This Review is part of a thematic series on Mitochondrial Dysfunction in Ischemia, which includes the following articles:

Role of the Mitochondrial Permeability Transition in Myocardial Disease
Evidence for Mitochondrial K⁺ Channels and Their Role in Cardioprotection
Primary and Secondary Signaling Pathways in Cardioprotection
Mitochondrial Death Pathways

Elizabeth Murphy, Guest Editor; Roberto Bolli, Editor

Role of the Mitochondrial Permeability Transition in Myocardial Disease
James N. Weiss, Paavo Korge, Henry M. Honda, Peipei Ping

Abstract—Mitochondria play a key role in determining cell fate during exposure to stress. Their role during ischemia/reperfusion is particularly critical because of the conditions that promote both apoptosis by the mitochondrial pathway and necrosis by irreversible damage to mitochondria in association with mitochondrial permeability transition (MPT). MPT is caused by the opening of permeability transition pores in the inner mitochondrial membrane, leading to matrix swelling, outer membrane rupture, release of apoptotic signaling molecules such as cytochrome c from the intermembrane space, and irreversible injury to the mitochondria. During ischemia (the MPT priming phase), factors such as intracellular Ca²⁺ accumulation, long-chain fatty acid accumulation, and reactive oxygen species progressively increase mitochondrial susceptibility to MPT, increasing the likelihood that MPT will occur on reperfusion (the MPT trigger phase). Because functional cardiac recovery ultimately depends on mitochondrial recovery, cardioprotection by ischemic and pharmacological preconditioning must ultimately involve the prevention of MPT. Investigations into this area are beginning to unravel some of the mechanistic links between cardioprotective signaling and mitochondria. (Circ Res. 2003;93:292-301.)

Key Words: mitochondria ■ programmed cell death ■ mitochondrial permeability transition ■ ischemia ■ reperfusion

Mitochondria are increasingly recognized as key players in cell survival, not only because of their traditional role as energy providers for vital cellular processes but also because of their critical involvement in programmed cell death via apoptosis. Cells die from necrosis when they cannot maintain adequate ATP levels, and they die from apoptosis when mitochondria participate in molecular signaling events, initiating activation of the caspase cascade, which requires preserved ATP levels. Caspase activation can occur via three routes: (1) the receptor (extrinsic) pathway involving death receptors, (2) the mitochondrial (intrinsic) pathway, and (3) an endoplasmic reticulum (ER) pathway. In the receptor pathway, the stimulation of cell death receptors (such as Fas and tumor necrosis factor receptors) directly triggers caspase 8 to initiate the cascade. Whether mitochondria are essential for apoptosis via the receptor pathway is controversial, but at the very least, they appear to be involved in cells with mitochondria, inasmuch as caspase 8 activation also cleaves the proapoptotic protein Bid to its truncated from, tBid. tBid promotes the permeabilization of the mitochondrial outer membrane (OM), causing release of proapoptotic signaling molecules, such as cytochrome c, apoptotic protease-activating factor 1, Smac/DIABLO, apoptosis-inducing factor (AIF), Endo G, and Htra2/Omi, which promote cell death via...
both caspase-dependent and caspase-independent (eg, AIF) mechanisms.4 In the mitochondrial pathway, mitochondria are directly stimulated by environmental factors such as reactive oxygen species (ROS) or ischemia/reperfusion to release the same signaling molecules to initiate apoptosis. Recently, the ER pathway of caspase activation has been described, triggered by ER stress, leading to the activation of caspase 12,2,5

Necrosis and apoptosis initiated by the mitochondrial and receptor pathways are both relevant to acute and chronic cardiac disease.1 For example, acute myocardial ischemia/reperfusion injury involves a variable mix of necrosis and apoptosis6 dependent on the experimental model, duration of ischemia, and other factors,1 triggered by irreversible mitochondrial injury and activation of the mitochondrial apoptotic pathway. In addition, the failing heart surviving a large myocardial infarction or other pathophysiological insults is subject to an elevated circulating level of chemokines, such as tumor necrosis factor-α, which may signal programmed cell death via the receptor pathway, leading to further loss of myocardial reserve.

The goal of the present review is to summarize the current state of knowledge about the mechanisms by which mitochondria signal cell death in cardiac tissue, with particular emphasis on mitochondrial permeability transition pore (mPTP) regulation in cardiac injury and cardioprotection. The interested reader is also referred to several excellent recent reviews.7-10

Mitochondrial Permeability Transition Pore
mPTPs are multiprotein complexes that are capable of forming large nonselective pores in the inner membrane (IM) of the mitochondria11 (Figure 1). How does mPTP opening cause mitochondrial injury and cell death? Mitochondria use electron transport to generate a large electrochemical gradient across the IM, consisting of membrane potential ($\Delta \psi_m$) ≈ 200 mV, the major component under normal aerobic conditions) and a proton gradient (ΔpH). This electrochemical gradient is then used by ATP synthase ($F_0$ – $F_1$, ATPase) to phosphorylate ADP to ATP. To sustain $\Delta \psi_m$ requires that the IM remain relatively impermeant to ions. The mPTP opening immediately depolarizes $\Delta \psi_m$, causing ATP synthase to operate in reverse, consuming ATP in a futile attempt to restore the proton gradient. Thus, mitochondrial permeability transition (MPT) converts mitochondria from ATP producers to ATP consumers, accelerating cellular energy depletion and hastening cell death.

It has been proposed that mPTP can open in two modes: low conductance and high conductance.11 Somewhat controversial is the existence of a reversible low-conductance mode allowing permeation of small solutes that depolarize $\Delta \psi_m$ transiently. The more well-defined high-conductance mode passes solutes up to 1.5 kDa and causes marked matrix swelling as the high oncotic pressure of the matrix proteins and equilibrated ions drives water influx. In the high-conductance mode, mPTP openings fall into two classes: transient and long-lasting, the latter often being irreversible. Because the surface area of the IM exceeds that of the OM, extensive matrix swelling with long-lasting mPTP opening can lead to the unfolding of cristae, causing the OM to rupture, irreversibly damaging mitochondria. In addition, OM rupture releases proapoptotic molecules residing in the intermembrane space, including cytochrome c, Smac/DIABLO, AIF, Endo G, and Htra2/Omi, which promote cell death via both caspase-dependent and caspase-independent (eg, AIF) mechanisms.4 Thus, under conditions in which MPT is not widespread enough to impair global energy production, it can induce apoptosis if a critical proportion of mitochondria has been damaged. This is consistent with the observation that cell death in ischemia/reperfusion injury is due to a mixture of necrosis and apoptosis (see review1).

The exact molecular composition of the mPTP is still under debate.4 The adenine nucleotide translocator (ANT) in the IM, cyclophilin D (CyP-D) in the matrix, and perhaps the voltage-dependent anion channel (VDAC, also called porin) in the OM appear to be the key structural components, but other proteins, such as the benzodiazepine receptor, hexokinase, and creatine kinase, may play regulatory roles (Figure 1). mPTPs are Ca$^{2+}$, redox, voltage, and pH sensitive, such that their open probability is increased by matrix-free [Ca$^{2+}$]. ROS, $\Delta \psi_m$ depolarization, and high pH (>7.0). In the physiological setting, Ca$^{2+}$ and ROS are the most important inducers of mPTP opening. Formation of disulfide bonds between critical thiol groups on the ANT have been implicated in allowing CyP-D binding to promote mPTP opening, and this may be the basis for the effects of ROS.4 Consistent with this idea, various oxidizing and SH group cross-linking agents are also potent artificial inducers of mPTP opening.
The conformation of the ANT in which its adenine nucleotide binding site faces the cytoplasm (c conformation) appears to promote mPTP opening, whereas the conformation with this binding site facing the matrix (m conformation) prevents mPTP opening. Other modifiers promoting mPTP opening include inorganic phosphate (P\textsubscript{i}), which enhances matrix Ca\textsuperscript{2+} uptake and may compete for adenine nucleotide binding sites on ANT; fatty acids, which enhance ROS production and inhibit and stabilize ANT in its c conformation\textsuperscript{14}; atractyloside, which binds to and stabilizes the ANT in its c conformation; and proapoptotic Bcl family proteins, such as Bax, Bak, Bad, Bid, Bim, Bok, Noxa, and Puma,\textsuperscript{2} which translocate to mitochondria and associate with OM components to promote mPTP opening by as-yet-unclear mechanisms.

The most potent inhibitor of mPTP opening in isolated mitochondria is the cyclophilin binding protein cyclosporin A (CsA), which chelates Cyp-D, thereby preventing its interaction with other PTP components. Although a potent inhibitor, its effects can be overcome by sufficiently large elevations in matrix Ca\textsuperscript{2+}, leading to the suggestion that the primary role of Cyp-D binding to mPTP components is to increase their sensitivity to Ca\textsuperscript{2+}. In addition, the efficacy of CsA in intact cells is inconsistent\textsuperscript{15} because of the inability to control other pro-MPT factors in the intact cytoplasm. This has been an important limitation in assessing the importance of MPT in ischemia/reperfusion injury.\textsuperscript{1} In addition, CsA is not completely specific and inhibits calcineurin, which also plays important roles in modulating mitochondrial death signals.\textsuperscript{16} Sanglifehrin A is also a potent inhibitor, which binds to a different site on Cyp-D and does not inhibit calcineurin.\textsuperscript{17} Other inhibitors of mPTP opening include bongkrekic acid (which stabilizes ANT in its m conformation), acidos, Mg\textsuperscript{2+}, and adenine nucleotides (which lower the Ca\textsuperscript{2+} sensitivity of mPTP), polyamines, and antiapoptotic Bcl proteins, such as Bcl-2 and Bcl-x\textsubscript{L}.

**Role of mPTP Opening in Ischemia/Reperfusion Injury**

Crompton et al\textsuperscript{18} were the first to propose that MPT may play a role in cardiac reperfusion injury. Figure 2 summarizes the hypothetical role of MPT in ischemia/reperfusion injury. It has been shown that MPT occurs on reperfusion of the ischemic heart, and mounting evidence indicates that MPT is an important cause of ischemia/reperfusion injury.\textsuperscript{19,20} For the heart to recover from ischemia/reperfusion, its mitochondria must return to full functionality. Irreversible depolarization of mitochondria by MPT ultimately induces necrotic cell death by impairing energy production. Even before this stage, however, other factors contribute to injury. During ischemia, cytosolic Ca\textsuperscript{2+} becomes elevated, and while the mitochondria are still polarized, Ca\textsuperscript{2+} is driven into the matrix through the Ca\textsuperscript{2+} uniporter. When the mitochondria depolarize, this accumulated matrix Ca\textsuperscript{2+} flows down its electrochemical gradient (through either the uniporter, Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, or mPTP) back into the cytoplasm, contributing to myofilament hypercontracture. This hypercontracture can be severe enough to mechanically disrupt the sarcolemmal membrane and cause contraction band necrosis characteristic of irreversibly damaged reperfused tissue. Occasionally, contraction band necrosis is localized to discrete regions of the myocyte, accompanied by locally swollen, ruptured mitochondria, suggesting that localized MPT has occurred.

Even if the overall damage to mitochondria is not widespread enough to cause necrosis, milder mitochondrial damage can still trigger apoptotic cell death by releasing enough cytochrome c to induce caspase activation via the mitochondrial pathway.\textsuperscript{8} Apoptosis initiated by this mitochondrial pathway requires maintained cellular ATP levels. Cytochrome c, apoptotic protease-activating factor 1, and procaspase 9 together form a complex, the apoptosome, which activates caspase 9 and, subsequently, downstream caspases. Moreover, cytochrome c can be released from the mitochondria via both mPTP-dependent and mPTP-independent mechanisms.\textsuperscript{5} With OM rupture due to MPT, cytochrome c is released directly. Cytochrome c release in the absence of mPTP opening is discussed in more detail below.

Although evidence that MPT plays a key role in reperfusion injury has been mounting, some investigators question its role as a primary mechanism of injury. For example, CsA, one of the most potent MPT inhibitors, conferred only limited protection against reperfusion injury and even promoted injury at high concentrations (>0.4 \mu mol/L).\textsuperscript{21} In general, however, potent MPT inhibitors such as CsA and sanglifehrin A, have been found to be protective, even when delivered only during reperfusion, although the extent of protection varies depending on the experimental species, whether the model is global ischemia/reperfusion or coronary ligation with regional ischemia/reperfusion, and other factors.\textsuperscript{23–25} At a more general level, the relative contribution of reperfusion to ischemic injury has been under debate for decades.\textsuperscript{26} Because therapy delivered at the time of reperfusion often has limited efficacy, it has been questioned whether reperfusion has an independent role in causing injury or just unmasks latent injury occurring during the ischemic period.

However, the MPT hypothesis of ischemia/reperfusion injury is compatible the idea that injury arises from both mechanisms. As ischemic time increases, latent susceptibility of mitochondria to MPT increases (which we will call the MPT priming component), yet the conditions of reperfusion can modulate whether or not MPT is induced (the MPT trigger component). This scenario is analogous to the recently

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**Figure 2. Hypothetical role of MPT in cell death during ischemia/reperfusion of the heart. LCFA indicates long-chain fatty acids. See text for details.**

\[ \text{MPT & cell fate during ischemia/reperfusion} \]

- **Ischemia** \( [\text{Ca}^{2+}], [\text{Pi}], \text{pH} \) LCFA, ROS
- **Reperfusion** \( + \) ROS burst, \( \text{pH} \)
- **Hypoxia**

- **MPT priming**
  - Minimal MPT
  - Recovery or Apoptosis (10-50%)
  - Full Recovery
- **MPT trigger**
  - Localized MPT
  - Generalized MPT
  - Necrosis

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\( \text{H11001} \)
described response of cultured cardiac myocytes to H2O2-induced injury, in which Akao and colleagues27,28 separated the process of cell death into distinct phases: a priming phase, consisting of ROS and Ca2+-dependent morphological changes in mitochondria (swelling and remodeled cristae) but with maintained Δψm; a depolarization phase, consisting of MPT-induced Δψm depolarization; and finally, a cell fragmentation phase, representing massive swelling with the release of cytochrome c and surface membrane alterations.

A major factor governing the MPT priming component may be MPT-independent cytochrome c loss and increased IM leakiness during the ischemic period resulting from the accumulation of long-chain fatty acids and ROS29,30 (see below). This may account for the failure of CsA to prevent reperfusion injury, because if mitochondria have become extensively depleted of cytochrome c and have leaky IM, their capacity to regenerate Δψm by electron transport, as required for ATP synthesis, may be too limited even if mPTPs are closed by CsA. In addition, MPT-independent cytochrome c release will trigger caspase activation, leading to apoptosis if ATP is not severely depleted.

The MPT trigger component, on the other hand, is influenced by the interplay between the MPT inducers/inhibitors present during reperfusion (particularly matrix-free Ca2+ and ROS levels) and electron transport capacity for regenerating Δψm. The latter is highly sensitive to the extent of cytochrome c loss and IM leakiness occurring during the preceding ischemia. We postulate the following scenario: Like other ion channels, PTPs open and close stochastically. Because PTP open probability is strongly voltage dependent, the rapidity with which electron transport regenerates Δψm when the PTP transiently closes will play a critical role in determining whether it remains closed or reopens. Intermembrane cytochrome c content and IM leakiness are both major determinants of controlling the rate at which electron transport can regenerate and maintain Δψm, so that cytochrome c loss and IM leak, by depressing the Δψm recovery rate, will increase the probability that a transiently closed mPTP will reopen. In addition, cytochrome c depletion increases oxidant stress, promoting mPTP opening, because it is an ROS scavenger, and in slowing electron transport, it also increases superoxide production by allowing reducing equivalents to accumulate.1 In the high-conductance mode of MPT, this interplay may thus determine whether PTP openings are transient, long-lasting, or irreversible.31 In this scenario, both PTP closure and recovery of proton pumping by electron transport are required for full recovery of mitochondrial function. The coordination of these two effects allows for the recovery of Δψm, which is ultimately required for the functional recovery of mitochondrial and cardiac function.

MPT Priming Component and Cytochrome c Loss

OM rupture due to MPT is one mode of cytochrome c release. However, cytochrome c and other proapoptotic factors can leave the intermembrane space in the absence of OM rupture, although the mechanisms are still debated.2 Recent studies indicate that the majority (≈85%) of cytochrome c resides in the intercristal spaces and is not directly accessible to the OM except via long, narrow (∼20-nm) tubular necks, as revealed by high-voltage electron microscopic tomographic imaging32,33 (Figure 3). Under proapoptotic conditions, these tubular necks widen to ∼60 nm, promoting access of cytochrome c to the OM.33 In addition, only ∼15% of cytochrome c is loosely bound (ie, releasable in response to increased ionic strength), with the majority more tightly bound to cardiolipin in the IM. Release of the latter component requires cardiolipin oxidation by ROS,34 which is well documented to occur during myocardial ischemia.35,36 Once mobilized for release, cytochrome c (molecular mass of 12.6 kDa) must then permeate the OM. Normally, the largest pore in the OM is porin (also called VDAC), permeant to molecules <5 kDa. Recently, Kuwana et al37 have shown that an OM channel permeable to molecules up to 2 MDa can be reconstituted in OM vesicles (containing porin) by the combination of Bid, Bax, and cardiolipin. This channel is inhibited by Bcl-xL, does not require any IM constituents, and therefore is independent of mPTP opening.

In studies using in vitro and in situ cardiac mitochondria,30 we have found that long-chain activated fatty acids and ROS progressively lower the threshold for MPT as a result of progressive MPT-independent cytochrome c loss and increased IM leakiness. Both long-activated fatty acids and ROS accumulate during acute ischemia in the intact heart35,36 and therefore may be important factors increasing the likelihood of MPT on reperfusion in intact cardiac tissue, both as a result of their direct MPT-inducing effects and the indirect effects described above. Consistent with their known cardioprotective roles, we found that both the mitochondrial ATP-sensitive K+ (mitoKATP) channel agonist diazoxide and ROS scavengers protected against the MPT-independent cytochrome c loss induced by long-chain activated fatty acids and that this protection was blocked by the mitoKATP antagonist 5-hydroxydecanoate (5-HD). Thus, fatty acid accumulation and ROS may be two important factors that depress the intrinsic threshold for MPT in the setting of ischemia. However, the effects of ROS are complex and may be both time and dose dependent. Although during prolonged ischemia, ROS prime the heart for MPT, when they are administered before prolonged ischemia (or induced by repetitive brief ischemia/reperfusion episodes before prolonged ischemia), they trigger cardioprotection by activating protective signaling mechanisms.40,41

MPT Trigger Component and Reperfusion

Current evidence suggests that mPTPs remain closed during ischemia and open during reperfusion.19,20 The strongest evidence is based on studies examining the trapping of tritiated 2-deoxyglucose (DOG), which readily enters the cytoplasm but is too large to access the mitochondrial matrix unless mPTPs open. The DOG content of mitochondria was markedly increased when mitochondria were isolated after reperfusion compared with the end of the ischemic period. Even though CsA did prevent increased DOG uptake during reperfusion, this was later attributed to reversible mPTP opening, inasmuch as ischemia/reperfusion episodes that were too brief to cause irreversible injury also led to significant DOG entrapment.8 Moreover, cardioprotective interven-
tions inhibited DOG entrapment after reperfusion.25,42 Thus, even though susceptibility to MPT may be increased, current evidence suggests that acidosis, elevated Mg$^{2+}/H^{+}$, and depressed electron transport tend to keep mPTPs closed during ischemia. During reperfusion, however, the resupply of oxygen leads to a dramatic burst of ROS and resumption of electron transport at a time when cytoplasmic free Ca$^{2+}$ and $P_i$ are elevated, acidosis quickly clears, and Mg$^{2+}/H^{+}$ returns toward normal. As depolarized mitochondria attempt to regenerate $\Delta\psi_m$, additional Ca$^{2+}$ is driven into the matrix when mPTP open probability is intrinsically high because of the partially recovered $\Delta\psi_m$. $\Delta\psi_m$ is essential for providing the driving force for Ca$^{2+}$ uptake into the matrix via the Ca$^{2+}$ uniporter, so that $\Delta\psi_m$ dissipation protects against MPT by reducing matrix Ca$^{2+}$ accumulation. However, $\Delta\psi_m$ dissipation also directly promotes MPT by increasing mPTP open probability. Thus, if mitochondria are depolarized before they have accumulated significant amounts of Ca$^{2+}$ [eg, by diazoxide or uncouplers like carbonyl cyanide $p$-(trifluoromethoxy)-phenylhydrazone, FCCP], they are protected from subsequent Ca$^{2+}$ uptake and MPT.43 However, if mitochondria are already loaded with Ca$^{2+}$, $\Delta\psi_m$ dissipation triggers MPT. This interplay may be particularly important during ischemia/reperfusion, when mitochondria become depolarized because anoxia is present at the same time that cytoplasmic free Ca$^{2+}$ is increasing. Even after $\Delta\psi_m$ dissipation, however, mitochondria may continue to accumulate Ca$^{2+}$ via mitochondrial Na$^+$/Ca$^{2+}$ exchange,44 and in isolated cardiac myocytes subjected to hypoxia, a rise in matrix-free [Ca$^{2+}$] to >300 to 400 mmol/L was associated with irreversible injury.45 The relative timing of these factors is critical with respect to whether mPTP opening occurs.43

Thus, whether MPT occurs during reperfusion is determined by the interplay between MPT inducers/inhibitors present during reperfusion (particularly matrix-free Ca$^{2+}$ levels) and electron transport capacity for regenerating $\Delta\psi_m$.43 The latter is highly sensitive to the extent of cytochrome c loss and IM leakiness occurring during the preceding ischemia. Consistent with its known cardioprotective role, we found that the mitoK$_{ATP}$ channel agonist diazoxide, as well as protein kinase C stimulation by phorbol 12-myristate 13-acetate (PMA, a phorbol ester), protected against Ca$^{2+}$-induced MPT in isolated and in situ mitochondria and that protection in both cases was blocked by the mitoK$_{ATP}$ antagonist 5-HD.29

**Cardioprotection and MPT**

If the MPT hypothesis is correct, then protection of the heart from ischemia/reperfusion injury, and perhaps other patho-

**Figure 3.** Morphological changes in mitochondria during cytochrome c (Cyt c) release. A and B, High-voltage electron microscopic tomographic slices of representative mitochondria before (A) and after (B) exposure to tBID induces Cyt c release. A’ and B’, Corresponding 3D tomographic reconstructions, with OM in red and IM in green, showing extensive matrix and crystal remodeling. Right panels, Schematic illustration of crista before and after tBID. After tBID, 85% of the Cyt c (red spheres) residing in the intercristal space is mobilized and gains access to the OM via the widened of tubular necks of the crista. e-Flow indicates electron flow. Reprinted from Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell. 2002;2:55–67, with permission from Elsevier, copyright 2002).
physiological stresses, must ultimately involve the prevention of MPT. Recent investigations demonstrating that cardioprotection can be influenced through modulating the MPT support this notion. A key issue, then, is whether the mitochondria are the proximate target of the cardioprotective interventions or a far downstream end effector. In the heart, the most potent method for reducing reperfusion injury is to precondition with brief ischemic episodes before subjecting the heart to prolonged ischemia. In addition to ischemic preconditioning (PC), other PC stimuli, such as transient exposure to hypoxia, elevated extracellular Ca\(^{2+}\), NO, or ROS, and a variety of pharmacological agents (see below) are comparably cardioprotective. Cardioprotection after PC interventions can be divided into early and late phases. The early phase involves begins immediately, lasts \(\approx 2\) hours, and is attributed to posttranslational modification of existing proteins. The late phase, which is less protective, appears after \(\approx 24\) hours, lasts \(\approx 72\) hours, and involves gene reprogramming and new protein synthesis. A distinction has been made between triggers versus mediators of ischemic PC. A trigger sets in motion a cascade of signaling events that results in cardioprotection even if the intervention is not present during prolonged ischemia. A mediator, on the other hand, must remain present during prolonged ischemia to be cardioprotective. Multiple signaling pathways have been implicated in early and late ischemic PC, with the most clearly defined being activation of protein kinase C (PKC) via G-protein–coupled receptors (adenosine, muscarinic, \(\alpha\)-adrenergic, opioid, angiotensin, endothelin, and bradykinin receptors). PKCe is a primary cardioprotective PKC isoform, whereas PKCe promotes injury. Tyrosine kinases (eg, Src and Lck), mitogen-activated protein kinases (p38, c-Jun N-terminal kinase, and extracellular signal–regulated kinase), heat shock protein 27, and NO signaling have also been shown to play roles whose relative importance differs among species.

By use of classic reductionist approaches, tremendous knowledge regarding the role of individual proteins in PC has been gained. However, although the reductionist approach is effective in characterizing the effects of single molecules, it has limited utility when a repertoire of proteins is involved. In cardioprotective signaling, the considerable crosstalk between signaling cascades has made it difficult to determine the precise upstream-downstream relationships using classic reductionist approaches alone. A promising new approach in addressing this limitation is the application of functional proteomics to investigate the function of subsets of proteins, subproteomes. It enables the delineation of a functional role of multiple molecules in parallel, thereby providing a holistic portrait (in contrast to a “single molecular view”) of a signaling system. This approach has contributed to the concept that cardioprotective signaling in the heart involves parallel interactions between modular signaling pathways, the so-called signaling module hypothesis. In cardioprotection, proteomic studies in combination with targeted genetic approaches have demonstrated that multiple proteins act in concert with PKCe and that it is the integrated effort from a battery of molecules that results in protection. Functional proteomic analyses of PKCe-associated proteins indicate that this kinase forms multiprotein signaling complexes with at least 93 proteins, which can be categorized into at least six groups: signaling proteins, stress activated proteins, structural proteins, transcription/translation factors, metabolic proteins, and PKC binding domain-containing proteins. These complexes are differentially regulated in protected versus unprotected hearts, suggesting that they represent a coordinated mechanism of signal transduction conferring cardioprotection.

The functional proteomics approach can be applied in investigating the subproteomes associated with specific organelles. To this end, recent studies have identified the association of PKCe with a variety of mitochondrial proteins, including mPTP components. This intriguing evidence raises the possibility that there may be a direct functional link between PKCe and the mitochondria, and perhaps the mPTP itself, inasmuch as PKC activation made isolated mitochondria less sensitive to Ca\(^{2+}\)--induced MPT. On the other hand, mitochondria isolated from preconditioned hearts are not less sensitive to pore opening even though they are in situ, suggesting that the effects of PKC signaling in ischemic PC may be indirect. The signaling mechanisms by which PKCe coordinates protection with these mitochondrial proteins are an exciting area for future investigation.

**MitoK\(_{\text{ATP}}\) Channels and Cardioprotection**

Ischemic PC can be mimicked pharmacologically by K\(^+\) channel openers (KCOs), which activate mitochondrial ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channels, and their protective effect on mitochondrial function after ischemia/reperfusion is well documented. Although it was initially assumed that cardioprotection by KCOs involved the energy-sparing effects of sarcolemmal K\(_{\text{ATP}}\) channels (which may still apply to rodent hearts, inasmuch as knockout of the sarcolemmal K\(_{\text{ATP}}\) channel abolishes PC), the observation that the mitoK\(_{\text{ATP}}\) channel–selective agent diazoxide was similarly protective focused attention on mitoK\(_{\text{ATP}}\) channels. In addition, ischemic PC can be prevented by the mitoK\(_{\text{ATP}}\) antagonist 5-HD, indicating that ischemic PC and pharmacological PC are closely linked. MitoK\(_{\text{ATP}}\) channels were originally characterized in lipid vesicles using flux studies and more recently have been successfully reconstituted in lipid bilayers. They are directly activated by diazoxide and blocked by 5-HD (even in the absence of ATP). However, it is important to emphasize that diazoxide and 5-HD are not completely specific for mitoK\(_{\text{ATP}}\) channels: Diazoxide inhibits succinate oxidation, 5-HD is a fatty acid derivative that can be actively metabolized, and the extent to which these effects are more important than mitoK\(_{\text{ATP}}\) channel actions in cardioprotection is controversial. Indeed, the very existence of mitoK\(_{\text{ATP}}\) channels as a distinct entity has been questioned. However, the idea that mitochondrial K\(^+\) channel activation is cardioprotective has recently been supported by the demonstration that activation of a different type of K\(^+\) channel, the mitochondrial Ca\(^{2+}\)--activated K\(^+\) channel, is also protective.

These observations have naturally led to a search for links between signaling pathways implicated in ischemic PC and mitoK\(_{\text{ATP}}\) channel activation. There is evidence that PKC activation sensitizes mitoK\(_{\text{ATP}}\) channels to activation by dia-
zoxide.\textsuperscript{72} Translocation of both PKC\textepsilon and PKC\delta to mitochondrial membranes has also been described,\textsuperscript{73,74} but whether mitoK\textsubscript{ATP} channels (whose molecular identity is still unknown) are targets of either the cardioprotective PKC\textepsilon isoform or the injury-promoting PKC\delta isoform\textsuperscript{46,50} is unknown. In contrast, sarcolemmal K\textsubscript{ATP} channels are known to be regulated by PKC.\textsuperscript{75,76} Finally, it is possible that these PKC isoforms act through other mechanisms besides mitoK\textsubscript{ATP} channel activation. For example, PKC-mediated phosphorylation of Bel-2\textsuperscript{27} and Bad\textsuperscript{52} regulates their apoptotic activities, and they could be targets of PKC in cardioprotection.

Other signaling pathways implicated in ischemic PC may also exert cardioprotective effects via mitoK\textsubscript{ATP} channels. NO has been reported to stimulate mitoK\textsubscript{ATP} channels.\textsuperscript{78} Conversely, mitoK\textsubscript{ATP} activation has been reported to stimulate mitochondrial ROS production, which may be important in its role as a trigger of ischemic PC.\textsuperscript{40,41} These studies present evidence that ROS induced by diazoxide lead to the activation of PKC, which subsequently triggers protection against subsequent prolonged ischemia. ROS bursts after preconditioning ischemic episodes may trigger protection by the same unified mechanism. MitoK\textsubscript{ATP} activation has been implicated in both trigger and mediator roles in ischemic PC, depending on the experimental model.\textsuperscript{40,41,70}

**MitoK\textsubscript{ATP} Channel Agonists and MPT**

Although it is clear that mitoK\textsubscript{ATP} channel agonists are cardioprotective and that cardioprotection is blocked by mitoK\textsubscript{ATP} channel antagonists, it is still controversial whether these drugs exert these effects via mitoK\textsubscript{ATP} channels or via other nonselective actions.\textsuperscript{66–69} Nevertheless, if the final event in mitochondrial injury is irreversible mPTP opening,\textsuperscript{7,19} then these agents must somehow influence mPTP opening, either via mitoK\textsubscript{ATP} channel activation or other effects. Experimental evidence suggests that mitoK\textsubscript{ATP} channels must stay open during ischemia and reperfusion for mitochondrial protection to occur.\textsuperscript{57,58} Accordingly, several mechanisms have been hypothesized to explain how mitoK\textsubscript{ATP} channel activation may protect against mPTP opening; these are relevant to both the MPT trigger and the MPT priming components.

The first hypothesis proposes that mitoK\textsubscript{ATP} activation induces \(\Delta \psi_m\) depolarization, reducing the driving force for \(Ca^{2+}\) uptake by mitochondria and thereby preventing mitochondrial matrix \(Ca^{2+}\) overload, a major trigger for MPT.\textsuperscript{80,81} This hypothesis was initially challenged by the observation that in energized mitochondria with normal \(\Delta \psi_m\) cardioprotective concentrations of mitoK\textsubscript{ATP} agonists that are capable of opening mitoK\textsubscript{ATP} channels (eg, 25 to 50 \(\mu\)mol/L diazoxide) had only a very modest depolarizing effect on \(\Delta \psi_m\). Subsequently, however, mitoK\textsubscript{ATP} agonists were shown to be much more effective at depolarizing \(\Delta \psi_m\) when mitochondria were studied under conditions more relevant to ischemia/reperfusion, ie, when the ability of electron transport to compensate for increased IM permeability due to mitoK\textsubscript{ATP} channel opening was limited.\textsuperscript{29} Indeed, an ideal cardioprotective agent would have little effect on normal mitochondria and exert its effects only under pathophysiological conditions. In the latter scenario, PKC activation by the phorbol ester PMA was also shown to protect against MPT by dissipating \(\Delta \psi_m\).\textsuperscript{29} Protection against MPT by both diazoxide and PMA was abolished by 5-HD, which is consistent with a direct role of PKC in modulating mitoK\textsubscript{ATP} channel function.\textsuperscript{72}

A second hypothesis, most relevant to the MPT priming component, is based on the observation that mitoK\textsubscript{ATP} channel activation causes mild mitochondrial matrix swelling, which is proposed to protect IM-OM contact sites and thereby limit adenine nucleotide depletion from the matrix.\textsuperscript{66} MitoK\textsubscript{ATP} activation thereby preserves electron transport capacity on reperfusion, which indirectly may reduce susceptibility to MPT.

A third hypothesis is that diazoxide and other KCOs protect by reducing ROS production during reoxygenation, possibly by actions unrelated to mitoK\textsubscript{ATP} activation, such as succinate dehydrogenase inhibition.\textsuperscript{67} However, diazoxide-triggered cardioprotection has also been shown to depend on its stimulation of ROS production in energized mitochondria, inasmuch as scavenging ROS eliminated cardioprotection by diazoxide.\textsuperscript{40,41}

Potentially related to the second and third hypotheses are recent observations that long-chain activated fatty acids (but not long-chain free fatty acids or short-chain [\(\leq 10\) fatty acids] both increase IM leakiness and cause MPT-independent cytochrome \(c\) loss in isolated nonenergized mitochondria.\textsuperscript{30} These effects could be prevented by substituting succrose for KCl in the extramitochondrial buffer, which is known to protect IM-OM contact sites, consistent with the second hypothesis. These effects could also be prevented by ROS scavengers, consistent with the third hypothesis, inasmuch as long-chain fatty acids are known to stimulate ROS production by mitochondria.\textsuperscript{82} Moreover, diazoxide was similarly protective against long-chain activated fatty acid–induced cytochrome \(c\) loss, and 5-HD abolished this protection.\textsuperscript{30} On the basis of observations that ROS are known to induce cytochrome \(c\) release\textsuperscript{34} and that ROS production by mitochondria is very sensitive to \(\Delta \psi_m\) dissipation by diazoxide, we postulated that modest \(\Delta \psi_m\) dissipation by diazoxide inhibited ROS production sufficiently to prevent the induction of cytochrome \(c\) loss in this setting.\textsuperscript{30}

Thus, by multiple mechanisms, diazoxide protects isolated mitochondria both against the MPT priming and MPT trigger components when studied individually. In addition, diazoxide is also protective when the MPT priming and trigger components are concurrently activated in in situ mitochondria subjected to anoxia/reoxygenation.\textsuperscript{43} In the intact heart, long-chain fatty acid accumulation\textsuperscript{38,39,85} and increased ROS production\textsuperscript{66} are well-documented consequences of acute ischemia. Therefore, it is reasonable to speculate that they may play an important role in increasing susceptibility to MPT on reperfusion by inducing cytochrome \(c\) loss and IM leakiness. This impairs the ability of mitochondria to maintain \(\Delta \psi_m\) and resist mPTP opening in the face of increased cytoplasmic free \(Ca^{2+}\) and the reperfusion-induced ROS burst.

**Mitochondria as an Excitable Medium—Death Waves?**

In addition to their roles in energy production and apoptosis, mitochondria are increasingly recognized as modulators of...
localized Ca\(^{2+}\) signaling because of their ability to store and release Ca\(^{2+}\).\(^{10}\) mPTP can be characterized as a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) channel, because under conditions in which the matrix has accumulated sufficient Ca\(^{2+}\) to trigger mPTP opening, \(\Delta \psi_m\) dissipation leads to rapid Ca\(^{2+}\) efflux. The released Ca\(^{2+}\) can then be taken up by adjacent well-polarized mitochondria via the rapid uptake mode of the Ca\(^{2+}\) uniporter, causing MPT to propagate regeneratively along the mitochondrial network, analogous to regenerative Ca\(^{2+}\) waves due to CICR from SR ryanodine receptors or ER inositol triphosphate receptors.\(^{88,89}\) Isolated mitochondria immobilized in a gel have been shown to propagate regenerative CsA-sensitive Ca\(^{2+}\) and \(\Delta \psi_m\) depolarization waves due to regenerative mPTP opening, termed mCICR waves.\(^{90}\) In addition, \(\Delta \psi_m\) depolarization and redox waves have been observed in intact isolated cardiac myocytes.\(^{91–93}\) Except for the study of Romashko et al.,\(^{92}\) these \(\Delta \psi_m\) depolarization waves were generally interpreted as passive responses to Ca\(^{2+}\) waves arising from SR CICR rather than as self-regenerative mCICR waves. However, a recent study\(^{94}\) has shown that after pretreatment with the proapoptotic agents C2 ceramide and ethanol, mCICR waves associated with \(\Delta \psi_m\) depolarization, matrix Ca\(^{2+}\) release, cytochrome c release, caspase activation, and nuclear apoptosis can be induced by Ca\(^{2+}\) loading in permeabilized and intact cardiac myotubes. These MPT waves traveled slowly (0.5 to 1 \(\mu\)m/s), required Ca\(^{2+}\) uptake by mitochondria, and were blocked by CsA, EGTA, Bcl-X\(_m\) overexpression, although they were still dependent on intact SR function.

These observations lead to the intriguing possibility that under conditions such as ischemia/reperfusion, when mitochondria are overloaded with Ca\(^{2+}\) and highly susceptible to MPT, regenerative \(\Delta \psi_m\) waves may increase the susceptibility to MPT. Further elucidation of the nature of these \(\Delta \psi_m\) depolarization waves and their relationship to cytochrome c depletion, IM leakiness, and MPT is an interesting area for future research.

**Summary and Clinical Implications**

Under well-oxygenated, energized conditions, isolated mitochondria have a tremendous capacity to accelerate electron transport in order to maintain \(\Delta \psi_m\) in response to modest changes in IM permeability due to proton leak or mitoK\(_{ATP}\) activation. The first priority of the mitochondria is to maintain \(\Delta \psi_m\), as indicated by their ready conversion from ATP producers to ATP consumers in a futile attempt to maintain \(\Delta \psi_m\) when challenged with potent protonophores. During ischemia, however, mitochondria are neither well oxygenated nor provided with abundant substrates, and their ability to accelerate electron transport to compensate for increased IM permeability is greatly reduced. Under these conditions, separable MPT priming and MPT trigger components that determine susceptibility to mitochondrial injury via MPT can be identified. We postulate that these elements contribute to ischemia/reperfusion injury in the following fashion: Ischemia sets the threshold for injury via MPT, and conditions during reperfusion provide the trigger. In addition to its negative impact on overall energy production, MPT promotes the release of proapoptotic signaling molecules from the intermembrane space and amplifies injury by triggering apoptosis.

From the above-described evidence, therapeutic interventions designed to prevent mPTP opening during ischemia/reperfusion hold major promise as a novel strategy for reducing cardiac injury from ischemia and reperfusion. Because ischemic heart disease remains the leading cause of death in western societies, effective therapies developed along these lines will represent a major advance in health care.

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**References**


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