Primary and Secondary Signaling Pathways in Early Preconditioning That Converge on the Mitochondria to Produce Cardioprotection

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Abstract—Cardioprotective mechanisms such as acute or early preconditioning activate several primary signaling pathways that seem to converge on mitochondrial targets, leading to altered cell metabolism and inhibition of apoptosis. Acute preconditioning leads to generation of agonists, which bind to G protein–coupled receptors, and initiates a signaling cascade that involves activation of phosphoinositide-3-kinase, endothelial NO synthase, protein kinase C, glycogen synthase kinase 3β, mitogen-activated protein kinases, and other signaling pathways. Activation of these signaling pathways along with generation of reactive oxygen species leads to alterations in the activity of key mitochondrial proteins such as mitochondrial ATP-sensitive K+ channels, the mitochondrial permeability transition pore, and bcl-2 family members. Alterations in these mitochondrial proteins results in altered metabolism and inhibition of cell death, thus resulting in cardioprotection. (Circ Res. 2004;94:7-16.)

Key Words: apoptosis • cardioprotection • signaling pathways • preconditioning • mitochondria

Over the years, several cardioprotective drugs and interventions have been described and studied. A major goal of this research effort has been to understand the mechanisms involved in cell death so that one can intervene to block cell death. The recent recognition of the role of apoptosis, a regulated form of cell death, in cardiac cells has enhanced the expectation that one can pharmacologically intervene to block or modulate cell death. Cell death is a primary factor in the pathogenesis of ischemia/reperfusion, as well as in the myocyte loss associated with heart failure. Most studies in cardioprotection have focused on reducing myocyte death during an acute episode of ischemia and reperfusion. Many drugs have been shown to be cardioprotective when given before ischemia1–4; however, because patients typically present after the onset of ischemia, many of these drugs are of limited clinical benefit. There is an obvious clinical need for drugs that reduce cell death and dysfunction when given at the time of reperfusion. There are a few reports that addition of cardioprotective agents at the immediate onset of reperfusion can reduce infarct size.6–10 These drugs may offer promise when given with angioplasty-associated reperfusion. It has also been suggested that a gene therapy approach to upregulate cardioprotective genes is a plausible strategy for protecting at-risk populations.11,12 A better understanding of the signaling mechanisms in cardioprotection and how they ultimately lead to reduced cell death will enhance our ability to make cardioprotection a useful clinical strategy.

This review focuses on the signaling pathways involved in cardioprotection. Cardioprotection can be divided conceptually into cardioprotection that requires new transcription or
translational and cardioprotection that occurs as a result of addition of a drug or intervention that modulates cell metabolism or signaling pathways directly or via posttranslational modification of proteins. This review focuses on acute cardioprotection, which does not require new protein synthesis. As mentioned, there are many drugs or agents that have been reported to be cardioprotective. These drugs initiate cardioprotection by modulating diverse signaling pathways. However, there is emerging evidence that these diverse signals converge on a few final common effectors or signaling pathways that ultimately ameliorate cell death (see the Figure). With analogy to the apoptosis pathway, activation of effector caspases are the mediators of apoptosis; however, many different signaling pathways can lead to caspase activation. Thus, this review is divided into two parts. The first part reviews some of the common primary signaling pathways or mediators of cardioprotection, and the second part focuses on how these primary signals may converge on secondary cardioprotective signals or the end effectors of cardioprotection.

**Primary Signaling Pathways in Cardioprotection**

This review focuses on cardioprotection related to signaling pathways resulting in posttranslational modification. Acute cardioprotective agents can protect either by decreasing the development of injury or by attenuating cell death pathways. Some approaches, such as reducing temperature, mediate cardioprotection by decreasing metabolism and the consumption of ATP. On the other hand, such effects may lead to protection by attenuating mechanisms of cell death. Most cardioprotective agents work by both decreasing the development of injury (eg, preserving ATP) and by inhibition of cell death mechanisms. For example, limiting calcium overload may serve both to conserve ATP and reduce mitochondrial-induced apoptosis. Drugs such as Na+/H+ exchange inhibitors reduce the development of injury if given before ischemia. Na+/H+ exchange inhibitors reduce the ischemia-reperfusion-induced rise in intracellular Na+ and intracellular Ca2+. The reduction in the rise in Ca2+ would be expected to reduce Ca2+ activation of proteases and to reduce ATP degradation related to stimulation of Ca2+ ATPases. The reduction in Ca2+ would also reduce Ca2+ accumulation by intracellular organelles such as sarcoplasmic reticulum and mitochondria, and decreased mitochondrial Ca2+ release would mediate cardioprotection via a reduction in mitochondrial-dependent apoptosis. Activation of the phosphoinositide-3-kinase (PI3K) or protein kinase C (PKC) pathways seems to protect primarily by inhibiting cell death mechanisms, although alterations in cell metabolism and energy utilization may also play a role.

Many of the cardioprotective signaling pathways have been elucidated through studies of preconditioning. Brief intermittent periods of ischemia and reperfusion, referred to as preconditioning (PC), have been shown to reduce injury during a subsequent sustained period of ischemia. The Figure shows signaling pathways involved in PC.

**Overview of PC Signaling**

Considerable data suggest that PC leads to release of hormones or agonists that bind to receptors and activate signaling pathways. Released factors include adenosine, opioids, and bradykinin. These agonists bind to G protein–coupled receptors and activate signaling pathways, and most of these agonists activate PI3K, which generates phosphoinositides that localize kinases, such as phosphoinositide-dependent kinase (PKD), with substrates, leading to activation of downstream kinases such as protein kinase B (PKB, also known as Akt) and p70S6 kinase (p70S6K). PI3K is also important in receptor internalization and endosomal signaling, which, as discussed below, may play a role in PC. Activation of PI3K has been reported to be upstream of PKC, GS3Kβ, p70S6K, generation of NO, and activation of mitochondrial ATP-sensitive K channels (mitoKATP). However, Qin et al have shown that different signaling pathways can vary somewhat depending on the agonist. Details of these signaling elements are discussed.
**G Protein–Coupled Receptor**

Administration of many G protein–coupled receptor (GPCR) agonists, especially those that signal via Go, such as adenosine, opioids, bradykinin, and acetylcholine, has been shown to reduce infarct size.1,2,4,19–22 Furthermore, inhibition of Go signaling by pertussis toxin (PTX) treatment blocks the protection afforded by PC in rabbits18 and in some28 but not all studies in rats.29 Activation of GPCR leads to signaling via Go and Gβγ. As discussed below, signaling through Gβγ can lead to activation of PI3K23 and also recruits G protein receptor kinase, which leads to receptor internalization. Activation of GPCR can also lead to activation of the mitogen-activated protein kinase (MAPK) pathway via G arrestin–dependent signaling, by transactivation of the epidermal growth factor (EGF) receptor, or via β-arrestin–dependent endosomal signaling.30 Pertussis toxin inhibition of PC suggests a possible role for Go, mediated signaling in PC. Go has been shown to inhibit adenylyl cyclase or to lead to activation of membrane channels.

**PI3K**

Activation of the PI3K pathway has been shown in many models to be cardioprotective.7,16,22,24,31–35 PC has been shown to lead to activation of PKB and p70S6K, kinases downstream of PI3K, consistent with a role for PI3K in PC.24,31 Furthermore, inhibitors of PI3K, such as wortmannin and LY294002, attenuate the protection afforded by PC.24,31,33

How is PI3K activated? GPCR can activate PI3K via release of Gβγ, which activates PI3Kγ.36 To test whether this mechanism was responsible for PC-induced activation of PI3K, studies were performed using mice with a cardiac-specific expression of βARKct, a peptide that sequesters Gβγ.37 Expression of βARKct inhibits Gβγ–dependent signaling and has been shown to inhibit GPCR activation of PI3Kγ.23 Using hearts from βARKct mice, Tong et al38 reported that sequestration of Gβγ blocks PC; however, sequestering Gβγ did not block PC-induced activation of kinases downstream of PI3K, such as PKB and p70S6K. These data suggest that activation of PKB and p70S6K kinase are not sufficient for protection; however, the data do not rule out the possibility that activation of these signals may be required.

Krieg et al22 have recently reported that acetylcholine-induced protection leads to activation of the PI3K pathway via GPCR transactivation of the EGF receptor. Krieg et al22 reported that acetylcholine-induced phosphorylation of PKB was blocked by the src inhibitor PP2 and the EGF receptor inhibitor AG-1478. Sequestration of Gβγ has been reported to inhibit transactivation of the EGF receptor.30 If this is the case in PC, one would expect that expression of βARKct would block transactivation of the EGF receptor and subsequent activation of PI3K. Additional studies will be necessary to resolve the precise mechanism of activation of PI3K in PC. It is worth noting that hydrogen peroxide has been reported to activate PI3K,39 suggesting that ROS may play a role in PI3K activation in PC.

What is the role of Gβγ in PC? Gβγ is known to be required for receptor internalization and associated endosomal signaling, and there are emerging data suggesting that inhibition of endosomal trafficking blocks activation of cytosolic extracellular-regulated kinase (ERK).30,40,41 Recent studies have shown also that inhibition of endosomal trafficking blocks PC.38

What are the signals downstream of PI3K and how does activation of PI3K mediate cardioprotection? Because there are no inhibitors of PKB, it is unclear whether activation of PKB, which has been shown consistently to accompany PC, is required for PC. The data showing that sequestration of Gβγ blocked PC-induced protection, but not activation of PKB or p70S6K, suggest that activation of PKB and p70S6K is not sufficient for protection.36 However, PKB is reported to phosphorylate and modulate several pathways involved in cardioprotection, suggesting that it may have a role in PC, even though its activation is not sufficient to induce PC. PKB phosphorylates and activates endothelial NO synthase (eNOS) and p70S6K and phosphorylates and inactivates GSK3β and the proapoptotic BAD (phosphorylation of BAD causes it to be sequestered by 14–3–3).32 NO and eNOS have been shown to be important in PC.43,44 NO has been shown to activate the mitoKATP channel.45 There are also data showing a role for GSK3β, a kinase downstream of PKB, in preconditioning and cardioprotection.25 GSK3β is phosphorylated and inactivated by PKB. Expression of a mutant GSK that cannot be phosphorylated blocked the antiapoptotic action of PI3K.46 Phosphorylation of GSK3β has been shown to occur during PC; thus, PC would lead to inactivation of GSK3β. Consistent with a role for inhibition of GSK3β in cardioprotection, Tong et al25 showed that pharmacological inhibition of GSK3β with SB216763 reduced infarct size and improved recovery of postischemic function. Opioid protection has also been shown to involve GSK3β.47 The targets downstream of GSK-3β in PC remain to be elucidated, but GSK-3β has been reported to mediate the antiapoptotic actions of PI3K.46

Activation of PI3K was also shown to be upstream of PKC.25 Wortmannin was shown to block the PC-induced translocation of PKCε. Furthermore, the protective effect of pharmacological activation of PKC with DOG was not blocked by inhibitors of PI3K. Also consistent with activation of PKC downstream of PI3K, DOG did not enhance phosphorylation of PKB.

P70S6K, another kinase downstream of PI3K, has also been reported to be involved in PC.36 PDK1 phosphorylation of p70S6K on thr 229 is required for activation of P70S6K (reviewed by Murphy et al48). Prior phosphorylation of thr 389 by mammalian target of rapamycin (mTOR) is necessary before PDK1 can phosphorylate thr 229. PKB is also reported to phosphorylate p70S6K. As discussed below, p70S6K may integrate signals from several diverse pathways.

**PKC**

Numerous studies have demonstrated a role for PKCε in cardioprotection and PC.5,49–54 Ping et al50 demonstrated that PC leads to translocation of PKCe. Inhibition of PKC has been shown by several investigators to block the protection afforded by PC, and pharmacological activators of PKC have been shown to be cardioprotective.5,49,52,53 Transgenic mice with cardiac-specific overexpression of PKCε or expression
of an activator of PKCe have been shown to exhibit endogenous protection.\textsuperscript{51,54,55} Taken together, there is strong evidence suggesting a role for PKC in PC.

Several mechanisms have been suggested to be involved in PC-induced activation of PKC. Reactive oxygen species (ROS), which are generated by PC, have been reported to be involved in activation of PKC.\textsuperscript{56} As discussed above, inhibition of PI3K blocks translocation of PKCe, suggesting a role for PI3K-dependent signaling. The precise role of PI3K in activation of PKC has not been determined. Phosphoinositide products have been reported to activate several isoforms of PKC.\textsuperscript{57} Also, Ping et al\textsuperscript{44} have reported that NO is involved in PC-induced translocation of PKCe; they also report that NO donors lead to translocation of PKCe and that inhibitors of NO, such as L-NAME, block the PC-induced translocation of PKCe. Because inhibition of PI3K was shown to reduce generation of NO,\textsuperscript{24} these data would be consistent with PI3K activation of PKCe via an eNOS-mediated mechanism.

Considerable effort has been directed toward elucidating the downstream targets of PKC in cardioprotection. PKC activation has been shown to be important in activation of the mitoK\textsubscript{ATP} channel.\textsuperscript{58} PKCe also forms a complex with components of the mitochondrial permeability transition (MPT) pore.\textsuperscript{17} However, the association of PKCe with components of the MPT does not demonstrate that this association is important in modulating the MPT or in cardioprotection. Additional studies are need to fill in the details. Baines et al\textsuperscript{33} have additionally reported that ERK and PKCe are contained in a multimeric mitochondrial signaling complex and that PKCe may lead to activation of ERK in this complex. It will be of interest to determine whether ERK has a role in activation of the mitoK\textsubscript{ATP} channel. PKC activation has also been shown to be upstream of 12-LO signaling, and both are upstream of mitoK\textsubscript{ATP}.\textsuperscript{59}

**ERK**

Most\textsuperscript{38,60–62} but not all\textsuperscript{63} investigators report that PC leads to activation of ERK. However, there are conflicting data regarding the role of ERK in preconditioning. Strohm et al\textsuperscript{62} reported that inhibition of the ERK pathway with PD98059 blocked PC, and Fryer et al\textsuperscript{69} similarly reported that PD98059 blocked protection by PC or opioid-induced protection.\textsuperscript{19} In contrast, Mocanu et al\textsuperscript{31} reported that PD98059 did not block PC-induced protection. Johassen et al\textsuperscript{7} have suggested that inhibition of ERK on reperfusion blocks cardioprotection.

How are the ERKs activated? Ping and colleagues\textsuperscript{61,64} reported that PC-induced phosphorylation of ERK is mediated by PKC. Gross et al\textsuperscript{65} report that delay preconditioning initiated by K\textsubscript{ATP} channel openers is abolished by inhibition of the ERK pathway using the MEK1/2 inhibitor PD 98059, suggesting that K\textsubscript{ATP} channel opening can lead to activation of ERK, likely secondary to ROS generated by opening of the mitoK\textsubscript{ATP} channel. Tong et al\textsuperscript{18} showed that ERK phosphorylation is blocked by sequestering G\textsubscript{8y}, inhibition of PI3K, or inhibition of endosomal trafficking, consistent with a role for receptor-mediated endosomal activation of ERK. Endosomal activation of ERK is not inconsistent with ERK activation by PKC, and it is also possible that endosomal-dependent signals lead to mitochondrial targeting of ERK. Regarding the mechanism by which the ERKs mediated protection, Baines et al\textsuperscript{64} suggested that ERK is part of a mitochondrial complex involved in phosphorylation of BAD.

**p70S6K**

Kinases from different pathways such as PI3K, PDK1, PKC, ERK, mTOR, and PKB are involved in the phosphorylation and activation of p70S6K. p70S6K therefore can integrate signals from diverse pathways. Kis et al\textsuperscript{26} recently reported that inhibition of PI3K or inhibition of mTOR with rapamycin blocked the second window or delayed PC. Johassen et al\textsuperscript{7} have reported that insulin administered at reperfusion is cardioprotective and that rapamycin abolished this protection. The role of p70S6K in acute PC has not been addressed.

**p38 MAPK**

The role of p38 MAPK in PC has been controversial and has been recently reviewed elsewhere.\textsuperscript{66} Briefly, there are data suggesting that inhibition of p38 MAPK with SB202190 or SB203580 blocks the protection afforded by PC; however, there are also data showing that p38 MAPK inhibitors do not block PC. Furthermore, some groups find that in the absence of PC, inhibition of p38 MAPK before and during ischemia is cardioprotective, whereas others find inhibition of p38 MAPK to have no effect. The reasons for these differences are not clear and are discussed elsewhere.\textsuperscript{66}

**12-Lipoxygenase**

12-Lipoxygenase (12-LO) metabolites have been reported in neuronal cells to activate K\textsubscript{ATP} channels.\textsuperscript{57} This observation led to studies to examine the role of 12-LO in PC. 12-LO metabolites are generated by PC, and inhibitors of 12-LO block the protection afforded by PC.\textsuperscript{59,68} Furthermore, transgenic mice that are null for the leukocyte 12-LO are not protected by PC.\textsuperscript{69} 12-LO has also been shown to be upregulated in an opioid model of PC.\textsuperscript{70} The 12-LO pathway has been shown to be downstream of PKC and upstream of mitoK\textsubscript{ATP}.\textsuperscript{59}

**Linear Versus Converging Pathways**

Several signaling molecules seem to be necessary for the protection afforded by PC (see the Figure). For example, there are data suggesting that adenosine, opioids, G\textsubscript{8y}, PI3K, PKC, 12-LO, ERK, NOS, GSK3\textbeta, and mitoK\textsubscript{ATP} channels are all required for acute PC. Although the signaling pathway in acute PC is frequently presented as linear, the data are consistent with branching and converging pathways, as illustrated in the Figure. There seem to be redundant mechanisms to activate some key molecules, such as PKC. For example, PKC may be activated by ROS as well as by PI3K/eNOS, and different pathways may predominate under different conditions. The diverse pathways seem to converge on mitochondrial signals that integrate signals from different pathways. However, the diverse pathways could also act on additional targets, and the protection afforded by PC may require the modulation of multiple targets, such as inhibition of cell death, alterations in the cytoskeleton to prevent plasma membrane rupture, and reduction in the rate of ATP utiliza-
tion. Also, PC activates signaling pathways that lead to the second window or delayed PC, and additional signaling pathways, such as inducible NOS, COX2, nuclear factor-κB, and JAK/STAT have been identified as components of the second window or delayed PC. In most cases, the role of these pathways in acute PC has not been established.

Compartmentation
Emerging data indicate that localization of signaling molecules is a key component in the phenotype of the signal. It has been recognized that PKC translocates to different compartments and that the signaling phenotype is dependent on the location of receptors for C kinase. Similarly, A-kinase anchoring proteins regulate and localize signaling for protein kinase A (PKA). Furthermore, it has been shown that global measurements of cAMP are not a reliable indicator of localized activation of PKA. Agonists can activate localized PKA and phosphorylate localized targets without a global increase in cAMP. Conversely, a global increase in cAMP does not always lead to PKA-dependent phosphorylation of all PKA targets in the cell. Also, recent data show that angiotensin 1a receptor signaling leads to activation of functionally distinct pools of ERK. Angiotensin II leads to activation of nuclear ERK via Gq/11-dependent signaling and activation of cytosolic ERK via β-arrestin signaling. Consistent with the importance of localized signaling, Ping and coworkers and Arrell et al have shown a role for large signaling complexes in PC. For example, Baines et al have reported that PKCe forms a complex with MAPK as well as a complex with voltage-dependent anion channel (VDAC), hexokinase II (HKII), and ANT1. Arrell et al have used a proteomic approach to show altered posttranslational modifications associated with addition of adenosine. Taken together, these data suggest that measurements of total cell changes in a signaling pathway will not necessarily reflect localized changes in signaling pathways and that the signaling in localized compartments is likely to be important in cardioprotection.

In summary, there are several signaling pathways that are involved in initiating cardioprotection. There seem to be redundant and multiple signaling branches that converge on common signaling pathways in the mitochondria. There may also be additional targets. Certainly the second window or delayed PC involves transcription factors that act on the nucleus to upregulated cardioprotective genes. Localized signaling is likely to be important, and these localized signals may not be reflected in a global measurement. It is also possible that there may be species variations in some of the signaling pathways or in their relative importance, and this may account for some of the discrepancies in the literature.

End Effectors of Cardioprotection
Cardioprotection is ultimately mediated by mechanisms that reduce cell death, both apoptotic and necrotic. Thus, the end effectors or secondary signaling pathways must somehow reduce cell death. Necrotic cell death is characterized by loss of plasma membrane integrity, whereas apoptotic cell death typically occurs initially with an intact plasma membrane via mitochondrial or FAS receptor–mediated activation of the caspase pathway. We will consider pathways that inhibit apoptosis as well as necrosis, because cardioprotective mechanisms seem to reduce death by both pathways. Thus, the cardioprotective signaling pathways must ultimately lead to secondary signals that maintain plasma membrane integrity and inhibit cell death. Agents that inhibit apoptosis, such as bcl-2 overexpression, also inhibit necrosis.

This is not surprising, because mitochondria are the primary organelles involved in ATP production, which is important in maintaining cell integrity, and mitochondria are also key players in apoptosis. There are data suggesting a role for many mitochondrial proteins as end effectors; the mitoKATP channel, apoptotic proteins such as BAD, and the mitochondrial permeability transition pore are all possible candidates as end effectors. Because of their common mitochondrial location, it is possible that the mechanisms involved in the regulation of these different mitochondrial proteins are related. As discussed, it is also possible that there are nonmitochondrial targets. For example, cytoskeletal or membrane proteins would be plausible targets, because they might help maintain cell integrity. In fact, cardioprotection may be mediated by the integration of multiple pathways rather than a final end-effector. For example, cardioprotection or preconditioning could modulate several targets, both mitochondrial and nonmitochondrial, to achieve protection. This review focuses on the mitochondrial targets.

MitoKATP
There are considerable data, which will be reviewed as part of this thematic series (O’Rourke, Evidence for Mitochondrial K+ Channels and Their Role in Cardioprotection), suggesting that activation of the mitoKATP channel is important in preconditioning and other mechanisms of cardioprotection. Selective activators of the mitoKATP channel that do not open the plasma membrane K ATP channel have been shown to be protective, and selective mitoKATP channel inhibitors block the protection afforded by PC. Several recent publications have questioned whether the cardioprotective effects of diazoxide are mediated via the mitoKATP channel. Diazoxide also inhibits succinate dehydrogenase (SDH), and it has been suggested that this may initiate cardioprotection, perhaps via generation of ROS. As discussed in detail elsewhere, the observation that other K+ channel activators that do not inhibit SDH are cardioprotective argues against diazoxide protection being mediated by SDH inhibition.

The mechanism by which activation of the mitoKATP results in cardioprotection is not well understood. Three mechanisms have been suggested by which activation of mitoKATP could reduce cell death. These include inhibition of mitochondrial calcium uptake, regulation of mitochondrial volume that could alter mitochondrial permeability of the VDAC or the MPT, and modulation of ROS.

Inhibition of Mitochondrial Ca2+ Uptake
Holmuhamedov et al showed that addition of K+ channel openers to isolated cardiac mitochondria depolarized the mitochondrial inner membrane and reduced mitochondrial Ca2+ influx. They additionally showed that pinacidil activated
release of Ca\(^{2+}\) from the mitochondria. Murata et al\(^{93}\) reported that diazoxide attenuated the accumulation of mitochondrial Ca\(^{2+}\) during simulated ischemia and reperfusion in rabbit ventricular myocytes, using rhod-2 to measure mitochondrial Ca\(^{2+}\). Ishida et al\(^{94}\) reported that diazoxide depolarized the mitochondrial membrane and attenuated ouabain-induced calcium overload in mitochondria in rat ventricular myocytes. These studies support a role for altered mitochondrial Ca\(^{2+}\) overload in mitochondria in rat ventricular myocytes. However, in these studies, one cannot distinguish whether diazoxide reduced injury, which in turn reduced mitochondrial Ca\(^{2+}\) overload, or whether a primary action of diazoxide was to reduce Ca\(^{2+}\) overload, which in turn reduced injury.

**Generation of ROS**

There are data showing that addition of diazoxide results in generation of ROS.\(^{95,96}\) Furthermore, inhibition of diazoxide-induced ROS by addition of antioxidants blocks the protection afforded by diazoxide, suggesting that the ROS generated by the \(\text{K}_{\text{ATP}}\) channel are required for protection.\(^{95,96}\) An alternative interpretation to consider is that an oxidized redox state may be important for the binding of diazoxide to the mito\(\text{K}_{\text{ATP}}\) channel, because the channel can be regulated by redox.\(^{97,98}\) Although most investigators find that diazoxide results in an initial increase in ROS generation, there are data suggesting that diazoxide addition to mitochondria reduced generation of ROS at reperfusion and improved ADP-dependent oxygen consumption.\(^{99}\) Interestingly, Ozcan et al\(^{99}\) report that the effects of \(K^+\) channel openers on ROS production were maintained in nominally \(K^+\)-free medium, suggesting that diazoxide is modulating reperfusion ROS by a mechanism that is independent of \(K^+\) channel activity. These studies were performed on isolated mitochondria, and it is possible that these isolated mitochondria are in a different state than in situ mitochondria (see the study by Dos Santos et al\(^{100}\)).

**Mito\(\text{K}_{\text{ATP}}\) andVDAC**

Dos Santos et al\(^{100}\) have suggested that opening the mito\(\text{K}_{\text{ATP}}\) channel results in slight mitochondrial swelling, which maintains mitochondrial structure and thus maintains VDAC in a low permeability state. Reduced VDAC permeability would reduce ATP entry into the mitochondria during ischemia and therefore reduce consumption of glycolytically generated ATP by the F1-F0 ATPase. However, there are recent data that report that the changes in mitochondrial volume do not correlate with protection.\(^{91}\) This is an important area in which future studies are needed. VDAC, also known as porin, is an abundant protein in the outer mitochondrial membrane that forms a channel, allowing passage of small metabolites such as ATP and ADP. In vitro, VDAC has been shown to decrease conductance when either a positive or negative potential is applied.\(^{101}\) VDAC in concert with the adenine nucleotide transporter (ANT) controls transport of adenine nucleotides into and out of the mitochondria. Mitochondrial generation of ATP occurs via the F1-F0 ATPase, which uses the proton gradient generated by electron transport to provide the driving force for converting ADP to ATP.\(^{102}\) Cell energetics depend on the appropriate transport of ADP into the mitochondria and the transport of ATP to the cytosol.

The hypothesis that activation of the mito\(\text{K}_{\text{ATP}}\) channel induces protection via modulation of VDAC is interesting in light of a recent study suggesting that overexpression of bcl-2 modulates cardioprotection via inhibition of VDAC.\(^{103}\) Cardiac-specific overexpression of bcl-2 has been shown to reduce myocyte death after ischemia and reperfusion.\(^{79,104}\) Bcl-2 overexpression, in addition to reducing ischemia-reperfusion injury, also reduced the rate of decline in ATP during ischemia and reduced ischemic acidification; these data are consistent with bcl-2-mediated inhibition of consumption of glycolytically generated ATP.\(^{103}\) During ischemia, when the lack of oxygen inhibits mitochondrial electron transport and mitochondrial generation of ATP, the F1-F0 ATPase can run in reverse and consume glycolytically generated ATP.\(^{105,106}\) The reduction in acidification and the rate of decline in ATP during ischemia was dependent on the activity of the mitochondrial F1-F0 ATPase in wild-type hearts but not in bcl-2-overexpressing hearts,\(^{103}\) suggesting that the bcl-2-induced reduction in the ischemic decline in ATP and pH was mediated by consumption of ATP via the F1-F0 ATPase. Bcl-2–mediated inhibition of consumption of glycolytically generated ATP could be accomplished by blocking ATP entry into the mitochondria by inhibition of VDAC or ANT or by direct inhibition of the F1-F0 ATPase. Consistent with a role for VDAC, there was an increased association of VDAC and bcl-2 in hearts overexpressing bcl-2.\(^{103}\) It is interesting that previous studies have ruled out a role for altered F1-F0 ATPase activity in the protection afforded by PC.\(^{107}\)

Emerging data suggest that proapoptotic family members such as BAX enter the outer mitochondrial membrane and form a large conductance channel that allows release of cytochrome \(c\).\(^{108-110}\) The precise mechanism by which BAX mediates release of cytochrome \(c\) is not well understood but has been suggested to involve channels formed by BAX or by BAX in association with other mitochondrial proteins, such as VDAC, or MPT, which is suggested to be composed of the ANT, VDAC, and cyclophilin. It has been suggested that antiapoptotic family members such as bcl-2 oppose release of cytochrome \(c\) either by binding and sequestering proapoptotic members\(^{108}\) or by binding to proteins such as VDAC and blocking the formation or opening of a cytochrome \(c\) release pathway.\(^{109,110}\) Bcl-2 is localized to the outer mitochondrial membrane, and bcl-2 family proteins have been reported to interact with VDAC.\(^{109}\)

Similar to the effects of bcl-2 overexpression, inhibition or reduction of VDAC activity might explain many of the changes associated with preconditioning, such as the reduced rate of decline in ATP, reduced acidification, and reduced cell death. Diazoxide addition has also been reported to reduce acidification during ischemia.\(^{95}\) and perhaps this effect is mediated via \(\text{K}_{\text{ATP}}\)-dependent closure of VDAC, consistent with the suggestion of Dos Santos et al.\(^{100}\) Dos Santos et al attributed the diazoxide-induced decrease in permeability of the mitochondria to ADP to the confirmation of VDAC conferred by diazoxide-induced mitochondrial swelling. However, in conflict with this hypothesis, Lim et al\(^{91}\) did not find diazoxide-induced mitochondrial swelling.
HKII has also been reported to bind to and reduce permeability of VDAC.\textsuperscript{17,111} Elevated glucose-6-phosphate (G-6-P) occurs during ischemia, which would reverse HKII closure of VDAC, and MPT. Interestingly, both PC and diazoxide have been reported to decrease G-6-P levels during ischemia,\textsuperscript{89} providing another mechanism by which PC and diazoxide might reduce permeability of VDAC. Future studies will be needed to prove or disprove this speculation. Diazoxide has also been reported to reduce apoptosis,\textsuperscript{86} and this could be mediated by closing of VDAC as part of the MPT. Alteration of VDAC by mitoK\textsubscript{ATP} or bcl-2 family members (eg, ERK phosphorylation of BAD) could conserve ATP levels during ischemia, preserve mitochondrial function, and inhibit cell death on reperfusion, adding up to cardioprotection. VDAC has also recently been reported to modulate mitochondrial Ca\textsuperscript{2+} uptake. Thus, decreased VDAC activity might explain decreased mitochondrial Ca\textsuperscript{2+} uptake observed with diazoxide.\textsuperscript{92,93} It is also interesting that VDAC is related to a bacterial channel that closes under conditions of cell stress. Thus, the closing of VDAC and other mitochondrial modifications may be evolutionarily conserved from bacteria.

**Phosphorylation of BAD or Modification of MPT**

BAD is a proapoptotic protein that binds to and sequesters bcl-2, such that bcl-2 can no longer inhibit apoptosis. Phosphorylation of BAD causes it to be bound to 14-3-3, thereby freeing bcl-2 to inhibit apoptosis. Baines et al\textsuperscript{64} have reported that mice overexpressing PKC\textepsilon contain a mitochondrial localized signaling complex containing PKC\textepsilon and MAPK; this signaling complex was associated with increased mitochondrial phosphorylation of BAD. Baines et al\textsuperscript{17} have also reported that PKC\textepsilon forms a complex with components of the MPT, including VDAC, ANT, and HKII. Modulation of the MPT by phosphorylation or complex formation is another potential end effector (see the study by Weiss et al\textsuperscript{85}). However, whether the association of these protein regulates pore opening requires additional study.

Using isolated mitochondria, Korge et al\textsuperscript{112} have reported that diazoxide prevents opening of the MPT induced by Ca\textsuperscript{2+}. Addition they additionally reported that PKC activation mimicked the effect of diazoxide on the MPT and was blocked by 5HD. Furthermore, VDAC is a component of the MPT; therefore, if mitoK\textsubscript{ATP} channel opening leads to inhibition of VDAC, this could promote inhibition of the MPT and might provide a mechanism by which diazoxide inhibits cell death.

**Nonmitochondrial Targets**

As discussed, there are strong data supporting several mitochondrial targets as the end effectors of PC. It is possible that these mitochondrial targets mediate all the effects of cardioprotection, because mitochondria have a key role in regulating cell death and ATP generation, key aspects of cardioprotection. It is also possible that the same signaling kinases that modify mitochondrial proteins also modify nonmitochondrial proteins and modulate other aspects of cell metabolism. Notably, PC has been reported to alter connexins\textsuperscript{113,114} and Ca\textsuperscript{2+} handling by sarcoplasmic reticulum.\textsuperscript{115,116} PC has been abolished by cytochalasin D,\textsuperscript{117} suggesting a role for actin cytoskeleton; however, neither vinculin\textsuperscript{118} nor a\textsubscript{B} crystallin\textsuperscript{119} seem to be involved in PC. Furthermore, PC is known to result in acute and delayed cardioprotection, and the delayed cardioprotection involves upregulation of gene function; this likely occurs via PC-induced signaling pathways that do not directly involve the mitochondria. By analogy with hormone agonist signaling, it maybe naive to assume that there is only one end effector. In fact, it is likely that PC has pleiotropic effects that are mediated via different end effectors. However, activation of the mitoK\textsubscript{ATP} channel with diazoxide has been shown to be as protective as PC, suggesting that the mitoK\textsubscript{ATP} channel may be the primary end effector. Alternatively, the mitoK\textsubscript{ATP} channel could function as a trigger as well as an end effector, consistent with data showing that opening mitoK\textsubscript{ATP} generates ROS, which can trigger protective signaling pathways, thereby explaining why mitoK\textsubscript{ATP} channel openers induce full protection.

**How Do Primary Signals Lead to Activation of Secondary Signals or the End Effector?**

In acute cardioprotection, the mechanisms by which activation of the trigger/mediator signaling pathways lead to activation of the end effectors, such as the mitoK\textsubscript{ATP} channel or other mitochondrial proteins, must involve posttranslational modification. Because many of the primary signaling pathways involve kinases, it is likely that phosphorylation plays a role. NOS is also activated by PC; therefore, nitrosylation or nitration of proteins could also be involved. In fact, both PKC and NO have been reported to regulate the activity of K\textsubscript{ATP} channels. Redox modulation of thiol groups could also be involved. Alterations in macromolecular complexes are also likely to modulate the function of mitochondrial proteins. Baines et al\textsuperscript{17} have reported that PKC\textepsilon interacts with VDAC, ANT, and HKII. They additionally showed that overexpression of PKC\textepsilon increased the formation of this complex, and they presented in vitro data showing that PKC\textepsilon can phosphorylate VDAC. These macromolecular complexes may modulate function directly or may modulate function via phosphorylation or other posttranslational modifications. VDAC activity has been shown to be regulated by binding to ANT, HKII, and PKB.\textsuperscript{120}

In summary, cardioprotective mechanisms such as PC activate several signaling pathways that seem to converge on a few mitochondrial targets, leading to altered cell metabolism and inhibition of apoptosis. Future studies will determine whether there are other end targets in addition to the mitochondria.

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**References**


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