002;**55**:534–543.
60. Korge P, Honda HM, Weiss JN. Protection of cardiac mitochondria by diazoxide and protein kinase C: implications for ischemic preconditioning. *Proc Natl Acad Sci USA* 2002;**99**:3312–3317.
61. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. *Circulation* 2005;**111**:194–197.
62. Ardehali H, O'Rourke B. Mitochondrial K(ATP) channels in cell survival and death. *J Mol Cell Cardiol* 2005;**39**:7–16.
63. Holmuhamedov EL, Wang L, Terzic A. ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria. *J Physiol* 1999;**519**: 347–360.
64. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS *et al*. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000;**87**:460–466.
65. Krenz M, Oldenburg O, Wimpee H, Cohen MV, Garlid KD, Critz SD *et al*. Opening of ATP-sensitive potassium channels causes generation of free radicals in vascular smooth muscle cells. *Basic Res Cardiol* 2002;**97**: 365–373.
66. Otani H. Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. *Antioxid Redox Signal* 2004;**6**:449–469.

67. Jaburek M, Costa AD, Burton JR, Costa CL, Garlid KD. Mitochondrial PKC epsilon and mitochondrial ATP-sensitive K+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. *Circ Res* 2006;**99**:878–883.
68. Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM *et al*. Mitochondrial PKCepsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCepsilon-MAPK interactions and differential MAPK activation in PKCepsilon-induced cardioprotection. *Circ Res* 2002;**90**:390–397.
69. Fryer RM, Wang Y, Hsu AK, Gross GJ. Essential activation of PKC-delta in opioid-initiated cardioprotection. *Am J Physiol Heart Circ Physiol* 2001; **280**:H1346–H1353.
70. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X *et al*. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 1997;**81**:404–414.
71. Gray MO, Karliner JS, Mochly-Rosen D. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* 1997;**272**:30945–30951.
72. Ping P, Song C, Zhang J, Guo Y, Cao X, Li RC *et al*. Formation of protein kinase C(epsilon)-Lck signaling modules confers cardioprotection. *J Clin Invest* 2002;**109**:499–507.
73. Saurin AT, Pennington DJ, Raat NJ, Latchman DS, Owen MJ, Marber MS. Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovasc Res* 2002;**55**:672–680.
74. Tong H, Chen W, Steenbergen C, Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ Res* 2000;**87**:309–315.
75. Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M *et al*. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 2001;**104**:330–335.
76. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000;**101**:660–667.
77. Miao W, Luo Z, Kitsis RN, Walsh K. Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. *J Mol Cell Cardiol* 2000;**32**: 2397–2402.
78. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* 2001;**15**:1406–1418.
79. Majewski N, Nogueira V, Robey RB, Hay N. Akt inhibits apoptosis downstream of BID cleavage via a glucose-dependent mechanism involving mitochondrial hexokinases. *Mol Cell Biol* 2004;**24**:730–740.
80. Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation* 2004;**109**:3042–3049.
81. Plas DR, Talapatra S, Edinger AL, Rathmell JC, Thompson CB. Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. *J Biol Chem* 2001;**276**:2041–2048.
82. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y *et al*. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997;**91**:231–241.
83. Tsuruta F, Masuyama N, Gotoh Y. The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. *J Biol Chem* 2002;**277**:14040–14047.
84. Jones RG, Parsons M, Bonnard M, Chan VS, Yeh WC, Woodgett JR *et al*. Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. *J Exp Med* 2000;**191**:1721–1734.
85. Matsuzaki H, Tamatani M, Mitsuda N, Namikawa K, Kiyama H, Miyake S *et al*. Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. *J Neurochem* 1999;**73**:2037–2046.
86. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995;**378**:785–789.
87. Gross ER, Hsu AK, Gross GJ. Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. *Circ Res* 2004;**94**:960–966.
88. Tong H, Imahashi K, Steenbergen C, Murphy E. Phosphorylation of glycogen synthase kinase-3beta during preconditioning through a phosphatidylinositol-3-kinase-dependent pathway is cardioprotective. *Circ Res* 2002;**90**:377–379.
89. Nishihara M, Miura T, Miki T, Sakamoto J, Tanno M, Kobayashi H *et al*. Erythropoietin affords additional cardioprotection to preconditioned hearts by enhanced phosphorylation of glycogen synthase kinase-3 beta. *Am J Physiol Heart Circ Physiol* 2006;**291**:H748–H755.
90. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;**281**:1322–1326.
91. Suzuki M, Youle RJ, Tjandra N. Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 2000;**103**:645–654.
92. Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. *Cell* 2000;**103**:839–842.
93. Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R *et al*. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002;**111**:331–342.
94. Gustafsson AB, Gottlieb RA. Bcl-2 Family Members and Apoptosis, Taken to Heart. *Am J Physiol Cell Physiol* 2006;**292**:C45–C51.
95. Zhu L, Yu Y, Chua BH, Ho YS, Kuo TH. Regulation of sodium-calcium exchange and mitochondrial energetics by Bcl-2 in the heart of transgenic mice. *J Mol Cell Cardiol* 2001;**33**:2135–2144.
96. Imahashi K, Schneider MD, Steenbergen C, Murphy E. Transgenic expression of Bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. *Circ Res* 2004;**95**:734–741.
97. Chen Z, Chua CC, Ho YS, Hamdy RC, Chua BH. Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *Am J Physiol Heart Circ Physiol* 2001;**280**:H2313–H2320.
98. Brocheriou V, Hagege AA, Oubenaissa A, Lambert M, Mallet VO, Duriez M *et al*. Cardiac functional improvement by a human Bcl-2 transgene in a mouse model of ischemia/reperfusion injury. *J Gene Med* 2000;**2**: 326–333.
99. Weisleder N, Taffet GE, Capetanaki Y. Bcl-2 overexpression corrects mitochondrial defects and ameliorates inherited desmin null cardiomyopathy. *Proc Natl Acad Sci USA* 2004;**101**:769–774.
100. Rouslin W, Erickson JL, Solaro RJ. Effects of oligomycin and acidosis on rates of ATP depletion in ischemic heart muscle. *Am J Physiol* 1986;**250**: H503–H508.
101. Vuorinen K, Ylitalo K, Peuhkurinen K, Raatikainen P, Ala-Rami A, Hassinen IE. Mechanisms of ischemic preconditioning in rat myocardium. Roles of adenosine, cellular energy state, and mitochondrial F1F0-ATPase. *Circulation* 1995;**91**:2810–2818.
102. Huang J, Nakamura K, Ito Y, Uzuka T, Morikawa M, Hirai S *et al*. Bcl-xL gene transfer inhibits Bax translocation and prolongs cardiac cold preservation time in rats. *Circulation* 2005;**112**:76–83.
103. Gil-Parrado S, Fernandez-Montalvan A, Assfalg-Machleidt I, Popp O, Bestvater F, Holloschi A *et al*. Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. *J Biol Chem* 2002;**277**:27217–27226.
104. Chen M, Won DJ, Krajewski S, Gottlieb RA. Calpain and mitochondria in ischemia/reperfusion injury. *J Biol Chem* 2002;**277**:29181–29186.
105. Condorelli G, Morisco C, Stassi G, Notte A, Farina F, Sgaramella G *et al*. Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat. *Circulation* 1999;**99**:3071–3078.
106. Jung F, Weiland U, Johns RA, Ihling C, Dimmeler S. Chronic hypoxia induces apoptosis in cardiac myocytes: a possible role for Bcl-2-like proteins. *Biochem Biophys Res Commun* 2001;**286**:419–425.
107. Gustafsson AB, Tsai JG, Logue SE, Crow MT, Gottlieb RA. Apoptosis repressor with caspase recruitment domain protects against cell death by interfering with Bax activation. *J Biol Chem* 2004;**279**:21233–21238.
108. Capano M, Crompton M. Bax translocates to mitochondria of heart cells during simulated ischaemia: involvement of AMP-activated and p38 mitogen-activated protein kinases. *Biochem J* 2006;**395**:57–64.
109. Hochhauser E, Kivity S, Offen D, Maulik N, Otani H, Barhum Y *et al*. Bax ablation protects against myocardial ischemia-reperfusion injury in transgenic mice. *Am J Physiol Heart Circ Physiol* 2003;**284**: H2351–H2359.
110. Chen M, He H, Zhan S, Krajewski S, Reed JC, Gottlieb RA. Bid is cleaved by calpain to an active fragment in vitro and during myocardial ischemia/reperfusion. *J Biol Chem* 2001;**276**:30724–30728.
111. Scarabelli TM, Stephanou A, Pasini E, Comini L, Raddino R, Knight RA *et al*. Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. *Circ Res* 2002;**90**:745–748.
112. Toth A, Jeffers JR, Nickson P, Min JY, Morgan JP, Zambetti GP *et al*. Targeted Deletion of Puma Attenuates Cardiomyocyte Death and Improves Cardiac Function during Ischemia/reperfusion. *Am J Physiol Heart Circ Physiol* 2006;**291**:H52–60.

113. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA *et al*. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ* 2007;**14**: 146–157.
114. Kubli DA, Ycaza JE, Gustafsson AB. Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. *Biochem J* 2007;**405**: 407–415.
115. Regula KM, Ens K, Kirshenbaum LA. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res* 2002;**91**:226–231.
116. Yussman MG, Toyokawa T, Odley A, Lynch RA, Wu G, Colbert MC *et al*. Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy, 2nd ed. *Nat Med* 2002;**8**:725–730.
117. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004;**305**:626–629.
118. Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999;**274**:11549–11556.
119. Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N *et al*. Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* 2004;**117**:773–786.
120. Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N *et al*. Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* 2005;**25**:1025–1040.
121. Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000;**102**:33–42.
122. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE *et al*. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;**102**:43–53.
123. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001;**8**:613–621.
124. van Empel VP, Bertrand AT, van der Nagel R, Kostin S, Doevendans PA, Crijns HJ *et al*. Downregulation of apoptosis-inducing factor in harlequin mutant mice sensitizes the myocardium to oxidative stress-related cell death and pressure overload-induced decompensation. *Circ Res* 2005; **96**:e92–e101.
125. Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N *et al*. AIF deficiency compromises oxidative phosphorylation. *EMBO J* 2004;**23**: 4679–4689.
126. Joza N, Oudit GY, Brown D, Benit P, Kassiri Z, Vahsen N *et al*. Muscle-specific loss of apoptosis-inducing factor leads to mitochondrial dysfunction, skeletal muscle atrophy, and dilated cardiomyopathy. *Mol Cell Biol* 2005;**25**:10261–10272.
127. Siu PM, Bae S, Bodyak N, Rigor DL, Kang PM. Response of caspase-independent apoptotic factors to high salt diet-induced heart failure. *J Mol Cell Cardiol* 2007;**42**:678–686.
128. Kim GT, Chun YS, Park JW, Kim MS. Role of apoptosis-inducing factor in myocardial cell death by ischemia-reperfusion. *Biochem Biophys Res Commun* 2003;**309**:619–624.
129. Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* 2001;**412**:95–99.
130. Bahi N, Zhang J, Llovera M, Ballester M, Comella JX, Sanchis D. Switch from caspase-dependent to caspase-independent death during heart development: essential role of endonuclease G in ischemia-induced DNA processing of differentiated cardiomyocytes. *J Biol Chem* 2006; **281**:22943–22952.
131. Irvine RA, Adachi N, Shibata DK, Cassell GD, Yu K, Karanjawala ZE *et al*. Generation and characterization of endonuclease G null mice. *Mol Cell Biol* 2005;**25**:294–302.
132. David KK, Sasaki M, Yu SW, Dawson TM, Dawson VL. EndoG is dispensable in embryogenesis and apoptosis. *Cell Death Differ* 2006;**13**:1147–1155.
133. Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F *et al*. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 2001;**1**:515–525.
134. Jagasia R, Grote P, Westermann B, Conradt B. DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. *Nature* 2005;**433**:754–760.
135. Karbowski M, Lee YJ, Gaume B, Jeong SY, Frank S, Nechushtan A *et al*. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol* 2002;**159**:931–938.
136. Germain M, Mathai JP, McBride HM, Shore GC. Endoplasmic reticulum BIK initiates DRP1-regulated remodelling of mitochondrial cristae during apoptosis. *EMBO J* 2005;**24**:1546–1556.
137. Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *J Biol Chem* 2006;**281**:29776–29787.
138. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* 2005;**120**:159–162.
139. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T *et al*. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ Res* 2007;**100**:914–922.
140. Yan L, Vatner DE, Kim SJ, Ge H, Masurekar M, Massover WH *et al*. Autophagy in chronically ischemic myocardium. *Proc Natl Acad Sci USA* 2005; **102**:13807–13812.
141. Kametani R, Miura T, Harada N, Shibuya M, Wang R, Tan H *et al*. Carvedilol inhibits mitochondrial oxygen consumption and superoxide production during calcium overload in isolated heart mitochondria. *Circ J* 2006;**70**:321–326.
142. Oliveira PJ, Bjork JA, Santos MS, Leino RL, Froberg MK, Moreno AJ *et al*. Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Toxicol Appl Pharmacol* 2004; **200**:159–168.
143. Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR *et al*. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N Engl J Med* 1996;**334**:1145–1149.
144. Lonn E, Yusuf S, Hoogwerf B, Pogue J, Yi Q, Zinman B *et al*. Effects of vitamin E on cardiovascular and microvascular outcomes in high-risk patients with diabetes: results of the HOPE study and MICRO-HOPE substudy. *Diabetes Care* 2002;**25**:1919–1927.
145. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000; **342**:154–160.
146. Arstall MA, Yang J, Stafford I, Betts WH, Horowitz JD. N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction. Safety and biochemical effects. *Circulation* 1995;**92**:2855–2862.
147. Sochman J, Vrbska J, Musilova B, Rocek M. Infarct Size Limitation: acute N-acetylcysteine defense (ISLAND trial): preliminary analysis and report after the first 30 patients. *Clin Cardiol* 1996;**19**:94–100.
148. Yesilbursa D, Serdar A, Senturk T, Serdar Z, Sag S, Cordan J. Effect of N-acetylcysteine on oxidative stress and ventricular function in patients with myocardial infarction. *Heart Vessels* 2006;**21**:33–37.
149. Miura T, Miki T. ATP-sensitive K+ channel openers: old drugs with new clinical benefits for the heart. *Curr Vasc Pharmacol* 2003;**1**:251–258.
150. Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I *et al*. Postconditioning the human heart. *Circulation* 2005;**112**:2143–2148.