Mechanisms Underlying Acute Protection from Cardiac Ischemia-Reperfusion Injury

Elizabeth Murphy[1] and **Charles Steenbergen**[2]

[1]Vascular Medicine Branch, NHLBI, NIH, DHHS, Bethesda, MD

[2]Johns Hopkins Medical Institute, Baltimore, MD

Abstract

Mitochondria play an important role in cell death and cardioprotection. During ischemia, when ATP is progressively deleted, ion pumps cannot function resulting in a rise in calcium (Ca^{2+}), which further accelerates ATP depletion. The rise in Ca^{2+} during ischemia and reperfusion leads to mitochondrial Ca^{2+} accumulation, particularly during reperfusion when oxygen is reintroduced. Reintroduction of oxygen allows generation of ATP; however damage to electron transport chain results in increased mitochondrial generation of reactive oxygen species (ROS). Mitochondrial Ca^{2+} overload, and increased ROS can result in opening of the mitochondrial permeability transition pore, which further compromises cellular energetics. The resultant low ATP and altered ion homeostasis result in rupture of the plasma membrane and cell death. Mitochondria have long been proposed as central players in cell death, since the mitochondria are central to synthesis of both ATP and ROS and since mitochondrial and cytosolic Ca^{2+} overload are key components of cell death. Many cardioprotective mechanisms converge on the mitochondria to reduce cell death. Reducing Ca^{2+} overload and reducing ROS have both been reported to reduce ischemic injury. Preconditioning activates a number of signaling pathways that reduce Ca^{2+} overload and reduce activation of the mitochondrial permeability transition pore. The mitochondrial targets of cardioprotective signals will be discussed in detail.

I. Introduction

Myocardial cell death due to ischemia-reperfusion is a major cause of morbidity and mortality in western nations. In the past few decades, it has become clear that the myocardial response to ischemia-reperfusion can be manipulated to delay injury, which has motivated intense study of the mechanisms of cardioprotection. The cardioprotective strategy of ischemic preconditioning (PC), first described in 1986, provided an indication of the magnitude of the possible cardioprotective effect and stimulated considerable research into the mechanisms involved (176). Over the past two decades we have learned a great detail about the signaling pathways involved in preconditioning. More recently, activation of signaling kinases at reperfusion, either by agonist addition (100) or by postconditoning (287), has been shown to be cardioprotective. In spite of the identification of these signaling pathways, the precise mechanism by which these pathways reduce cell death is only beginning to be understood. In parallel with the intense study of cardioprotective mechanisms, the past few decades have also seen intense research into mechanisms involved in regulating apoptosis and necrosis (18, 51). It has recently been appreciated that necrosis is also regulated and can be inhibited by many "anti-apoptotic" agents. In the past few years, studies of both cardioprotection and cell death have focused on the role of the mitochondria

Address for correspondence: E. Murphy, Room 7N112, Building 10, 10 Center Drive, Bethesda, MD, Phone: 301-4965828, Fax: 301-402-0190, murphy1@niehs.nih.gov.

as regulators of energetics and cell viability. This review will examine what protects against myocardial ischemia-reperfusion injury and what promotes injury, and what we can learn from the comparison. Section II will discuss mechanisms involved in cell death in cardiac ischemia-reperfusion. The relative roles of ischemia and reperfusion in irreversible injury will be discussed. The involvement of apoptosis and necrosis will also be considered. Section III will discuss alterations in calcium and ROS and their involvement in cell death. Section IV will discuss cardioprotective mechanisms with emphasis on the signaling pathways involved in pre and post-conditioning. This review will focus on acute cardioprotection, which is primarily mediated by activation of signaling pathways and post-translational modification of proteins. We will not discuss the mechanisms involved in the second window or delayed preconditioning, which are primarily mediated by gene induction and protein synthesis; the reader is referred to other excellent reviews on this topic (237, 282) Section V will summarize how the acute cardioprotective signaling pathways activated by pre- and postconditioning might reduce cell death. Section V will also consider future directions in cardioprotection.

II. Why myocytes die following ischemia-reperfusion?

A. Relative roles of Ischemia versus Reperfusion in Irreversible Myocyte Injury

It has been debated whether cardiomyocytes suffer irreversible injury primarily during ischemia, which may be revealed at the start of reperfusion, or whether additional injury occurs during reperfusion (reperfusion injury). This point has important clinical implications, because if additional injury occurs on reperfusion this would allow an opportunity to intervene with cardioprotective drugs on reperfusion. There have been a large number of basic studies which suggest that introduction of cardioprotective drugs or strategies at the very start of reperfusion can significantly reduce infarct size. Postconditioning (287), Na-H exchange inhibitors (128), activation of kinases (99), perfusion with erythropoietin (95), inhibition of PKCδ (114), inhibitors of the mitochondrial permeability transition pore (MPT) (98), inhibition of GSK-3β (89) and other interventions have been reported to protect when added at the start of reperfusion. Taken together these data suggest that events occurring at the start of reperfusion can impact cell fate, and that interventions at this time can be cardioprotective. However, as is clear from a number of failed clinical trials (30, 69, 250), it is imperative to initiate the intervention during the first seconds of reperfusion. Such early intervention is only practical before cardiac surgery or with angioplasty. It appears that the window of opportunity during reperfusion is very limited. Interventions initiated later than 5 to 10 minutes after the start of reperfusion do not appear to provide significant protection (279). This narrow window for intervention is consistent with the release of intracellular enzymes such as creatine kinase during early reperfusion. It is also of note that studies showing protection with interventions at the start of reperfusion typically employ relatively short durations of ischemia (248) that may not mimic the clinical setting in which patients present after longer durations of ischemia.

Although protection can be initiated at reperfusion, injury also occurs during ischemia, and the relative proportion of injury occurring during ischemia versus reperfusion likely depends on the duration of ischemia. Casapases are activated during ischemia (238) and ion dysfunction occurs during ischemia (236). If cardioprotective strategies can be initiated before or during ischemia, it is likely that they will enhance protection, especially with longer durations of ischemia. In addition, events during ischemia can enhance the opening of the MPT and thus the initiation of death at reperfusion. Thus, it is important to administer cardioprotective agents as soon as possible.

B. Mechanisms of Death: Role of Necrosis, Apoptosis and Autophagy

Cell death following ischemia-reperfusion has been reported to have features of apoptosis, autophagy, and necrosis. The precise proportion of each form of death may depend on the model (adult versus neonatal, cultured cells versus in vivo). The mechanisms regulating apoptosis, autophagy, and necrosis have been recently reviewed (51, 78, 83, 94, 127, 247, 290) and will not be covered in detail here.

Apoptotic cell death results in apoptotic bodies that contain cellular components, and these apoptotic bodies are phagocytized by other cells; because there is no release of intracellular components, there is little or no inflammatory response. Apoptosis as originally described by Kerr et al is characterized by chromatin condensation and fragmentation, cell shrinkage and plasma membrane budding with release of apoptotic bodies that are phagocytized (129). Although these morphological descriptions do not fully apply to the majority of cell death in the setting of ischemia-reperfusion, apoptotic mechanisms have been implicated in ischemia-reperfusion injury (8, 9, 51, 141). Apoptotic cell death typically occurs via activation of caspases which cleave DNA and other cell components. Deletion of pro-apoptotic proteins or increased expression of anti-apoptotic proteins have been shown to reduce ischemia-reperfusion mediated cell death. Mice lacking the pro-apoptotic protein Bax have been reported to have reduced ischemia-reperfusion injury (106). Mice that lack Fas have also been reported to have smaller infarcts following ischemia and reperfusion (141). Overexpression of the anti-apoptotic protein Bcl-2 has been shown in a number of studies to reduce infarct size (42, 113). However it is likely that anti-apoptotic proteins can reduce necrotic and autophagic death as well as apoptotic cell death (18, 38, 106, 113, 229). Studies have shown in wild type hearts subjected to 20 minutes of global ischemia and 2 hours of reperfusion that 38% of the heart was dead as measured by triphenyl-tetrazolium chloride staining, but only 4% of the death was apoptotic as assessed by TUNEL. Cardiac specific Bcl-2 overexpression reduced total cell death (from 38% to 18%) and apoptotic cell death from 4.3% to 1.2% (113). Thus, the decrease in apoptotic cell death is not sufficient to account for the reduction in cell death, suggesting that Bcl-2 overexpression reduces necrotic as well as apoptotic cell death. Similar data were obtained by others (158). Interestingly, cardiac specific overexpression of Bcl-2 has been shown to reduce the rate of fall in ATP during ischemia and to reduce ischemic acidification (113). The mechanisms responsible for these metabolic effects of Bcl-2 are not clear, but appear to involve effects of Bcl-2 on either reducing ATP entry into the mitochondria or reducing breakdown of ATP by the $F_1F_0ATPase$ (see (173)).

Activation of caspases is thought to be a major mechanism of apoptotic cell death. The role of caspases in ischemia-reperfusion injury has been debated (14, 38, 52, 84). Some have reported that inhibition of caspases results in only a modest reduction in infarct size, less than that observed with overexpression of antiapoptotic proteins (84). However, a large number of studies have reported that addition of caspase inhibitors reduces infarct size, suggesting an important role for caspase activation in ischemia-reperfusion injury (164, 238, 280). Caspase 9 has been reported to be activated during ischemia, whereas caspase 8 and 9 are activated during reperfusion(238). It has also been suggested that inhibition of caspases promotes necrotic cell death (264), and autophagy (284).

Caspases cleave a large number of targets that contribute to cell death. However, cleavage of what substrate(s) would quickly (within 1 to 2 hours, a time of significant cell death) result in the immediate loss of cell viability and release of intracellular components such as creatine kinase and troponin? There has been a lot of focus on caspase cleave of DNA as an important mediator of cell killing. Although loss of DNA will eventually lead to cardiac cell death, it is not clear that cleavage of DNA is the immediate cause of cell death in cardiomyocytes which is observed within 1 to 2 hours of reperfusion following ischemia.

Caspases target a number of mitochondria proteins and these targets could promote rapid cell death. Caspase cleavage of the cytoskeleton or plasma membrane constituents are also possible targets that could lead to rupture of the plasma membrane. Although as originally defined apoptosis does not result in rupture of the plasma membrane. However, in the setting of ischemia-reperfusion, where multiple forms of death occur, caspase cleavage of structural proteins could contribute to plasma membrane rupture.

Autophagy is another form of death which has been suggested to play a role in ischemia-reperfusion injury. Autophagy is a physiological mechanism that is used to remove damaged organs, such as mitochondria or endoplasmic reticulum. Autophagy is also initiated by starvation to provide nutrients. However, extensive autophagy can cause cell death. There are conflicting data as to whether the increased autophagy that occurs during ischemia and reperfusion is beneficial or detrimental. There are studies showing that inhibition of autophagy during ischemia or anoxia is detrimental, suggesting a beneficial role for stimulation of autophagy during ischemia (60, 277). However, in contrast to these studies a decrease in beclin1 (a protein that stimulates autophagy) reduces ischemia-reperfusion mediated autophagy and myocyte death (247). Also consistent with a detrimental role for autophagy in ischemia-reperfusion, in HL-1 cells an increase in beclin1, which increased autophagy, was also shown to decrease activation of BAX, and knockdown of beclin1 decreased activation of BAX (93). Thus the role of autophagy in cell death during ischemia-reperfusion is still somewhat unclear, although it seems that inhibition of autophagy during ischemia and reperfusion (as opposed to ischemia alone) is beneficial.

There are a number of connections between autophagy and other forms of cell death. An increase in calcium, as occurs during ischemia, has been shown to increase autophagy (107). Activation of calpain, a calcium activated protease has been reported to cleave Atg5, a protein involved in autophagy; cleaved Atg5 translocates to the mitochondria where it is reported to bind Bcl-2 and thereby stimulate apoptosis (94, 247). Taken together these data suggest that an increase in calcium will activate autophagy and at the same time convert some of the autophagy machinery to apoptosis. Activation of phosphoinositide-3-kinase (PI3K) class I, which is cardioprotective, decreases beclin1 leading to a decrease in autophagy. PI3K activation of the mammalian target of rapamycin (mTOR) results in inhibition of autophagy. Activation of class III PI3K, which is involved in regulating vesicular trafficking, stimulates autophagy. A decrease in Bcl-2 has been shown to result in an increase in beclin1. Also activation of JNK has been reported to increase beclin1. It is also clear that there is considerable cross-talk through calcium, calpain, caspases, Bcl-2 family members, and signaling kinases, between autophagy and the other death pathways active in ischemia-reperfusion.

Necrotic cell death is characterized by cell swelling leading to irreversible rupture of the plasma membrane with release of cytosolic components, which result in an inflammatory response. An inflammatory response is an important component of ischemia-reperfusion injury. The release of troponin and creatine kinase that occur during ischemia-reperfusion are likely due to necrotic release of these intracellular components. Until recently, necrosis was thought to be an unregulated process, but recent studies suggest that necrosis can be regulated and that interventions can reduce necrotic cell death (83, 290). Since rupture of the plasma membrane is a prominent feature of necrosis and ischemia-reperfusion injury and is a lethal event, it is worth considering what might lead to rupture of the plasma membrane. Rupture of the plasma membrane could be facilitated by calpain or some other protease cleavage of the cytoskeleton. Complete loss of ATP would also inhibit ion pumps, which would result in swelling perhaps rupturing the plasma membrane, particularly if the cytoskeleton has been weakened. ATP falls to very low levels within about 15 to 20 minutes of global ischemia in a rat heart, a time when there is very little irreversible injury. Thus

there is usually a poor correlation between ATP levels and irreversible injury. For example, Neely and Grotyohann reported that the ability of the heart to recover was inversely related to the build-up of metabolic endproducts (lactate, NADH etc) and not the levels of ATP (186). The lag between loss of ATP and irreversible injury is consistent with the loss of ATP initiating some process (or inhibiting some process) that ultimately results in cell death.

It is possible that a combination of protease activation with loss of ATP and ion dysregulation and cell swelling all conspire to rupture the plasma membrane. The question then arises as to what causes activation of proteases and the total loss of ATP? A rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) has been consistently observed in ischemia and early reperfusion and most cardioprotective protocols reduce the rise in $[Ca^{2+}]_i$ during ischemia. A rise in $[Ca^{2+}]_i$ will lead to activation of calpains which could be involved in cleaving proteins leading to plasma membrane rupture. Calpain also activates the pro-apoptotic BID and calpain cleaves Atg5 shifting the balance from autophagy to apoptosis. An increase in $[Ca^{2+}]_i$ and ROS can lead to activation of an inner mitochondrial large conductance channel known as the mitochondrial permeability transition (MPT). Opening of this channel would lead to loss of ATP and mitochondrial function, which would quickly lead to mitochondrial swelling and release of cytochrome c, which could activate apoptosis. If a large number of mitochondria in a cell undergo MPT the cell will lose the capacity to make ATP, the cell will lose ion homeostasis resulting in cell swelling, membrane rupture and cell death.

Death following ischemia-reperfusion injury appears to be a mixture of apoptotic, autophagy, and necrotic cell death and it can have features of all three. The distinction between the modes of death may not be important since recent data suggest that all three forms of cell death can be regulated and are inter-related (83, 290). With ischemia and reperfusion, it may therefore be more useful to discuss mechanisms of death without attempting to precisely define the mode of death. The important issue is that cell death during ischemia-reperfusion appears to be an active process, which can be inhibited with appropriate interventions. Taken together the mitochondria are emerging as an important mediator and regulator of all forms of cell death in ischemia-reperfusion. In particular, the MPT appears to be a major regulator of both apoptotic and necrotic cell death. Recent studies have shown that inhibition of MPT by knockdown of cyclophilin D (a regulator of the MPT) results in a significant reduction in infarct size in ischemia reperfusion, but loss of cyclophilin D did not block BAX mediated apoptosis. These and other data suggest a major role for the MPT in ischemia-reperfusion injury. The MPT is activated by elevated calcium and reactive oxygen species (ROS), and both of these are elevated during ischemia and reperfusion. In the next section we will focus on mechanisms that regulate Ca^{2+} and ROS during ischemia since Ca^{2+} and ROS appear to be the primary activators of MPT during ischemia-reperfusion.

III What Activates the Mitochondrial Permeability Transition?

A. Role of Calcium

1) Ca^{2+} rises during ischemia?—Studies from over 2 decades ago showed that a rise in cytosolic $[Ca^{2+}]_i$ preceded irreversible myocardial injury and that drugs or protocols that reduced or delayed the rise in cytosolic $[Ca^{2+}]_i$ also reduced or delayed myocardial death (112, 138, 174, 196, 234–236). Although some early studies reported that a rise in $[Ca^{2+}]_i$ was not important in the genesis of injury (44, 133), the majority of recent studies have shown that a rise in $[Ca^{2+}]_i$ precedes irreversible injury and that mechanisms that block the rise in $[Ca^{2+}]_i$ attenuate or delay the onset of irreversible injury (155, 162, 183, 196, 234). Each systole is initiated by Ca^{2+} entry via the L-type Ca^{2+} channel resulting in Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum, and the combined increase in $[Ca^{2+}]_i$ leads to

contraction. The Ca^{2+} that enters via L-type Ca channel is removed from the cell primarily by Na-Ca exchange (NCX) with a very small contribution from the sarcolemmal Ca^{2+} ATPase (25, 67). The Ca^{2+} released from the sarcoplasmic reticulum is reaccumulated into the sarcoplasmic reticulum (SR) via SR/ER Ca^{2+} ATPase (SERCA). Studies show that much of the rise in cytosolic Ca^{2+} during ischemia is due to Ca^{2+} entry by reverse mode NCX secondary to the rise in $[Na^+]_i$ during ischemia (see figure 1) (112). This occurs because of increased generation of protons during ischemia, which are extruded from the cell via Na-H exchange (NHE), resulting in an increase in intracellular Na^+ (174). Na has also been shown to enter the cell during ischemia on non-inactivating Na^+ channels (172). This rise in intracellular Na^+, coupled with the depolarized plasma membrane results in a reversal of NCX to bring Ca^{2+} into the myocyte. NHE inhibitors, such as amiloride derivatives and the more specific NHE inhibitor, cariporide, have been shown in animal studies to attenuate the rise in $[Na^+]_i$ during ischemia and the subsequent rise in $[Ca^{2+}]_i$, and to reduce ischemia-reperfusion injury. The animal data clearly showed that these inhibitors were beneficial when administered prior to ischemia (128, 174). Inhibition of NCX has also been shown to be protective (174, 183), and hearts from mice lacking the NNH or mice lacking the NCX have been shown to have reduced ischemia-reperfusion injury (112, 269). In addition to NCX, Ca^{2+} entry via the L-type Ca^{2+} channel can contribute to the rise in Ca^{2+} during ischemia (41).

2) Ca^{2+} during reperfusion—During ischemia intra- and extracellular pH are acidic; however during reperfusion extracellular pH rapidly returns to normal. However, initially the intracellular pH is still acidic and this pH gradient facilitates extrusion of H^+ from the cell in exchange for Na^+ on NHE. The increased cytosolic Na^+ can be extruded by Na-K-ATPase or NCX in exchange for Ca^{2+}, thereby raising (at least transiently) $[Ca^{2+}]_i$. Arrhythmias can be triggered by altered Ca^{2+} homeostasis and are a major cause of death in ischemia-reperfusion. In the absence of arrhythmias, $[Ca^{2+}]_i$, returns quickly to near normal levels in myocytes that survive. However, there can be $[Ca^{2+}]_i$, oscillation (64, 198), which can lead to hypercontracture which will contribute to further decline in ATP and deterioration of the myocyte. In some myocytes, $[Ca^{2+}]_i$, remains high during reperfusion; this condition typically results in irreversible injury to the myocyte. What determines whether $[Ca^{2+}]_i$, returns to normal, oscillates or stays elevated? This depends on myocyte ATP levels (which may in turn depend on activation of MPT), intracellular Na^+ levels, and damage to Ca^{2+} handling proteins such as the ryanodine release channel in the SR. Thus if there is SR dysfunction, $[Ca^{2+}]_i$, oscillations can occur, which further deplete ATP and can contribute to arrhythmias. The Ca^{2+} released from the SR can also be taken up by the mitochondria leading to activation of the MPT. Improving SR Ca^{2+} handling has been shown to be an important target for reducing ischemic injury (55, 197, 291). Interestingly, adenoviral mediated overexpression of SERCA was shown to reduce infarct size and improve function following ischemia (55).

Treatment with NHE inhibitors given immediately at the start of reperfusion provides some protection, although usually somewhat less than that observed when NHE inhibitors are given during ischemia as well as reperfusion (172). A large clinical trial was performed to test the beneficial effects of cariporide (an NHE inhibitor). Consistent with the animal studies, NHE inhibitors were protective only in the setting of CABG surgery (30, 250). A follow up study to specifically test the effects of NHE inhibitors was undertaken using a high dose of the drug and was discontinued due to toxicity (30).

3) Role of mitochondrial calcium—Ca^{2+} uptake into mitochondria occurs via the mitochondrial calcium uniporter, which uses the energy of mitochondrial membrane potential ($\Delta\psi$) (see figure 2); thus $\Delta\psi$ used to transport Ca^{2+} is not available to synthesize ATP from ADP. The mitochondrial uniporter is inhibited by ruthenium red and related

compounds. Surprisingly, the Ca^{2+} uniporter has not been cloned. Ca^{2+} uptake into the mitochondria will act to dissipate $\Delta\psi$, unless electron transport is activated to re-synthesize the gradient. Thus during ischemia, with inhibited electron transport (due to lack of oxygen), one would expect Ca^{2+} uptake into the mitochondria to dissipate $\Delta\psi$, ultimately limiting Ca^{2+} uptake into the mitochondria. Ca^{2+} is released from the mitochondria in exchange for Na^+ (on the mitochondrial NCX; see figure 2). The matrix Na^+ level has been reported to be regulated by mitochondrial NHE and thus the pH gradient across the inner mitochondrial membrane. In energized mitochondria, the Na^+ gradient is the inwardly directed and $[Na^+]_i$ is reported to be lower in the matrix (58, 124).

During simulated ischemia, most studies suggest that there is a small rise in mitochondrial Ca^{2+} (87, 163, 170, 183, 208). Interestingly, Griffiths et al observed that the rise in mitochondrial Ca^{2+} during ischemia was inhibited by clonazepam (an inhibitor of mitochondrial NCX), thus suggesting a role for mitochondrial NCX operating in the reverse mode to increase mitochondrial matrix Ca^{2+} (87). It is suggested that the Na^+ gradient decreases as a result of the decrease in pH gradient and this reduced inwardly directed Na^+ gradient, along with the rise in $[Ca^{2+}]_i$, may contribute to Ca^{2+} entry into the mitochondria on Na-Ca^{2+} exchange. However clonazepam increased the rise in mitochondrial $[Ca^{2+}]$ during reperfusion, suggesting that during reperfusion when the mitochondrial pH gradient and the Na^+ gradient is restored, the mitochondrial NCX extrudes Ca^{2+} from the mitochondria (87). Interestingly Bcl-2 has been reported to modulate mitochondrial NCX (288). Cardioprotective agents such as diazoxide and sarcolemmal NCX inhibitors are reported to reduce mitochondrial Ca^{2+} overload (170, 183).

Griffiths et al (88) reported that 0.1 μM cyclosporin A (CsA), an MPT inhibitor, protected cells subjected to simulated ischemia and reperfusion; protection was defined as ability to recover rod shape and respond to electrical stimulation. Interestingly, the mitochondrial Ca^{2+} concentration in the surviving myocytes was higher in the CsA treated myocytes than in untreated; these data are consistent with the hypothesis that CsA mediated protection is by reducing the Ca^{2+} activation of the MPT. Under these conditions, CsA does not reduce the Ca^{2+} loading of mitochondria, it reduces the Ca^{2+} sensitivity of the MPT. The myocytes were able to survive with higher levels of mitochondrial Ca^{2+}. Higher concentrations of CsA were found to reduce mitochondrial Ca^{2+} during simulated ischemia, an effect that was attributed to CsA inhibition of calcineurin.

Miyamae et al (162) measured mitochondrial $[Ca^{2+}]$ in perfused hearts with indo-1 and surface fluorescence, using Mn^{2+} to quench cytosolic Ca^{2+}. They found an inverse correlation between mitochondrial $[Ca^{2+}]$ during ischemia and recovery of LVDP on reperfusion. Miyamae et al reported that ruthenium red attenuated the increase in mitochondrial $[Ca^{2+}]$ and improved recovery of LVDP. However the effects of ruthenium red on mitochondrial $[Ca^{2+}]$ have been very diverse, perhaps reflecting the fact that ruthenium red inhibits many Ca^{2+} channels in the cell. Taken together, reducing the rise in cytosolic and mitochondrial $[Ca^{2+}]$ during ischemia has been shown to be cardioprotective. However, cardioprotection can also be achieved, as in the case of CsA, not by lowering mitochondrial $[Ca^{2+}]$ but by inhibition of Ca^{2+} activation of the MPT. Thus there are multiple pathways to cardioprotection.

On reperfusion, when oxygen is re-introduced and $\Delta\psi$ is regenerated, if $[Ca^{2+}]_i$ is elevated, one would expect significant Ca^{2+} uptake into the mitochondria via the Ca^{2+} uniporter. Consistent with this, on re-oxygenation (removal of metabolic inhibition), there appears to be an initial additional rise in mitochondrial $[Ca^{2+}]$, which then falls toward baseline (87). As discussed, this rise in mitochondrial $[Ca^{2+}$ during reperfusion has been suggested to be a trigger for activation of the MPT.

B. Role of reactive oxygen species (ROS)

1) Does ROS rise during ischemia?—The role of reactive oxygen species (ROS) in ischemia-reperfusion injury has been extensively studied. ROS has been measured by electron spin resonance using spin traps added to the perfusate to trap the short lived radicals that are released into the perfusate (13, 31, 79, 293). Electron spin resonance has also been used to measure signals from the spin traps in the perfused heart (293). Free radicals have also been measured directly by electron spin resonance by grinding frozen samples (294). However caution must be used since radicals can be generated artifactually by the grinding (231). Other studies have used fluorescent indicators to measure ROS (23, 130, 263); these studies have typically been performed on isolated myocytes using simulated ischemia (23, 263) although some studies have used surface fluorescence of perfused hearts (130). These studies have consistently demonstrated a burst of ROS generation on the start of reperfusion (13, 31, 79, 130, 263, 293, 294). Whether there is an increase in ROS during ischemia in an intact heart, however, is less clear. Studies in isolated myocytes have shown an increase in ROS generation during anoxia or simulated ischemia (23); however it is difficult to totally remove oxygen from solutions. Oxygen levels available are a balance between supply and demand. There is usually some oxygen in the buffer, but in a perfused heart with global ischemia the high density of cells rapidly consumes the small amount of oxygen available. In isolated myocytes the oxygen demand is low (due to low density of cells) and can be met in part by the low contaminant oxygen in the surrounding buffer (which in many cases is quite large). Thus the generation of ROS in hypoxic isolated myocytes could be due to small amounts of oxygen not present in intact ischemic myocardium. ROS has also been measured during global ischemia using surface fluorescent measurement of dihydroethidium (130). The surface of the heart would be exposed to ambient oxygen and the surface has been shown to behave differently than the interior core of the heart during ischemia (37). ROS has also been measured during low flow ischemia (13), but again oxygen is present during low flow ischemia. An early study using ESR measured ROS during ischemia in frozen ground heart and observed an increase in ROS during ischemia (294). Taken together these data suggest that generation of ROS in heart during ischemia is likely to be heterogeneous depending on the level of tissue oxygenation, which will depend on factors such as collateral flow. Even in a global model of ischemia oxygen does not immediately fall to zero so there is initially some oxygen to generate ROS. However the levels of ROS generated during ischemia are low and the pathological significance of them is uncertain.

2) ROS during Reperfusion—During reperfusion, with the return of oxygen, a large burst of ROS has been consistently shown to occur (13, 31, 79, 130, 263, 293, 294). The increase in ROS during ischemia and reoxygenation is thought to be due to damage to electron transport chain components resulting in inefficient transfer of electrons, generating superoxide. It was proposed that ROS, particularly ROS generated during early reperfusion, would lead to extensive oxidative damage to the cell resulting in loss of cell viability. This hypothesis was extensively tested in the 1980s. Studies consistently demonstrated a role for ROS, particularly ROS generated during reperfusion, in myocardial stunning. Treatment of in vivo and in vitro hearts with antioxidants reduced ROS and stunning (29). In contrast to the general agreement regarding ROS and stunning, there is considerable controversy and disagreement regarding the role of ROS in reducing infarct size. Although a number of studies find that addition of antioxidants or scavengers such as superoxide dismutase and catalase reduce infarct size (6, 43, 132, 184), there are a similar number of studies that find no reduction in infarct size (62, 77, 187, 190, 261, 267). Others have suggested that antioxidants can delay but not prevent manifestations of infarction (159). Based on the beneficial effects reported in some of these studies, clinical trials were initiated to test whether superoxide dismutase would be beneficial in patients undergoing angioplasty following acute myocardial ischemia. These clinical trials failed to show a beneficial effect

of superoxide dismutase on ventricular function (69, 171). The negative studies do not necessary mean that ROS is not an important determinant of infarct size; only that agents such as superoxide dismutase and catalase administered intravascularly are not protective. It is likely that ROS generated during reperfusion initiates injury before it can be scavenged by exogenous superoxide dismutase and catalase. Also consistent with the hypothesis, overexpression of manganese superoxide dismutase reduced infarct size in mice (122). Kim et al have recently reported that ROS generated during early reperfusion is the primary activator of the MPT and cardiomyocyte death (133). It has been reported that some recently developed, intracellularly targeted scavengers provide some reduction in infarct size (2, 27, 228). Antioxidants such as vitamins C and E have also been suggested to scavenge ROS and reduce ischemic injury (199), although the result of clinical trials do not suggest reduced overall mortality with vitamin E supplementation (1, 151, 285).

These studies reinforce the importance of localization and timing in cardioprotection. The concept that ROS is an important contributor to ischemia-reperfusion injury is likely to be correct; however the ability to translate this knowledge into a clinical therapy is very complex. Delivery of the antioxidants (superoxide dismutase) or NHE inhibitors to the right compartment in the right time period is very difficult to achieve in controlled animal studies and even more difficult in patients. As a result of the controversy in the animal studies and the failed clinical trials it is often concluded that inhibition of ROS will not influence infarct size. A more realistic assessment is that to have a significant benefit in reducing infarct size requires the correct delivery (at the start of reperfusion) of mitochondrial targeted antioxidants perhaps in conjunction with other therapies (reduction in Ca, inhibition of MPT etc). However, oxidants can also have a cardioprotective signaling function, and antioxidants can inhibit cardioprotection (40, 71, 191).

3) Mitochondrial ROS—Mitochondria are thought to be both a major source of ROS as well as a major target for ROS damage. Mitochondrial electron transport is one of the primary sources of reactive oxygen species (ROS) in the cell. ROS is generated during electron transport at complex I and III. There is a positive correlation between mitochondrial membrane potential ($\Delta\psi$) and production of ROS by electron transport. It has been suggested that even though only low levels of ROS are generated during ischemia, this ROS can lead to damage to the electron transport chain (143, 209); this damage is then thought to lead to increased ROS production because of inefficient transfer of electrons. Inhibition of electron transport at complex I during ischemia reduces ROS generation during ischemia. Inhibition of electron transport (particularly complex I) and mitochondrial uncouplers have been shown to reduces ischemia-reperfusion injury (143, 209); it is proposed that this protection is mediated by reduction in ROS during ischemia.

However, not all ROS is detrimental and ROS generation has been shown to be part of the protective signaling pathway of preconditioning (40). Diazoxide, an activator of the mitoKATP channel has been shown to increase ROS (71, 191), and addition of antioxidants has been shown to block the protection afforded by diazoxide as well as PC.

The burst of ROS at the start of reflow will also lead to damage to the electron transport chain. ROS generation on reflow has also been shown to be a major activator of MPT. Taken together it appears that low levels of ROS generation are important in signaling cardioprotection. However high levels of ROS, as occur at the start of reperfusion, can lead to damage to the electron transport chain and activation of the MPT. As discussed later, S-nitrosation of proteins, as can occur with PC, can protect proteins from further oxidative damage (244).

IV. What protects and how does it protects?

A. Preconditioning

PC, originally described by Murry et al, consists of 4 cycles of 5 minutes of ischemia and 5 minutes of reperfusion just prior to a sustained period of ischemia (see figure 3) (176). Others have used different protocols, all of which have in common brief periods of ischemia separated by brief periods of reperfusion. PC has been shown to reduce infarct size, reduce the generation of lactate and to reduce the rate of fall in ATP. Later studies, by other groups, showed that PC also reduced arrhythmias and reduced contractile dysfunction. As discussed below, over the subsequent 20 years, we have learned a lot about the signaling pathways activated by PC. However we are just beginning to understand how activation of these signaling pathways leads to protection.

There are several interesting aspects of PC that provide potential insight into the mechanisms. The protection afforded by PC is lost if the time between the initial PC protocol and the sustained period of ischemia is extended beyond ~1 hour. A "second window" of preconditioning, which involves upregulation of genes, occurs ~24 hours after preconditioning (203, 281). The initial "early" preconditioning does not appear to depend on new protein synthesis because of the rapid onset and because since inhibition of protein synthesis does not block early PC (251); although this may need to be re-examined (239). Because the second window is mediated by new gene expression rather than post-translational modification of proteins, the mechanisms of the second window are different and will not be covered in this review. The reader is referred to other excellent reviews on this area (237, 281).

In the original characterization of PC it was noted that PC hearts have less anaerobic glycolysis during the sustained period of ischemia; that is, they make less lactate and have less ischemic acidosis (i.e. a higher intracellular pH during ischemia) (178, 236). Interestingly, cardiac specific overexpression of the antiapoptotic protein Bcl-2, addition of diazoxide, which mimics PC reportedly by opening a mitochondrial K_{ATP} channel, and adenosine pretreatment have also been shown to reduce acidosis during ischemia (71, 73, 113). The mechanism responsible for reduced ischemic acidosis by these cardioprotective agents is unknown. In addition to reduced anaerobic glycolysis, the rate of ATP consumption during ischemia is slower in PC hearts. There is an initial decrease in ATP during the PC protocol, such that PC hearts start with a lower ATP than non PC hearts. However, ATP falls more slowly in PC hearts (178, 236). Taken together these data suggest that PC reduces the rate of ATP hydrolysis during ischemia, since they have less anaerobic glycolysis to generate ATP (which is the primary source of ATP during global ischemia) and ATP levels fall more slowly. The mechanism by which PC reduces ATP breakdown during ischemia is still unknown. Interestingly, cardiac specific overexpression of the antiapoptotic protein Bcl-2, overexpression of the cardioprotective PKC-ε, and adenosine pretreatment have all been shown to slow the rate of ATP breakdown during ischemia (50, 73, 113).

An early hypothesis to account for the reduced ATP hydrolysis was that PC might inhibit ATP breakdown by reverse mode of the F_1F_0ATPase. Under normoxic conditions, the protons extruded from the mitochondrial matrix by electron transport re-enter the matrix via the F_1F_0ATPase which couples the proton gradient to synthesis of ATP. When the mitochondrial membrane potential ($\Delta\psi$) falls, the F_1F_0ATPase can reverse and consume ATP to generate $\Delta\psi$. It has been reported that as much as 35–50% of the ATP generated by glycolysis during ischemia is consumed by the reverse mode F_1F_0ATPase (see figure 1) (90, 118). Di Lisa et al used the fluorescent potential sensitive dye, JC1 to measure mitochondrial $\Delta\psi$ in anoxic rat cardiomyocytes and showed a biphasic decline in $\Delta\psi$ (56).

Di Lisa et al showed that glycolytically generated ATP was used to maintain $\Delta\psi$, since $\Delta\psi$ was shown to decline more rapidly during ischemia in the presence of oligomycin, an inhibitor of the F_1F_0ATPase (see Figure 1). Leyssens et al obtained similar results using JC1 to measure $\Delta\psi$ in rat cardiomyocytes metabolically inhibited with cyanide and 2-deoxyglucose (144). They found $\Delta\psi$ declined in metabolically inhibited myocytes, but the decline in $\Delta\psi$ was enhanced when the F_1F_0ATPase was inhibited with oligomycin. These data support the conclusion that the F_1F_0ATPase is a major consumer of ATP during ischemia and/or metabolic inhibition and they further demonstrate that this consumption of glycolytic ATP is used to maintain $\Delta\psi$. Consistent with the hypothesis that PC slows the rate of ATP breakdown by reverse mode of the F_1F_0ATPase, Ylitalo et al have shown that during ischemia $\Delta\psi$ depolarized to a greater extent in PC hearts than in non-PC hearts (283).

Measurements of mitochondrial membrane potential in perfused rat hearts during ischemia and reperfusion have recently been reported (157). These studies show a decrease in $\Delta\psi$ during ischemia confirming studies of simulated ischemia in isolated myocytes. They further reported that on reperfusion a larger number of cells undergo loss of $\Delta\psi$, consistent with activation of MPT on reperfusion.

There are data suggesting that during ischemia a mitochondrial ATPase inhibitor subunit (IF1) binds to and inhibits the mitochondrial ATPase thus conserving ATP (205–207). It was proposed that perhaps PC promoted earlier binding of the IF1 to the F_1F_0ATPase. However, studies by two different groups, using submitochondrial particles, found no evidence supporting inhibition of the ATPase in PC hearts (85, 266). However, other groups have reported that PC and diazoxide enhance IF1 binding to the F_1F_0ATPase (4, 46, 47). A recent study has also reported that pharmacological PC with adenosine results in increased phosphorylation of the beta subunit of the ATP synthase; however the functional effects of phosphorylation of the ATPase were not addressed (12).

Because the protection afforded by PC is lost within 1–2 hours (149, 161, 177) it was suggested that PC was mediated by some time dependent intermediate, which is either lost or regenerated with time. It was proposed that perhaps glycogen was the memory in PC (273). Glycogen is significantly reduced during the PC protocol, so anaerobic glycolysis is limited and less lactate can be generated during the sustained period of ischemia in PC hearts (236). Less anaerobic glycolysis results in less acid generation during ischemia, which would reduce the rise in Na (via Na-H exchange) and reduce the rise in Ca (via Na-Ca exchange) (174). If the time between PC and the sustained ischemia was extended, glycogen would be resysnthesized and the protection lost. However, a number of studies showed that depletion of glycogen per se does not result in cardioprotection (135, 232, 265).

Recent studies suggest that PC activates a number of signaling pathways and that the activation of these pathways likely decays with time, leading to loss of protection. We will therefore consider the signaling cascades in detail with attention to how each component might lead to protection. PC causes release of agonists such as adenosine, bradykinin, and opioids which bind to G-protein coupled receptors (GPCR) and activate a cardioprotective signaling cascade. As shown in figure 4, activation of GPCRs leads to activation of the phosphoinositide-3-kinase (PI3K) pathway, which via phosphoinositide-dependent protein kinase 1 (PDK1) results in activation of downstream targets such as Akt, p70S6K, and signaling through βarrestin and endosomal signaling which results in activation of extracellular regulated kinase (ERK) and other kinases and phosphatases. In addition Akt, p70S6K, protein kinase C (PKC) and ERK all modulate a number of downstream signaling pathways, which are discussed in detail.

1) Phosphoinositide-3-kinase (PI3K)—PI3K is a lipid and protein kinase. As a lipid kinase, PI3K catalyzes the phosphorylation of the inositol ring of phosphoinositides at the D3 position. As a protein kinase, PI3K has been shown to phosphorylate non-muscle tropomyosin which is involved in β-adrenergic receptor endocytosis (179). Activation of PI3K has been associated with cytoprotection and reduced apoptosis. PI3K is inhibited by wortmanin (WM) and LY294002 (see figure 5). Inhibition of PI3K blocks the protective effect of PC (254).

What are the targets of PI3K and how might it mediate protection?: The action of PI3K is usually attributed to generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) which facilitates PDK1 phosphorylation of substrates (see figure 5). PDK1 has been reported to phosphorylate Akt, p70S6K, PKC, serum and glucocorticoid regulated kinase (SGK), p90 ribosomal S6 kinase (RSK), and protein kinase A (PKA). It is not clear that all of these targets are phosphorylated in vivo. Akt, p70S6K, and RSK were not phosphorylated in response to agonist in cells lacking PDK1, suggesting that they are in vivo substrates of PDK1 (272). Despite its name, PDK1 is reported to be constitutively active (188). PIP3 generated by PI3K is thought to bring Akt to the membrane and to alter Akt conformation to facilitate phosphorylation by constitutively active PDK1. The details of how PI3K enhances PDK1 mediated phosphorylation of other substrates such as p70Sk6 or SGK is not clearly understood (see (168)). PDK1 phosphorylation of p70S6K and SGK appears to be dependent on prior phosphorylation of a hydrophobic motif and PI3K appears to somehow enhance phosphorylation at this site. However, there are also data showing that PI3K can enhance the activity of PDK1 (271).

The phosphoinositides such as PIP3 generated by PI3K are degraded by the phosphatases PTEN (phosphatase and tensin homolog deleted on chromosome ten)(154) and SHIP (SH2 domain-containing inositol polyphosphate 5-phosphatase) (268). PTEN and SHIP thus oppose the action of PI3K (see figure 5). Regulation of these phosphatases is another means by which PC might mediate protection. PTEN has been suggested to be involved in cardioprotection (167). PTEN is degraded during a PC protocol in the rat heart consisting of 15 min of ischemia and 30 minutes of reperfusion (36). The loss of PTEN, which increases phosphorylation of Akt (by increasing PIP3 levels), appears to be mediated by the proteosome and dephosphorylation since loss of PTEN is blocked by a proteosome inhibitor MP32 and a phosphatase inhibitor, okadaic acid. It is also interesting that receptor internalization, which has been shown to be important in PC (256), is blocked by PTEN (63). Degradation of PTEN during PC might be involved in stimulating receptor recycling. PTEN is regenerated with extended reperfusion; this would be consistent with the loss of PC with extended reperfusion.

2) Akt—It has been shown that PC results in increased phosphorylation of Akt and that PC activation of Akt is inhibited by WM and LY294002, suggesting a role for PI3K in activation of Akt during PC (254). Recent data have shown that in hearts with reduced PDK1 activity, PC does not result in an increase in phosphorylation of Akt and does not afford protection (34).

What are the targets of Akt and how might it mediate protection?: Akt is thought to mediate protection by phosphorylation of a number of target proteins (see figure 6) such as glycogen synthase kinase (GSK)-3β, endothelial nitric oxide synthase (eNOS), the pro-apoptotic Bcl-2 family member BAD, caspase 9, the ubiquitin ligase murine double minute 2 (mdm2), and others (see(68)). Phosphorylation of BAD targets it to 14-3-3 protein where it is sequestered, thereby blocking its pro-apoptotic role. Overexpression of Akt blocked hypoxia mediated activation of caspase 3 and 9(260). Akt also phosphorylates and activates a ubiquitin ligase, mdm2. Myocytes overexpressing mdm2 have been reported to be resistant

to hypoxiareoxygenation cell death (257). It has been reported that the pro-apoptotic p53 can be inactivated by binding to phosphorylated mdm2 (166). Mdm2 is also recruited to a βarrestin signaling complex (82, 227), and the βarrestin signaling complex appears to be involved in the protection afforded by preconditioning (256). Akt phoshorylates and activates eNOS resulting in an increase in nitric oxide (NO) production(254). Akt also phosphorylates and inactivates GSK-3β (254). Inactivation of GSK-3β has been reported to be important in cardioprotection in a number of models (89, 123, 255); this will be discussed in detail below.

3) P70 Ribosomal S6 kinase (P70S6K)—P70S6K is another major target of PDK1, which appears to be activated by PC in a WM sensitive manner (256). As shown in figure 7, P70S6K is phosphorylated on a number of sites, and there are data suggesting that kinases from different pathways such as PKC, ERK, mammalian target of rapamycin (mTOR), Akt and PDK1 are involved in the phosphorylation and activation of p70S6K. Thus, p70S6K has a role in integrating signals from diverse pathways.

Inhibition of PI3K with WM blocks PC mediated phosphorylation of p70S6K and blocks protection (256). Although inhibition of PI3K blocks PC, the relative role of PDK1 activation of p70S6K, versus other targets, such as Akt, have not been delineated. Rapamycin, an inhibitor of mTOR (which phosphorylates p70S6K on thr 389) has been used to examine the effects of p70S6K (see figure 7). Rapamycin added before ischemia has been reported to block opioid mediated cardioprotection (89). Addition of rapamycin alone prior to ischemia had no effect on infarct size (89). Studies have also reported that rapamycin added at the start of reperfusion blocks PC (99). In another study, addition of insulin at the onset of reperfusion reduced infarct size and resulted in an increase in phosphorylation of p70S6K and Akt; the protection was abrogated by addition of rapamycin concomitant with insulin (120). In contrast to these finding, a recent study has reported that acute treatment with rapamycin per se is cardioprotective (131). Khan et al suggested that perhaps acute addition of rapamycin might activate Akt due to crosstalk between the pathways.

These studies with rapamycin have largely been taken as evidence for a role for p70S6K in PC; however the effects of rapamycin on PC may also be mediated by mTOR. In support of a direct role of p70S6K in protection, Jonassen et al have shown that insulin mediated protection in Girardi cells was blocked by anti-sense oligodeoxynucleotides targeted against p70S6K (119). Additional studies on the role of p70S6K and mTOR in PC will be needed.

What are the targets of p70S6K and how might it mediate protection?: As shown in figure 7, GSK3β can be phosphorylated by p70S6K (245). P70S6K has also been reported to phosphorylate BAD (97). Autophagy has been recently suggested to play a role in ischemic injury (93) and p70S6K, has recently been suggested to positively regulate autophagy, in contrast to the inhibition of autophagy by mTOR (223).

4) Mammalian target of rapamycin (mTOR)—As discussed above, rapamycin, an inhibitor of mTOR, has been shown to block the protective effects of PC and its effects have largely been attributed to inhibition of phosphorylation of p70S6K by mTOR. However, mTOR has some effects that could be important in cardioprotection, which are not related to its effects on phosphorylation of p70S6K. mTOR is activated by growth factors which activate PI3K. mTOR appears to function in a complex with either raptor (mTORC1; a rapamycin sensitive complex) or rictor (rapamycin insensitive companion of TOR) (mTORC2). These two complexes appear to have different functions. As mentioned, mTOR phosphorylates and contributes to the activation of p70S6K, and rapamycin has been shown to block the phosphorylation of thr 389 of p70S6K. However there are p70S6K mutants lacking the carboxyl terminal domain that can be phosphorylated on thr 389 in a rapamycin

insensitive manner (5, 211). Data have shown that rapamycin blocks phosphorylation of p70S6K by mTOR complexed with raptor (mTORC1), but rapamycin does not block phosphorylation of p70s6K by mTOR complexed with rictor (mTORC2)(5). mTORC2 (mTOR complexed with rictor) has been reported to phosphorylate Akt on ser473 (the putative PDK2 site) (213).

What are the targets of mTOR and how might it mediate protection?: mTOR phosphorylates and inactivates eukaryotic translation initiation factor eIF4E-binding protein-1 (4EBP1) resulting in an increase in protein synthesis; this may be involved in delayed PC, but since inhibition of protein synthesis does not consistently block acute PC, this increase in protein synthesis is not likely to be required for acute PC protection. mTOR has been reported to phosphorylate PKCε. Recent studies in Jurkat cells have shown that mTOR complexed with raptor increases mitochondrial oxygen consumption and mitochondrial $\Delta\psi$, whereas mTOR complexed with rictor decreases mitochondrial oxygen consumption (217, 218). The mTOR-raptor complex also regulates the balance between ATP generated by oxidative phosphorylation versus glycolysis. Addition of rapamycin to Jurkat cells for 12 hours decreased oxygen consumption and reduced phosphorylation of several mitochondrial proteins such as the voltage dependent anion channel (VDAC) and several mitochondrial dehydrogenases (218). mTOR was also shown to localize with mitochondria (218). Whether rapamycin would alter oxygen consumption, $\Delta\psi$ and phosphorylation of proteins in a more rapid time scale (consistent with time frame of PC) is unknown. Many cardioprotective agents appear to decrease $\Delta\psi$ and oxygen consumption; it is therefore somewhat difficult to reconcile that rapamycin, which reduces mitochondrial membrane potential and oxygen consumption, blocks PC. However, as mentioned, acute addition of rapamycin has been reported to be protective (131). Prolonged rapamycin treatment has been reported to inhibit assembly of TORC2 and inhibit phosphorylation of Akt (212). It will be important to better define the time dependent changes mediated by rapamycin on Akt, mitochondrial function and other signaling pathways important in cardioprotection. Clearly this is a complex area, but one that warrants further study.

5) Glycogen synthase kinase (GSK)—As shown in figure 4, GSK-3β is phosphorylated and inhibited during PC in a WM sensitive manner (255). The activity of GSK-3β is regulated by phosphorylation and by interaction with proteins. In unstimulated cells, GSK-3β is active and phosphorylates downstream substrates (see figure 8). Phosphorylation of GSK3β results in its inactivation. As shown in figure 8, Akt, p70S6K, PKC, PKA and p90RSK have all been reported to phosphorylate and inactivate GSK-3β. FrzA, a secreted antagonist of the Wnt/Frizzled pathway is reported to decrease phosphorylation of GSK-3β, independent of activity of Akt. Overexpression of FrzA has been reported to block the PC mediated increase in phosphorylation of GSK-3β and block the protection afforded by PC (21). ERK has recently been reported to phosphorylate GSK-3β and prime it for phosphorylation and inactivation by p70S6K (57). GSK-3β is also regulated by dephosphorylation and protein phosphatase 2A is suggested to be the primary phosphatase regulating dephosphorylation of GSK-3β.

Phosphorylation and inactivation of GSK-3β has been reported to be antiapoptotic. Tong et al showed that PC results in increased phosphorylation of GSK-3β, which is blocked by inhibitors of PI3K (254). Tong et al also showed that addition of inhibitors of GSK-3β prior to ischemia was as protective as PC. Using a cardiomyocyte model, Juhaszova et al showed that many different cardioprotective agents result in increased phosphorylation of GSK-3β (123). Gross et al showed that inhibition of GSK-3β is involved in cardioprotection induced by addition of opioids and they further showed that GSK-3β inhibitors were protective even when added just before reperfusion (89).

What are the targets of GSK-3β and how might it mediate protection?: A large number of transcription factors, kinases and other enzymes are reported to be substrates for GSK-3β (175). GSK-3β has a strong preference for substrates that have been primed by previous phosphorylation by another kinase (e.g. primed substrates, see figure 9). This preference for primed substrates provides a means to integrate multiple signaling pathways. Glycogen synthetase was the first substrate describe for GSK-3β and it is from this substrate that GSK 3β takes its name. Phosphorylation and inhibition of GSK-3β results in reduced phosphorylation and thereby greater activity of glycogen synthetase leading to increased glycogen synthesis. Although PC has been reported to reduce glycogen breakdown, this is likely regulated by glycogen phosphorylase, which is not regulated by GSK-3β. However there is coordinate regulation of glycogen synthesis and breakdown. β-catenin is another established substrate for GSK3β. Addition of an adenoviral vector containing a constitutively active β-catenin decreased infarct size measured 7 days after a coronary occlusion (91). PC was also reported to increase β-catenin accumulation along with an increase in β-catenin mediated transcriptional activity and an increase in capillary density measured at 4 days post-myocardial infarction (125). Thus the beneficial effects of β-catenin appear to be primarily mediated by altered gene expression and therefore may have limited impact on acute PC. Juhaszova et al showed that inhibition of GSK-3β delayed the time to opening of MPT initiated by ROS. The mechanism by which inhibition of GSK-3β reduces MPT is unclear but it is plausible that GSK-3β alters MPT by altering phosphorylation of target substrates (175). GSK-3β is reported to phosphorylate the pro-apoptotic protein, BAX, and target BAX to the mitochondria; thus inhibition of GSK-3β would reduce phosphorylation of BAX. GSK-3β phosphorylates a number of enzymes involved in metabolism, such as acetyl CoA carboxylase, ATP citrate lyase, and pyruvate dehydrogenase. Obviously, with so many substrates, it will be challenging to define the mechanisms involved in the cardioprotection afforded by inhibition of GSK-3β. Because inhibition of GSK-3β has been shown to reduce MPT, a focus on mitochondrial targets of GSK-3β may be useful. However, the link between GSK-3β and MTP may be indirect, and may involve cytosolic intermediates.

6) Endothelial nitric oxide synthase (eNOS)/Nitric oxide(NO)—Nitric oxide (NO) has been shown to have an important role in PC and cardioprotection (28, 121). NO can be generated by eNOS (see figure 10), which is phosphorylated and activated by Akt. In addition, during ischemia with low intracellular pH and oxygen, NO may also be generated from nitrite by deoxymyoglobin or xanthine oxidoreductase (108, 145, 230). Nitrite has been shown to reduce ischemia-reperfusion injury (66).

What are the targets of eNOS and how might it mediate protection?: eNOS generates nitric oxide (NO) and it is thought that the action of eNOS (as well as nNOS and iNOS) is mediated by NO. Early studies suggested that NO increased activity of the mitoK$_{ATP}$ channel (214). Recent studies suggest that the effects of NO on mitoK$_{ATP}$ channel are mediated via protein kinase G and PKCε (see figure 10). eNOS generates NO, which results in activation of guanylyl cyclase, which via protein kinase G is reported to activate a mitochondrial PKCε, which results in opening of the mitoK$_{ATP}$ channel (48). As shown in figure 10, NO can also result in post-translational modification of proteins such as S-nitrosation (also know as S-nitrosylation). S-nitrosation is a reversible modification that can protect thiol groups from further oxidation. S-nitrosation can also alter the activity of enzymes and transporters. NO has also been shown to result in S-nitrosation of mitochondrial complex I (35). Although it seems counterintuitive, there are data suggesting that in the setting of ischemia-reperfusion, inhibition of complex I can reduce ischemic injury (39, 70). Inhibition of complex I has been suggested to reduce activation of the MPT (70). However long term inhibition of complex I has been shown to be detrimental. NO has

also been shown to lead to S-nitrosation and inhibition of the cardiac L-type Ca^{2+} channel (243). Inhibition of the L-type Ca^{2+} channel would reduce Ca^{2+} loading during ischemia. NO has also been reported to modulate cell metabolism (156, 200). The anti-apoptotic effects of thioredoxin are enhanced by S-nitrosylation of thioredoxin at Cys-69 (249).

7) Receptor recycling and endosomal signaling—Activation of GPCRs has been shown to result in receptor internalization which was originally described as a mechanism to desensitize receptor signaling, but which has recently been shown to initiate novel endosomal signaling pathways (142). Recent studies suggest that homologous desensitization of GPCRs that is triggered by G-protein receptor kinase 2 (GRK2) mediated phosphorylation and β-arrestin binding, targets receptors to endosomes through an internalization process. β-arrestin has recently been shown to be a scaffolding protein that brings other signaling molecules in contact with GPCRs in the endosome during receptor recycling. There is increasing evidence that in addition to leading to receptor desensitization, β-arrestin mediated interaction can lead to activation of kinases such as ERK (22, 142, 226, 252). Inhibition of endosomal trafficking, and receptor recycling has also been shown to block the protective effects of preconditioning (256). Preconditioning was blocked in hearts treated with bafilomycin or monodansylcadaverine or when receptor recycling was blocked by sequestration of Gβγ (which inhibits receptor phosphorylation by GRK2) (256). In addition to elimination of protection, inhibition of endosomal/receptor recycling also inhibited PC mediated activation of ERK (256), consistent with a role for GPCR mediated endosomal signaling in PC. Interestingly, inhibition of microtubules with nocodazole or colchicine also blocked the protection afforded by PC, but did not increase infarct size in non-preconditioned hearts (115, 181). Arrestins have recently been shown to bind to microtubules (96).

PC has been reported to result in translocation of a number of important signaling molecules to specific intracellular locations, particularly the mitochondria. Connexin 43 (26), hexokinase (292), and Akt (3) have been suggested to translocate to the mitochondria during preconditioning. The molecular mechanisms regulating this translocation are not well understood. In general, translocation is thought to be regulated by alterations in post-translational modifications such as phosphorylation, which by altering conformation exposes sites that bind to scaffolding or anchoring proteins on specific organelles. However, it is likely that signaling molecules are also localized to the mitochondria by the cytoskeleton or other localizing mechanisms. It is tempting to speculate that perhaps endosomal signaling might be involved in targeting some of these kinases and other signaling molecules to the mitochondria or other intracellular locations.

8) Protein kinase Cε (PKCε)—Activation of PKCε has been shown in numerous studies to have an important role in cardioprotection. PKCε translocation to the membrane fraction has been used as a measure of activation. PC mediated translocation is blocked by WM suggesting it is downstream of PI3K (256). Ping et al showed that NO donors caused translocation of PKCε suggesting that NO (or eNOS) was upstream of PKCε (194). eNOS can be phosphorylated and activated by Akt, suggesting a mechanism that would place PKCε downstream of PI3K (254). Recently it has been shown that NO activates GC which in turn activates mitochondrial PKCε (48). PKCε also appears to be activated by the ROS generated during the brief preconditioning cycles of ischemia and reperfusion (15).

What are the targets of PKCε and how might it mediate protection?: PKCε has been shown using immunoblotting and immunofluorscence to form a mitochondrial localized signaling complex with mitogen-activate protein kinases (MAPKs) (20). Increased phosphorylation of mitochondrial ERK was only observed in mice expressing active PKCε, but not inactive PKCε. PKCε has also been reported to coimmunoprecipitate with VDAC,

adenine nucleotide translocator (ANT) and hexokinase II (19). In vitro studies showed that PKCε can phosphorylate VDAC (19), although the functional effect of this phosphorylation was not explored. PKC activation has also been shown to activate the mito K_{ATP} channel (150). PKCε and the mitochondrial K_{ATP} channel co-purify, suggesting they associate in the mitochondria as a signaling complex (7). When this purified fraction was reconstituted into liposomes, a PKC activator produced a mito KATP dependent K flux; this effect was reversed by addition of the protein phosphatase 2A (7).

9) ERK—Preconditioning has been shown to increase phosphorylation of ERK (74, 195). ERK can be phosphorylated by PKC and Baines et al showed that mitochondrial ERK phosphorylation occurred in mice expressing active PKCε (20). Receptor mediated endosomal signaling appears to be involved in PC mediated activation of ERK since ERK phosphorylation was blocked by inhibitors of endosomal recycling (256). MitoK$_{ATP}$ channel openers have also been reported to activate ERK, an effect that was blocked by the antioxidant N-acetyl cysteine (210). Most groups report that treatment with the MEK-1 inhibitor, PD 98059 blocks the protective effects of PC (53, 75, 195, 201, 240, 256). However, a few groups reported that PD 98059 did not block PC (134, 165). Mocanu et al (165) reported that although PD 98059 infused during PC, blocked PC mediated phosphorylation of ERK, it did not block PC mediated reduction in infarct size. However, as discussed later, this same group found that PD 98059 addition during reperfusion blocked the protection afforded by PC(100). Taken together ERK activation during ischemia and reperfusion appear to be important in mediating PC.

What are the targets of ERK and how might it mediate protection?: Activated ERK is known to modulate transcription factor activity; this would be important for delayed PC, but it is less likely to play a major role in acute PC, which appears to be mediated by post-translational modification of proteins.. Activation of ERK, particularly on reperfusion (195), has been shown to result in inhibition of the MPT. ERK has been shown to phosphorylate the pro-apoptotic protein BAD; phosphorylated BAD is sequestered by 14-3-3 and is therefore unable to bind and inhibit anti-apoptotic protein Bcl-2. ERK has also been shown to be involved in endosomal signaling pathways, which have been shown to be activated by PC. It is interesting to speculate that perhaps this endosomal signaling targets ERK to the proper compartment. This is particularly intriguing because PC has been shown to cause ERK translocation to different compartments.

10) p38 MAPK—p38 MAPK is a serine-threonine kinase that is activated by stress stimuli (241). The role of p38 MAPK in PC and cardioprotection is controversial (11, 182, 219, 233, 270). There are considerable data suggesting that inhibition of p38 MAPK is cardioprotective (152, 153, 219). There also are data in the literature suggesting that inhibition of p38MAPK blocks the protective effects of preconditioning (182, 270) and other data suggesting that inhibition of p38MAPK has no effect on PC (219). The reasons for this discrepancy are unclear and have been discussed in detail elsewhere. Briefly the discrepancy might be due to timing of activation of p38MAPK, activation of different isoforms (216), or differential localization (233).

What are the targets of p38MAPK and how might it mediate protection?: Activated p38 MAPK phosphorylates and activates ATF1 and 2, cAMP response element binding protein (CREB), and MAPK-activated protein (AP) kinase 2, which in turn phosphorylates substrates, including heart shock protein 27 (HSP27). Armstrong et al did not find a PC mediated increase in phosphorylation of HSP27, although they did find a PC induced increase in phosphorylation of p38MAPK (11). p38 MAPK is also reported to enhance uptake of the glucose analog 2-deoxyglucose into cells (253). Inhibition of p38 MAPK

kinase has also been shown to increase contractility (219). Consistent with this finding, p38 MAPK has recently been reported to depress sarcomeric tension development and ATPase activity (262)

11) JNK—JNK is another member of the MAPK family that has been shown to be activated by stress. Early studies suggested that activation of JNK was pro-apoptotic (275). However, it is clear the effect of JNK on cell survival is very complex. Most studies report that PC results in activation of JNK; however there is disagreement regarding the role of JNK in PC. Sato et al (215), but not Iliodromitis et al (111) find that inhibition of JNK blocks the protective effects of PC. Milano et al (160) recently reported that addition of a new JNK inhibitor reduced ischemia-reperfusion injury, suggesting a detrimental role for JNK. Three different mouse models with reduced JNK activity in the heart were all found to have less ischemia-reperfusion injury and less apoptosis (126). However, the same study examined the effect of sustained JNK activation by generating mice with increase MKK7 (the kinase that phosphorylates JNK) in heart; hearts from these mice were also protected from ischemia-reperfusion injury (126). Thus, the effects of JNK in the heart in the setting of ischemia-reperfusion appear to be complex and likely depend on localization, timing, and which isoform is activated.

What are the targets of JNK and how might it mediate protection?: JNK has been reported to form a complex with the apoptosome and delay activation of caspase 9 (258). JNK is also reported to mediate re-activation of AKT (225). JNK has also been shown to phosphorylate transcription factors such as c-jun (for which it is named) and AP-1.

12) Mitochondrial Targets of PC

Mitochondrial K_{ATP} channels: Early PC studies showed that inhibitors of K_{ATP} channels such as glibenclamide blocked the protection afforded by PC and that activators of K_{ATP} channels mimicked PC. Studies reviewed elsewhere suggested that it was a mitochondrial rather than a sarcolemmal K_{ATP} channel that mediated protection (80, 189). The mitochondrial K_{ATP} channel (mitoK_{ATP}) was described by Garlid as a mitochondrial channel involved in regulating mitochondrial matrix volume; potassium influx results in matrix swelling (see (80, 81)). Pharmacological drugs such as diazoxide open mitoK_{ATP} and 5-hydroxydecanoic acid has been used as an inhibitor of mitoK_{ATP} (189). In PC, the mitoK_{ATP} channel has been shown to be activated by NO and PKCε; as mentioned it has been suggested that NO activates guanylyl cyclase which leads to activation of PKCε which in turn (by an unknown mechanism) activates mitoK_{ATP} (see figure 3). Activation of a mitochondrial Ca activated K channel has also been shown to lead to cardioprotection (276).

In spite of intense effort, the molecular structure of the mitoK_{ATP} channel is ill defined. It has been reported to be composed of succinate dehydrogenase, phosphate carrier, ANT, ATP synthase, and the ATP binding cassette protein 1 (10). Activation of mitoK_{ATP} has been measured in myocytes by monitoring an increase in mitochondrial flavoprotein fluorescence (189). In isolated mitochondria, activation of mitoK_{ATP} is frequently measured by monitoring an increase in mitochondrial volume using absorbance (80, 81). In many studies in intact heart, a role for mitoK_{ATP} is defined by the ability of 5-hydroxydecanoic acid to block the protection. Questions have been raised about the specificity of 5-hydroxydecanoic acid and diazoxide (147).

How does activation of mitoK_{ATP} mediate protection?: It has been shown that activation of the mitoK_{ATP} channel by diazoxide will reduce ischemia-reperfusion injury (189). It has been suggested that activation of the mitoK_{ATP} channel will slightly reduce the mitochondrial membrane potential which would reduce mitochondrial Ca uptake during

ischemia (170). Activation of mitoK$_{ATP}$ has been suggested to result in an increase in mitochondrial volume, which has been suggested to alter the K_m for ADP entry into the mitochondria, perhaps by altering the conformation of VDAC (59). A decrease in ATP/ADP entry into the mitochondria during ischemia has been suggested to occur with overexpression of Bcl-2 and has been suggested to reduce the consumption of glycolytically generated ATP, which is used to maintain the mitochondrial $\Delta\psi$ (113); thus inhibition of ATP entry into the mitochondria during ischemia would reduce the $\Delta\psi$ and thereby reduce the uptake of Ca into the mitochondria (see Figure 1). In addition to acting as an "end-effector", activation of the mitoK$_{ATP}$ channel has been suggested to result in release of small signaling levels of ROS (71, 191) which may further enhance activation of PKCε and amplify the cardioprotective signaling. Clearly further studies will be needed to better define the molecular structure of the mitoK$_{ATP}$ channel and how it mediates cardioprotection.

Connexin-43: Mice heterozygous for connexin 43 (Cx-43) are not protected by PC (221). The role for Cx-43 in preconditioning does not involve gap junction formation between cells, because the reduction in Cx-43 blocks protection in isolated myocytes, a model in which there are no cell-cell connections and therefore no gap junctions (146). It has been shown that PC results in translocation of Cx-43 to the mitochondria and that mitochondrial Cx-43 is important for the protection afforded by PC (204). Interestingly the diazoxide mediated increase in ROS which seems to be involved in signaling cardioprotection, does not occur in Cx-43 deficient mice (104).

Inhibition of MPT: There are considerable data suggesting that inhibitors of MPT, a large conductance channel, reduce ischemia-reperfusion injury (86, 92, 117, 224). The MPT has been suggested to be a multiprotein complex comprised of the ANT and VDAC, and which is modulated by cyclophilin (24, 246, 274). However, it appears that neither VDAC nor ANT are required components of MPT (17, 137). Cyclosporin which binds to and inhibits cyclophilin has been shown to inhibit the MPT (49, 72). Cyclosporin reduced injury in perfused hearts during ischemia-reperfusion and cardiomyocytes subjected to anoxia (86, 185). The effects of ANT inhibitors are complex. Bongkrekic acid and carboxyatractylase both inhibit ANT; however bongkrekic acid inhibits ANT in c-state, in which the binding pocket faces the cytosol and carboxyatractylase inhibits ANT in m-state in which the binding pocket faces the matrix (136). Bongkrekic acid prevents dissociation of ATP/ADP from ANT and causes mitochondrial contraction similar to that observed with addition of adenine nucleotides, whereas carboxyatractylase reverses the ADP/ATP induced contraction of mitochondria. Interestingly bongkrekic acid inhibits whereas carboxyatractylase activates MPT (140, 180). This would be consistent with inhibition of MPT by adenine nucleotides. Mice with cardiac specific loss of cyclophilin were shown to be less sensitive to calcium activation of MPT and were also shown to have significantly smaller infarcts following ischemia-reperfusion (16).

The MPT opens under conditions of high Ca^{2+}, ROS, high NADH, depletion of adenine nucleotides and loss of $\Delta\psi$ (103, 109, 110); conditions that occur during ischemia-reperfusion (24, 65). Low pH, as occurs during ischemia, inhibits MPT. Intracellular pH is rapidly restored on reperfusion, thus allowing activation of the MPT. Furthermore, the reduced $\Delta\psi$ during ischemia would limit uptake of mitochondrial Ca^{2+}, but the re-introduction of oxygen on reperfusion would reconstitute $\Delta\psi$ (stimulating Ca^{2+} uptake into the mitochondria) and the introduction of oxygen will also allow generation of ROS. Thus the conditions that exist right at the start of reperfusion are ideal to stimulate opening of the MPT. This may explain why strategies that inhibit MTP during the first few seconds of reperfusion can reduce infarct size. Opening of the MPT causes rapid loss of $\Delta\psi$ and robust mitochondrial swelling which can be followed by changes in absorbance in isolated mitochondria. The extensive mitochondrial swelling results in rupture of the outer

mitochondrial membrane with loss of cytochrome c which can initiate apoptosis, although with full opening of MPT, it is likely cell death will occur before the apoptotic program is completed. Thus sustained activation of the MPT is incompatible with viability due to loss of mitochondrial function and resultant loss of ATP. It is interesting that many cardioprotective strategies would reduce opening of MPT. Protocols that reduce calcium loading of the cell reduce MPT and reduce infarct size. A reduction in cytosolic Ca^{2+} would reduce Ca^{2+} uptake into the mitochondria during ischemia and early reperfusion and thus reduce MPT. A reduction in ROS during ischemia and reperfusion would also reduce opening of MPT. Inhibition of complex I, which has been reported to be cardioprotective (39), has also been reported to reduce opening of MPT (70).

Thus reducing Ca^{2+} and ROS during ischemia and reperfusion and maintaining acid pH during the very start of reperfusion would all inhibit MPT. Although a large decrease in $\Delta\psi$ is a trigger for MPT, $\Delta\psi$ is also the driving force for Ca2+ uptake into the mitochondria and a high $\Delta\psi$ enhances generation of ROS. Thus a moderate reduction in $\Delta\psi$, particularly during ischemia has been suggested to be protective (39, 170). Activation of the reperfusion injury salvage kinases (RISK) at the start of reperfusion has been reported to reduce MPT (100). It is less clear how the MPT is modulated by PC or activation of the RISK pathways (100, 101); several potential mechanisms are summarized in figure 3. One hypothesis is that the activation of cardioprotective kinases results in activation of the mito K_{ATP} channel which reduces $\Delta\psi$, which as discussed would reduce the uptake of mitochondrial Ca^{2+} (170) and the generation of ROS. A number of kinases have also been reported to result in phosphorylation of mitochondrial proteins that might be involved in the MPT. PKCε has been reported to phosphorylate VDAC (19), a purported component of the MPT. The functional effects of PKCε phosphorylation of VDAC have not been delineated. It is likely that the kinases activated by cardioprotection have a number of undiscovered mitochondrial targets by which they mediate their protection. This is an area for future study.

B. Reperfusion Injury Survival Kinases - RISK Pathways

The RISK pathway was originally used to describe activation of survival kinases by addition of agonists such as adenosine and insulin at the start of reperfusion (102). It was shown that addition of these agonists at the start of reperfusion caused a rapid activation of PI3K and ERK resulting in cardioprotection. The activation of these survival kinases at the start of reperfusion is thought to mediate protection by inhibition of the MPT. Yellon and coworkers further demonstrated that preconditioning results in activation of ERK and Akt at the start of reperfusion and that inhibition of ERK and PI3K at the start of reperfusion blocks the protective effects of PC (101). These data suggest that at least part of the protection afforded by PC is mediated by activation of survival kinases at the start of reperfusion (100). There are data suggesting that activation of some kinase pathways during ischemia may initiate activation of survival kinases on reperfusion. For example, addition of erythropoietin (EPO) before ischemia is cardioprotective. Hanlon et al found that addition of PKC inhibitors, but not PI3K inhibitors, during ischemia, blocked the protective effects of EPO (95). However, addition of PI3K inhibitors at the start of reperfusion block the protection provided by EPO added prior to and during ischemia.

C. Postconditioning

In 2003 Zhao et al. reported that intermittent reperfusion, after ischemia, can also reduce infarct size (287). The precise postconditioning protocol (the number episodes and the duration of intermittent ischemia and reperfusion) required for protection appears to vary depending on the species. Yang also reported that if postconditioning was begun after 10 minutes of reperfusion, it was not protective (279). Most (32, 45, 105, 192, 193, 259, 278, 279, 287), but not all (61, 222) investigators report that postconditioning reduces infarct

size. It has also been suggested that cardioprotection by postconditioning is limited to coronary occlusion durations less than 45 minutes (248). Interestingly, it has been shown that the protection afforded by postconditioning occur via activation of many of the same signaling kinases that are involved in PC mediated protection (101). Furthermore the protection afforded by PC and postconditioning are not additive (259). As shown in figure 3b, postconditioning has been suggested to involve activation at the time of reperfusion of PI3K, Akt, eNOS, PKG, PKCe, ERK and mitoKATP.

1) Adenosine Receptors—A number of groups have reported that adenosine receptor antagonists will block the protective effects of postconditioning. Morrison et al (169) reported that the protection afforded by postconditioning was lost in hearts from mice with deletion of the A2A adenosine receptor. The phosphorylation of ERK and Akt that occur during reperfusion in postconditioned heart was also blocked in A2A adenosine receptor knock-out mice. Yang et al (278) reported that treatment of rabbit heart with the adenosine receptor inhibitor (8-p (sulfophenyl) theophylline (SPT)) blocked the protection afforded by postconditioning. Philipp et al (193) reported that an adenosine A2b antagonist MRS 1754 blocked the postconditioning mediated reduction in infarct size. Philipp et al (193) further showed that infusion 5 minutes before reperfusion of an adenosine A2b agonist ((N-ethylcarboxamido)adenosine; NECA) was as protective as postconditioning.

2) PI3K—Tsang et al (259) demonstrated in rat heart that postconditioning activates Akt, eNOS, and p70S6K, and that activation is blocked by inhibitors of PI3K. Tsang et al also showed that postconditioning mediated reduction in infarct size is blocked by addition of inhibitors of PI3K administered during the first 15 minutes of reperfusion. Consistent with a role for PI3K in postconditioning, Yang et al (278) showed that treatment of rabbit hearts with inhibitors of PI3K added for 20 minutes starting 5 minutes before reperfusion blocked the infarct reduction associated with postconditioning. Bopassa et al (32) also found that addition of PI3K inhibitors added at the start of reperfusion blocked postconditioning mediated improvement in both postischemic function measured by rate pressure product and enzyme release. PI3K inhibition was also shown to block postconditioning in a remodeled myocardium (289). However, Darling et al (54) reported that addition of a PI3K inhibitor during reperfusion did not block postconditioning in the perfused rabbit heart. Schwartz and Lagranha (222) find that postconditioning leads to activation of Akt and ERK, but does not result in a reduction in infarct size in pigs.

3) PKC—Philipp et al (193) showed that the PKC inhibitor, chelerythrine blocked the postconditioning mediated reduction in infarct size. Philipp et al also reported that infusion 5 minutes before reperfusion of a PKC activator PMA was as protective as postconditioning. Furthermore, the protection afforded by PMA addition on reperfusion was blocked by either MRS 1754 or wortmannin, a PI3K inhibitor (193). These data suggest that protection afforded by PKC activation on reperfusion is mediated by activation of adenosine receptors and PI3K. Interestingly, this same group (139) has suggested that the activation of PKC that occurs in PC increased the sensitivity of the adenosine A(2b) receptor to adenosine. Zatta et al (286) also showed that the postconditioning mediated reduction in infarct size is blocked by inhibition of PKCε. Zatta et al further showed that phosphorylated PKCε was higher in postconditioned hearts and that translocation of phosphorylated PKCδ to the mitochondria was less in postconditioned hearts.

4) MAPK—Yang et al (279) reported that infusion, beginning 5 minutes before reperfusion, of PD98059, an inhibitor of ERK activation, abrogated the postconditioning mediated protection. Darling et al(54) reported that postconditioning resulted in an increase in phosphorylation of ERK and was blocked by PD98059. Sun et al (242) reported that

hypoxiareoxygenation results in activation of JNK and p38MAPK; postconditioning reduces apoptosis in cardiomyocytes and also reduced activation of JNK and p38MAPK. Furthermore, addition of anisomycin, a JNK/p38MAPK activator, eliminated the inhibition of apoptosis by postconditioning.

5) PKG/NO—Yang et al (278{Yang, 2004 #320, 279) reported that infusion beginning 5 minutes before reperfusion of an inhibitor of NOS or inhibitors of guanylyl cyclase (ODQ) block the protection afforded by postconditioning. L-NAME, an inhibitor of NOS, was also shown to block postconditioning (279).

6) Mitochondria—Inhibition of mitoKATP channels has been reported to block the protection afforded by postconditioning (279). Interestingly, although loss of connexin 43 blocks preconditioning, it does not block the ability of postconditioning to reduce infarct size (105, 220). Hearts from mice lacking cyclophilin D, a component of the MPT, exhibit smaller infarcts than wild type mice and show no additional protection by postconditioning; thus the effect of loss of cyclophilin D and postconditioning are not additive, suggesting a role for the inhibition of MPT in postconditioning (148). Also consistent with a role for postconditioning mediated inhibition of MPT are recent data that suggest that postconditioning maintains acidosis during the start of reperfusion, which inhibits MPT (45, 76). Reperfusion with pH 7.7 buffer blocked protection induced by postconditioning (45).

V. Summary

Figure 11 summarizes the working hypothesis as to why cells die and how we might stop them. Data suggest that during ischemia, there is a rise in $[Ca^{2+}]_i$ (due to increased $[Na^+]_i$ and reverse mode NCX and Ca^{2+} entry via L-type Ca^{2+} channels and reduced Ca^{2+} efflux via Ca^{2+} ATPase). There is also ATP depletion, but this fall in ATP and the increase in $[Ca^{2+}]_i$ during ischemia do not appear to immediately result in rupture of the plasma membrane and irreversible injury, at least not during moderate durations of ischemia. As much as 50% of the ATP consumed during ischemia is via reversal of the $F_1F_0ATPase$, energy which is used to generate mitochondrial membrane potential ($\Delta\psi$). Maintaining $\Delta\psi$ will allow uptake of Ca^{2+} during ischemia and this Ca^{2+} when present at the start of reperfusion (when pH is restored) will initiate activation of the MPT. Furthermore, on reperfusion, oxygen is returned to the cell resulting in generation of ROS, regeneration of $\Delta\psi$ with uptake of potentially large amounts of Ca^{2+} into the mitochondria. This increase in matrix Ca^{2+}, coupled with increased ROS and the restoration of a normal pH (acid pH inhibits MPT) results in activation of the MPT, which in turn totally depletes the cell of ATP, activates the mitochondrial apoptotic pathways including caspases. The activation of these proteolytic pathways coupled with the total loss of ATP and ROS mediated damage synergize, leading to rapid loss of plasma membrane integrity. Cardioprotective pathways appear to work primarily by inhibition of the MPT directly or by preventing the conditions that promote MPT opening. Lowering $[Ca^{2+}]_i$ inhibits MPT. Inhibiting complex I inhibits MPT. Reducing ROS inhibits MPT. Lowering $\Delta\psi$ reduces mitochondrial Ca^{2+} uptake which would reduce MPT. As shown in figure 3, PC initiates a number of signaling pathways some of which reduce injury during ischemia and some of which activate survival kinases at reperfusion that protect primarily by inhibition of the MPT. Pre- and postconditioning both share activation of survival kinases. Postconditioning also slows recovery of extracellular pH, thus providing a lower intracellular pH that will also inhibit MPT. It is likely that postconditioning mediated maintenance of an acidic pH at the start of reperfusion keeps the MPT inhibited thus allowing time for the activation of the of the survival kinases to inhibit MPT.

The signaling cascades initiated by pre- and postconditioning also appear to inhibit MPT directly, likely by post-translational modification of mitochondrial proteins. There are data suggesting that activation of PI3K results in phosphorylation and inactivation of GSK-3β which by some ill defined mechanism reduces MPT. ERK has also been shown to increase phosphorylation of BAD which would reduce apoptosis. PKC which is activated by the survival kinases has also been shown to phosphorylate VDAC. Unfortunately our understanding of the MPT is very limited. The precise molecular make-up of the MPT is poorly understood. The role of the MPT in normal cell physiology (if there is one) is not understood. Clearly a better understanding of MPT is imperative if we are to better learn how to inhibit it to reduce ischemic death.

The similarity in protection between pre- and postconditioning in many models of ischemia (particularly at relatively short times of ischemia) suggest that under these conditions activation of survival kinase at reperfusion is sufficient to inhibit MPT. However, with longer periods of ischemia, preconditioning can modify conditions during ischemia which inhibit the MPT and this added protection may allow PC to protect against injury with longer durations of ischemia. However, the ability to elicit protection at the start of reperfusion is a much more relevant clinical application.

The protection afforded by postconditioning suggests that treatment at the start of reperfusion can be cardioprotective. How do we reconcile this with the large number of clinical trials that failed to show cardioprotection. Reasons for this discrepancy have been discussed in detail elsewhere (30). However, many of the failed clinical trials included patients in which reperfusion had already started. Thus the drugs were not administered before or at the very start of reperfusion. Indeed in the Guardian trial, administration of NHE inhibitors was protective in the group receiving coronary artery bypass surgery. It appears that it is important to apply cardioprotective drugs no later than then start of reperfusion. Furthermore, as discussed elsewhere (30), it is important that basic studies (prior to clinical trials) demonstrate protection in clinical relevant models using clinically relevant outcomes. It is also important to demonstrate protection using clinically relevant durations of ischemia.

Where do we go in cardioprotection?

Many drugs and protocols protected when added at the start of reperfusion. However given the history of previous failed clinical trials in cardioprotection one needs to be cautious. It is important to assess protection after longer, more clinically relevant periods of ischemia. It is also imperative that treatments be initiated before, or immediately at the start of reperfusion, for example with cardiac surgery or angioplasty. It is worth considering a cocktail (Na-H exchange inhibitors, antioxidant, NO donors, erythropoietin, caspase inhibitors and MPT inhibitors) that blocks multiple mechanisms in cell death. Perhaps this cocktail can be taken, similar to aspirin at the onset of a heart attack, while the patient is taken to the hospital.

Acknowledgments

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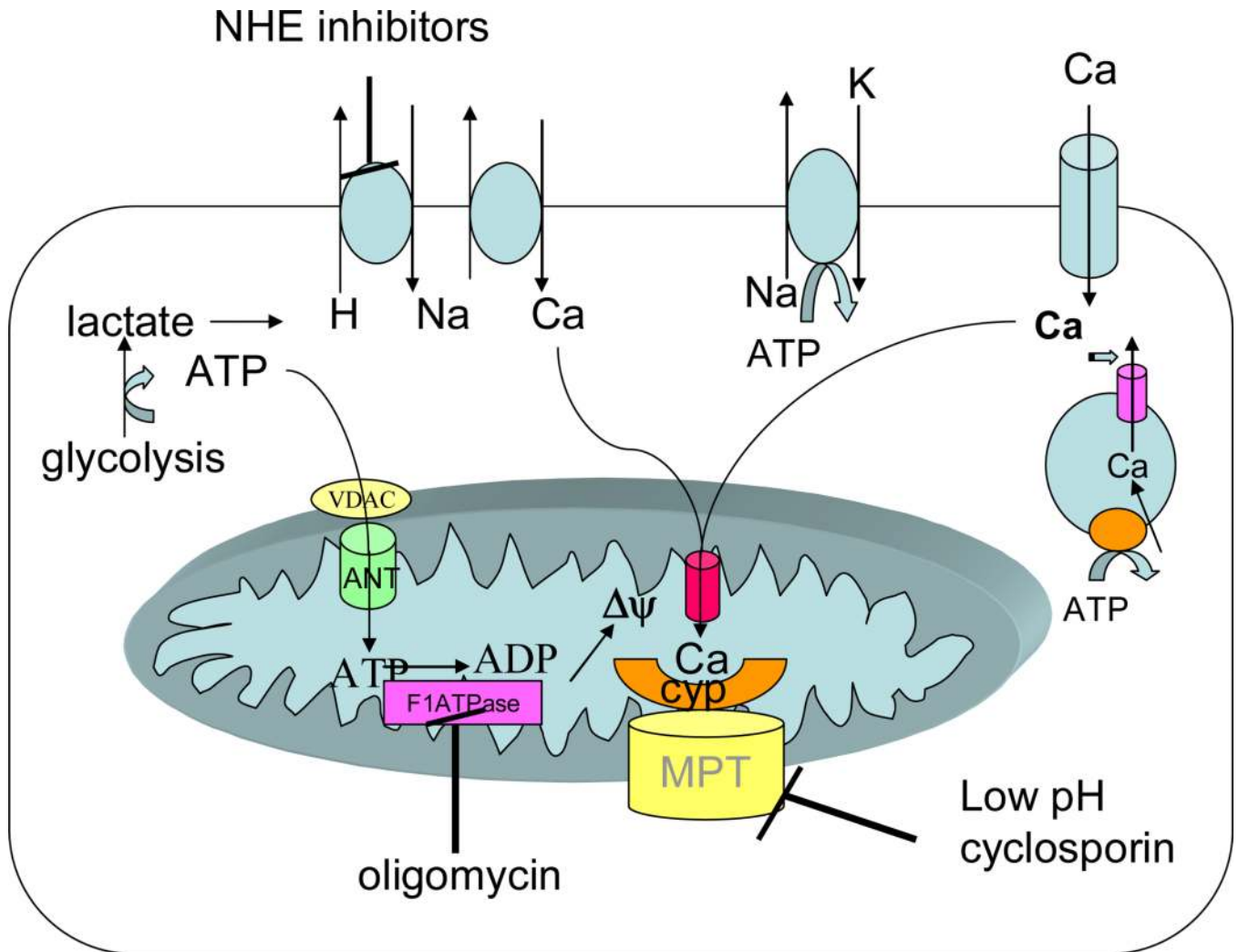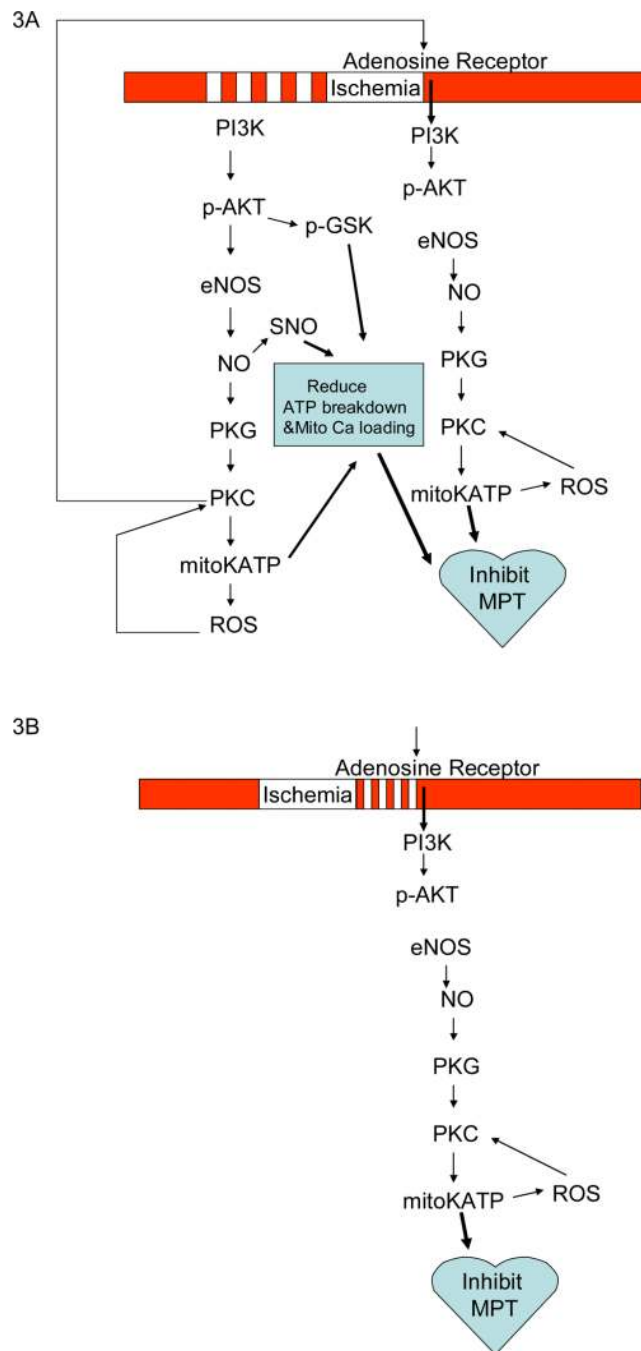
Figure 1.
Changes in ions and metabolites during ischemia. During ischemia ATP declines resulting in a decrease in pH due to anaerobic glycolysis. The increase in proton stimulates Na-H exchange and Na-Ca exchange resulting in an increase in cytosolic Ca. Much of the ATP generated by glycolysis is consumed by the reverse mode of the mitochondrial F1F0ATPase which uses the energy to generate $\Delta\psi$. The $\Delta\psi$ can then be used to take up Ca into the mitochondria. This increase in mitochondrial Ca can activate the MPT, but the low pH due ischemia inhibits MPT so that it is not activated until reperfusion when the pH is restored to normal.

Na ~ 10 mM
pH = 7.2

Na~1.25 mM
pH = 7.8
$\Delta\psi$ = -180 mV
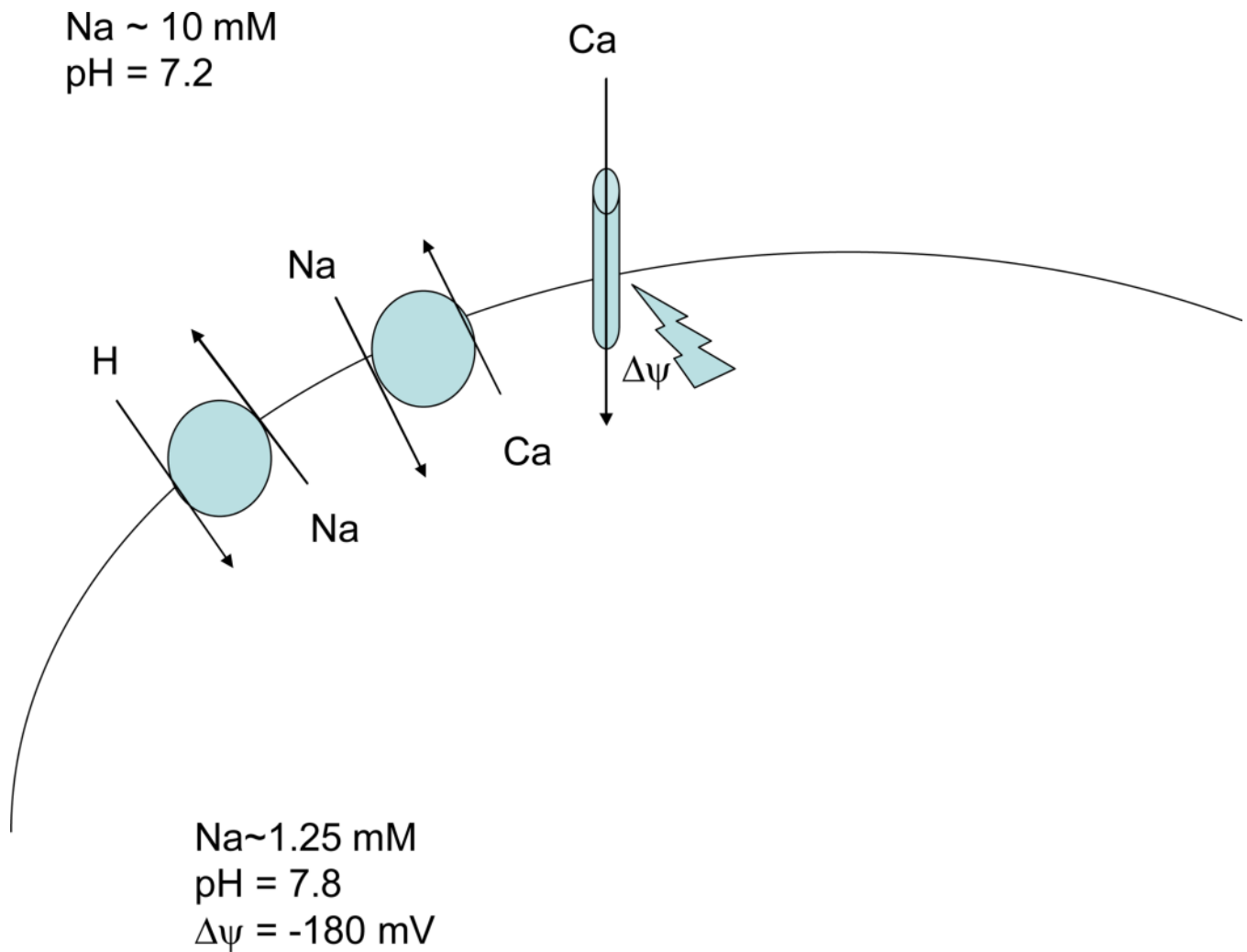
Figure 2.
Regulation of mitochondrial Ca^{2+}. Ca^{2+} enters mitochondria on the uniporter, driven by the negative mitochondrial membrane potential. Ca^{2+} efflux from the mitochondrial matrix is via the mitochondrial Na-Ca exchanger.

Figure 3.
Panel 3A illustrates the signaling pathways activated by PC during ischemia and at reperfusion. Panel 3B show the signaling pathways activated by postconditioning.
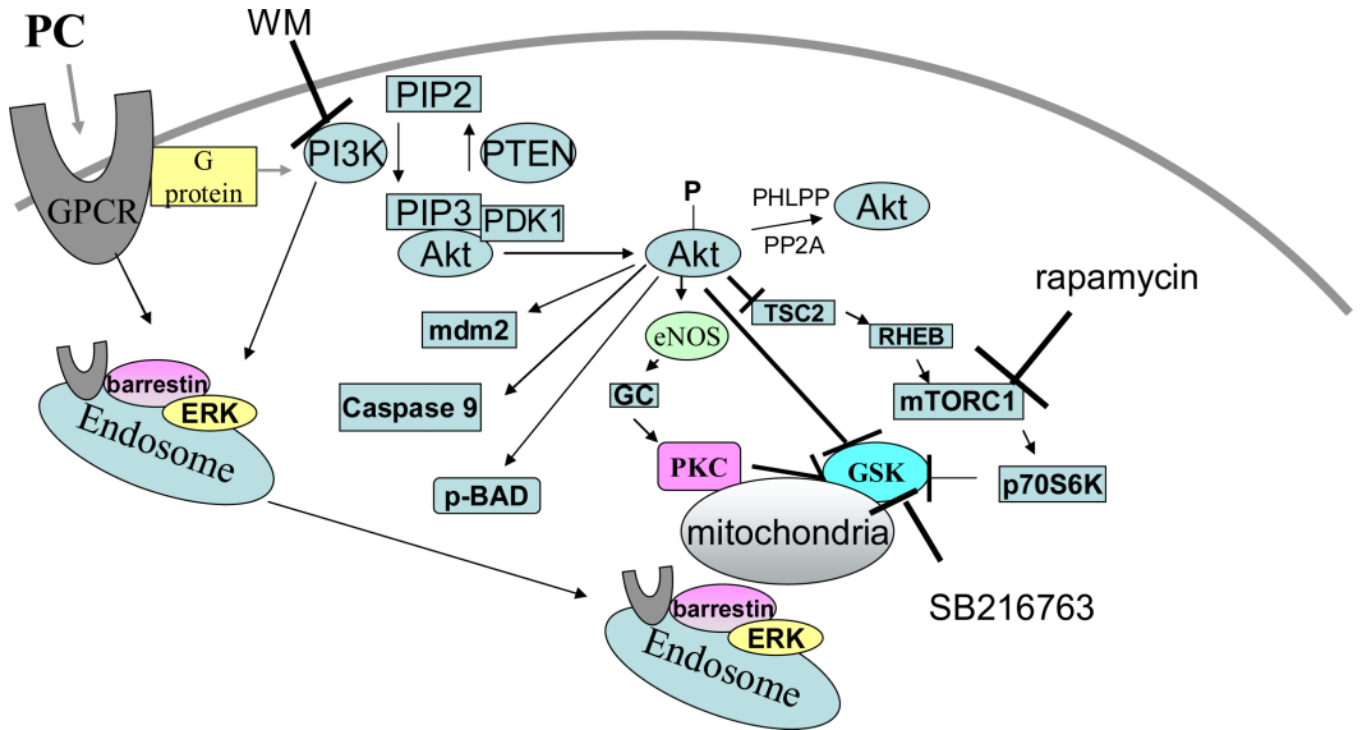
Figure 4.
Details of the signaling pathways activated by PC. PC leads to release of agonist that activate GPCR, leading to activation of PI3K, which in turn activates AKT and P70S6K. Internalization of GPCRs involves sequential binding of β-arrestin, the clathrin adaptor (adaptor protein 2; AP-2), and clathrin. PI3K and its phosphoinositide products play a critical role in recruitment of β-arrestin GPCR complexes to endosomes. Many of these pathways converge on the mitochondria where the result in activation of the mitoK$_{ATP}$ channel and inhibition of the MPT.
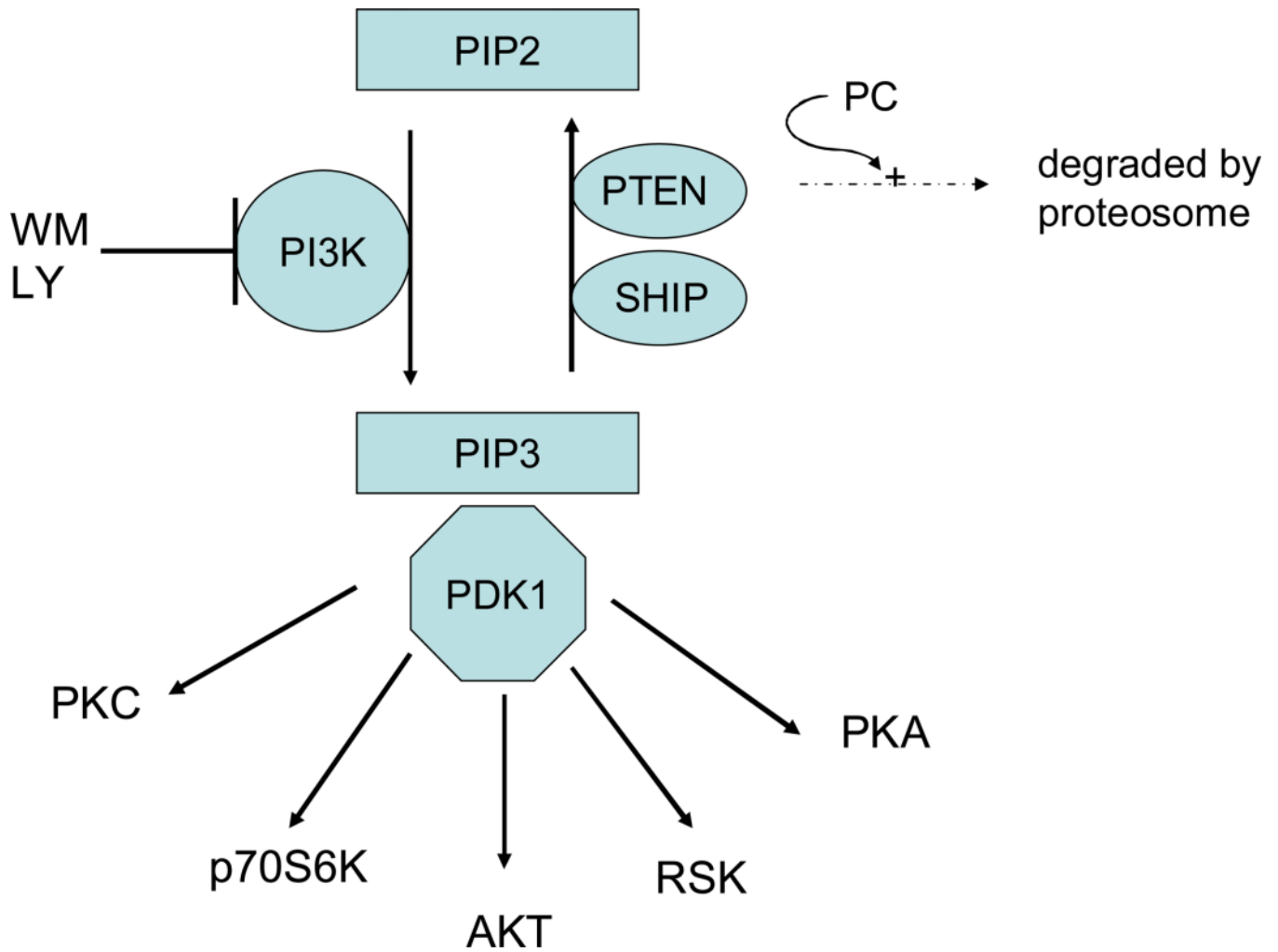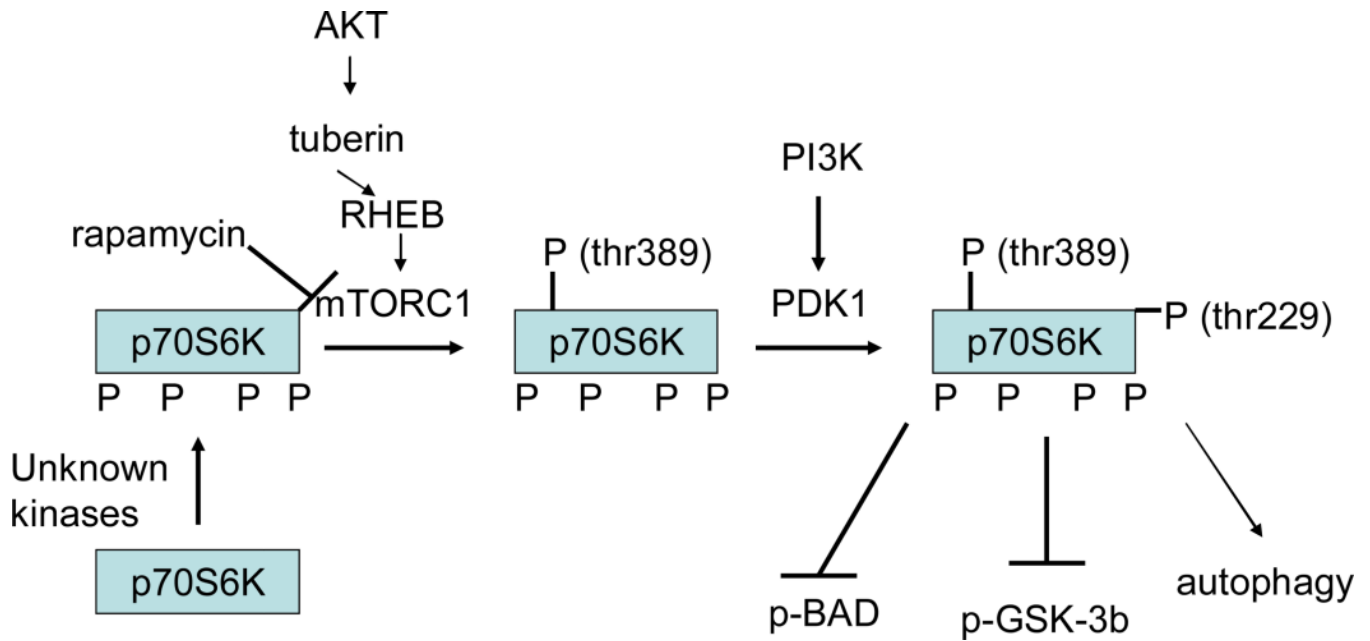
Figure 5.
PI3K signaling. PI3K generates PIP3 which recruits AKT to the membrane where it can be phosphorylated by PDK. Newly synthesized PKC is attached to the plasma membrane where it is phosphorylated by PDK1. PDK1 phoshorylation of PKCs in the activation loop is required for PKC activation and stability. However, for most PKC isoforms, the rate of PKC processing by PDK1 is not dependent on phosphoinositides generated by PI3K and thus the rate of PKC processing is not inhibited by wortmannin (188). PDK can also phosphorylate and activate PKA, RSK and p70S6K.
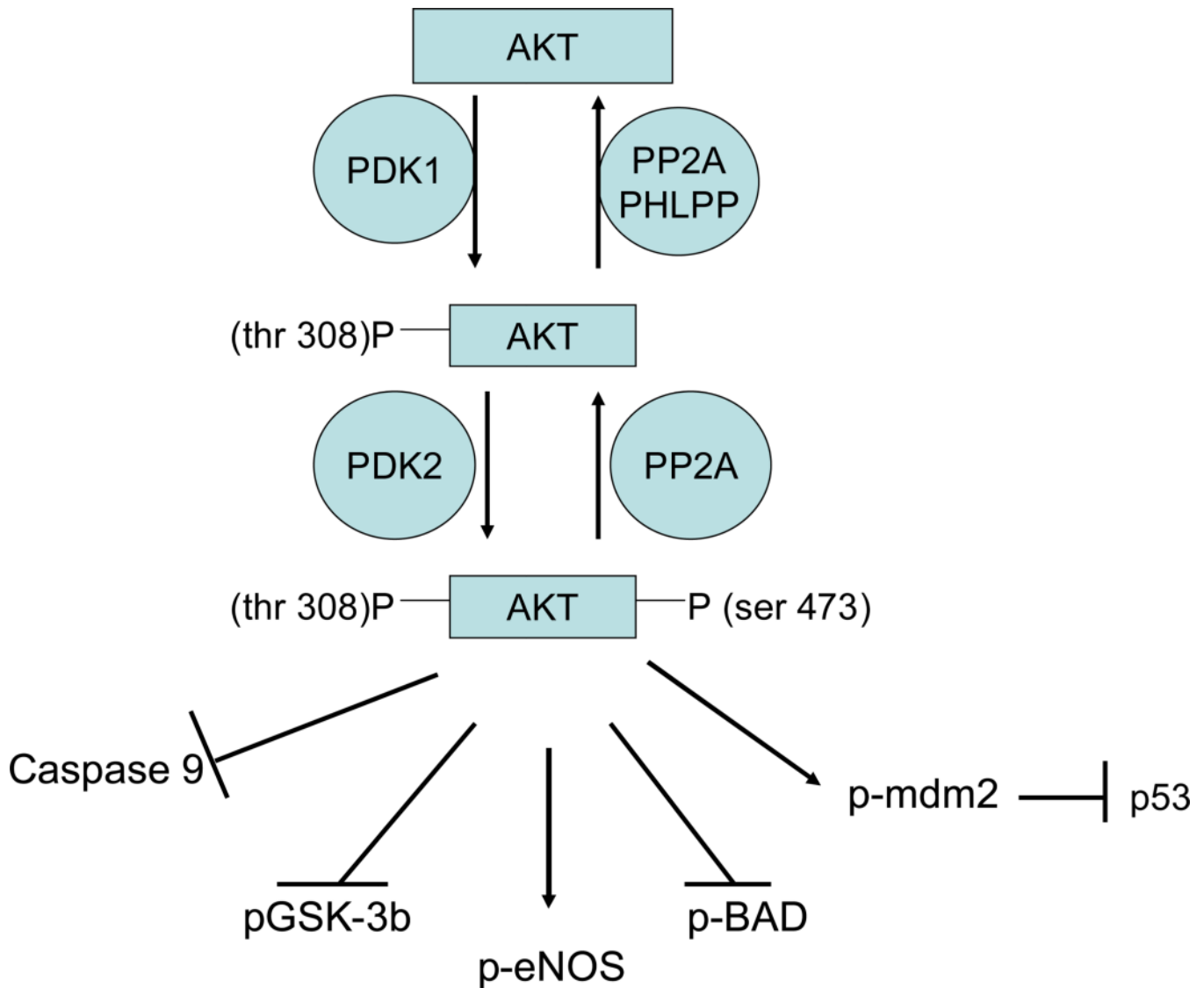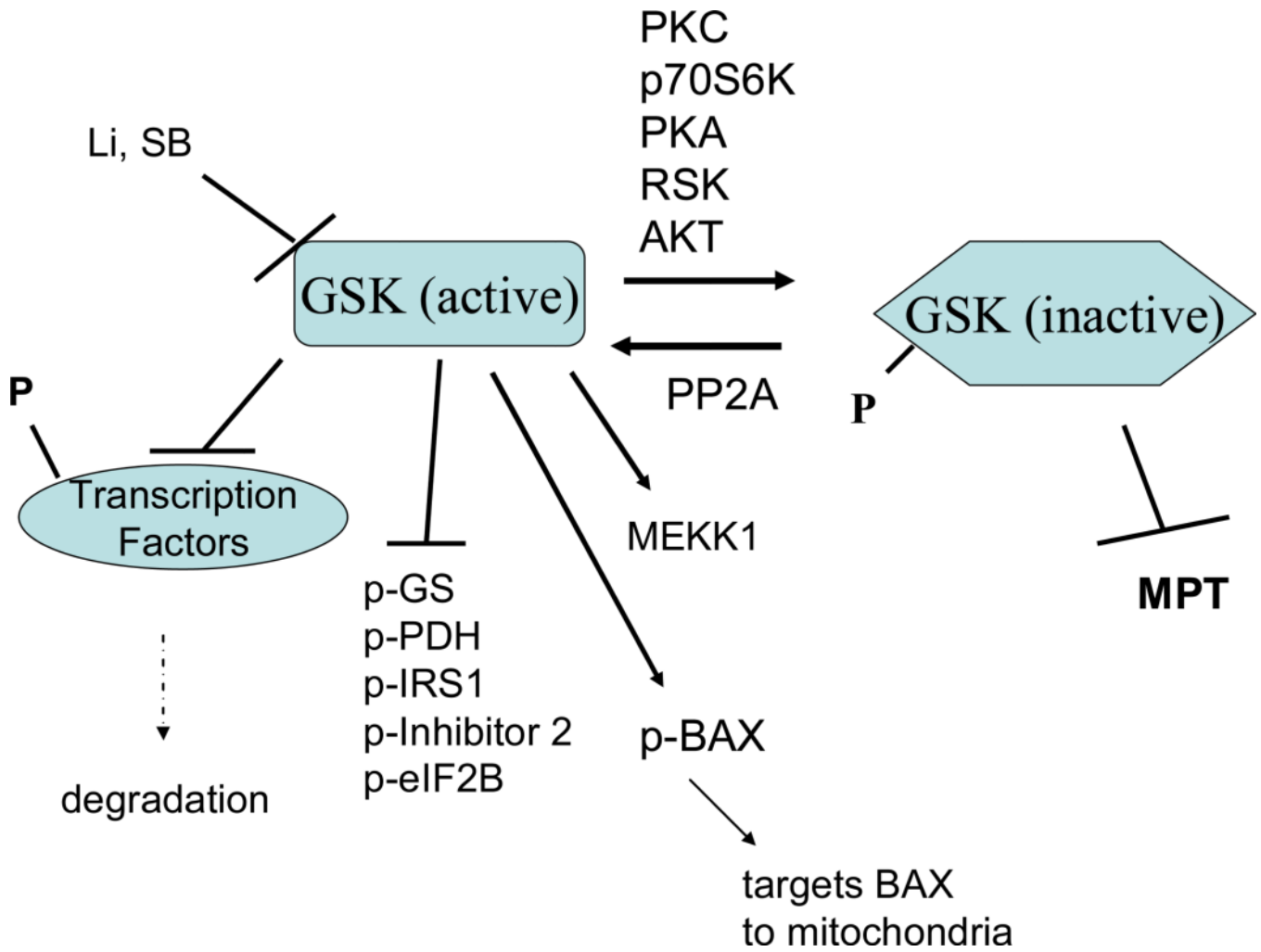
Figure 6.
AKT signaling. Akt is activated by phosphorylation of thr 308 by PDK1 and by phosphorylation of ser 473 by a poorly defined kinase. Phosphorylation of Akt at thr 308 is mediated by binding of PI3K generated lipid (PIP3) to the pleckstrin homology domain of Akt thereby facilitating translocation and association of Akt with PDK1. PIP3 binding to Akt also exposes the PDK1 phosphorylation site thus facilitating phosphorylation of Akt (188). A large number of kinases have been proposed to phosphorylate ser 473, including Akt autophosphorylation, PDK1, ILK1, MAPKAPK2, PKCbII, PIKK, ATM, DNA-PK, and TORC2 (see (68)). Increasing data seem to suggest a role for TORC2 in phosphorylating ser 473 on Akt (116). Akt can also be regulated by dephosphorylation, and PP2a (202) and PHLPP (33) have been reported to be important phosphatases regulating Akt phosphorylation.

Figure 7.
Regulation of p70S6K phosphorylation and signaling. P70S6K is phosphorylated by PDK1 on thr 229 and by mTOR on thr 389. Prior phosphorylation of thr 389 is reported to be necessary before PDK1 can phosphorylate thr 229. Phosphorylation of thr 389 appears to be rate-limiting and correlates with activity; however activation of mTOR depends on Akt activation. P70S6K is also phosphorylated on the pseudosubstrate or auto-inhibitory domain at ser 411, ser 418, thr 421 and ser 424, but the kinases responsible for phosphorylating these sites are debated. Phosphorylation of these sites appears to be necessary but not sufficient for activation. The activation of mTOR appears to be mediated by activation of Akt. Akt phosphorylates and inhibits tuberin (TSC2), a GTPase, which results in activation of RHEB, which results in phosphorylation and activation of mTOR. Akt is also reported to directly phosphorylate mTOR on serine 2448, but phosphorylation of this site does not appear to regulate activity of mTOR. mTOR activity is also inhibited by AMP-kinase; thus under conditions of increased AMP (decreased ATP) activation of AMP-kinase will lead to inhibition of mTOR
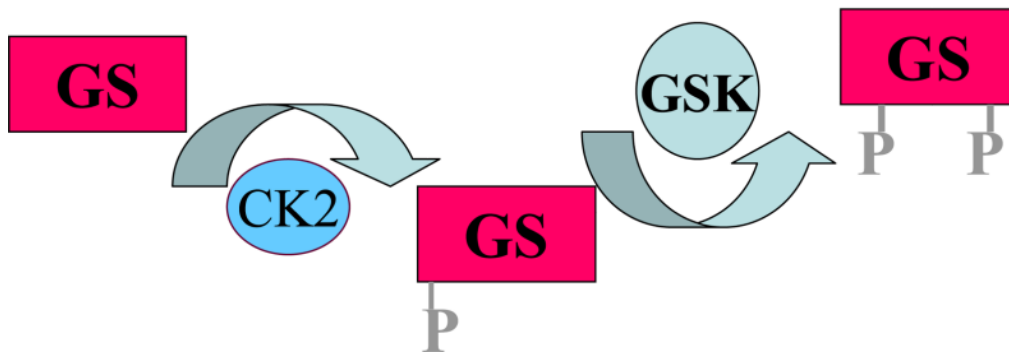
Figure 8.
GSK-β signaling in cardioprotection. In unstimulated cells, GSK-3β is unphosphorylated and active and it phosphorylates and usually inactivates its downstream targets. GSK-3β is phosphorylated and inactivated by a large number of kinases. Inactive GSK-3β has been suggested to be cardioprotective.
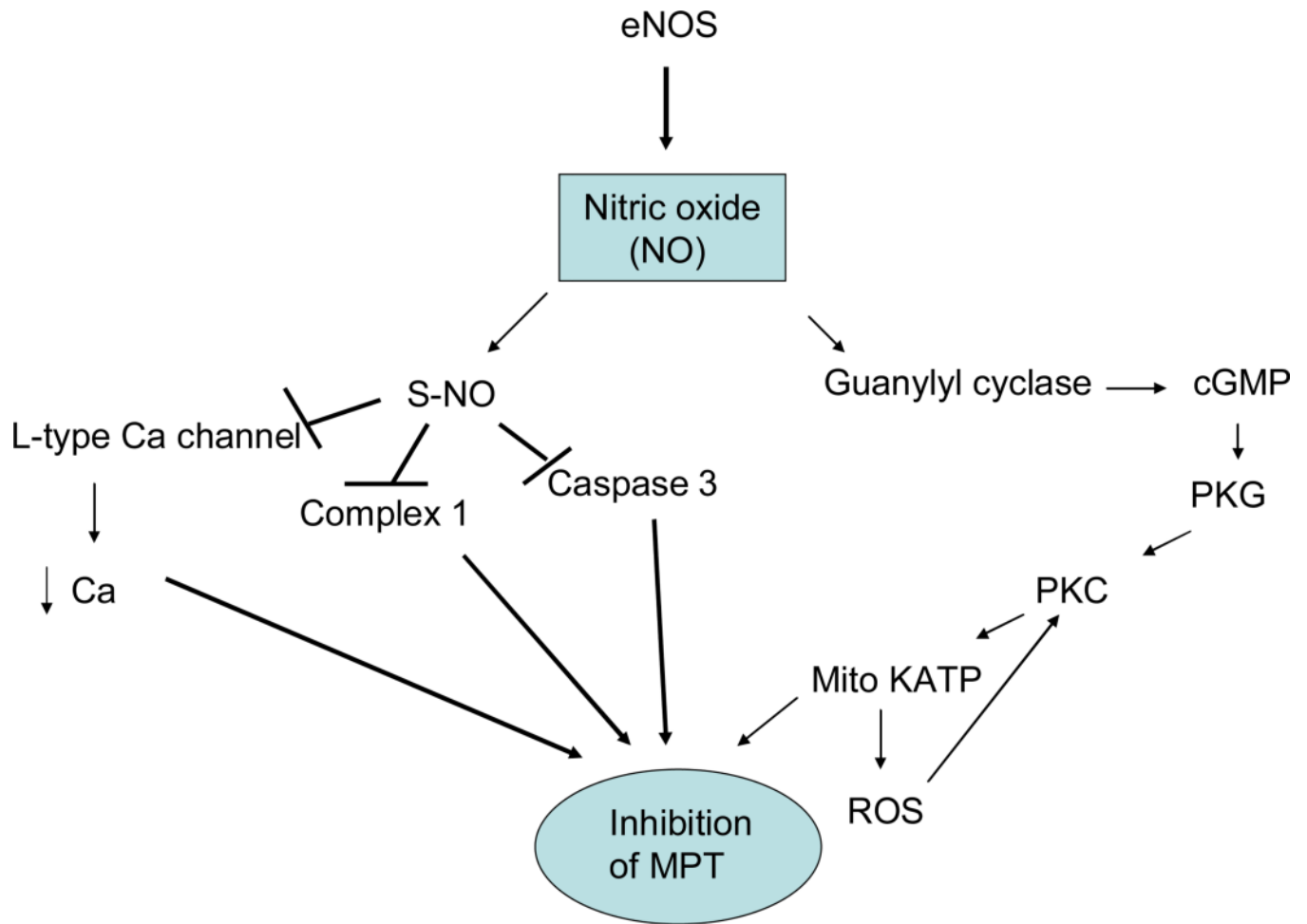
- ## Primed substrates increases efficiency of GSK by 100 to 1000 fold.

- Ser/thr-X-X-X-ser/thr-P

Figure 9.
GSK-3β has a preference for primed substrates.

Figure 10.
Nitric oxide signaling in cardioprotection. Nitric oxide (NO) is generated by nitric oxide synthases (NOS). NO can activate guanyly cyclase resulting in activation of protein kinase G. Alternatively NO can result in s-nitrosation of a number of proteins.
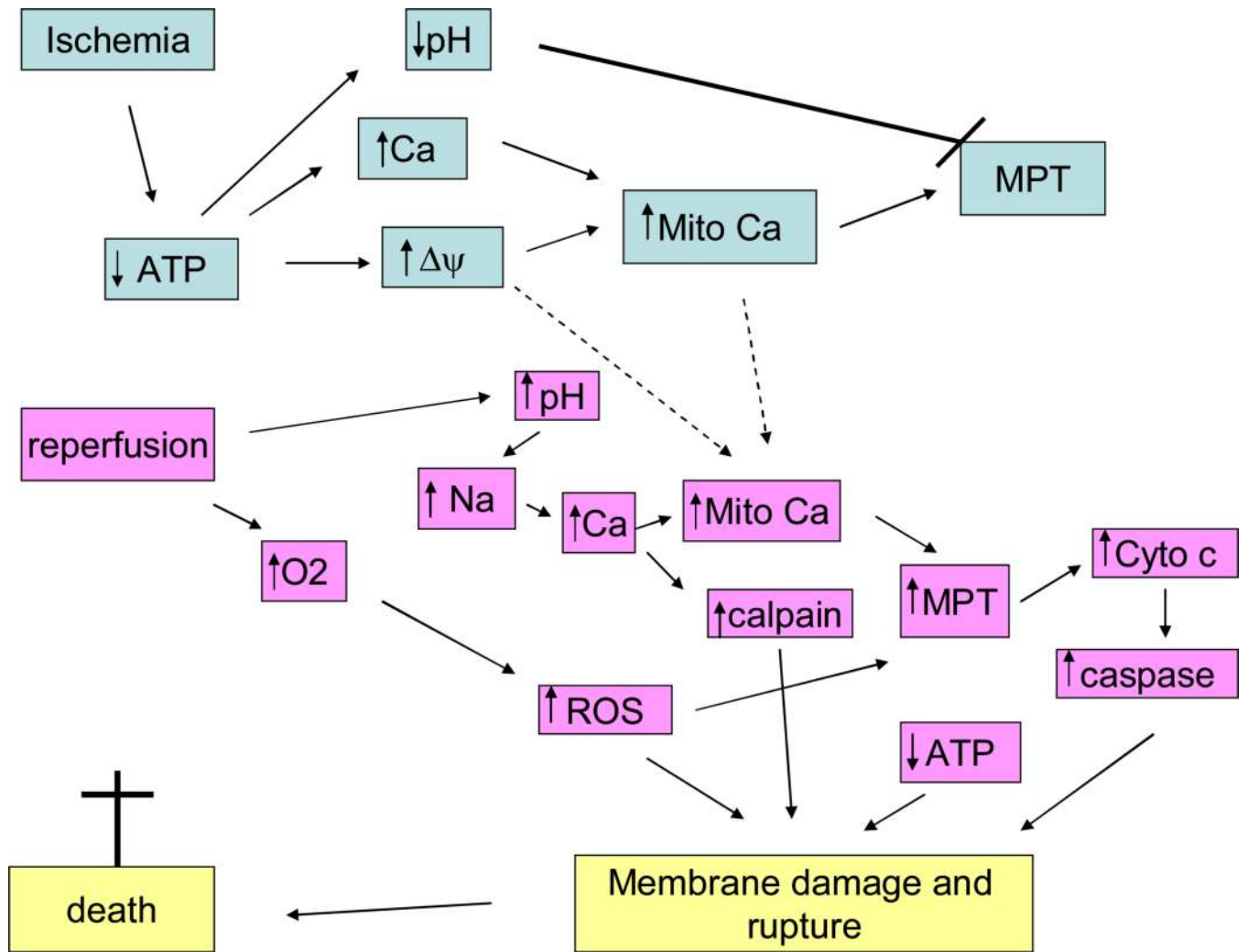
Figure 11.
Pathways Leading to Cell Death in Ischemia-Reperfusion.