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Comprehensive Invited Review

Ischemic Preconditioning: From Molecular Mechanisms to Therapeutic Opportunities

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ABSTRACT

Ischemia/reperfusion (I/R) injury is a major contributory factor to cardiac dysfunction and infarct size that determines patient prognosis after acute myocardial infarction. Considerable interest exists in harnessing the heart's endogenous capacity to resist I/R injury, known as ischemic preconditioning (IPC). The IPC research has contributed to uncovering the pathophysiology of I/R injury on a molecular and cellular basis and to invent potential therapeutic means to combat such damage. However, the translation of basic research findings learned from IPC into clinical practice has often been inadequate because the majority of basic research findings have stemmed from young and healthy animals. Few if any successful implementations of IPC have occurred in the diseased hearts that are the primary target of viable therapies activating cardioprotective mechanisms to limit cardiac dysfunction and infarct size. Therefore, the first purpose of this review is to facilitate understanding of pathophysiology of I/R injury and the mechanisms of cardioprotection afforded by IPC in the normal heart. Then I focus on the problems and opportunities for successful bench-to-bedside translation of IPC in the diseased hearts. *Antioxid. Redox Signal.* 10, 207–247.

I. INTRODUCTION

Myocardial infarction (MI) is a leading cause of death and disability in industrialized countries. The mortality and morbidity in patients with MI is correlated with infarct size, which is determined by the severity of ischemia/reperfusion (I/R) injury. Therefore, inhibition of I/R injury is of prime importance in improving the prognosis after MI. Thrombolytic therapy and coronary interventions are the therapeutic strategies to reduce infarct size to achieve rapid recanalization of occluded coronary arteries after MI, aimed at reducing infarct size. Pharmacologic approaches such as β-blockers and nitrates and mechanical supports such as intraaortic balloon pumping to reduce energy demand and increase coronary flow of the infarcted myocardium are also common practices to minimize infarct size. An alternative approach to minimize infarct size is to stimulate endogenous mechanisms for cardioprotection against I/R injury. Ischemic preconditioning (IPC) has been exploited as a powerful endogenous form of cardioprotection. IPC was first discovered by Murry and associates (230), who demonstrated that a brief period of repetitive cardiac I/R exerts a protective effect against subsequent lethal periods of ischemia. It is now evident that IPC has two distinct phases: an early phase, which lasts from a few minutes to 2–3 h, and a late phase, termed late IPC, which develops after 12 h, peaks between 24 and 48 h, and lasts for 72–96 h (27, 38, 359). Because the cardioprotective effect of IPC was found to be consistent beyond species including humans, numerous investigators have studied the mechanism of cardioprotection afforded by IPC and have attempted to apply it to clinical practice. Although the IPC research has the potential to open a new avenue in the management of MI and ischemic heart diseases, no established therapeutic strategies are based on IPC. The lack of opportunities to practice IPC may be responsible for inadequate understanding of the mechanism of IPC.

The aim of this review is to provide better understanding of preconditioning, which can provide feedback to activate basic research and clinical practice for cardioprotection against I/R injury. To this end, I briefly overview the pathophysiology of cardiac I/R injury that is necessary for fundamental under-

standing of the molecular and cellular biology of cardiac preconditioning, with a special focus on redox signaling triggered by generation of reactive oxygen species (ROS). In addition, I shed a light on preconditioning-mimetic phenomena that are also sensitive to redox signaling. Then I discuss potential mechanisms by which diseased hearts become refractory to preconditioning protection. Although preconditioning protection against I/R injury is successful in a majority of animal studies *in vitro* and *in vivo*, the translation of basic research findings about preconditioning into clinical practice has often been inadequate, because a majority of basic research findings have stemmed from young and healthy animals. Few if any successful implementations of preconditioning have been made in diseased hearts that are the primary target of viable therapies, activating cardioprotective mechanisms to limit cardiac dysfunction and infarct size. Finally, future directions for cardioprotection research and therapeutic opportunities to prevent I/R injury and ischemic heart diseases are discussed on the basis of what we have learned from preconditioning.

II. THE MECHANISM OF I/R INJURY AND PROTECTION BY IPC

I/R injury of the heart is characterized by reversible contractile dysfunction, known as stunning, and irreversible injury leading to cardiomyocyte death and MI. Here, I discuss the mechanism of myocardial stunning and cardiomyocyte death; apoptosis, oncosis, and autophagy.

A. Myocardial stunning and IPC

Postischemic dysfunction, or myocardial stunning, is defined by the mechanical dysfunction that persists after reperfusion, despite the absence of irreversible damage and despite restoration of normal or near-normal coronary flow (37). The two essential points of this definition are (a) that postischemic dysfunction, no matter how severe or prolonged, is a fully reversible abnormality; and (b) that the dysfunction is not caused by a primary deficit of myocardial perfusion. In accordance with this definition, myocardial stunning is a relatively mild, sublethal injury that must be kept quite distinct from MI. It is unknown whether these two conditions share a common mechanism, and therefore, data obtained in models of infarction should not be extrapolated to models of stunning. The phenomenon of postischemic dysfunction was initially described by Heyndrickx and associates (126), who demonstrated that regional mechanical function remained depressed for >3 h after a 5-min coronary occlusion and for >6 h after a 15-min occlusion in conscious dogs. The term *myocardial stunning* was coined in 1982 (47) and has become the focus of increasing interest after the development of thrombolytic therapies and coronary interventions for acute MI.

Two major hypotheses have been raised as the mechanism of myocardial stunning (44): the oxyradical hypothesis, in which myocardial stunning is caused by the generation of ROS; and the calcium (Ca^{2+}) hypothesis, in which it is caused by a transient Ca^{2+} overload on reperfusion. The final lesion responsible for the contractile depression appears to be a decreased responsiveness of contractile filaments to Ca^{2+}. Recent evidence suggests that Ca^{2+} overload may activate calpains, Ca^{2+}-dependent neutral proteases, resulting in selective proteolysis of myofibrils. The time required for resynthesis of damaged proteins would explain in part the delayed recovery of function in stunned myocardium. The oxyradical and Ca^{2+} hypotheses are not mutually exclusive and are likely to represent different facets of the same pathophysiologic cascade. For example, increased ROS formation could cause cellular Ca^{2+} overload, which would damage the contractile apparatus of the cardiomyocytes. ROS generation could also directly alter contractile filaments in a manner that renders them less responsive to Ca^{2+} (*e.g.*, oxidation of critical thiol groups). However, it remains unknown whether ROS play a role in all forms of stunning and whether the Ca^{2+} hypothesis is applicable to stunning *in vivo*. Nevertheless, it is clear that the lesion responsible for myocardial stunning occurs, at least in part, after reperfusion, so that this contractile dysfunction can be viewed, in part, as a form of "reperfusion injury." An important implication of the phenomenon of myocardial stunning is that so-called chronic hibernation may be the result of repetitive episodes of stunning, which have a cumulative effect and cause protracted postischemic dysfunction.

Besides direct effects of ROS and Ca^{2+} on Ca^{2+}-regulatory proteins and contractile machinery, ROS and intracellular Ca^{2+} overload modulate cardiomyocyte contractility through phosphorylation of contractile proteins. A variety of protein kinases are activated in response to oxidative stress, and this process is pivotal in regulating the function of contractile machinery. Particularly, protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK) have been implicated in the pathogenesis of ischemic contractile dysfunction (177, 345).

Whether early IPC reduces stunning is not entirely clear (142, 272), because the IPC stimulus by itself causes stunning, and difficulty exists in discriminating an antistunning effect from reserved contractile function as a result of a larger remaining contractile mass after a lethal period of ischemia. In contrast to early IPC, late IPC has demonstrated significant protection against myocardial stunning. Thus, an initial episode of stunning triggers a delayed adaptive response that renders the myocardium more resistant to subsequent episodes of stunning 24–72 h later (41).

B. Mitochondria and cell death

Two basic patterns of cell injury progressing to cell death are known: cell injury with shrinkage, known as apoptosis, and cell injury with a primary increase in cell membrane permeability, known as oncosis. Apoptosis and oncosis are the distinct forms of cell death that are distinguished by both morphologic and biochemical criteria (198). The former is defined by the occurrence of internucleosomal fragmentation of genomic DNA associated with a sealed plasma membrane, whereas the latter is characterized by early plasma membrane rupture and disruption of cellular organelles, including mitochondria. Myocardial I/R is known to produce both cardiomyocyte apoptosis and oncosis of a variable degree. Although apoptosis does not contribute to infarct size during the early phase of reperfusion when the sarcolemmal membrane remains intact, apoptotic cardiomyocyte death becomes an independent contributing vari-

able of infarct-size development during the late phase of ischemia and reperfusion when secondary necrosis occurs in apoptotic cardiomyocytes (147). It has been demonstrated that cardiomyocyte apoptosis is a rare event during a relatively brief period (<1 h) of ischemia but is accelerated on reperfusion (90). However, the form of cardiomyocyte death induced by I/R is not uniform, consisting of apoptosis, oncosis, and necrosis, which is an ultimate form of cell death by any causes (286). Conversely, the form of cardiomyocyte death during reperfusion after a relatively brief period of ischemia is only oncosis with DNA fragmentation (242).

Although such variability in the form of cardiomyocyte death during I/R is at least in part responsible for the lack of uniform criteria to differentiate between apoptosis and other types of cell death, it is also attributed to the lack of understanding of the critical event that determines the form of cardiomyocyte death during reperfusion.

Mitochondrial dysfunction is a common denominator of apoptosis and oncosis. Oncosis is associated with loss of mitochondrial function and ATP. During I/R, intracellular Ca^{2+} is increased, and excessive Ca^{2+} is taken up by mitochondria at the expense of the mitochondrial membrane potential ($\Delta\Psi m$), leading to abrogation of oxidative phosphorylation and ATP generation *via* F_1/F_0 ATPase (236). Excessive entry of Ca^{2+} into mitochondria in concert with oxidant stress and accumulation of inorganic phosphate that likely to occur during sustained ischemia provokes the opening of the mitochondrial permeability transition pore (mPTP), a nonspecific channel of the inner mitochondrial membrane. Opening of the mPTP is also attributed to the collapse of $\Delta\Psi m$ and loss of mitochondrial function (87). The critical role of the mPTP opening, as a mediator of myocardial I/R injury, has been confirmed in studies demonstrating that mice lacking cyclophilin-D, an important regulatory component of the mPTP, showed increased resistance to the mPTP opening, and sustained smaller myocardial infarcts (19, 232). Emerging studies implicate the suppression of the mPTP opening as a potential end-effector of cardioprotection in IPC (123, 141).

In contrast to oncosis, apoptosis is an energy-requiring process. The opening of the mPTP triggers cytochrome *c* release from the intermembrane space (50, 114), which in the presence of Apaf-1 and ATP or dATP activates caspase-9, which in turn activates caspase-3, the executioner responsible for activation of caspase-activated DNase and the resultant DNA fragmentation specific for apoptosis (52). Thus, depletion of cellular energy inhibits caspase-3 activation and switches cell death from apoptosis to oncosis, indicating that oncosis and apoptosis represent different outcomes of the same pathway induced by the mPTP opening.

C. Autophagy and cell death

Autophagy is another potential form of cell death that can be seen under a variety of adverse environments. The role of autophagy in cardiac I/R injury has been investigated in an *in vivo* model of chronic ischemia (352) and I/R (206). Autophagy is a cellular degradation process responsible for the turnover of unnecessary or dysfunctional organelles and cytoplasmic proteins. However, unlike oncosis and apoptosis, which result in cell death without regeneration, autophagy is not simply a destructive phenomenon, because the salvaged amino acids can be used to build new proteins. Thus, autophagy can be considered a protective mechanism against cell death. However, the signal-transduction pathway and the consequence of autophagy are different between ischemia and reperfusion. Activation of autophagy during ischemia is mediated by AMP-activated protein kinase and is cardioprotective, whereas that induced by reperfusion is mediated by beclin 1, a molecule involved in autophagosome formation, and is detrimental (206). The role of autophagy has also been investigated in isolated cardiomyocytes subjected to anoxia and reoxygenation (79). The inhibition of autophagy with *N*-3-methyladenine during anoxia–reoxygenation caused an increase in the number of necrotic cells and a decrease of the live-cell population. Moreover, simultaneous inhibition of both autophagy and apoptosis (*N*-3-methyladenine and caspase-3 inhibitor application) in anoxia–reoxygenation led to a dramatic increase of necrotic cardiomyocytes and a concomitant decrease in the number of living cells. These observations suggest that the cytoprotective mechanism of autophagy seems to conserve a cellular energy level and ATP to avoid oncotic cell death. Further studies are required to determine the exact roles of autophagy in cardiac injury and protection associated with I/R.

D. IPC and cardioprotection

1. IPC prevents apoptosis. IPC is known to protect cardiomyocyte apoptosis during reperfusion. The mechanism of protection from cardiomyocyte apoptosis by IPC appears to be due to protection of mitochondria. It has been shown that IPC reverses many aspects of mitochondrial dysfunction induced by I/R, including loss in the activity of the redox-sensitive Krebs cycle enzyme α-ketoglutarate dehydrogenase, declines in NADH-linked ADP-dependent mitochondrial respiration, insertion of the pro-apoptotic Bcl-2 protein Bax into the mitochondrial membrane, and release of cytochrome *c* into the cytosol (193). This protection of mitochondria by IPC is mediated by activation of survival signaling that converges on prevention of the mPTP opening (11, 69, 346). The survival signaling includes the PKC-ε–mitochondrial K_{ATP} (mitoK_{ATP}) channel pathway (181), the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (122), the Janus kinase (JAK)/signal transducers, and activators of transcription (STAT) pathway (116). The cardioprotective signal-transduction pathways and complex cross-talks between these signaling pathways are discussed in detail in the later sections.

2. IPC prevents oncosis. Unlike plasma membranes of other cell types and isolated cardiomyocytes that do not undergo mechanical stress, a unique feature of myocardial reperfusion is a sudden increase in mechanical stress on the sarcolemmal membrane, associated with reintroduction of contractile activity or development of supercontracture. The resultant disruption of the sarcolemma abrogates mitochondrial function and severely depletes cellular energy, leading to oncosis. Although cardiomyocytes become oncotic without reperfusion when the period of ischemia is prolonged, it has been demonstrated that permeability of the sarcolemma is not increased in the majority of cardiomyocytes after a brief period (30–40 min) of ischemia by itself (143). Thus, it appears that

sudden reintroduction of mechanical force executes cardiomyocyte oncosis during the early phase of reperfusion. It has been demonstrated that blocking contractility during the early phase of reperfusion switched cardiomyocyte oncosis to apoptosis associated with increased caspase-3 activity, whereas increased mechanical stress at the time of reperfusion increased oncotic cardiomyocytes and decreased apoptotic cardiomyocytes associated with inhibition of caspase-3 activation (250). These observations are consistent with the notion that mechanical stress is a critical determinant of the form of cardiomyocyte death during the early phase of reperfusion.

The most salient cardioprotective effect of IPC is the inhibition of cardiomyocyte oncosis and MI. Such a preconditioning effect emerges immediately after reperfusion. Accumulating evidence indicates that cardioprotective signal transduction derived from IPC converges on protection of mitochondria (113, 229, 248, 310). Although mitochondrial protection is a principal mechanism of protection against apoptosis and oncosis, the molecular link between mitochondrial protection and prevention of oncosis afforded by IPC remains an enigma. Because sarcolemmal fragility is a cause of oncosis during reperfusion, inhibition of sarcolemmal fragility during reperfusion was considered a primary mechanism of cardioprotection mediated by IPC (16).

The pathogenesis of sarcolemmal fragility during reperfusion has been a target of extensive research for many years. In the normal heart, the sarcolemma is tightly attached to the sarcomere through the Z-band (Fig. 1). However, shortly after reperfusion, the sarcolemma is broken at the site of Z-band attachment. Consecutive breakage of the Z-band increases permeability through the sarcolemma and produces a subsarcolemmal bleb, which is typically observed in oncotic cardiomyocytes. The alteration of proteins that link sarcolemma and cytoskeleton has been implicated in this pathogenesis (15, 99). The contractile force generated by actin–myosin interaction is transmitted to the sarcolemma at the lateral costamere junction. Therefore, the physical stress imposed on the fragile membrane causes its breakage at the site of Z-band attachment. The lateral costamere junction contains a number of structural proteins that link the Z-band and the sarcolemma (15, 99). Of these, dystrophin serves as a mechanically strong physical linkage between the sarcolemma and the costameric cytoskeleton in the cardiac muscle (283). Dystrophin stabilizes the sarcolemma against shear stresses imposed during eccentric muscle contraction by acting as a shock absorber (267, 307). The absence of the dystrophin gene is associated with vulnerability of the sarcolemma to mechanical force (215, 308).

It has been demonstrated that sarcolemmal dystrophin is translocated from the sarcolemma to the cytoplasmic region during ischemia and is subsequently lost during reperfusion (154, 166, 309). Reintroduction of contractile activity during reperfusion produced oncosis in cardiomyocytes depleted of sarcolemmal dystrophin but not in cardiomyocytes replenished with sarcolemmal dystrophin, suggesting that loss of sarcolemmal dystrophin is causally related to reperfusion injury. Therefore, we hypothesized that mitochondrial protection may culminate in restoration of sarcolemmal dystrophin during reperfusion, and this mechanism may represent a missing link between mitochondrial protection and protection from oncosis afforded by IPC (154, 166) (Fig. 2).

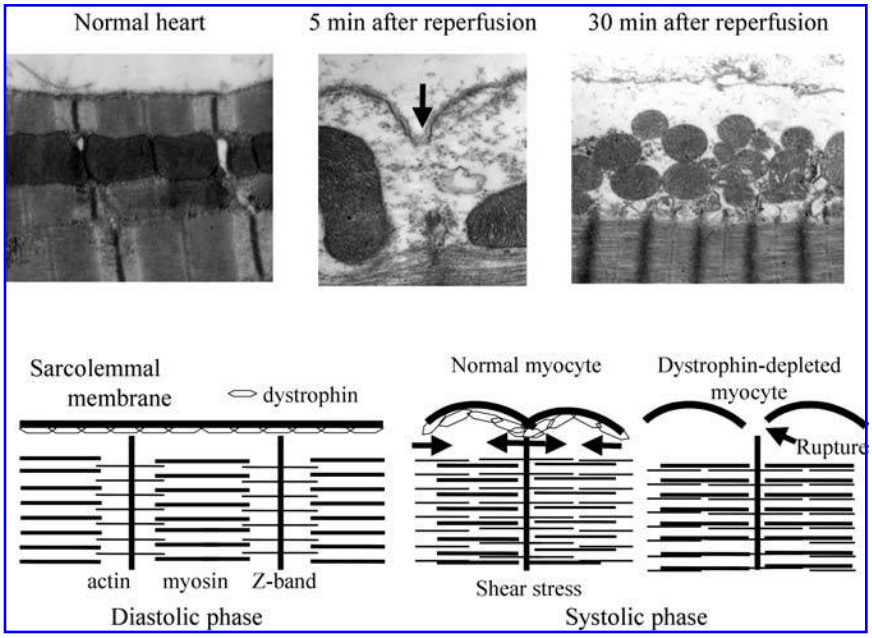

FIG. 1. Oncosis as a characteristic feature of myocardial reperfusion injury. Oncosis, defined by a primary increase in permeability of cell membrane, is a characteristic feature of myocardial reperfusion injury. In the normal heart (electron micrography, left panel, ×10,000), the sarcolemma is tightly attached to the sarcomere at the Z-band. However, shortly after reperfusion (middle panel, ×15,000), the sarcolemma is broken at the site of Z-band attachment (*arrow*). Consecutive breakage of the Z-band increases permeability through the sarcolemma and produces a subsarcolemmal bleb, which is typically observed in oncotic cardiomyocytes (right panel, ×8,000). The hypothetical mechanism of sarcolemmal fragility is illustrated in the lower panels. Dystrophin plays a pivotal role in stabilizing sarcolemmal membrane against physical stress. Contractile force generated by interaction of actin and myosin is transmitted to the sarcolemma at the site of Z-band attachment. The normal myocyte contains dystrophin that connects the sarcolemma to the Z-band and spans the inner surface of the entire sarcolemma, thereby protecting the sarcolemma from injury at the site of Z-band attachment by shear stresses produced during each cycle of contraction and relaxation. However, when sarcolemmal dystrophin is lost under certain pathologic conditions, such as ischemia/reperfusion, the sarcolemma becomes fragile and is ruptured at the site of Z-band attachment on contraction during the systolic phase.

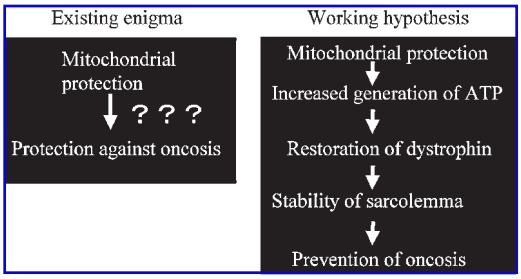

FIG. 2. A hypothetical mechanism of protection against oncosis during reperfusion. An enigma exists as to why oncosis is induced on physical stress in cardiomyocytes depleted of ATP and why protection of mitochondria confers protection against oncosis. It is hypothesized that mitochondrial protection results in increased generation of ATP during reperfusion, which facilitates restoration of dystrophin to the sarcolemma and stabilization of the membrane against oncosis.

This hypothesis was tested by elucidating the relation between mitochondrial function and localization of dystrophin. When the isolated and perfused rat hearts were subjected to 30 min of global ischemia, the loss of sarcolemmal dystrophin and myocardial ATP was similar in the control and the IPC hearts (Fig. 3A and B). Similar loss of sarcolemmal dystrophin and myocardial ATP was observed when the nonischemic perfused hearts were treated with 2,4-dinitrophenol (DNP), an uncoupler of mitochondrial respiration, or oligomycin, an inhibitor of mitochondrial F_1F_0-ATPase. However, the IPC heart increased sarcolemmal dystrophin during reperfusion associated with an increase in $\Delta\Psi m$, as demonstrated by increased uptake of tetramethylrhodamine ethylester (an indicator of $\Delta\Psi m$), and myocardial ATP (Fig. 3C and D). The increase in relocalization of sarcolemmal dystrophin and myocardial ATP during reperfusion was inhibited by treatment with DNP or oligomycin. The role of mitochondria in the distribution of dystrophin was further investigated by using isolated mitochondria. When mitochondria were incubated under an ATP-generating condition, the ischemic IPC heart mitochondria increased relocalization of dystrophin from the insoluble (cytoskeletal) to the soluble (membrane) fractions associated with increased ATP generation in a DNP and oligomycin-sensitive manner (Fig. 4A and B). More important, increased relocalization of sarcolemmal dystrophin in the IPC heart during reperfusion was associated with inhibition of several parameters of cardiomyocyte oncosis (Evans blue dye accumulation, creatine kinase release, and infarct size) (Fig. 5A–C). These results suggest that enhanced relocalization of dystrophin to the sarcolemma during reperfusion may be a mechanistic link between improved generation of ATP by mitochondria and protection against oncosis conferred by IPC. Furthermore, we found that temporary contractile arrest by reperfusion with a contractile blocker, 2,3-butanedione monoxime (BDM), completely prevented cardiomyocyte oncosis in the IPC heart. Although it is still premature to conclude that restoration of sarcolemmal dystrophin during reperfusion is an obligatory end effect of cardioprotection conferred by IPC, a temporal relation between restoration of sarcolemmal dystrophin and prevention of contractile force–induced oncosis during reperfusion, in conjunction with *in vitro* evidence that redistribution of dystrophin to the sarcolemma depends on mitochondrial oxidative phosphorylation, lends support to the hypothesis that dystrophin is an end target of mitochondrial protection that confers protection against mechanical stress–induced oncosis during the early phase of reperfusion.

Here, I propose a hypothetical mechanism by which temporary blockade of contractility enhances an antioncotic effect of IPC (Fig. 6). In intact cardiomyocytes, normal contractile activity does not break the sarcolemma because of sarcolemmal localization of dystrophin in the presence of abundant ATP generated by mitochondria. However, sarcolemmal dystrophin is lost during ischemia because of the loss of ATP, but sarcolemmal injury is not yet induced because of the absence of contractile activity. On reperfusion, sudden reintroduction of contractile activity breaks the sarcolemma depleted of dystrophin that allows unlimited entry of extracellular solutes such as Na^+ and Ca^{2+}, leading to oncosis. However, when contractility is blocked on reperfusion, sarcolemma remains intact so that oncotic death is prevented and mitochondria are capable of generating ATP to replenish the sarcolemma with dystrophin. In such a cardiomyocyte, no injury of the sarcolemma is induced after reintroduction of contractility.

3. IPC and chloride channels. Another potential mechanism of protection mediated by IPC is activation of chloride channels. Chloride channels play a crucial role in cell-volume regulation on cell swelling (262). Diaz and associates (76) proposed the hypothesis that cell-volume regulation is enhanced by IPC through activation of chloride channels and may account for the majority of sarcolemma protection against reperfusion-induced massive cell swelling with cell-membrane rupture. However, chloride channels regulate not only chloride flux but also superoxide generation. It has been demonstrated that chloride channel inhibition prevents superoxide-dependent apoptosis induced by I/R in mouse cardiomyocytes (344). It has also been demonstrated that superoxide flux in endothelial cells *via* the chloride channels mediates intracellular signaling (124). Because superoxide plays an essential role in redox signaling in cardioprotection mediated by IPC, the exact role of chloride channels and cell-volume regulation in IPC-induced cardioprotection remains elusive.

4. IPC and antioxidant defense systems. The cardiovascular system is continuously exposed to both ROS and reactive nitrogen species. Oxygen, although essential for tissue survival, can be injurious when produced in excess or when innate antioxidant defenses are overwhelmed during I/R. Several groups of investigators measured ROS generation in *in vivo* models of regional myocardial I/R, and showed that potent ROS, which are produced during the early minutes of reperfusion, play a critical role in myocardial injury in models of myocardial stunning and infarction (43, 106, 216). A number of ROS sources have been found in reperfused myocardium, such as the infiltration of polymorphonuclear leukocytes (PMNs) into the infarcting tissue, xanthine oxidase, activation of the arachidonate cascade, autoxidation of catecholamines, and activation of various NAD(P)H oxidases (369). Of these, accumulation of PMNs in the postischemic heart has been implicated in the pathogenesis of reperfusion injury. In the reperfused region, PMNs adhered to the endothelium can injure endothe-

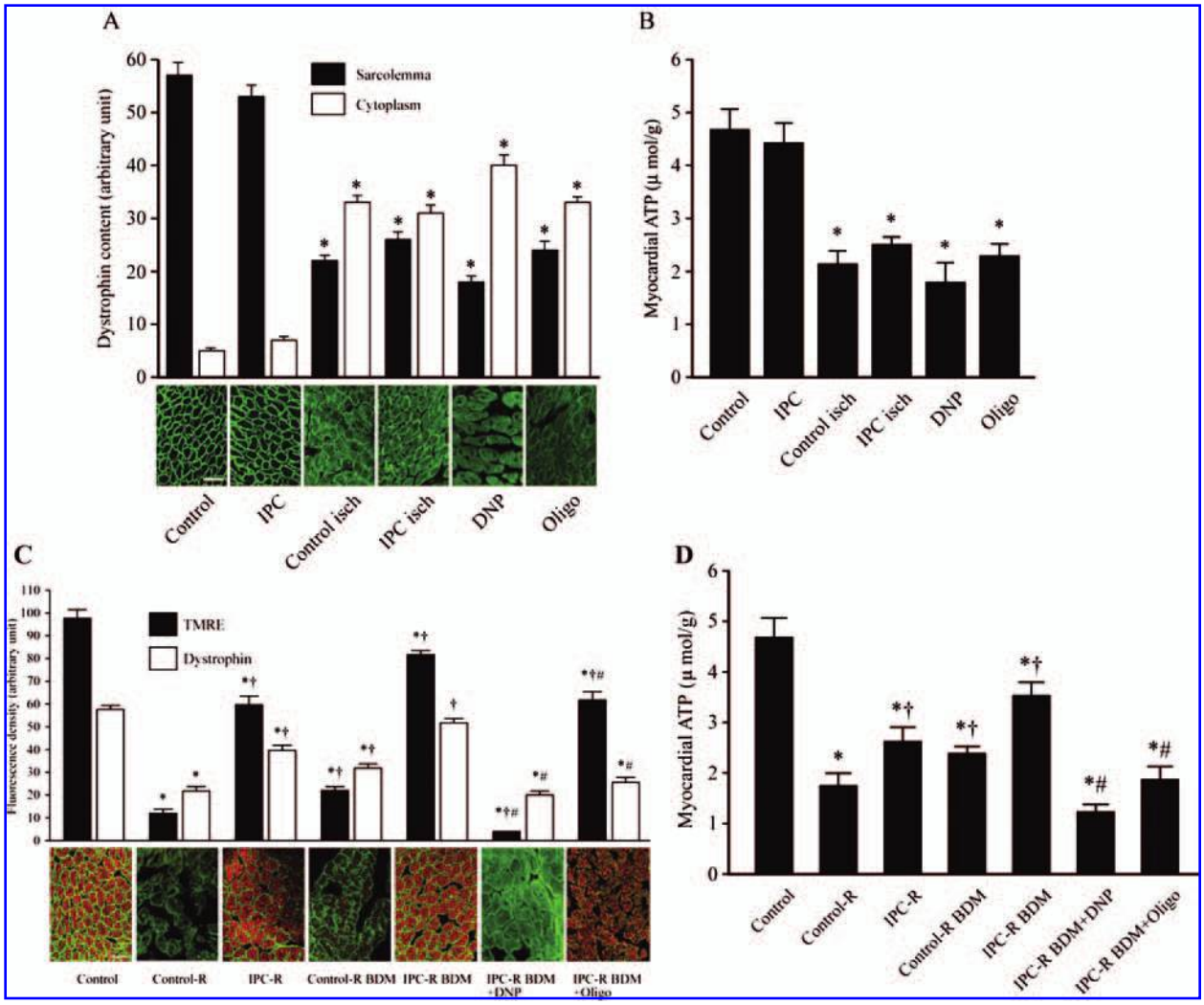

FIG. 3. Relation between myocardial ATP and distribution of dystrophin. (**A**) Immunohistochemical analysis of sarcolemmal (*solid boxes*) and cytoplasmic (*open boxes*) dystrophin and (**B**) myocardial ATP content. Control, nonischemic control heart; IPC, ischemic preconditioning heart; Control isch, control ischemic heart; IPC isch, IPC ischemic heart; DNP, treatment of nonischemic heart with 0.1 m*M* 2,4-dinitrophenol (DNP) for 30 min; Oligo, treatment of nonischemic heart with 5 μM oligomycin for 30 min. Bar indicates 20 μm. Each bar graph represents mean ± SEM of five experiments. *$p < 0.05$ compared with Control. (**C**) Immunohistochemical analysis of sarcolemmal dystrophin and tetramethylrhodamine ethylester (TMRE) uptake, and (**D**) myocardial ATP content. Control, nonischemic control heart; Control-R, control heart after reperfusion with normal Krebs-Henseleit bicarbonate (KHB) buffer; IPC-R, IPC heart after reperfusion with KHB; Control-R BDM, control heart after reperfusion with 2,3-butanedione monoxime (BDM); IPC-R BDM, IPC heart after reperfusion with BDM; IPC-R BDM+DNP, IPC heart after reperfusion with BDM and DNP; IPC-R BDM+Oligo, IPC heart after reperfusion with BDM and oligomycin. Bar indicates 20 μm. Each bar graph represents mean ± SEM of five experiments. *$p < 0.05$ compared with Control; †$p < 0.05$ compared with Control-R; #$p < 0.05$ compared with IPC-R BDM. Adapted from Kyoi *et al*. (166).

lial cells and cardiomyocytes mechanically by occlusion of a large number of capillaries and chemically by releasing ROS. In line with this hypothesis, a large number of studies of *in vivo* regional I/R models in a number of species have demonstrated protection with therapeutics designed to prevent leukocyte adhesion or complement activation (339, 347). In this type of reperfusion injury, nuclear factor-κB (NF-κB), a transcription factor known to be activated through redox-sensitive signal transduction, becomes a proinjurious pathway by activating inflammatory responses (334). Activation of NF-κB induces gene programs leading to transcription of factors that promote inflammation, such as leukocyte adhesion molecules, cytokines, and chemokines. However, NF-κB also mediates cardioprotective pathways in IPC by upregulating cytoprotective proteins before I/R. IPC has been shown to increase NF-κB nuclear translocation before I/R but decreased it after I/R associated with inhibition of inflammatory cytokine expression, thereby reducing infarct size (127).

An alternative mechanism by which IPC mitigates ROS-mediated reperfusion injury is inhibition of burst generation of ROS originated from mitochondria during reperfusion. It has been shown that mitochondrial generation of ROS increases

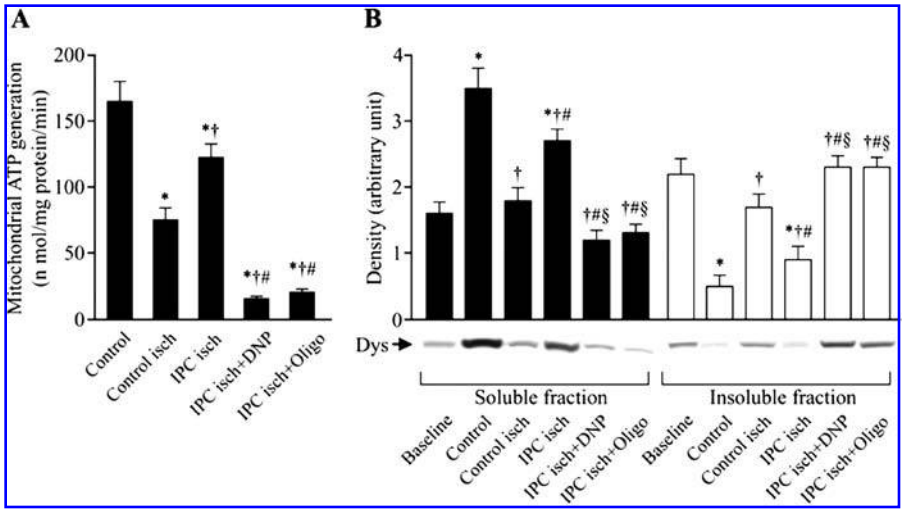

FIG. 4. Relation between mitochondrial function and distribution of dystrophin. Mitochondria isolated from the ischemic preconditioned (IPC) heart after 30 min of ischemia increases ATP generation and relocalization of dystrophin from the insoluble to the soluble fractions. (**A**) Mitochondrial ATP generation. (**B**) Immunoblot analysis for dystrophin. Baseline, before ATP generation; Control, nonischemic control mitochondria; Control isch, control ischemic mitochondria; IPC isch, IPC ischemic mitochondria; IPC isch+DNP, IPC ischemic mitochondria treated with 2,4-dinitrophenol; IPC isch+Oligo, IPC ischemic mitochondria treated with oligomycin. Each bar graph represents mean ± SEM of five experiments. In (**A**), $*p < 0.05$ compared with Control, $\dagger p < 0.05$ compared with Control isch, $\#p < 0.05$ compared with IPC isch. In (**B**), $*p < 0.05$ compared with Baseline; $\dagger p < 0.05$ compared with Control; $\#p < 0.05$ compared with Control isch; $\S p < 0.05$ compared with IPC isch. Adapted from Kyoi *et al*. (166).

during reoxygenation *in vitro* with a time window comparable to the development of reperfusion injury (252), and IPC can mitigate oxidative stress during reperfusion by inhibiting mPTP (119). The tetravalent reduction of molecular O_2 by the mitochondrial electron-transport chain (mETC) is necessary for generating most biologic energy. To accomplish this, four mitochondrial complexes (I–IV) are involved in energy conservation. The reduction is not 100% efficient, however, and 1–4% of available oxygen is normally incompletely reduced and leaks from the mETC in the form of superoxide, which is produced at complex I (NADH coenzyme Q reductase, also called ubiquinone oxidoreductase) and complex III (ubiquinol cytochrome C reductase). This process becomes greatly accelerated at supranormal O_2 tensions or after mitochondrial injury and is believed to be the primary intracardiac source of ROS during I/R. Cellular hypoxia decreases the activity of complex IV (cytochrome oxidase); when O_2 is reintroduced, leakage of free radicals from more proximal complexes is greatly accelerated. Although it was previously believed that ROS formation occurred primarily or solely at reoxygenation after ischemia, it is now known that significant formation of ROS occurs during ischemia from residual O_2.

An important additional concept regarding ROS burst in mitochondria is "ROS-induced ROS release" described by Zorov and associates (368). Working with isolated adult rat cardiomyocytes, they created "triggering" or "inducing" ROS (likely to be singlet oxygen or superoxide) *via* intracellular photoactivation of tetramethylrhodamine compounds. These triggering ROS were associated with mitochondrial depolarization along with mPTP opening. Observed simultaneous with mPTP opening was a large burst of ROS from individual mitochondria; suggesting a positive-feedback loop of ROS-induced ROS release. Supporting this concept was the observation that bongkrekic acid, an mPTP-opening inhibitor, prevented the large burst of ROS release in a dose–response manner despite the presence of the triggering ROS. This suggests that triggering ROS alone are not sufficient for ROS release, and mPTP opening may also be required for burst generation of ROS. The significance of ROS-induced ROS release in I/R injury remains to be determined.

Endogenous antioxidant systems counteract the potential for injury to cellular structures by regulating the balance of individual ROS and their reactants. Most of superoxide is dismutated by manganese-superoxide dismutase (MnSOD) in the mitochondrial matrix to H_2O_2, which readily diffuses through mitochondrial membranes. The remainder exits the mitochondria through anion (chloride) channels in the mitochondrial membrane and is then rapidly converted to H_2O_2 in the cytoplasm, either spontaneously, or when catalyzed by copper/zinc superoxide dismutase (Cu/ZnSOD). H_2O_2 is reduced to H_2O and O_2 by catalase and glutathione peroxidase. Alternatively, H_2O_2 reacts with transition metals, particularly Fe^{2+} (the Fenton reaction), to generate hydroxyl radical ($OH^{\cdot}$), which is a particularly reactive radical, such that it will react with virtually all biomolecules at diffusion-limited rates. This has important consequences, as $OH^{\cdot}$ will react at the site of its formation, whereas more stable species, such as superoxide and H_2O_2, can react at more-distant locations. Endogenous antioxidants are upregulated when exposure of the cell to ROS is increased. However, under pathologic conditions such as I/R, ROS formation can rapidly overcome antioxidant defenses, and cellular injury ensues. For example, during cardiopulmonary bypass for open heart surgery when the heart is electively exposed to ischemia, endogenous antioxidant systems become activated to high levels in response to increased ROS, but these rapidly become depleted so that cardiopulmonary bypass leads to oxidant damage (211).

The antioxidative defense system during reperfusion of the ischemic heart is upregulated by IPC. This facilitated upregulation of the antioxidant defense system by IPC is critical in eliminating injurious ROS and preventing reperfusion injury. Das and associates (68) investigated the effect of IPC on ex-

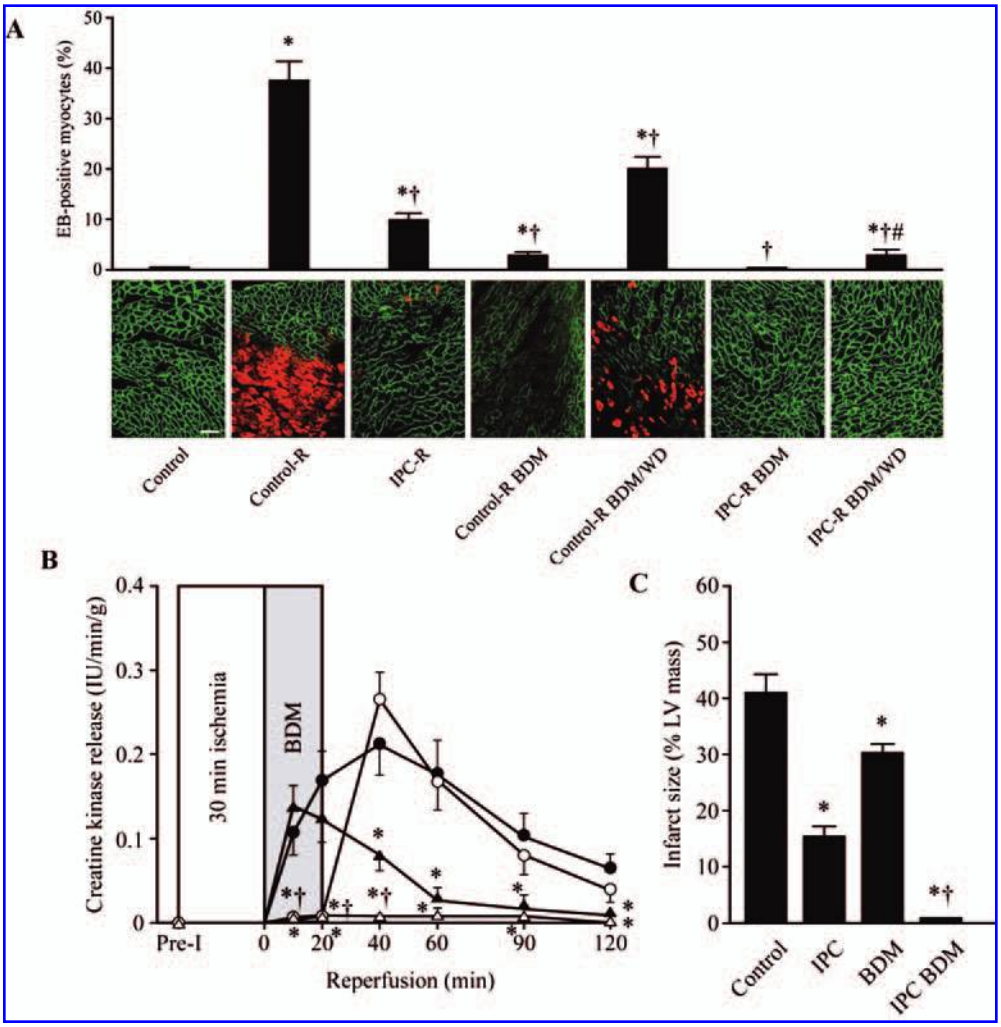

FIG. 5. Ischemic preconditioning replenishes sarcolemmal dystrophin during reperfusion and prevents oncosis. (**A**) Distribution of dystrophin and Evans Blue (a membrane-impermeable dye) uptake. Control, nonischemic control heart; Control-R, control heart after reperfusion without 2,3-butanedione monoxime (BDM); IPC-R, ischemic preconditioning (IPC) heart after reperfusion without BDM; Control-R BDM, control heart after reperfusion with BDM; Control-R BDM/WD, control heart after reperfusion with BDM followed by its discontinuation; IPC-R BDM, IPC heart after reperfusion with BDM; IPC-R BDM/WD, IPC heart after reperfusion with BDM followed by its discontinuation. Bar indicates 50 μm. Each bar graph represents mean ± SEM of five experiments. *$p < 0.05$ compared with control; †$p < 0.05$ compared with Control-R; #$p < 0.05$ compared with Control-R BDM/WD. (**B**) Creatine kinase (CK) release. Pre-I indicates preischemia. *Solid circle*, control heart; *open circle*, IPC heart; *solid triangle*, control heart reperfused with BDM; *open triangle*, IPC heart reperfused with BDM. Each symbol represents mean ± SEM of six experiments. *$p < 0.05$ compared with control heart; †$p < 0.05$ compared with IPC heart. (**C**) Infarct size. Control, control heart after reperfusion; IPC, IPC heart after reperfusion; BDM, control heart after reperfusion with BDM followed by perfusion with Krebs-Henseleit bicarbonate (KHB) buffer; IPC BDM, IPC heart after reperfusion with BDM followed by perfusion with KHB buffer. *$p < 0.05$ compared with Control; †$p < 0.05$ compared with IPC. Each bar graph represents mean ± SEM of six experiments. Adapted from Kyoi *et al*. (166).

pression of stress-related and antioxidative genes and proteins. In their study, IPC hearts showed the induction of heat-shock protein (HSP) 27, HSP 70, and HSP 89 mRNAs, as well as catalase and MnSOD mRNAs. The activities of three major antioxidative enzymes, MnSOD, peroxisomal catalase, and glutathione peroxidase, but not Cu/ZnSOD, cytosolic catalase, and glutathione reductase, were enhanced after 60 min of reperfusion in IPC hearts. Another example of upregulation of antioxidant defense systems by IPC is the enhanced expression of thioredoxins (Trx), which are critical for redox regulation of protein function and signaling *via* thiol redox control. Trx are reduced by electrons from NADPH via Trx reductase. A recent study demonstrated that Trx-1 plays an important role in redox signaling and in transmitting survival signal in ischemic myocardium (199). Trx-1 is downregulated after I/R but upregulated in the preconditioned myocardium. Inhibition of Trx-1 abrogated the cardioprotective effects of IPC, as evidenced from impaired postischemic ventricular recovery and increased myocardial infarct size and cardiomyocyte apoptosis. The role of Trx-1 in transmitting survival signals was supported further

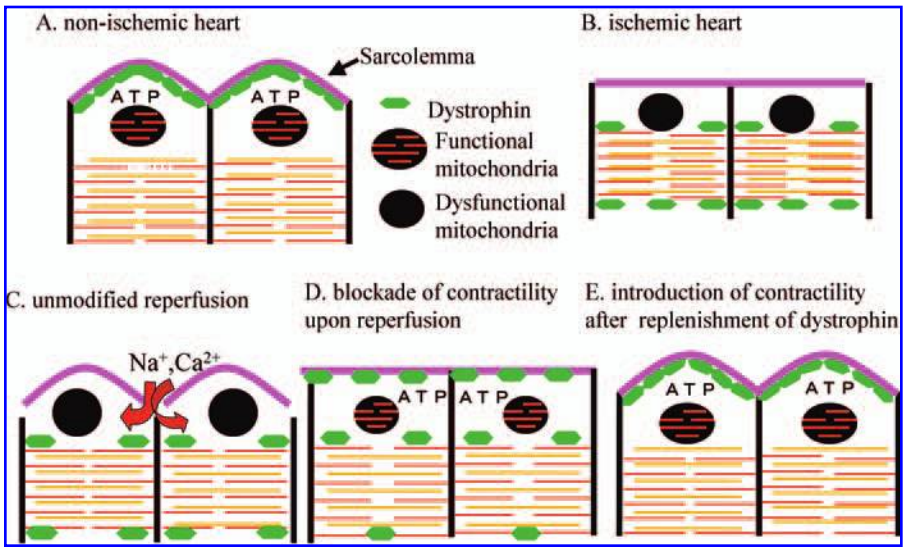

FIG. 6. Model of a preventive effect of temporary blockade of contractility on reperfusion in cardiomyocytes with protected mitochondria on oncotic death. (**A**) Nonischemic heart contains intact mitochondria and sufficient ATP for sarcolemmal distribution of dystrophin. (**B**) Ischemic heart contains dysfunctional mitochondria and is depleted of ATP, which results in redistribution of dystrophin to the cyto-skeletal fraction. (**C**) Unmodified reperfusion of the ischemic heart introduces contractility that disrupts sarcolemma depleted of dystrophin, leading to unlimited entry of Na^+ and Ca^{2+} and oncotic death. (**D**) Blockade of contractility on reperfusion prevents oncotic death and allows the ischemic preconditioning-protected mitochondria to generate ATP, resulting in restoration of dystrophin to the sarcolemma. (**E**) Introduction of contractility after replenishment of sarcolemmal dystrophin does not disrupt sarcolemma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

from the evidence that transgenic mouse hearts with extra copies of Trx-1 were resistant to apoptotic cardiomyocyte death (330). The exemplification of enhanced expression of stress-inducible and antioxidant genes and stimulation of antioxidant enzyme activities by IPC may reflect the preconditioned heart's response, enabling it to survive against ischemic stress by eliminating the oxidative assault.

Although ROS alone are injurious, superoxide in conjunction with nitric oxide (NO) generates peroxynitrite, a more powerful mediator of myocardial injury during reperfusion (369). NO is generated by nitric oxide synthase (NOS) by using l-arginine, NADPH, and oxygen, as well as the cofactor tetrahydrobiopterin (BH_4). To date, three major NOS isoforms have been identified. NOS1, termed nNOS, primarily in neurons, and NOS3, termed eNOS, primarily in endothelial cells are constitutive and dependent on Ca^{2+} and calmodulin. The third isoform, NOS2, termed iNOS, is inducible and Ca^{2+} independent and is primarily involved in inflammation. NOS acts as a double-edged sward that plays protective and detrimental roles, depending on the bioavailability of NO. IPC may inhibit NOS-mediated oxidative stress during reperfusion by eliminating ROS. Because BH_4 can be readily depleted by oxidants, oxidation of BH_4 could result in a switch of NOS from NO to superoxide generation, a phenomenon termed NOS uncoupling. Conversely, increased bioavailability of NO can inhibit burst generation of ROS and Ca^{2+} uptake by mitochondria during reperfusion by modestly inhibiting mETC (273). Increased NO generation during reperfusion may also prevent microcirculatory disturbance mediated by leukocyte–endothelium interactions (132).

5. IPC enhances angiogenesis and prevents infarct expansion and remodeling. Although the initial concept of IPC was the delay of ischemic injury, and reperfusion was thought to be necessary to elicit the infarct size–limiting effect of IPC, accumulating evidence suggests that IPC can salvage permanently ischemic myocardium. Coronary occlusion without reperfusion results in MI in the center of the ischemic region. However, the border area of infarction can be supplied with more or less oxygen from the surrounding coronary circulation, although the amount of oxygen is not sufficient for survival or normal contractile function. Recent advances in therapeutic angiogenesis have demonstrated that salvageable myocardium exists in infarcted myocardium after permanent coronary artery occlusion (190). The degree to which the border area of myocardium can be salvaged critically depends on the rate of angiogenesis. Hypoxic preconditioning and IPC have been shown to inhibit left ventricular (LV) remodeling and enhance ventricular contractile functional reserve in animal models of chronic MI by promoting angiogenesis (96, 149, 287). Thus, IPC has emerged as a promising approach for therapeutic angiogenesis.

III. SIGNAL TRANSDUCTION IN IPC

As discussed before, mitochondria are the main target of protection against I/R injury. A variety of signal-transduction pathways converge on mitochondria during IPC. In this section, I discuss the mechanism of cardioprotective signal transduction in IPC.

A. Role of surface receptors

1. G protein–coupled receptors. Activation of cardiomyocyte surface receptors is presumed to be an initial event induced by ischemia or hypoxia. It is now evident that IPC is triggered through a G protein–coupled receptor (GPCR)-dependent and -independent mechanism. GPCRs are either pertussis toxin (PTX) sensitive or insensitive. Former receptors are coupled with $G_{i/o}$, which consists of heterotrimeric G protein $G_{\alpha\beta\gamma}$, whereas later receptors are coupled with a G_q family,

which consists of the heterotrimeric G protein, $G_{\alpha q}$. These receptors fulfill both distinctive and overlapping functions in mediating cellular responses. Activation of $G_{i/o}$-coupled receptors induces dissociation of G_{α} from $G_{\beta\gamma}$, which activates phospholipase C (PLC) and a resultant generation of the second messengers D-myoinositol 1,4,5-triphosphate and diacylglycerol, leading to activation of PKC, an essential step for mediating IPC (51, 303). Conversely, signaling pathways linked with activation of $G_{\alpha q}$ also converge at the level of PLC (75). Involvement of $G_{i/o}$-coupled receptors in IPC has been well documented. Thornton and associates (319) first demonstrated that a PTX-sensitive G protein mediates the protective effects of preconditioning. Subsequent studies supported the role of $G_{i/o}$-coupled receptors in mediating IPC in various animal species (221, 276, 294). However, a conflicting report has been provided by Liu and Downey (182), who demonstrated that preconditioning against infarction does not involve a PTX-sensitive G protein in the intact rat heart. Such a discrepant observation strongly suggests the involvement of both PTX-sensitive and -insensitive G proteins in IPC. Ninomiya and associates (238) demonstrated that PTX-sensitive adenosine receptors and PTX-insensitive P2y purinoceptors play a complementary role in mediating IPC.

General agreement exists that adenosine is a major GPCR in triggering IPC (63). Myocardial ischemia causes abrupt deprivation of oxygen and the energy substrate from cardiomyocytes and endothelial cells. A subsequent decrease in the adenylate energy charge [(ATP + 1/2 ADP)/ATP + ADP + AMP] may trigger the activation of cytosolic 5′-nucleotidase and enhance adenosine production (138). It has also been reported that 5′-nucleotidase is present as ecto-5′-nucleotidase bound to the membrane (93). Extracellular adenine nucleotides, which are the substrates for ecto-5′-nucleotidase, can be derived from endothelium, sympathetic nerves, and erythrocytes (264). Kitakaze and associates (157) hypothesized that ecto-5′-nucleotidese is activated by PKC as a result of α_1-adrenergic stimulation during ischemia *in vivo*. A further study using isolated intact cardiomyocytes demonstrated that α_1-adrenergic stimulation increased adenosine production from these cells in a PKC- and ecto-5′-nucleotidese–dependent manner (156), suggesting that adenine nucleotides released from cardiomyocytes by themselves are responsible for increased adenosine production on sympathetic nerve stimulation during ischemia in IPC. However, preconditioning can be induced in isolated cardiomyocytes by ischemia alone without activation of α_1-adrenergic stimulation (12), indicating that α_1-adrenergic stimulation is not indispensable in adenosine generation.

Like other Gq-coupled receptors, the activation by autacoid in preconditioned tissue triggers a multiple kinase cascade through phosphorylation reactions, leading to the rapid post-translational modifications of proteins that regulate cellular resistance to I/R injury (107, 361). Among the autacoids, kinins have been implicated in myocardial IPC (26). The most abundant of these, bradykinin, was originally shown by Wall and associates (342) to be an obligatory mediator of IPC in rabbit myocardium because the B_2-receptor antagonist, icatibant (HOE140) abolished the protection afforded by IPC. A subsequent work confirmed both the ability of exogenously administered bradykinin to act as a preconditioning mimetic and the participation of endogenous bradykinin in IPC in several species, including humans (26).

2. Growth-factor receptors. In addition to GPCR-coupled agonists, it was found that growth-factor receptor (GFR) agonists that are coupled with receptor tyrosine kinase (RTK) are equally effective in cardioprotection. Several independent groups of investigators demonstrated that treatment with insulin-like growth factor-II and acidic as well as basic fibroblast growth factors mimics the cardioprotective effects of IPC (131, 258, 340). The mechanism of GFR-mediated preconditioning effects is not well understood but may share common intracellular signaling pathways to GPCRs. These pathways ultimately converge on activation of end-effector systems, which are discussed later, because GFR activation is also known to be coupled with activation of PLC and resultant activation of PKC (84). Moreover, recent studies suggest that GPCR activation induces transactivation of GFRs *via* a redox-sensitive mechanism (161, 245). Thus, it is conceivable that coordinated activation of GPCR and GFRs provokes efficient cardioprotective signal transduction in IPC.

B. Signal-transduction pathways downstream of surface receptors

1. Role of ROS. As in all known biologic events evoked by any kinds of stress, the occurrence of IPC also was found to be dependent on the redox-based mechanism (59). A growing body of evidence suggests that ROS play a crucial role in signal transduction mediated by IPC. Vanden Hoek and associates (336) first demonstrated the loss of preconditioning protection with antioxidants in cardiomyocytes. In their study, isolated cardiomyocytes were protected from cell death by 10-min IPC just before 1 h of I/R. Oxidant generation was observed to occur during the preconditioning ischemia that could be attenuated with antioxidants, 2-mercaptopriopionyl glycine (MPG), and mitochondrial inhibitors, myxothiazol. When MPG was added only during the preconditioning period in an effort to inhibit this oxidant generation only during the preconditioning stimulus, the protection of preconditioning before ischemia was lost. Under these conditions, increased cell death is the result of adding an antioxidant that has been protective when administered to the non-preconditioned cardiomyocytes during reperfusion. This observation has been confirmed in several other laboratories that have reported the mechanism of ROS generation during IPC and the interfering effect of antioxidants against cardioprotection when added during the preconditioning phase (6, 153). The "triggering" role for ROS in the induction of late IPC has been established in the whole-animal model (315). The addition of MPG was able to abrogate late IPC, whereas exogenous oxidants were able to mimic late IPC in the conscious rabbit model of myocardial infarction, thus confirming the central roles of ROS in cardioprotective signal-transduction pathways in both early and late IPC.

2. Potential sources of ROS in IPC. Several potential sources of ROS exist in IPC. One potential candidate for ROS production during IPC is xanthine oxidase, which generates superoxide in the presence of hypoxanthine. Xanthine

oxidase can be activated during ischemia by enhanced degradation of adenine nucleotides to hypoxanthine, as well as conversion of the cytosolic enzyme xanthine dehydrogenase to xanthine oxidase in a Ca^{2+}-calmodulin–dependent manner (212). However, the fact that IPC can be induced in animals that are devoid of xanthine oxidase precludes this enzyme as an essential source of ROS triggering early-phase IPC (81).

Nonmitochondrial NADH oxidase has recently emerged as a source of ROS in the heart. It has been proposed that membrane-bound NADH oxidase activity linked to cytosolic NADH reductase is a major source of superoxide production when lactate concentrations and oxygen tensions are high (223). Although reperfusion after temporary ischemia is likely to induce such a condition, the contribution of this enzyme system in the induction of IPC remains unknown.

NOS can be an alternative source of ROS, as mentioned earlier. eNOS is expressed constitutively in the endothelial cells and cardiomyocytes (150) and is, therefore, a candidate for ROS generation. However, because ROS generation through eNOS requires uncoupling of NO generation through oxidation of BH4 (338), eNOS-uncoupling–mediated ROS require oxidative stress that exists upstream of eNOS. Thus, NOS are unlikely to be a primary source of ROS in IPC.

NADPH oxidase may represent an upstream source of ROS generated in response to surface receptor stimulation in IPC. Because NADPH oxidase activity depends on PKC activation (172), a sequential relation may exist between surface-receptor stimulation, PKC activation, and ROS generation after the IPC challenge. The study conducted by Bell and associates (32) suggests that NADPH oxidase plays a pivotal role in generating ROS that act as a trigger in the cardioprotective signal transduction in IPC. Although this study also suggests that adenosine A_1 receptor–mediated cardioprotection is independent of NADPH oxidase and ROS, whether or not pharmacologic preconditioning (PPC) with exogenous adenosine A_1 receptor agonist shares the same downstream signal-transduction mechanism for cardioprotection as IPC remains unknown. It is possible that certain preconditioning techniques activate PKC and protect mitochondria by bypassing the activation of NADPH oxidase. Nevertheless, an NADPH-independent mechanism of cardioprotection does not necessarily mean that this mechanism is entirely independent of redox signaling but may require ROS derived from mitoK_{ATP} channels, as discussed later. Accordingly, NADPH oxidase–dependent and –independent mechanisms of cardioprotection mediated by preconditioning are illustrated in Fig. 7.

Although mETC is a major source of ROS under the stationary condition, it is unknown whether ROS generation through nonspecific electron leak from mETC plays a role in IPC. Instead, activation of mitoK_{ATP} channels has been shown to be responsible for mitochondria-derived ROS generation in IPC (56, 92). The role of mitoK_{ATP} channels as a trigger of IPC and PPC has been demonstrated in isolated as well as *in vivo* hearts by using the specific mitoK_{ATP} channel opener diazoxide (108). How mitoK_{ATP} channel opening is linked to ROS production is unclear. However, site III mETC, most likely originating from the cytochrome *b-c1* segment, can be an important source of ROS during mitoK_{ATP} channel activation (358), although it is not clear whether ROS will be produced from an increase or decrease in flux through the mETC. Unlike increased generation of ROS during hypoxia, which is attributed to downstream block of electron transfer, leading to shunting of electrons to ROS production, the opening of mitoK_{ATP} channels induces mitochondrial depolarization and stimulates electron transport by uncoupling of the respiratory chain, thereby increasing ROS generation because state 4 respiration is associated with more intense ROS production than state 3 respiration in ischemic mitochondria (252). This possibility was supported by the study performed by Brennan and associates (48), who demonstrated that in the isolated rat heart, partial mitochondrial uncoupling with a low-dose FCCP [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone], a mitochondrial protonophore that bypasses mitoK_{ATP} channels, significantly improved postischemic functional recovery through an ROS-dependent pathway. In addition, the fact that ROS scavengers blocked diazoxide-induced protection (92, 259) lends support to the hypothesis that mitoK_{ATP} channels play a crucial role in the generation of redox signaling during IPC.

Conversely, the exclusivity of mK_{ATP} channel activation in the mitochondrial perturbations described has been questioned. It has been demonstrated that the ROS-attenuating effects of mitoK_{ATP} channel openers in the I/R phase were maintained in a nominally K^+-free medium, suggesting that a K^+ conductance–independent pathway may be activated by the mitoK_{ATP} openers to confer mitochondrial protection (256). Thus, it is possible that mitoK_{ATP} channel–independent perturbation of

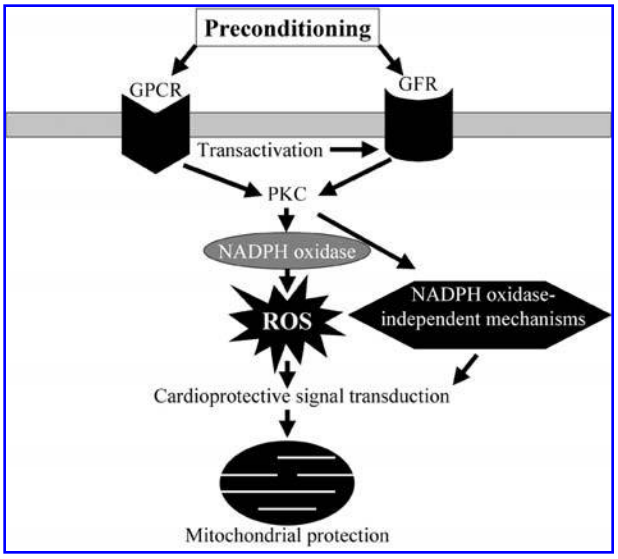

FIG. 7. Schematic drawing of ROS-dependent and -independent mechanisms of cardioprotection mediated by preconditioning. In the reactive oxygen species (ROS)-dependent model of cardioprotection, G protein–coupled receptor (GPCR) stimulation and/or transactivation of growth factor receptors (GFR) during ischemic preconditioning (IPC) challenges promote NADPH oxidase activation through activation of protein kinase C (PKC). ROS derived from NADPH oxidase act as a trigger in activating the cardioprotective signal transduction involved in protection of mitochondria from ischemia/reperfusion injury. In the ROS-independent model of cardioprotection by IPC, GPCR-mediated activation of PKC is directly linked to cardioprotective signal transduction, culminating in mitochondrial protection.

ETC also contributes to the generation of ROS during the IPC challenges.

3. Role of PKC-ε and mitoK$_{ATP}$ channels in the memory of cardioprotection. The unique feature of IPC is the memory of Cardioprotection, which lasts for up to 2 h after the discontinuation of the preconditioning stimulus (360). The underlying mechanisms for memory of cardioprotection generated by IPC have been a subject of extensive research for many years. It has been suggested that mitoK$_{ATP}$ channels and PKC create a self-perpetuating cycle during the memory of IPC (332). Recent studies (146, 306) raised the hypothesis that ROS generated through the activation of mitoK$_{ATP}$ channels play a pivotal role in the memory of cardioprotection. In line with this hypothesis, Juhaszova and associates (146) suggested that the characteristic memory of IPC is mediated by moderate, reversible, and sustained mitochondrial swelling, which causes increased generation of ROS and consequent redox activation of PKC-ε, a novel PKC isoform that has consistently been implicated in the cardioprotective signal transduction (104, 180).

A growing body of evidence indicates that PKC-ε and PI3K play a crucial role in cardioprotective signal transduction mediated by IPC (13). Important cross-talk between PKC-ε and PI3K exists in mediating the memory of cardioprotective signal transduction in IPC. It has been demonstrated that PI3K exists upstream of PKC in cardioprotection mediated by IPC in the isolated and perfused rat heart model (324). However, more recent study (244) suggests that PKC-ε and PI3K exist in parallel positions, and the activities are regulated at least in part by each other's kinase. The hypothetical mechanism for intensification and memory of cardioprotective signal transduction generated by the self-perpetuating cycle of redox signaling consisting of PKC-ε, PI3K, and mitoK$_{ATP}$ channels during preconditioning is illustrated in Fig. 8.

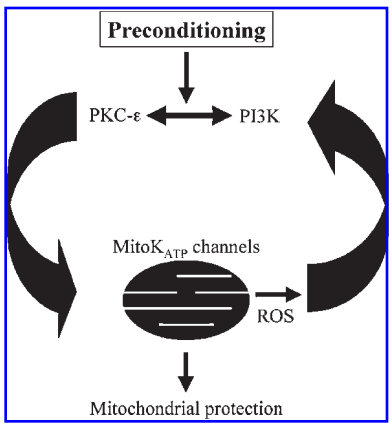

FIG. 8. Model of self-perpetuating cycle of redox signaling for the memory of cardioprotection mediated by ischemic preconditioning. Ischemic preconditioning (IPC) activates protein kinase C (PKC)-ε and phosphatidylinositol 3-kinase (PI3K) in an interdependent manner. This signaling complex activates mitochondrial K$_{ATP}$ channels. ROS derived from mitoK$_{ATP}$ channels feed back to activate the PKC-ε and PI3K signaling complex, creating a self-perpetuating cycle. MitoK$_{ATP}$ channels then act as mediators of mitochondrial protection.

4. Glycogen synthase kinase-3β as a point of integration of protective signaling. IPC is known to activate both classic and novel isoforms of PKC, dependent on the degree of IPC challenges. Low-grade IPC activates only PKC-α, whereas high-grade IPC can increase the membrane association of PKC-δ and PKC-ε (192). High-grade IPC was associated with better recovery of postischemic LV function and smaller infarct size compared with low-grade IPC. However, the exact roles of PKC-δ and PKC-ε in high-grade IPC-induced cardioprotection have not been defined. As discussed earlier, translocation of PKC-ε to mitochondria is thought to induce opening of the mitoK$_{ATP}$ channels that play a role in creating the self-perpetuating cycle of redox signaling and protecting mitochondria from the opening of the mPTP. Besides this action, translocation of PKC-ε to mitochondria may inhibit opening of the mPTP independent of the activation of mitoK$_{ATP}$ channels. It has been demonstrated that glycogen synthase kinase-3β (GSK-3β) is an important downstream effector of PKC-ε–mediated protection in cardiac mitochondria through inhibition of the mPTP (20).

GSK-3β serves as a point of integration, a master switch immediately proximal to the mPTP complex (146). GSK-3β is highly active in unstimulated cells and becomes inactivated in response to mitogenic stimulation (65). A recent study demonstrated that pharmacologic inhibition of GSK-3β reduced infarct size and improved postischemic LV function (325). GSK-3β is a substrate for numerous kinases, including PKC-ε and the reperfusion-injury salvage kinase (RISK) that has been proposed as a family of survival kinases specifically protecting the heart from reperfusion injury (122). RISK consists of Akt and a MAPK family, extracellular signal-regulated kinase (ERK), and a crucial role of RISK in protecting the heart from reperfusion injury was demonstrated by studies of ischemic postconditioning, which is induced by multiple brief ischemias during reperfusion after a lethal period of ischemia (328). Accumulating evidence indicates that IPC and ischemic postconditioning exert their cardioprotective effects through the recruitment of the RISK pathway at the time of reperfusion and that the protection in these settings is mediated through the inhibition of the mPTP opening at this time (122).

In contrast to PKC-ε, general agreement exists that the effect of PKC-δ activation during I/R is detrimental to cardiomyocyte survival. This conclusion is derived from the experiment demonstrating that acute inhibition of PKC-δ translocation to mitochondrial targets at the onset of reperfusion after *in vivo* or *in vitro* ischemia dramatically improves functional recovery, reduces infarct size, and limits postischemic apoptotic cell death (137). Moreover, the beneficial effect of PKC-δ inhibition was found to be mediated by inhibition of GSK-3β. This observation is consistent with the hypothesis that inhibition of GSK-3β mediates convergence of protection signaling to inhibit the mPTP complex (146).

Although PKC-δ is activated during IPC and may play a trigger role in cardioprotective signal transduction, in light of the detrimental effect of activation of PKC-δ on cardiomyocyte survival during I/R, it is conceivable that IPC downregulates PKC-δ during I/R. The role of PKC-δ in IPC has been investigated in PKC-$\delta^{-/-}$ mice. It was shown that IPC-mediated cardioprotection is associated with increased superoxide production in PKC-$\delta^{+/+}$ hearts, but no increase in ROS during IPC and no

cardioprotection was observed in PKC-$\delta^{-/-}$ hearts (210). A trigger role of PKC-δ activation was further supported by the fact that the volatile anesthetic sevoflurane preconditioning-induced protection was triggered by increased Ca^{2+} influx *via* the Na^+/Ca^{2+} exchanger and subsequent activation of PKC-δ (46). These observations suggests that the transient activation of PKC-δ, which occurs during the IPC challenge by transient Ca^{2+} overload, acts as a trigger of cardioprotection by generating redox signaling, but PKC-δ may become deactivated during the sustained ischemia and reperfusion, thereby inhibiting the GSK-3β and superoxide generation responsible for mPTP opening and cardiomyocyte death. Whether transient mPTP opening during the IPC challenge by activation of PKC-δ plays a role in generating superoxide and redox signaling remains to be answered.

5. Integrated pharmacologic preconditioning to reproduce the memory of cardioprotection. Appreciation of GPCR as a trigger of IPC has prompted investigators to use various GPCR agonists to reproduce cardioprotective effects of IPC. Because implementation of IPC before myocardial infarction is limited in clinical practice, PPC may represent an ideal alternative to IPC. GPCR agonists that have so far been effective in exerting cardioprotection against I/R injury are adenosine, acethylcholine, phenylephrine (an α_1-adrenergic receptor agonist), bradykinin, opioids, and angiotensin II (64, 329).

PPC also uses other putative triggers of IPC, such as mitoK_{ATP} channel openers and NO donors. However, the efficacy of early PPC with a single drug is variable. Several investigators reported that single-agent PPC was as effective as IPC (64, 188, 234, 259, 320); however, other studies, including our own, showed only marginal cardioprotection by the single-PPC strategy and requires a combined PPC technique using multiple agents to elicit optimal cardioprotection (31, 57, 331, 357). Such conflicting observations may be attributed to differences in species and experimental models, but more important, may be related to the strength and the duration of cardioprotective signaling activity.

We previously demonstrated that the memory of cardioprotection that mimics IPC cannot be achieved by simply increasing the dose of each three putative triggers of IPC [*i.e.*, a GPCR agonist adenosine, a mitoK_{ATP} channel opener diazoxide, and an NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP)], but is successfully reproduced by combining these drugs (251). Because cardioprotective memory through combined PPC was thought to be mediated by coordinated interaction of cardioprotective signaling, we developed the concept of "integrated PPC," which uses multiple triggers of IPC (331). The experiments performed by Uchiyama and associates (331) suggest that combined PPC may be effective in generating the memory of cardioprotection by persistently activating mitoK_{ATP} channels (Fig. 9). When flavoprotein fluorescence was used as an index of mitoK_{ATP} channel activity (184), treatment with 5-hydroxydecanoate (5-HD), a selective mitoK_{ATP} channel blocker, modestly decreased flavoprotein fluorescence (<5% of DNP-induced flavoprotein oxidation) in the normally perfused rat heart, indicating that only trivial mitoK_{ATP} channel activity occurred under the baseline condition. Flavoprotein fluorescence decreased during each cycle of 5 min of ischemia and returned above the baseline during each reperfusion. Maximal flavoprotein oxidation was usually obtained between 10 and 15 min after the last reperfusion. Treatment with 5-HD before and during IPC abrogated the increase in flavoprotein oxidation induced by IPC, suggesting that IPC-induced flavoprotein oxidation was mediated by activation of the mitoK_{ATP} channels. Diazoxide promptly increased flavoprotein fluorescence, which reached a plateau within 5 min. The magnitude of flavoprotein oxidation was comparable to that induced by IPC. The increase in flavoprotein oxidation induced by diazoxide was abrogated by pretreatment with 5-HD. Adenosine treatment induced a transient decline of flavoprotein oxidation but did not increase flavoprotein oxidation thereafter, nor did it enhance diazoxide-induced flavoprotein oxidation. Subsequent addition of SNAP also had no appreciable effect on diazoxide-induced flavoprotein oxidation. Quantitative analysis showed that maximal flavoprotein oxidation induced by IPC and combined PPC was not significantly different from that induced by diazoxide alone. Flavoprotein oxidation induced by IPC, diazoxide, and combined PPC was significantly inhibited by pretreatment with 5-HD. Because flavoprotein oxidation is influenced by various metabolic alterations during IPC challenges and the effect of 5-HD is not necessarily attributed to inhibition of mitoK_{ATP} channels, these observations should be interpreted with caution.

The ability of combined PPC to generate the memory of cardioprotective signal transduction was confirmed in the cultured cardiomyocyte model (244). This study demonstrated that combined PPC with adenosine, diazoxide, and SNAP confers the memory of cardioprotection by sustained activation of PKC-ε and PI3K. The sustained increase in PKC-ε and PI3K activity and cardioprotective memory could not be achieved by simply increasing the dose of each drug; however, it was successfully reproduced by combining these drugs. These results reinforce the hypothesis that the sustained activation of PKC-ε and PI3K and the memory of cardioprotection are mediated by combined PPC with adenosine, diazoxide, and SNAP.

Although adenosine alone can activate mitoK_{ATP} channels and eNOS through the activation of PKC and PI3K, respectively (291, 351), the study conducted by Okada and associates (244) suggests that coordinated interaction of distinct and overlapping downstream signaling generated by diazoxide and SNAP is necessary to amplify PKC and PI3K activities (Fig. 10). Adenosine stimulates adenosine A_1, A_2, and A_3 receptors, which are coupled with $G_{i/o}$ proteins (246). $G_{i/o}$ protein activation results in membrane translocation and phosphorylation of PKC in a Ca^{2+}-dependent (in case of the classic PKC isoforms) and -independent (in case of the novel PKC isoforms) manner by generating the lipid second messenger diacylglycerol (72). The heterotrimeric G proteins can also activate PI3K *via* the transactivation of receptor tyrosine kinases through the generation of ROS in certain cell types (194, 333). Although several $G_{i/o}$-coupled receptor agonists can generate ROS through the activation of mitoK_{ATP} channels in the heart (64), ROS generation by $G_{i/o}$-coupled receptor stimulation may be transient, and the redox signaling is attenuated over time. Persisted generation of ROS can be obtained by diazoxide administration through the opening of mitoK_{ATP} channels (183). This mechanism is also essential for diazoxide to activate PKC (155). Sato and associates (291) demonstrated that the primary effect of PKC activation by adenosine on mitoK_{ATP} channel activation

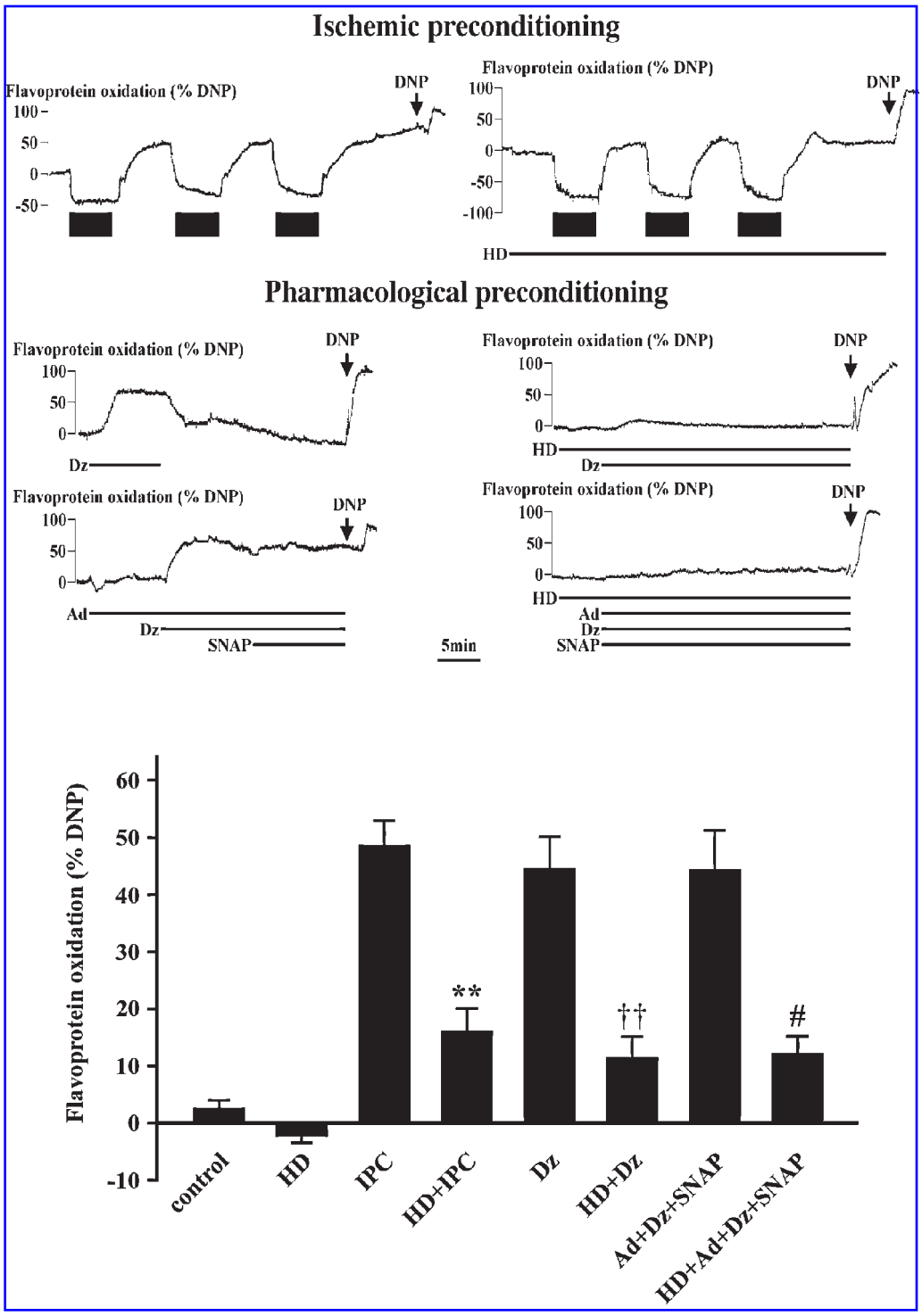

FIG. 9. Integrated pharmacologic preconditioning and the memory of cardioprotection. Flavoprotein oxidation during ischemic preconditioning (IPC) and pharmacologic preconditioning (PPC). Flavoprotein fluorescence was measured as an index of mitoK_{ATP} channel activity (291). The upper panel shows representative traces of flavoprotein fluorescence during IPC or PPC with diazoxide (Dz) alone or in combination with adenosine (Ad) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) in the presence or absence of 5-hydroxydecanoate (HD). Flavoprotein oxidation was expressed as a percentage of the dinitrophenol (DNP)-induced fluorescence. *Solid boxes* represent ischemia, and a *scale bar* indicates 5 min. The lower panel shows quantitative analysis of maximal flavoprotein oxidation. Each bar graph represents the mean ± SEM of six experiments. $^{**}p < 0.01$ *vs.* IPC; $\dagger\dagger p < 0.01$ *vs.* Dz; and $\#p < 0.01$ *vs.* Ad + Dz + SNAP. Adapted from Uchiyama *et al.* (331).

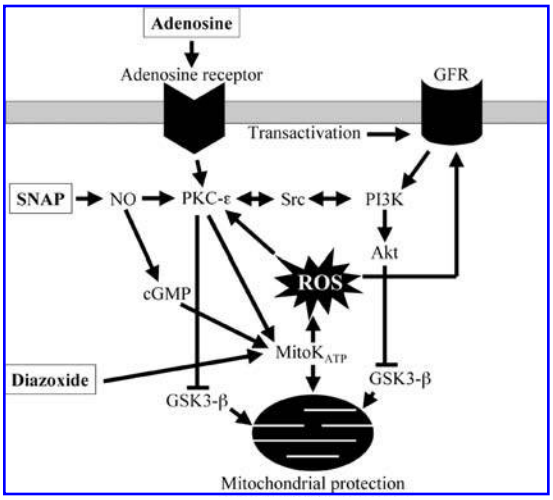

FIG. 10. Schematic drawing of signal-transduction pathways mediated by integrated pharmacologic preconditioning. Adenosine stimulates adenosine A_1, A_2, and A_3 receptors, which are coupled with $G_{i/o}$ proteins. $G_{i/o}$ protein activation results in membrane translocation and phosphorylation of protein kinase C (PKC). The heterotrimeric G proteins can also activate phosphatidylinositol 3-kinase (PI3K) *via* the transactivation of receptor tyrosine kinases. However, redox signaling produced by adenosine is insufficient for sustained activation of PKC-ε and PI3K after the withdrawal of adenosine, and additional sources of reactive oxygen species (ROS) and nitric oxide (NO) are necessary to provoke positive-feedback and feed-forward amplification of PKC-ε and PI3K activities. Diazoxide treatment promotes persistent generation of ROS through the opening of mitochondrial K_{ATP} (mitoK$_{ATP}$) channels. *S*-nitroso-*N*-acetylpenicillamine (SNAP) is an NO donor. NO-induced activation of PKC-ε occurs through ROS-dependent tyrosine nitration of PKC-ε or cGMP-dependent activation of mitoK$_{ATP}$ channels. Cardioprotective signal transduction induced by NO also proceeds *via* the formation of the PKC-ε–Src kinase signaling module, which may interdependently upregulate each other's kinase activity. Src kinase then leads to PI3K activation through protein–protein interaction. The positive-amplification-loop activation of PI3K/Akt and PKC-ε converges on mitochondrial protection by inhibiting glycogen synthase kinase (GSK)3-β and facilitating the mediator role of mitoK$_{ATP}$ channels.

was to prime and enhance the channel activation by diazoxide but not to open the channels by itself. Conversely, NO-induced activation of PKC-ε occurs through ROS-dependent tyrosine nitration of PKC-ε (22) or cGMP-dependent activation of mitoK$_{ATP}$ channels (350). Moreover, cardioprotective signal transduction induced by NO proceeds *via* the formation of the PKC-ε–Src kinase signaling module (341), which may interdependently upregulate each other's kinase activity (118). Src kinase then leads to PI3K activation through protein–protein interaction (4). Thus, it seems likely that ROS and NO signaling produced by adenosine is insufficient for sustained activation of PKC-ε and PI3K after the withdrawal of adenosine, and additional sources of ROS and NO are necessary to provoke positive-feedback and feed-forward amplification of PKC-ε and PI3K activities. The positive-amplification-loop activation of PI3K/Akt and PKC-ε converges on mitochondrial protection by inhibiting GSK3-β and facilitating the mediator role of mitoK$_{ATP}$ channels. However, it has been shown that acetylcholine and adenosine provoke sustained activation of PI3K after the discontinuation of these drugs in the isolated rabbit heart (162). In that model, acetylcholine and adenosine each alone appears to be capable of producing the memory of cardioprotection. Therefore, in certain experimental models but not in others, GPCR agonists alone can produce ROS and NO or alternative molecules at an amount sufficient to integrate the positive amplification loop for sustained activation of PKC-ε and PI3K.

It should be noted that sustained activation of PI3K signaling may be mediated not only by PI3K activation but also by superoxide-induced inhibition of a lipid phosphatase, PTEN (phosphatase and tensin homologue deleted on chromosome 10) (178), which dephosphorylate phosphatidylinositol-3,4,5-triphosphate, a substrate for PI3K to phosphorylate and activate Akt. Thus, the memory of cardioprotection is mediated by a complex array of redox-sensitive activation of protein kinases, inactivation of phosphatases, and yet undetermined mechanisms.

6. Role of Src and Ras in MAPK activation. In contrast to PKC, the role of MAPKs in IPC has been a considerable matter of debate. MAPKs comprise a superfamily of serine/threonine protein kinases; ERK, p38 MAPK, and c-Jun-NH_2-terminal kinase (JNK). Src tyrosine kinase plays a role not only in the generation of the PKC-ε/PI3K signaling complex but also in signal transduction through MAPK in IPC (314). In the classic ERK pathway, transactivation of growth-factor receptors initiates sequential recruitment and activation of Src and Ras at the inner side of the cell membrane; GTP-activated Ras triggers a protein kinase cascade that typically includes Raf and MAPK kinase (MKK)-1/2, an immediate upstream kinase of ERK. Src may also exist upstream of p38 MAPK and JNK. However, in contrast to ERK, activation of p38 MAPK and JNK is linked to distinct downstream substrates of Src, such as apoptosis signal-regulating kinase (ASK)-1, which has been implicated in cytokine- and stress-induced cell death, survival, and differentiation (207). ASK-1 is a ubiquitously expressed MAPK kinase kinase (MAPKKK) that leads to the recruitment/activation of MKK-3/6 and MKK-4/7, which in turn activate p38 MAPK and JNK, respectively (136).

7. Role of ERK as a survival kinase. As is the case for Akt, activation of ERK may be necessary for cardioprotection during reperfusion. It has been proposed that IPC activates the prosurvival kinases Akt and ERK-1/2 at reperfusion, after a period of lethal ischemia, and protects the heart against I/R injury (121). The crucial role of ERK in cardioprotection was substantiated by specific activation of this kinase as the RISK pathway during reperfusion by ischemic postconditioning (355). A subsequent study using PI3K inhibitor LY-294002 or the ERK1/2 antagonist PD-98059 during reperfusion and inhibition of the infarct size-limiting effect of postconditioning with PD-98059 but not with LY-294002 implicated the involvement of ERK1/2 rather than PI3K/Akt in cardioprotection achieved with postconditioning (67). However, a conflicting observation was provided by Schwartz and Lagranha (296), who demonstrated that IPC during reperfusion activates Akt and ERK with-

out protecting against lethal myocardial I/R injury in pigs. Thus, although a growing body of evidence generally argues in favor of a cardioprotective role of ERK against reperfusion injury, activation of the members of the RISK pathway alone may not be sufficient, and additional factors may be required to fulfill a cardioprotective effect of IPC and ischemic postconditioning. In addition, cross-talk occurs between PKC-ε, ERK, and Akt. PKC-ε–mediated ischemic tolerance may require activation of the ERK pathway (170). ERK has been shown to activate Akt. Thus, it is reasonable to assume that the RISK activities are regulated by coordinated interaction of each member of the RISK pathway.

8. Dual role of p38 MAPK in myocardial injury and protection. In contrast to that of ERK, the role of p38 MAPK in myocardial protection against I/R injury remains a controversial issue (1, 268). Activation of p38 MAPK during IPC may be involved in triggering cardioprotective signal transduction (94, 209). In addition, some studies suggested that p38 MAPK activation may play a mediator role of cardioprotection afforded by IPC (222, 233). Conversely, others demonstrated no correlation between p38 MAPK activation and cardioprotection mediated by IPC (14, 111, 201). These conflicting observations are attributed to the differences in species and experimental models, as well as the dose and selectivity of p38 MAPK inhibitors, but more important, could arise from dual involvement of this kinase in cardiomyocyte death and survival. Consistent with a possible detrimental role of p38 MAPK, studies using p38 MAPK inhibitors and manipulating p38 MAPK genes provided evidence that ischemia-induced activation of p38 MAPK is involved in myocardial injury (23, 203, 253, 292, 316). Although the mechanism by which p38 MAPK activation induces cell death is not fully understood, it has been shown that p38 MAPK mediates mitochondrial dysfunction and triggers activation of the cell-death pathway (60, 101, 364). p38 MAPK activation triggers apoptosis by activating the mitochondrial cell-death pathway, including mPTP opening and cytochrome *c* release from the intermembrane space (60, 101, 364). In contrast to the general agreement that p38 MAPK plays a proapoptotic role during hypoxia or I/R (62, 196, 197), p38 MAPK activation may confer an antioncotic effect (187, 196, 292, 309). Signal-transduction pathways downstream of p38 MAPK involve activation of MAPKAP kinase-2 (MK2) (233), which phosphorylates several HSP family members, such as HSP27 and α-B-crystallin (134, 148). Translocation of these HSPs from the cytosol to the cytoskeleton occurs along with their phosphorylation. Accumulating evidence suggests that phosphorylation of these HSPs is cytoprotective against a wide variety of cellular stress. It has been demonstrated that overexpression of a nonphosphorylatable form of HSP27 is much less effective in mediating protection in Chinese hamster CCL39 cells (109). Furthermore, HSP27 phosphorylation-mediated cytoprotection is mediated by actin polymerization and physical reinforcement of the plasma membrane actin cytoskeleton in the murine fibrosarcoma cell lines (134), suggesting that HSP27 phosphorylation and subsequent reorganization of the actin cytoskeleton may be a crucial step for cytoprotection. Conversely, transgenic overexpression of wild-type HSP27 or nonphosphorylatable HSP27 provided equal protection against I/R injury in the mouse heart (129), suggesting that either form of HSP27 plays a cardioprotective role, although the mechanism of cardioprotection may be different. The nonphosphorylated form of HSP27 acts as F-actin cap-binding protein and inhibits actin polymerization, hence providing a mechanism to explain the *in vivo* observations that phosphorylation of HSP27 regulates actin polymerization and modulates filament stability (168). This reorganization of F-actin appears to be involved in resistance against actin fragmentation and cell death induced by oxidative stress (135). Similarly, the importance of the actin cytoskeletal reorganization in maintaining cell structure and function was proposed by Aoudjit and associates (10), who demonstrated that phosphorylation of HSP27 is protective against complement-induced disruption of the actin cytoskeleton in glomerular epithelial cells. A recent study (243) using cultured neonatal cardiomyocytes also points to the conclusion that, although p38 MAPK activation during I/R promotes apoptosis, physical reinforcement of the actin cytoskeleton by p38 MAPK-mediated F-actin reorganization confers resistance against osmotic fragility and oncosis during reoxygenation. Such dual roles of p38 MAPK in promoting apoptosis but inhibiting oncosis are illustrated in Fig. 11.

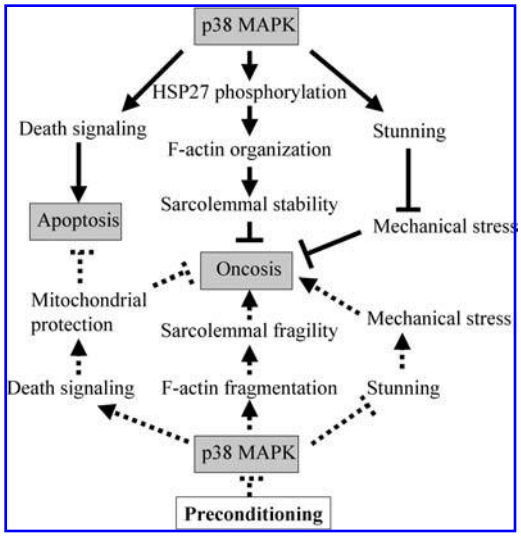

FIG. 11. Schematic drawing of the dual role of p38 mitogen–activated protein kinase in cardiomyocyte death. p38 Mitogen–activated protein kinase (MAPK) activation during ischemia and reperfusion promotes mitochondria-mediated death signaling, leading to apoptosis. At the same time, p38 MAPK activation results in phosphorylation of heat-shock protein 27 (HSP27) and F-actin organization, leading to the stabilization of the sarcolemmal membrane and inhibition of oncosis. Furthermore, p38 MAPK activation causes a negative inotropic effect (acute myocardial stunning), which is also preferable for inhibition of oncosis by reducing mechanical stress on the sarcolemma weakened by depletion of dystrophin. Ischemic preconditioning (IPC) is associated with transient activation of p38 MAPK during the preconditioning challenge, but its inactivation during sustained ischemia and reperfusion. Inactivation of p38 MAPK by IPC inhibits mitochondria-mediated death signaling but promotes cardiomyocyte oncosis by exacerbating the instability of the sarcolemmal membrane and by increasing contractility through the inhibition of stunning.

In line with the role of HSP27 phosphorylation in cytoprotection against physical stress in the cytotoxic environment, it was assumed that sarcolemmal membrane fragility during I/R may be prevented by p38 MAPK activation and HSP27 phosphorylation. On the contrary, inactivation of p38 MAPK during reperfusion may facilitate cardiomyocyte oncosis by inhibiting HSP27 phosphorylation and weakening the sarcolemmal membrane, despite the possible protection of mitochondria. p38 MAPK has been implicated in myocardial stunning (177). Therefore, the pro-oncotic effect of p38 MAPK inactivation may be unmasked by simultaneous occurrence of an antistunning effect. It has been demonstrated that the p38 MAPK inhibitor SB203580 increases cardiomyocyte oncosis associated with an increase in contractility during reperfusion and that temporary blockade of contractility during reperfusion converts SB203580 from the pro-oncotic to the antioncotic agent (309). As mentioned before, sarcolemmal fragility is a characteristic feature of reperfusion injury in the heart, and the loss of sarcolemmal dystrophin may be involved in this pathogenesis. Because p38 MAPK activity was increased by IPC but was inactivated during sustained ischemia and reperfusion (201), the ability of temporary blockade of contractile activity during reperfusion to potentiate IPC-induced cardioprotection is attributed not only to the restoration of sarcolemmal dystrophin in a mitochondrial function-dependent manner (154) but also to inhibition of the antistunning effect–induced oncosis mediated by p38 MAPK inactivation. Thus, sarcolemmal fragility induced by loss of sarcolemmal dystrophin may be augmented by p38 MAPK inactivation during the early phase of reperfusion, and the antistunning effect of IPC may paradoxically increases cardiomyocyte oncosis despite the inhibition of death signaling and the protection of mitochondria (Fig. 11). This assumption reinforces the necessity of mechanical interventions that block contractility during the early phase of reperfusion in the preconditioned heart until the sarcolemmal dystrophin is restored in a mitochondria-dependent manner.

9. JNK as a possible signal transducer in IPC-mediated cardioprotection. The role of JNK in IPC is much less understood compared with ERK and p38 MAPK. Activation of p55 JNK during ischemia and reperfusion is somewhat different in the time course from that of p38 MAPK. Although p38 MAPK activity was increased during ischemia and during the early phase of reperfusion in the isolated rat heart (309), ischemia had no effect on JNK activity, but reperfusion for 5–30 min gave a dramatic increase in activation of p55 JNK but not p46 JNK in a canine model of *in vivo* ischemia (362). In an *in vivo* rat model, IPC and PPC with the opioid receptor agonist, TAN-67, did not increase p38 MAPK dual phosphorylation (95). However, these preconditioning protocols significantly increased p46/p54 JNK dual phosphorylation subsequent to I/R (5–30 min) with a loss of this increase subsequent to 60 min of reperfusion. In a perfused rat heart model, I/R increased p46/p54 JNK dual phosphorylation as compared with that in control oxygenated hearts (289). IPC also increased the kinase activation as compared with the control, oxygenated group. I/R of the IPC hearts decreased JNK dual phosphorylation as compared with that in the preconditioned, oxygenated group. The reason for such discrepant observations with respect to the effect of IPC on JNK activity remains unclear.

JNK has been shown to participate directly or indirectly in cardiomyocyte survival and death during oxidative stress and simulated I/R (9, 98). However, activation of JNK could promote survival of cardiomyocytes after oxidative stress induced by hypoxia and reoxygenation (80). The cardioprotective role of JNK in IPC was suggested by administration of JNK inhibitors, which attenuated the cardioprotection afforded by IPC (95, 289). However, because JNK inhibitors used in these studies are not selective for JNK, further studies are needed to determine the exact role of JNK in IPC.

10. Role of JAK/STAT in IPC-mediated cardioprotection. The JAK-STAT pathway is a stress-responsive mechanism that transduces signals from the cell surface to the nucleus, thereby modulating gene expression. Recent studies have demonstrated that myocardial I/R induces various members of the cytokine superfamily, such as interleukin (IL)-6 (71) and tumor necrosis factor (TNF)-α (214), and induces rapid activation of the JAK-STAT pathway (7). Although an immediate effect of JAK/STAT signaling during ischemia and reperfusion may be detrimental to cardiomyocyte survival and cardiac function (204), activation of this signaling pathway ultimately promotes cytoprotection (237). Emerging evidence suggests that JAK/STAT signaling plays an important role in the development of the cardioprotective phenotype associated with IPC. Specifically, ligand binding of cytokines to their cognate receptors after brief episodes of myocardial I/R induces heterodimerization with gp130, leading to phosphorylation and activation of JAK1 and JAK2, followed by recruitment of STAT1 and STAT3, resulting in transcriptional upregulation of cardioprotective genes. Hattori and associates (116) demonstrated that IPC exerted extensive phosphorylation of JAK2 and STAT3, which induced upregulation of the antiapoptotic gene bcl-2 and downregulation of the proapoptotic gene bax associated with reduction of cardiomyocyte apoptosis and myocardial infarction, and that the JAK kinase inhibitor tyrphostin AG490 blocked IPC-mediated cardioprotection by switching the IPC-mediated survival signal into a death signal. PPC with TNF-α activates the JAK/STAT pathway at reperfusion without involving classic prosurvival kinases, Akt and ERK (173). The cardioprotective effect of the JAK/STAT pathway is not confined to early IPC but is also an essential component of late IPC, as described later. Activation of the JAK/STAT pathway during the preconditioning challenge promotes upregulation of iNOS and cyclooxygenase (COX)-2, which then mediate the infarct-sparing effects of the late IPC (42). Thus, the JAK/STAT pathway appears to represent a distinct cardioprotective signal transducer that emerges relatively late during reperfusion by modulating gene expression regulating cell survival and death.

11. Role of connexin43. Connexin43 (Cx43) is a gap-junctional protein that plays a crucial role in electrical and chemical coupling between the adjacent cells. IPC is known to reduce ventricular arrhythmia through electrical uncoupling. Cx43 has been shown to be dephosphorylated and translocated from the gap junction associated with electrical uncoupling (30). Jain and associates (139) demonstrated that IPC markedly retards the intracellular translocation of Cx43 and greatly diminishes Cx43 dephosphorylation during ischemia. They also found that changes in the kinetics of uncoupling and the sub-

cellular distribution and phosphorylation state of Cx43 are regulated by activation of K_{ATP} channels and PKC during preconditioning.

The pivotal role of K_{ATP} channels and PKC in mediating cardioprotection by IPC has raised the question whether Cx43 is involved in the signal-transduction mechanism of IPC. It has been shown that transgenic mice underexpressing Cx43 cannot be preconditioned (295). Because gap junctions allow spreading of cell death during I/R in the myocardium, it has been hypothesized that the protection afforded by IPC is mediated by effects on gap junction–mediated intercellular communication. However, Padilla and associates (257) showed that the role of Cx43 in the protective effect of IPC is not mediated by a reduction of gap-junction permeability and is independent of a limited gap junction–mediated spreading of cell death during I/R. Moreover, Lin and associates (179) reported that the forced expression of Cx43 has a protective effect against cell death, independent of any effect of Cx43 overexpression on gap-junction function in astrocytes. These studies argue against the hypothesis that the protection afforded by IPC is mediated by effects on electrical coupling and raise a possibility that Cx43 could have effects on cell survival, independent of changes in cell-to-cell communication. In line with this notion, a more recent study (36) demonstrated that Cx43 is localized at cardiomyocyte mitochondria and that IPC enhances mitochondrial localization of Cx43. Although this finding is interesting, little is known about the mechanism of intracellular trafficking of Cx43 to mitochondria and their regulatory functions involved in cardioprotection.

IV. LATE IPC AND CARDIOPROTECTIVE PROTEINS

Late IPC develops 12–24 h after the initial IPC stimulus and lasts for ~48 h (165, 202). Because of the long-lasting nature of cardioprotection, extensive efforts have been made to exploit the mechanism of this adaptive response to protect the ischemic myocardium. Late IPC is triggered by NO, generated by the eNOS and acting *via* the formation of ROS, and activates a broad array of redox-sensitive transcription factors such as NF-κB, activating protein-1 (AP-1), and STAT families (70), which mediate late cardioprotection by increasing the synthesis of cardioprotective proteins such as MnSOD (130), iNOS (42, 366), COX-2 (300), aldose reductase (297), and HSPs (202). MnSOD can scavenge superoxide radical, which is generated on reperfusion, thereby mitigating myocardial injury (130). iNOS appears to play an essential role in late IPC-mediated cardioprotection.

Accumulating evidence indicates that mitoK_{ATP} channels are the distal mediators of iNOS in the late IPC (33, 313), indicating that mitoK_{ATP} channels are the common distal mediators of early as well as late IPC, although other unidentified cardioprotective effects of NO independent of activation of mitoK_{ATP} should be considered. However, iNOS may not be a sole mediator of the late IPC. It has been demonstrated that activation of COX-2 and resultant generation of prostaglandins is an obligatory co-mediator of late IPC (299), although the exact mechanism by which prostaglandins protects myocardium from I/R injury has not been clarified. Similarly, the role of aldose reductase in modifying I/R injury has not been completely understood but may be related to inhibition of lipid peroxidation (297). The role of HSPs in cardioprotection afforded by late IPC is controversial. This is in part because upregulation of HSPs during late IPC or PPC was not consistently demonstrated (34, 363), and the changes in myocardial content of HSPs by IPC do not correlate with protection against infarction (271). Therefore, the significance of HSP overexpression in late IPC remains undetermined.

Other candidate proteins that are potentially important in late IPC-induced cardioprotection are uncoupling proteins (UCPs), which are integral membrane proteins localized to the inner mitochondrial membranes. Theoretically, transient modest mitochondrial depolarization, whether *via* modulation in mETC flux or *via* pharmacologic uncoupling, confers protection against I/R injury by attenuating ROS generation and Ca^{2+} overload without disrupting the capacity to generate cardiac ATP production during I/R (284). It has been shown that UCP overexpression confers tolerance to oxidative stress *via* diminished mitochondrial Ca^{2+} overload and reduced ROS generation (318). Upregulation of UCPs is evident in the late preconditioned rat heart, and this is associated with increased GDP-sensitive proton leak in the presence of ROS. These mitochondria generate less ROS at reperfusion after a prolonged anoxic insult (213), suggesting the involvement of redox signaling in preconditioning-induced upregulation of UCPs. The hypothesis that transient activation of UCPs plays a cardioprotective role against I/R injury in the preconditioned heart is indeed intriguing and a subject of future studies.

V. PRECONDITIONING AND ANGIOGENESIS

Hypoxia results from an imbalance between the supply and consumption of oxygen. Coronary artery occlusion causes abrupt cessation of the blood supply, leading to anoxia or hypoxia of the affected myocardium, dependent on the availability of collateral circulation. Collateral circulation develops in the myocardial region with limited blood supply as a result of vasculogenesis and angiogenesis. Development of collateral circulation helps to rescue hypoxic endothelial cells and cardiomyocytes from death, thereby contributing to inhibition of the loss of functional mass of myocardium and compensatory hypertrophy of remaining viable cardiomyocytes. Although angiogenesis and hypertrophy of cardiomyocytes are initially compensatory phenomena, this adaptive response becomes maladaptive when the concomitant angiogenic response is attenuated. Subsequent exaggeration of hypoxia results in further loss of cardiomyocytes, leading to uncompensated loss of cardiac pump function (*e.g.*, heart failure). Thus, facilitation of angiogenesis in hypertrophic or infarcted myocardium represents an important therapeutic means to prevent remodeling of the heart and the development of heart failure.

Hypoxia is a powerful stimulus of angiogenesis. Tissue hypoxia exerts a proangiogenic action through various angiogenic factors, the most notable being vascular endothelial growth factor (VEGF). Hypoxia has been found to be the strongest in-

ducer, both *in vitro* and *in vivo*, of VEGF (301), which serves as a major angiogen in normal cardiac development. VEGF is associated mainly with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells. Flt-1 (VEGF receptor-1) and Flk-1/KDR (VEGF receptor-2) are the endothelium-specific tyrosine kinase receptors of VEGF through which its effects are primarily mediated (218). Two other angiogenic factors, the angiopoietins 1 and 2 (Ang-1 and Ang-2), have been found to regulate the maturation of new blood vessels from the proliferated endothelial cells (348). Tie-1 and Tie-2 compose another family of endothelial-specific receptor tyrosine kinases (RTK), Ang-1 and Ang-2 being the specific ligands for Tie-2. The upregulation of VEGF, Flt-1, and Flk-1 expression in response to hypoxia *in vitro* and *in vivo* (219) and to ischemia *in vivo* (115) is well established, although conflicting reports exist with regard to Flk-1 *in vitro*, suggesting the involvement of another factors, such as adenosine acting as a paracrine mediator through the A_2 receptor (49). Hypoxia was found to increase the levels of both monomer and dimer forms of VEGF (279). The increase in expression of the homodimer was more pronounced when compared with that of the VEGF monomer. On the basis of this finding, it may be suggested that the VEGF dimer plays a predominant role in myocardial angiogenesis. The abundance of VEGF expression continued to be strong and persistent, even after 4 h of hypoxia, stressing the importance and continuous nature of its role in angiogenesis. This would tend to suggest that not only is VEGF important during the initial stages of angiogenesis, but it also plays a crucial role in the subsequent maturation and maintenance of the new vasculature.

The biologic functions of VEGF, triggered by external stimuli, are initiated through the activation of intracellular signal-transduction cascades involving specific kinases. It is reported that a rapid increase in VEGF expression is due to the presence of hypoxia-inducible factor (HIF)-1–sensitive elements located in the VEGF promoter, which upregulates the transcription of VEGF (61). Furthermore, endothelial cells detect external angiogenic stimuli *via* oncogene activation (226). Formation of a receptor complex between VEGF and its tyrosine kinase receptor Flk-1 activates c-Src, which plays an important role in coordinating the effects of VEGF on cell adhesion and cell motility (125). The physiologic role of VEGF in increasing vascular permeability is also exerted through the Flk-1 receptor and involves MAPK activation mediated mainly by PKC (312).

Recent studies demonstrated that hypoxic preconditioning induced VEGF and increased capillary density even before myocardial infarction (279, 288). The relative time course of protein expression in response to hypoxic preconditioning, as indicated in the previous experiment (279), seems to suggest the involvement of the VEGF system as well as the Ang-Tie system in the early angiogenesis. The *in vivo* early angiogenic response to systemic hypoxemic hypoxia in adult rat myocardium appears to be mediated not only through the induction of VEGF and its receptors Flk-1 and Flt-1, but also through the concurrent induction of Ang-1, Ang-2, Tie-1, and Tie-2 (279). The increased presence of Ang-2 may actually be proangiogenic in nature by effecting dissolution of surrounding matrix, thereby setting up a suitable environment in which endothelial cell migration and capillary sprouting can occur. Examination of non-MI left ventricle (border-zone tissue) by anti-CD31, a marker of endothelial cells, revealed significant increase in the capillary density in the hypoxic preconditioned group compared with that in the control nonhypoxic group, confirming that modulation of angiogenic factors and their receptors by hypoxic preconditioning was able to stimulate capillary proliferation even in the non-MI animals (288). Such a method of preconditioning was also able to reduce infarct size after 24 h of MI.

Although the signal-transduction pathways of hypoxia-induced angiogenesis have not been fully elucidated, ROS appear to be involved in redox-sensitive upregulation of transcription factors, including HIF-1, AP-1, and NF-κB (288). Both *in vitro* and *in vivo* studies indicate that the angiogenic response in vascular tissue is triggered by ROS signaling in a highly coordinated manner. It appears that massive amounts of ROS produced during I/R in the vascular tissue, especially in the heart, cause significant injury to the cardiomyocytes and endothelial cells. However, during reperfusion, the same ROS potentiate a repair process and trigger a signal-transduction cascade leading to angiogenesis. Although several other factors are likely to be involved in such an angiogenic response, ROS certainly play a crucial role, as evident from their direct role as a mediator of angiogenesis and inhibitor of angiogenesis with free radical scavengers and/or antioxidants. Angiogenesis is regulated by redox-sensing transcription factors such as NF-κB, oxidants such as H_2O_2, and free radical molecules such as NO, which may function as second messengers in this highly coordinated process. Furthermore, expression of many angiogenic genes including those for VEGF, fibroblast growth factor, platelet-derived growth factor, and receptors such as Flt-1, Flk-1, Ang-1, and Ang-2 are likely to be regulated by redox signaling. It is tempting to speculate that the angiogenic response is under the autocrine and/or paracrine control of one or more cytokines, which in turn is redox regulated. Through angiogenesis, ROS appear to pave the way for repair of the vascular tissues, which have been damaged during I/R. The mechanism of angiogenesis mediated by preconditioning and inhibition of ventricular remodeling and heart failure after MI is illustrated in Fig. 12.

VI. LATE-IPC MIMETIC EVENTS MEDIATING CARDIOPROTECTION

Regular administration of nitroglycerin has been considered a therapeutic means to reproduce late cardioprotection (128). However, continuous administration of NO-releasing agents, such as organic nitrates, results in the rapid development of tolerance to their vasodilator and antiischemic effects. The development of nitrate tolerance is an important factor limiting the therapeutic efficacy of nitroglycerin in the treatment of patients with ischemic heart disease. Nitrate tolerance is, at least in part, caused by oxidative stress mediated by PKC-dependent activation of NADPH oxidase and uncoupling of eNOS (228). Despite the development of nitrate tolerance, nitroglycerin can faithfully recapitulate the infarct-sparing effects of the late IPC (128). This finding is consistent with the contention that peroxynitrite generated through a reaction of NO with superoxide, but not NO alone, is responsible for the development of late IPC.

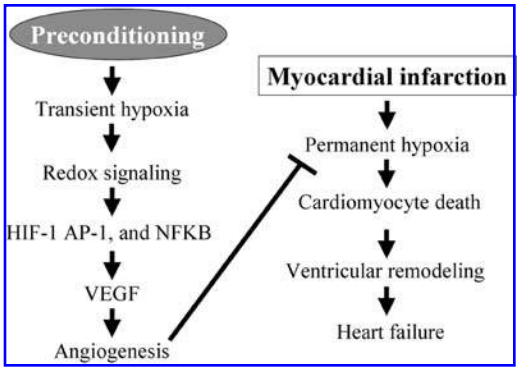

FIG. 12. Schematic drawing of preconditioning-mediated angiogenesis and inhibition of ventricular remodeling and heart failure after myocardial infarction. Ischemic preconditioning (IPC) causes transient hypoxia that upregulates transcription factors including hypoxia-inducible factor-1 (HIF-1), activating protein-1 (AP-1), and nuclear factor-kappa B (NF-κB) through activation of redox signaling. Upregulation of these transcription factors enhances expression of vascular endothelial growth factor (VEGF), which induces angiogenesis in the permanently hypoxic tissue after myocardial infarction and inhibits cardiomyocyte death, leading to prevention of ventricular remodeling and development of heart failure.

Because of the limitation for prolonged administration of organic nitrates, alternative strategies have been developed to recapitulate the infarct-sparing effects of the late IPC. A growing body of evidence suggests that any events that enhance NO bioavailability through activation of eNOS can mimic late IPC (144). It has been demonstrated that statins, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II type 1 (AT1)-receptor blockers, and phosphodiesterase-5 inhibitors produce salubrious effects in experimental models of myocardial infarction *via* their enhancement of NO bioavailability (145, 241, 353, 356), suggesting that the cardioprotective properties of these pharmacologic agents may be exerted *via* a late preconditioning effect. Resveratrol, a red wine and grape derived polyphenolic antioxidant, was also found to cause a late preconditioning effect through an NO-dependent mechanism (117).

Among the maneuvers that induce a late preconditioning effect without pharmacologic tools, regular exercise may represent the most convenient, economical, and effective means. Akita and associates (5) recently demonstrated that activation of eNOS triggered by activation of the cardiac sympathetic nerve (CNS) and oxidative stress can induce a late preconditioning effect by upregulating iNOS. When mice were trained by treadmill exercise for 1 h at an estimated work rate of 60–70% maximal O_2 uptake, reduced glutathione (GSH)/oxidized glutathione (GSSG) was temporarily reduced. The exercise-induced decrease in GSH/GSSG was blocked by topical application of phenol, which is known to ablate the CNS or by treatment with MPG, suggesting that the CNS plays a pivotal role in ROS generation during exercise. ROS generation induced by stimulation of the CNS during exercise was found to be associated with activation of eNOS, which was blocked by topical application of phenol or by treatment with MPG during exercise. Topical application of phenol or treatment with MPG during exercise also significantly inhibited upregulation of iNOS at 7 days after daily exercise. The reduced GSH/GSSG level induced by exercise was not affected in both $eNOS^{-/-}$ and $iNOS^{-/-}$ mice, suggesting that exercise-induced oxidative stress exists upstream of eNOS activation and iNOS expression. In addition, exercise did not increase expression and activity of iNOS in $eNOS^{-/-}$ mice 24 h after the last exercise session, suggesting that eNOS plays a crucial role in upregulation of iNOS induced by daily exercise. When coronary artery occlusion was performed 24 h after the last exercise session, the infarct size was significantly smaller in exercised animals. Topical application of phenol or treatment with MPG during exercise blocked the infarct size–limiting effect of exercise. In contrast to control mice, daily exercise did not reduce infarct size in $eNOS^{-/-}$ or $iNOS^{-/-}$ mice or in mice treated with an iNOS-selective inhibitor 1400W just before coronary artery occlusion. Thus, the role of eNOS as a trigger and iNOS as a mediator of exercise-induced late preconditioning is consistent with the hypothesis that NO is a common trigger and a mediator of late preconditioning induced by ischemic challenges or by pharmacologic agents (39, 40, 365). Efforts should be exerted to exploit ideal late preconditioning approaches that use the same mechanism of action.

VII. ISCHEMIC TOLERANCE AND REFRACTORINESS TO IPC IN DISEASED HEARTS, AGED HEARTS, AND IMMATURE HEARTS

A. Ischemic tolerance and refractoriness to IPC in diseased hearts

Oxidative/nitrosative stress is increased in the heart under various pathologic conditions such as inflammation, hypertension, and cardiomyopathy (189). Enhanced oxidative/nitrosative stress through the formation of peroxynitrite results in cardiomyocyte hypertrophy and the apoptotic and oncotic death of cardiomyocytes that are responsible for the development of heart failure. However, recognition of late IPC raises the idea that exposures to oxidative/nitrosative stress render the diseased hearts tolerant to I/R injury. Conversely, cardioprotective effects of IPC may be compromised by prolonged exposure to oxidative/nitrosative stress in the diseased hearts. A question exists whether IPC is a healthy heart phenomenon and IPC can be induced in diseased hearts such as diabetic, hypertensive, and aged hearts (86). Although most studies on IPC have been undertaken in healthy animal models in which ischemia is imposed in the absence of other disease processes, cardiac complications after coronary artery occlusion are more frequent in those diseased hearts. In this section, I provide models of diseased hearts that are tolerant to I/R injury but refractory to IPC.

1. Ischemic tolerance in cardiomyopathic heart before onset of heart failure. The role of oxidative/nitrosative stress in the tolerance to I/R injury was investigated by Kyoi and associates (167) by using BIO14.6 cardiomyopathy hamster hearts at 6 weeks of age when no significant morphologic change (Fig. 13) and LV dysfunction appeared (data

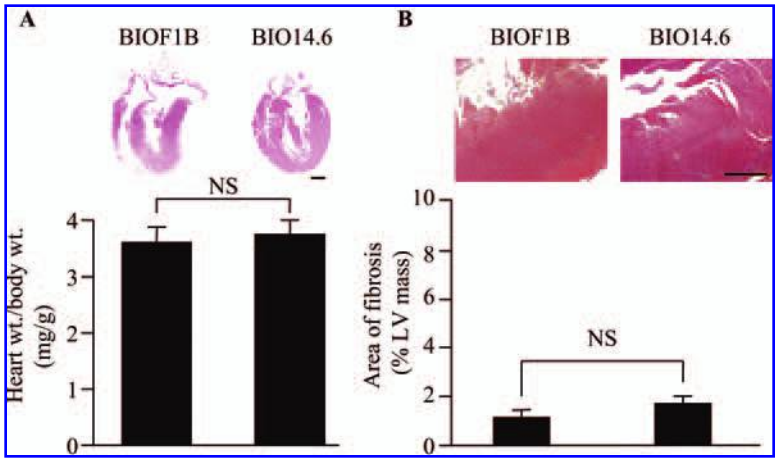

FIG. 13. Basic morphologic characteristics of cardiomyopathic hamster heart. (**A**) Hematoxylin-eosin staining of the heart and heart-weight/body-weight ratio. (**B**) Masson trichrome staining of the heart and the area of fibrosis. Bars indicate 1 mm. BIOFIB, BIOFIB wild-type hamster; BIO14.6, BIO14.6 cardiomyopathy hamster. Each bar graph represents mean ± SEM of five experiments. NS, not significant.

not shown). Expression and activity of iNOS, nitrotyrosine (NT) formation, and PKC-ε activity were increased in these hearts, and it was found that the redox-sensitive upregulation of iNOS activates PKC-ε upstream of mitoK$_{ATP}$ channels (Fig. 14). When the BIO14.6 hamster heart was isolated and subjected to 40 min of global ischemia, it showed smaller myocardial necrosis and greater recovery of LV function during reperfusion compared with the wild-type hamster heart (Fig. 15). All of these effects were abrogated by prolonged treatment with MPG. Although brief preischemic treatment with MPG ameliorated I/R injury in the wild-type hamster heart, the same treatment abrogated the tolerance to I/R injury in the BIO14.6 hamster heart. The preischemic treatment with MPG or the iNOS inhibitor 1400W abrogated NT formation and activation of PKC-ε and inhibited the tolerance to I/R injury in the BIO14.6 hamster heart. Brief preischemic treatment with the PKC inhibitor chelerythrine or the mitoK$_{ATP}$ channel blocker 5-HD and nonselective K$_{ATP}$ channel blocker glibenclamide had no effect on iNOS activation and NT formation but inhibited the tolerance to I/R injury in the cardiomyopathic heart. These observations suggest that the cardiomyopathic hamster heart is tolerant to I/R injury through the exposure to ROS that act at upstream and downstream of iNOS to mediate the tolerance to I/R injury. In line with this hypothesis, ROS generated *via* a mitoK$_{ATP}$ channel–independent mechanism (*e.g*, NADPH oxidase) are involved in upregulating iNOS, whereas at a distal step, ROS produced *via* a mitoK$_{ATP}$ channel–dependent mechanism in concert with iNOS-derived NO activate redox signaling, including the activation of PKC-ε to mediate cardioprotection against I/R injury, at least in part by inhibiting the generation of injurious ROS derived from mitochondria (58, 175). Collectively, it is suggested that a relatively small amount of ROS, generated presumably by NADPH oxidase in a specific plasma membrane domain, promote signaling cascades that prevent a catastrophic increase in mitochondria-derived ROS during I/R by activating mitoK$_{ATP}$ channels.

The temporal and the spatial differences in ROS formation and the target molecules of ROS in mediating cardioprotection and I/R injury remain to be investigated. Here, I delineate a schema in which the cardiomyopathic heart acquires tolerance to I/R injury through oxidative/nitrosative stress–mediated cardioprotective cascades in the BIO14.6 hamster heart (Fig. 16). Further studies are warranted to address whether the same mechanism takes place for the acquisition of tolerance to I/R injury under various pathologic cardiovascular conditions.

2. IPC in cardiomyopathic heart with overt heart failure. Although cardiomyopathic hearts are tolerant to I/R injury before the onset of heart failure, these hearts may not be tolerant to the same injury after the decompensated stage and may not be preconditioned in an appropriate manner. This issue was elegantly addressed by Miki and associates (217), who investigated whether postinfarction ventricular remodeling interferes with the preconditioning mechanism. The investigators created MI to induce remodeling by permanently ligating the left coronary artery in rabbits 2 weeks before isolation of the hearts. The isolated buffer-perfused hearts were subjected to 30-min global ischemia/2-h reperfusion, and infarct size was expressed as a percentage of the LV volume, excluding the scar area that had been created by permanent coronary ligation. Although the percentage of infarct size was similar in sham-operated and remodeled hearts, IPC with two episodes of 5-min ischemia protected sham-operated but not remodeled hearts, indicating that the failing heart is not tolerant to I/R injury and not adequately preconditioned. Infusion of valsartan, an AT1-receptor blocker, for 2 weeks after MI, prevented the ventricular remodeling and preserved the response to IPC, although valsartan alone did not change the percentage of infarct size/LV. Diazoxide protected both sham-operated and remodeled hearts. Based on these observations, the investigators concluded that the myocardium remodeled after MI is refractory to IPC, which is probably due to interruption of cellular signaling by IPC upstream of the mitoK$_{ATP}$ channels. An AT1-receptor blocker is beneficial not only for suppression of ventricular remodeling but also for preservation of the IPC mechanism. This study provides an important clinical implication, in that diseased hearts with overt heart failure may not be preconditioned unless heart failure is properly managed.

3. IPC in diabetic hearts. It is known that diabetes is a significant risk factor for increased mortality after MI. An experimental study demonstrated that hyperglycemia is a major determinant of the extent of MI (152). In addition, acute hy-

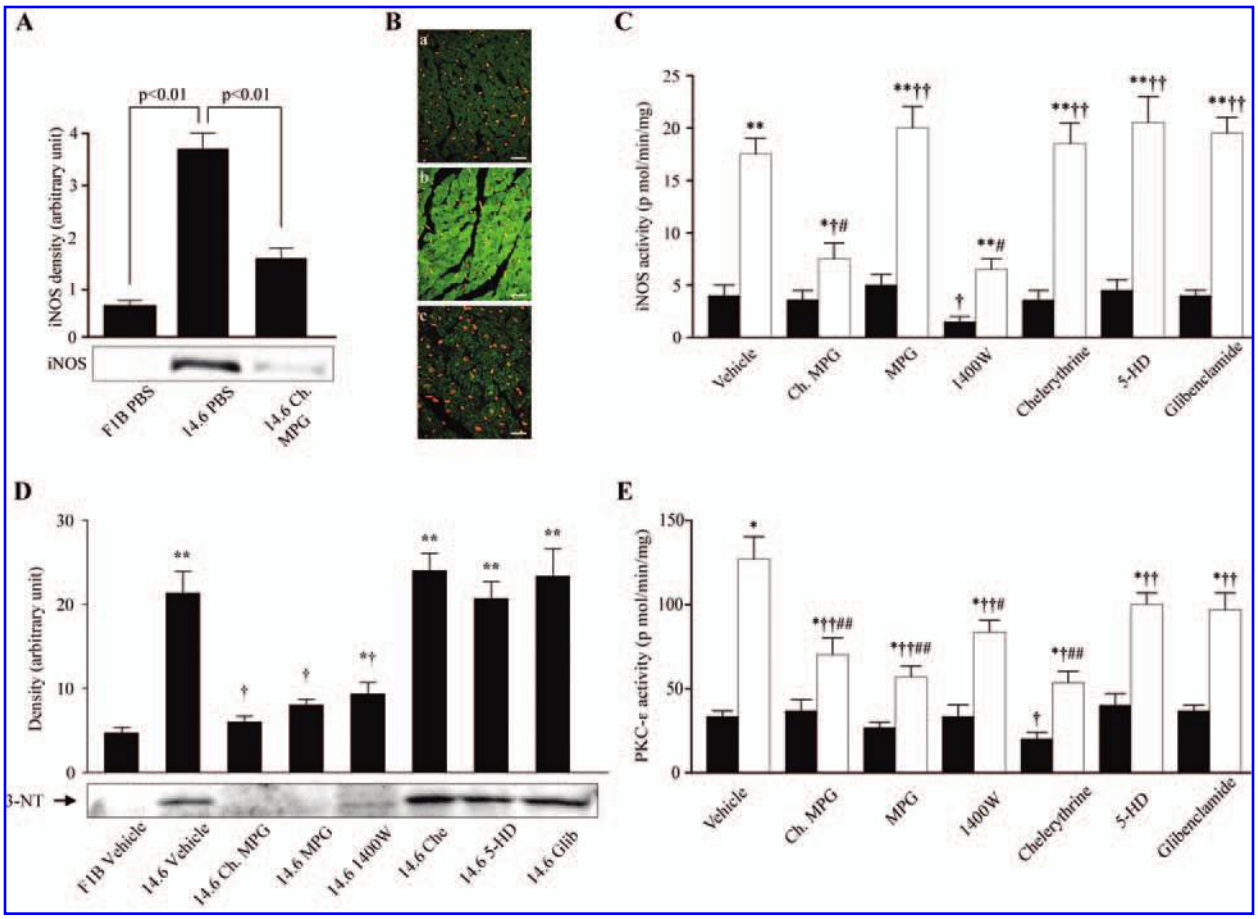

FIG. 14. Redox-sensitive upregulation of iNOS activates protein kinase C-ε upstream of mitochondrial K_{ATP} channels in BIO14.6 cardiomyopathic hamster heart. Vehicle: BIOF1B and BIO14.6 hamsters were treated (i.p.) with 0.2 ml PBS for 5 days, and the isolated hearts were treated with dimethylsulfoxide (0.01%). Ch. MPG: BIOF1B and BIO14.6 hamsters were treated (i.p.) daily with 100 mg/kg *N*-(2-mercaptopropionyl)-glycine (MPG) dissolved in 0.2 ml PBS for 5 days. MPG, 1400W, Chelerythrine, a protein kinase C (PKC) inhibitor (Chel.), 5-hydroxydecanoate (5-HD), and glibenclamide, a nonselective K_{ATP} channel inhibitor (Glb.): isolated and buffer-perfused BIOF1B and BIO14.6 hamster hearts were treated with 0.3 m*M* MPG, 10 m*M* 1400W, 5 μ*M* chelerythrine, 0.5 m*M* 5-HD, or 10 μ*M* glibenclamide for 15 min. (**A**) Immunoblotting for iNOS protein in BIOF1B (F1B) and BIO14.6 (14.6) hamster hearts. Each bar graph represents the mean ± SEM of five experiments. (**B**) Representative images of iNOS immunofluorescence. **a**, BIOF1B hamster treated (i.p.) with PBS for 5 days; **b**, BIO14.6 hamster treated with PBS for 5 days; **c**, BIO14.6 hamster treated (i.p.) with MPG for 5 days. Bars indicate 20 μm. (**C**) iNOS activity assay. *Solid bars* and *open bars* indicate BIOF1B and BIO14.6 hamster heart, respectively. Each bar graph represents mean ± SEM of five experiments. $*p < 0.05$, $**p < 0.01$ between BIOF1B and BIO14.6 hamster heart in each treatment; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with BIOF1B hamster heart treated with the vehicle; $\#p < 0.01$ between BIO14.6 hamster heart treated with the vehicle and BIO14.6 hamster heart treated with respective drugs. (**D**) Nitrotyrosine formation. 3-Nitrotyrosine (3-NT) was detected and quantified by immunoblot analysis. Each bar graph represents mean ± SEM of five experiments. $*p < 0.05$, $**p < 0.01$ compared with BIOF1B hamster heart treated with the vehicle; $\dagger p < 0.01$ compared with BIO14.6 hamster heart treated with the vehicle. (**E**) PKC-ε activity assay. *Solid bars* and *open bars* indicate BIOF1B and BIO14.6 hamster heart, respectively. Each bar graph represents mean ± SEM of five experiments. $*p < 0.01$ between BIOF1B and BIO14.6 hamster hearts in each treatment. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with BIOF1B hamster hearts treated with the vehicle; $\#p < 0.05$, $\#\#p < 0.01$ between BIO14.6 hamster heart treated with vehicle and BIO14.6 hamster heart treated with respective drugs. Adapted from Kyoi *et al.* (167). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

perglycemia blocks the cardioprotection afforded by early as well as late IPC (82, 151). Nevertheless, a number of experimental studies suggest that diabetic hearts are tolerant to I/R injury, suggesting that diabetic hearts are equipped with cardioprotective machinery beyond a deleterious hyperglycemic effect. It has been shown that streptozotocin-induced insulin-dependent diabetic rat hearts are tolerant to I/R injury (185). The ischemic tolerance in the diabetic hearts was not attributed to hyperglycemia by itself nor did it depend on increased collateral vessel formation (112). The reason for discrepant observation between clinical outcome and experimental observations in diabetic hearts with MI has not been answered.

The increased severity of acute MI in diabetic hearts may be attributed to comorbidities such as hypertension and advanced atherosclerosis, and the effect of sulfonylurea derivatives that block K_{ATP} channels may negatively affect the prognosis of MI

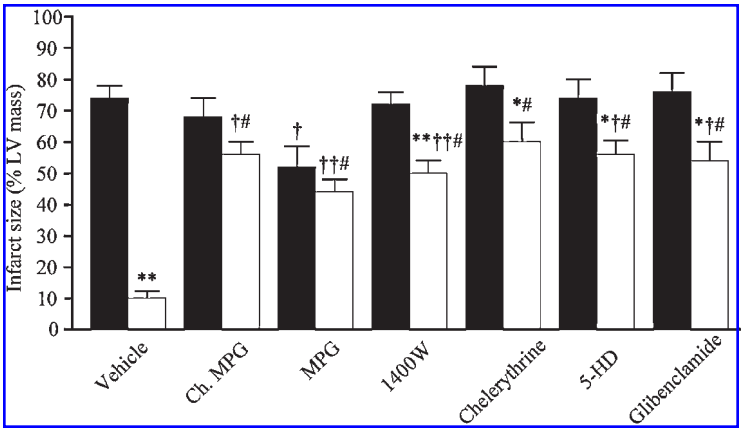

FIG. 15. BIO14.6 cardiomyopathy hamster heart without overt heart failure is tolerant to ischemia/reperfusion injury in a manner dependent on reactive oxygen species, iNOS, protein kinase C, and mitochondrial K_{ATP} channels. Isolated and buffer-perfused BIOF1B hamster and BIO14.6 hamster hearts were subjected to 40 min of global ischemia followed by 120 min of reperfusion. Infarct size was measured with a triphenyltetrazolium chloride staining method, as described (154). The experimental groups are as same as those in Fig. 14. *Solid bars* and *open bars* indicate BIOF1B and BIO14.6 hamster hearts, respectively. Each bar graph represents mean ± SEM of five experiments. $*p < 0.05$, $**p < 0.01$ between BIOF1B and BIO14.6 hamster hearts in each treatment. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with BIOF1B hamster heart treated with the vehicle; $\#p < 0.01$ between BIO14.6 hamster heart treated with the vehicle and BIO14.6 hamster heart treated with respective drugs.

in diabetic patients (100). More important, animal studies suggest that the worse prognosis of diabetic hearts after acute MI may be attributed to the duration of diabetes. The streptozotocin-induced diabetic rat hearts were resistant to postischemic ventricular dysfunction and arrhythmias in the early phase of diabetes (within 2 weeks after injection with streptozotocin), and this protective effect was not observed in the late phase of diabetes (4 to 9 weeks after injection with streptozotocin) (277, 326). Most of diabetic patients with acute MI have a prolonged diabetic history, whereas animal experiments usually use hearts with a relatively brief period of diabetes. Moreover, the majority of patients with acute MI have type 2 diabetes, whereas most of the animal experiments use those with type 1 diabetes, although a recent study (163) suggests that the type 2 diabetic rat heart is also tolerant to I/R injury.

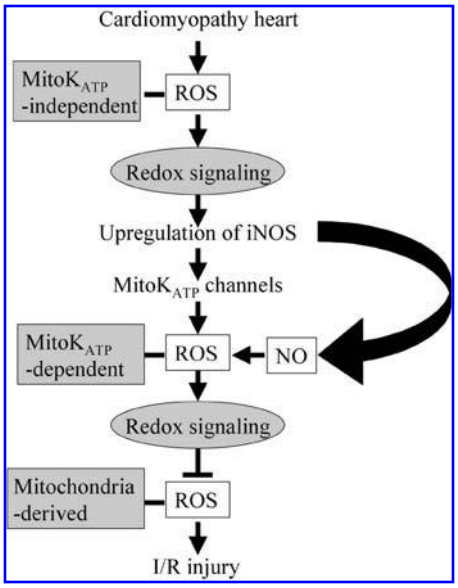

FIG. 16. Model of tolerance to ischemia/reperfusion injury in cardiomyopathy heart. Reactive oxygen species (ROS) generated *via* a mitochondrial K_{ATP} (mitoK_{ATP}) channel–independent mechanism are involved in upregulating iNOS, whereas at a distal step, ROS produced *via* a mitoK_{ATP} channel–dependent mechanism in concert with iNOS-derived NO activate redox signaling to mediate cardioprotection against ischemia/reperfusion (I/R) injury at least in part by inhibiting the generation of injurious ROS derived from mitochondria.

Whether diabetic hearts can be preconditioned is also a considerable matter of debate (263). Available evidence demonstrated further protection (185) or no protection (152, 278, 326) by IPC. The refractoriness of diabetic hearts to IPC is observed not only for early IPC but also for late IPC (74, 82). The mechanism of loss of protection by IPC in diabetic hearts remains undetermined. The rationale for diabetic hearts being more tolerant to I/R injury than normal myocardium is based on a result of reduced production of glycolytic metabolites during sustained ischemia and the concomitant attenuation of intracellular acidosis (317). However, the mechanism for tolerance of diabetic hearts to I/R injury does not appear to be simply attributed to metabolic effects but is more critically attributed to activation of the cardioprotective signal-transduction pathway.

PKC has been shown to be involved in the mechanism of tolerance of diabetic hearts to I/R injury (225), suggesting that IPC and diabetes use a common signaling pathway, culminating in K_{ATP} channel activation in mediating Cardioprotection, as in early IPC and late IPC (227). Thus, it is inferred that refractoriness of the diabetic heart to IPC is due to a defect in cardioprotective signal transduction, as is the case for the failing heart. Although PI3K is important in cardioprotective signal transduction in IPC (120, 121, 328), it has been shown that the PI3K pathway is defective in diabetes (133). Indeed, it has been shown that a mild degree of IPC cannot stimulate the PI3K/Akt pathway, and a higher degree of IPC stimuli is necessary to activate this pathway and to achieve a threshold for cardioprotection in the diabetic heart (327). Alternatively, failure to precondition the diabetic heart may be due to dysfunction of the mitoK_{ATP} channels (102) in human heart tissues and in sarcolemmal K_{ATP} channels in diabetic sheep hearts (74). Thus, it is possible that when the PI3K/Akt pathway remains intact and K_{ATP} channel function is maintained in diabetic hearts, IPC can enhance the tolerance to I/R injury in these hearts.

4. IPC in hypertensive hearts. Hypertension is another common disease condition that affects approximately half of the adult population in industrialized countries. Because hypertension is generally associated with atherosclerosis and a risk of MI, it is imperative to develop a strategy to mitigate myocardial injury after MI. Hypertension-induced LV hypertrophy (LVH) is well recognized as a precursor of heart failure, but evidence suggests that, even before a decline in contractile function occurs, subtle biochemical and metabolic perturbations predispose hypertrophied myocardium to augmented responses to I/R injury (28, 97, 195). Therefore, the high prevalence of LVH and heart failure led several investigators to examine the preconditioning response in these relatively common conditions. Several previous studies demonstrated that, in the stage of compensated LVH, hypertensive and thyroid hormone–induced LVH is associated with maintenance of the classic IPC response. For example, Ebrahim and associates (304) reported that IPC in deoxycorticosterone acetate (DOCA)-salt hypertensive rats *in vivo* elicited an infarct size–limiting effect. Subsequent studies in isolated heart preparations confirmed the ability of early IPC to elicit a cardioprotective response in other rodent models of LVH, including the spontaneously hypertensive rat strain (45), abdominal aortic banding (261), the (mREN-2)27 transgenic hypertensive rat (274), the Dahl salt-sensitive hypertensive rat (54), the hyperthyroid rat (260), and the DOCA-salt hypertensive rat (83). Although the efficacy of IPC as a cardioprotective intervention is maintained in the compensated stage of LVH and the threshold for induction of IPC (*i.e.*, the extent of preconditioning ischemia) is unchanged, it should be kept in mind that the majority of studies undertaken to date in hypertensive hearts have described the preservation of the cardioprotective early IPC mechanisms when no evidence of heart failure is found. Indeed, loss of the IPC response may occur in pressure-overload hypertrophy of long standing in aged animals or in heart failure. Animals with increasing age and heart size have attenuated responsiveness to anesthetic preconditioning with sevoflurane, even in younger guinea pigs (280). In addition, IPC does not protect hypertrophied myocardium in genetically hypertensive rats (224). Furthermore, IPC has detrimental effects on ischemic derangement of intracellular Ca^{2+} homeostasis in severely failing papillary muscles (73).

Currently, it is unknown why failing hypertrophied myocardium is refractory to preconditioning. However, several recent studies suggest that signal transduction for cardiomyocyte survival may be impaired in failing hypertrophied myocardium. Myocardial hypertrophy is induced by physical forces such as cardiomyocyte stretch, and activation of GPCR agonists, such as catecholamines, angiotensin II, prostaglandin $F_{2\alpha}$, or endothelin-1, binding to transmembrane GPCRs, which leads to activation of cytoplasmic signaling for hypertrophy. Accordingly, a likely mechanistic interpretation for LV decompensation may now emerge, in which initial forward signaling through these cascades exerts an early hypertrophic remodeling phase, often characterized by stable cardiac function, and that failure of these hypertrophy/survival pathways to inhibit cardiomyocyte apoptosis ultimately signals a critical step toward decompensation due to dramatic loss of contractile units, dilation of the ventricles, and finally, loss of contractile force. The biologic processes leading to cardiomyocyte hypertrophy and apoptosis remain the center of attention for future biologically targeted therapies. Traditionally, these two processes have been approached as reinforcing biologic circuits that are not necessarily mutually exclusive. Available evidence suggests that during the progression of hypertrophy to heart failure, an endogenous negative-feedback loop to a forward-signaling cascade for hypertrophy becomes activated, and this process plays a role in promoting enhanced cell death, favoring heart failure (335). Indeed, prolonged Akt activation induces feedback inhibition of PI3K activity through both proteasome-dependent degradation of insulin receptor substrate (IRS)-1 and inhibition of transcription of IRS-1, as well as that of IRS-2 (231), suggesting a mechanism by which prolonged Akt activation can become maladaptive. Restoration of PI3K rescued function and reduced I/R injury, indicating that PI3K-dependent but Akt-independent effectors are required for full cardioprotection. Whether this intriguing hypothesis is a unifying mechanism to explain the loss of tolerance to I/R injury and refractoriness to IPC in failing hearts remains to be addressed.

5. Is prolonged exposure to oxidative stress a mechanism for loss of tolerance to I/R injury and refractoriness to IPC in the failing heart? According to the available evidence that demonstrates that the failing heart is susceptible to I/R injury and refractory to preconditioning, I envision a hypothetical mechanism of a defect in cardioprotective signal transduction in the failing heart (Fig. 17). Refractoriness of the failing heart to IPC may be partially explained by the fact that such a heart is tolerated to I/R injury by activating the same mechanism as IPC. However, when the diseased hearts deteriorated because of heart failure, tolerance to I/R injury is lost. This is not due to interruption of oxidative stress and absence of redox signaling but is due to a yet-unidentified defect in the cardioprotective signal-transduction pathway. Heart failure is associated with an increase in variety of growth factors and neurohumoral factors that activate RTK, containing GFRs, GPCRs and cytokine receptors. Although transient exposure to NADPH oxidase–derived ROS has been hypothesized to play a triggering role in IPC-mediated cardioprotection, prolonged activation of NADPH oxidase blocks the cardioprotective signal transduction, presumably at the site between RTK and PI3K as a result of ROS-induced serine phosphorylation of IRS-1, which is known to be involved in inhibition of the PI3K/Akt pathway in type 2 diabetes–mediated insulin resistance (239). Inhibition of the PI3K/Akt-mediated cardioprotective signal-transduction pathway also unmasks death pathways that are concomitantly activated in the failing heart as a result of molecular switch from PI3K/Akt to death pathways containing Grb2/p38 MAPK, leading to cardiomyocyte death and exacerbation of heart failure. In contrast, although cytokine-receptor activation of gp130 is coupled with activation of PI3K/Akt, this signal-transduction pathway can bypass IRS-1. Such an IRS-independent pathway is particularly important when the IRS/PI3K/Akt becomes dysfunctional, because activation of PI3K can activate yet unidentified Akt-independent survival pathways (231). If dysfunction of IRS-1 is indeed the cause for the defect in cardioprotective signal transduction in failing hearts, direct activation of PI3K by cytokine therapy, such as the use of erythropoietin (88), may represent a promising approach for heart failure.

Prolonged exposure to oxidative stress may also impair the angiogenic response in hypertrophied or infarcted hearts. As dis-

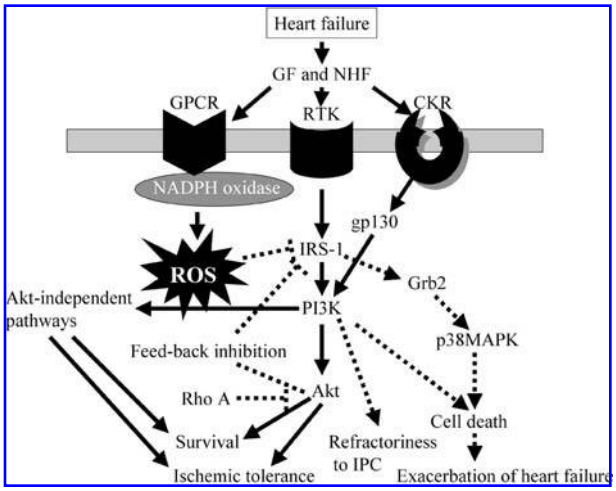

FIG. 17. Schematic drawing of the mechanism for loss of ischemic tolerance and refractoriness to ischemic preconditioning in the failing heart. Heart failure is associated with an increase in variety of growth factors (GFs) and neurohumoral factors (NHFs) that activate receptor tyrosine kinase (RTK)-containing growth-factor receptors, G protein–coupled receptors (GPCRs), and cytokine receptors (CKRs). Prolonged activation of NADPH oxidase blocks the cardioprotective signal transduction, presumably at the site between RTK and phosphatidylinositol 3-kinase (PI3K), as a result of reactive oxygen species (ROS)-induced serine phosphorylation of insulin-receptor substrate-1 (IRS-1). Inhibition of the PI3K/Akt-mediated cardioprotective signal-transduction pathway also unmasks death pathways that are concomitantly activated in the failing heart as a result of molecular switch from PI3K/Akt to death pathways containing Grb2/p38 MAPK, leading to cardiomyocyte death and exacerbation of heart failure. In contrast, although CKR activation of gp130 is coupled with activation of PI3K/Akt, this signal-transduction pathway can bypass IRS-1. Such an IRS-independent pathway is particularly important when the IRS/PI3K/Akt becomes dysfunctional, because activation of PI3K can activate yet unidentified Akt-independent survival pathways. *Solid lines*, pathways toward cell survival, ischemic tolerance; *dotted lines*, pathways toward cell death, refractoriness to ischemic preconditioning, and heart failure.

cussed, angiogenesis is initially an adaptive response of the hypoxic myocardium through redox-sensitive upregulation of HIF-1 and a resultant increase in VEGF expression. However, recent study (285) suggests that the angiogenic response is attenuated during the transition from myocardial hypertrophy to heart failure by accumulation of the tumor-suppressor protein p53 that inhibits HIF-1 activity and VEGF expression. Although the mechanism of accumulation of p53 in hypertrophied myocardium remains unclear, oxidative/nitrosative stress has been implicated in the upregulation of p53 (164). Therefore, redox-sensitive upregulation of p53 by long-term exposure to oxidative stress may represent another example of loss of tolerance to hypoxia or ischemia and loss of angiogenic response to IPC in the failing heart.

B. IPC in aged hearts

It is also a matter of debate whether the preconditioning response is conserved in senescent populations. In a clinical study (2), it was reported that no significant difference was found between elderly and younger adult patients in the incidence of in-hospital death, congestive heart failure, or shock, and the combined end points by previous angina before acute MI, which is thought to be the clinical equivalent of experimental IPC. This observation suggests that the cardioprotection afforded by angina in younger adult patients may involve the occurrence of IPC, which seems to be lost in senescent patients. Contradictory evidence was provided by Kloner and associates (158), who demonstrated that patients aged 60 years or older received benefit from preinfarction angina in the TIMI (Thrombolysis in Myocardial Infarction) 4 study. The underlying cause of the discrepant observation remains uncertain. Further studies, both in animals and human subjects, provided evidence for (191, 270) or against (24, 85, 293) the existence of the IPC response in the aging hearts. Burns and associates (53) demonstrated that the cellular pathways involved in the preconditioning response are well preserved in senescent myocardium in the *in vivo* sheep heart. Conversely, Abete and associates (3) demonstrated by using an isolated and perfused rat heart model that IPC significantly reduced postischemic dysfunction in younger adult but not in senescent hearts. This differential response to IPC was attributed to differences in IPC-induced norepinephrine release and α-adrenergic receptor stimulation, because exogenous norepinephrine was able to mimic preconditioning in both younger adult and senescent hearts, and IPC induced an increase in norepinephrine release in younger adult but not in senescent hearts. Because α-adrenergic receptor stimulation is not a necessary component of IPC, it may not be norepinephrine release by itself that is responsible for IPC, but signal transduction downstream of α-adrenergic receptor stimulation is impaired in senescent hearts. It is known that aged hearts are subjected to oxidative stress. The age-associated increase in ROS generation by mETC dysfunction may cause an elevated basal level of the p38 MAPK stress-response pathway activity. Defective signal transduction upstream of K_{ATP} channels as a possible cause of loss of cardioprotection by IPC in aged hearts is suggested by a study (174) demonstrating that treatment with nicorandil, a K_{ATP} channel opener, elicited cardioprotection in elderly patients in glibenclamide-sensitive manner. PKC-ε is involved in cardioprotective signal transduction in IPC in young animals, but this is not a case for aged animals (269). In addition, increased expression of PKC-δ was noted, and no difference was found in the expression of HSPs despite limited postischemic functional recovery in aged humans (25). Currently, no concrete evidence exists as to the site and the mechanism of the defect in cardioprotective signal transduction mediated by IPC in aged hearts. However, one can postulate that the PI3K/Akt pathway may be a site of impairment in IPC-mediated cardioprotective signaling, because in aged hearts, NADPH oxidase activity is increased as a result of increased AT1-receptor activation (110, 176, 255), and there is abundant RhoA (354), a small GTPase that inactivates Akt through activation of Rho kinase (220, 349). Thus, in diseased hearts with overt heart failure, not only increased ROS but also endogenous suppressors of growth and survival may interfere with development of cardioprotective signal transduction mediated by IPC. In this context, it was hypothesized that reduced IRS-1 expression represents a feedback mechanism in response to prolonged and repetitive stimulation of this signaling pathway by the complex

array of cytokines and growth factors increased in heart failure (200), many of which are capable of activating the IRS/PI3K/Akt signaling.

Another important aspect of age-related alteration of cardioprotective signal transduction is accumulation of PKC-δ and depletion of PKC-ε in mitochondria (160). Aged hearts exhibited upregulation of PKC-δ that accumulated in mitochondria and cytosol before ischemia. PKC-δ was translocated from the cytosol to the mitochondria and nucleus during reperfusion. Postischemic brief treatment with a PKC-δ–inhibitory peptide improved recovery of LV function during reperfusion, concomitant with a reduction of infarct size. This cardioprotection was associated with attenuation of mitochondrial and nuclear PKC-δ levels during reperfusion. This study suggests that age-related upregulation of PKC-δ and enhanced redistribution of PKC-δ to mitochondria and the nucleus is an important causal factor for age-related susceptibility to I/R injury. Collectively, age-related loss of tolerance to I/R injury and refractoriness to IPC may be caused at least in part by depletion of PKC-ε and accumulation of PKC-δ in mitochondria through impaired IRS/PI3K/Akt signaling that renders cardiomyocytes susceptible to mPTP opening on I/R.

Although early IPC may be impaired in aged hearts, late cardioprotection by IPC and PPC by using morphine (265) and δ-opioid (298) appears to be preserved in those animals. However, a lack of clinical evidence exists as to whether early and late IPC are preserved in aged hearts in humans, at least in part because of a small subset of aged subjects that have clinical manifestations of preinfarction angina. Therefore, subanalysis of the effect of preinfarction angina on the prognosis after acute MI in aged subjects is required to address this issue.

C. IPC in immature hearts

In contrast to IPC in mature and aged hearts, much less is known about IPC response in immature hearts. Protection by IPC develops within 7 days after birth, and the inability of neonatal hearts (<7 days old) to precondition is not due to insufficient stimulus or extended ischemia (17). The mechanism of loss of protection by IPC in immature hearts has not been clearly defined. This is probably due to unique properties of neonatal hearts in anaerobic metabolism and antioxidant defense systems. It is known that, relative to adult hearts, newborn hearts are more tolerant to hypoxia or ischemia, as a consequence of the greater capability for anaerobic glycolysis (247). Conversely, newborn hearts may be susceptible to ROS-mediated injury during I/R (249), although decreased oxidant production potential by xanthine oxidase, increased scavenging capacity (catalase), and augmented HSP 72 in newborn hearts has been reported (282). However, none of these properties can explain the lack of IPC response in immature hearts. A possible explanation for the lack in response to IPC challenges in immature hearts is that chronically hypoxic infant rabbit hearts are already resistant to ischemia compared with age-matched normoxic controls, and thus additional cardioprotection by IPC may not be possible (21). eNOS protein and NO are increased in chronically hypoxic infant hearts to protect against ischemia. Chronic hypoxia from birth also increases cardioprotection of infant hearts by increasing association of HSP90 with eNOS. Normoxic infant hearts also generate more superoxide by an *N*ω-nitro-L-arginine methyl ester–inhibitable mechanism than do chronically hypoxic hearts, indicating that normoxic infant hearts are more susceptible to oxidative stress and eNOS uncoupling. Thus, eNOS appears to be critically important in adaptation of infant hearts to chronic hypoxia and in resistance to subsequent ischemia by regulating the production of ROS and reactive nitrogen species. The critical importance of eNOS in mediating cardioprotection in neonatal hearts may explain why these hearts are generally refractory to early IPC but responsive to PPC, because eNOS is not an obligatory component of IPC (248) but is indispensable for opioid-mediated PPC and late IPC (40). Such an eNOS-dependent mechanism of tolerance to I/R injury in infant hearts is different from that in diseased and aged hearts, which are dependent on iNOS, but more akin to ischemic tolerance in the presence of "preventive antioxidants," as discussed in the later sections.

VIII. PRECONDITIONING AND POSTCONDITIONING AS THERAPEUTIC OPPORTUNITIES FOR CARDIOPROTECTION

A. Preconditioning in the real world

The warm-up phenomenon, first described >50 years ago in patients with effort angina, refers to the improved performance exhibited by more than half of all patients with coronary artery disease after their first exercise test (322). A form of myocardial adaptation to ischemia akin to IPC has been implicated in the mechanism of this phenomenon (321). For both logistic and ethical reasons, no clinical study can meet the strict conditions of experimental studies on IPC, with infarct size as the end point. Nevertheless, the demonstration of adaptation to ischemia observed during *in vitro* studies on human atrial muscles (55) and in patients in the setting of coronary bypass surgery (266) strongly suggests that IPC occurs in humans. However, whether the myocardium in patients with acute MI can be preconditioned independent of collateral flow development remains a controversial issue. Earlier studies (159, 254) suggested the possible occurrence of IPC in patients with acute MI undergoing thrombolytic therapy. However, later studies (8, 323) questioned this beneficial effect of preinfarction angina to be solely attributable to the preconditioning effect, originally defined as a delay in lethal myocardial injury, and rather suggested that earlier recanalization of occluded coronary arteries and myocardial reperfusion may be a more likely explanation for the limited infarct size seen in those patients. Consistent with this notion is the fact that when the protective effects of preinfarction angina were evaluated in acute MI patients treated with thrombolytic therapy or percutaneous coronary interventions (PCI), such as balloon angioplasty and stenting, only patients treated with thrombolytic therapy received a benefit from preinfarction angina. Moreover, when the cardioprotective effect of preinfarction angina was investigated in acute MI patients undergoing primary PCI, its beneficial effect was limited to the improvement of LV wall motion but not infarct size, indicating that these hearts were not truly preconditioned by preinfarction angina (240). Indeed, a more recent study (311) sug-

gested that a protective effect of preinfarction angina is mediated by inhibition of microcirculatory damage after reperfusion. Thus, available evidence in the clinical setting of IPC indicates that cardiomyocytes are not effectively preconditioned in human subjects. Although this inability of preinfarction angina to reproduce the cardioprotective effect of IPC in animal studies may be due to variability of the degree of IPC in the clinical arena, it may also be attributed to the fact that most of the human subjects with acute MI are old and have comorbidities such as hypertension, diabetes, and hyperlipidemia that render the heart refractory to IPC, as discussed earlier. Better understanding of the pathophysiology and molecular biology of aging and these morbidities is necessary to develop strategies that allow the human subjects to be responsive to preconditioning protection against acute MI.

B. Postconditioning as an emerging strategy for cardioprotection

As recently described and reviewed by Zhao and Jakob Vinten-Johansen (367), "postconditioning" is a series of brief mechanical interruptions of reperfusion following a specific prescribed algorithm applied at the very onset of reperfusion. Postconditioning, theoretically, might be more clinically applicable than preconditioning because postconditioning would not have to be administered before an ischemic episode, but could be administered at the time of reperfusion. Many studies, but not all, have demonstrated an infarct size–limiting effect of ischemic postconditioning that is equally potent as IPC. This cardioprotective effect was associated with improvement in endothelial function, a reduction in tissue superoxide generation, a reduction in cardiac apoptosis, and a decrease in microvascular injury. Ischemic postconditioning has been reported to occur in dogs, rabbits, mice, and rats, but not in the pig model (367). A few studies examined whether postconditioning can occur in humans. Laskey (171) reported a study of 17 patients undergoing percutaneous coronary intervention for acute MI that demonstrated significantly improved blood-flow velocity reserve in "conditioned" hearts. The results of this study were intriguing, but a larger study was needed to assess this approach for actually reducing MI size or improving clinical outcomes in patients. Staat and associates (305) recently published a pioneering study in which 30 patients admitted for coronary angioplasty for acute MI were assigned to reperfusion with direct stenting alone (control group) or were subjected to a postconditioning protocol after reperfusion by stenting. The postconditioning protocol consisted of 1 min of reflow followed by 1 min of angioplasty balloon inflation and 1 min of balloon deflation, times 4. The area at risk was determined from an LV angiogram. The two groups were equally matched for area at risk, collateral flow assessed by coronary angiography, and duration of ischemia. However, myocardial infarct size, estimated by the area under the curve of creatine kinase release, was 36% lower in the postconditioning group. Blush grade, a marker of microvascular patency after reperfusion, was also better in the postconditioning group. This is an important study, in that it is the first carefully performed, systematic approach to postconditioning patients with a coronary angioplasty balloon that demonstrates that postconditioning may reduce myocardial infarct size in patients with equally matched risk zones.

The mechanisms responsible for ischemic postconditioning and IPC have similarities and differences. As is the case for IPC, adenosine was implicated in ischemic postconditioning because adenosine blockers could inhibit the benefit. Another mechanism that has been implicated in the postconditioning phenomenon is activation of eNOS followed by generation of NO and activation of guanylyl cyclase, leading to activation of mitoK_{ATP} channels and inhibition of the mPTP. Activation of the RISK pathway is a well-known molecular mechanism of postconditioning that culminates in inhibition of mPTP (122), but whether this pathway depends on NO and mitoK_{ATP} channels in ischemic postconditioning has not been definitely answered. Nevertheless, because adenosine and the mitoK_{ATP} channels have been implicated in postconditioning in animal models, it is theoretically possible that the clinical benefits of nicorandil and adenosine in the setting of reperfusion for acute MI in humans are the first examples of the clinical use of postconditioning mimetic drugs. Future large clinical trials using postconditioning mimetic drugs are warranted to evaluate the clinical use during primary PCI for acute MI.

IX. A NEW PARADIGM FOR ANTIOXIDANT THERAPY

A. Genuine antioxidants and the "antioxidant dilemma"

Exposure to oxidative stress is an underlying mechanism by which diseased hearts are tolerant to I/R injury but are refractory to preconditioning. Because a wide variety of age-associated diseases are intimately related to oxidative stress, the use of antioxidants has become a common practice. However, the use of genuine antioxidants may not be an ideal choice for this purpose, because indiscriminate removal of ROS may concomitantly abolish the tolerance to I/R injury in diseased hearts. Although it is unclear the degree to which genuine antioxidants can affect morbidity and mortality in patients with acute MI, lessons learned from preconditioning research raise a word of caution about the use of genuine antioxidants that may negatively influence the outcome of ischemic heart disease. Despite many studies and a wide array of antioxidant agents, no clinical indications exist for the routine use of an antioxidant in the setting of cardiac I/R. It is not for lack of trying, as multiple clinical trials have attempted to use antioxidants in a wide variety of settings. Flaherty and associates (89) studied the administration of human SOD in acute MI patients who were undergoing percutaneous transluminal coronary angioplasty and found no benefit. A large trial of vitamin E and β-carotene likewise failed to show any protective cardiovascular effects when smokers with acute MI were treated over the long term with these agents (275). The risk of fatal coronary heart disease increased in the groups that received either β-carotene or the combination of vitamin E and β-carotene; a nonsignificant trend of increased deaths was noted in the vitamin E group. Several possible explanations have offered insight into the failure of clinical studies of antioxidant therapies. It may be argued that more than one antioxidant is required for clinical effectiveness. The rationale is that antioxidants exist as a "network" wherein both

lipid-soluble (like vitamin E) and water-soluble (ascorbate, glutathione, dihydrolipoic acid) molecules work in a network for the removal of oxidant stress plus the regeneration of oxidant defenses. To date, clinical trials have generally not used synergistic combinations of antioxidants, despite the theoretic advantages and basic science demonstrations of effectiveness.

Another cited explanation for the failure of clinical antioxidant studies is that by inhibiting the normal production of ROS, another "toxic" condition may be produced if these agents or byproducts lead to reduced oxidative phosphorylation and ATP production (302). For example, the β-carotene cleavage products have been shown to inhibit state 3 respiration strongly in isolated liver mitochondria (302). Persistent decreased ATP production may be an important consideration after I/R. However, a more realistic assumption to explain the "antioxidant dilemma" by prolonged administration of genuine antioxidants would be the elimination of ischemic tolerance in patients with coronary artery disease.

B. Cardioprotection by site-specific removal of ROS

As opposed to vitamin E and other genuine antioxidants, which are considered "symptomatic" antioxidants that have a limited potential to scavenge already formed oxidants and may even be deleterious to the heart that has acquired tolerance to I/R injury, recently emerging pharmacologic tools used for treatment of hypertension, diabetes, and hyperlipidemia have a potential to eliminate oxidative stress while preserving the tolerance to I/R injury and the responsiveness to preconditioning. Such drugs are, therefore, designated as a class of "preventive antioxidants" enabling causal therapy against oxidative stress. For example, it can be suggested that interrupting the overproduction of superoxide by the mETC without interrupting the formation of superoxide necessary for redox signaling would switch the death pathways involved in the development of I/R injury or heart failure to the survival pathways involved in cardioprotection. Site-specific inhibition of ROS generation and preservation of redox signaling is exemplified by ACE inhibitors and AT1-receptor blockers.

The renin–angiotensin system is locally activated in the heart under various disease conditions (337). Angiotensin II, acting through high-affinity cell-surface AT1 receptors, modulates cardiovascular homeostasis by exerting vasoconstriction and promoting myocardial hypertrophy and fibrosis, thereby contributing to pathologic remodeling during the development of heart failure (205). Accumulating evidence suggests that such detrimental effects of AT1 activation are mediated at least in part by oxidative stress through the activation of the NADPH oxidase system (105).

It has been demonstrated that AT1-receptor blockers inhibit oxidative stress in the hypertensive heart (29, 78). This raises the concern that the use of AT1-receptor blockers may abrogate tolerance to I/R injury in the hypertensive heart, which is chronically subjected to oxidative stress by activation of AT1 receptors. Indeed, a large number of studies have demonstrated the role of AT1 receptors in cardiac preconditioning against myocardial I/R injury (77, 186, 235). However, at the same time, many studies have indicated that preischemic treatment with AT1-receptor blockers confers Cardioprotection, as demonstrated by improved postischemic cardiac function and reduced infarct size (91, 140, 290). Although the mechanism of cardioprotection mediated by pretreatment with AT1-receptor blockers before I/R has not been completely understood, activation of angiotensin II type 2 receptors and the resultant generation of bradykinin have been implicated as a potential mechanism for AT1-receptor blocker–mediated cardioprotection (140, 169). Activation of eNOS is a downstream effect of increased bradykinin generation (18, 343). Therefore, it is hypothesized that AT1-receptor blockade can mediate cardioprotection *via* activation of eNOS despite inhibition of oxidative stress and resultant iNOS expression.

Another example of causal therapy against oxidative stress in patients with ischemic heart disease is the inhibition of adipocyte-derived proinflammatory cytokine release, which plays a crucial role in obesity-related vascular disorders, including hypertension, diabetes, atherosclerosis, and insulin resistance in metabolic syndrome (208). Recent studies have shown that fat tissue is not simply an energy-storage organ, but exerts important endocrine and immune functions. These are achieved predominantly through release of adipocytokines, which include several novel and highly active molecules released abundantly by adipocytes, like leptin, resistin, adiponectin, or visfatin, as well as some more classic cytokines released possibly by inflammatory cells infiltrating fat, like TNF-α, interleukin (IL)-6, monocyte-chemoattractant protein-1 (MCP-1), and IL-1. All of those molecules may act on immune cells, leading to local and generalized inflammation, and may also affect endothelial function by decreasing NO generation and increasing superoxide release *via* upregulation and activation of NADPH oxidase and the resultant uncoupling of eNOS. The antidiabetic thiazolidinediones (TZDs) are drugs that act *via* peroxisome proliferator–activated receptors (PPAR)γ which belong to the nuclear receptor superfamily of transcription factors in adipocytes, resulting in improved insulin sensitivity in skeletal muscles and liver (103). In addition to the insulin-sensitizing effect, TZDs possess antiinflammatory properties by inhibiting the release of proinflammatory cytokines from adipocytes (66), suggesting that TZDs can preserve the ability of the heart to be preconditioned despite elimination of oxidative stress. Thus, TZDs may also be a promising tool for cardioprotection in patients with metabolic syndrome comorbid with type 2 diabetes and coronary artery disease at a high risk of MI.

Cardiovascular protection by hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, is mediated by lipid-lowering and pleiotropic effects. The efficacy of statins has been established in patients with cardiovascular diseases and also more recently in patients who have acute coronary events, including acute MI (281). In addition, perioperative statin administration has been shown to improve both short-term and long-term cardiac outcome after noncardiac and coronary bypass graft surgery (35). Although the clinical benefits with HMG-CoA reductase inhibitors are seen more in patients with acute coronary syndromes or acute myocardial infarction than in those with stable coronary heart disease, HMG-CoA reductase inhibitors facilitate repair of ruptured or ulcerated atherosclerotic plaque, facilitate plaque stabilization, and/or reduce thrombus formation on ruptured plaque. The rapid onset of clinical benefit and weak correla-

tions between plasma low-density lipoprotein (LDL)–cholesterol levels and coronary lumen change or cardiovascular events indicate that this cardioprotection may be independent of improved hemodynamics because of a positive effect on plaque stability. Treatment with HMG-CoA reductase inhibitors improved endothelial dysfunction in patients with hypercholesterolemia, and this improvement in endothelial function was not correlated with reduction in total serum cholesterol levels (281). Similarly, reduction in endothelial pre-proendothelin mRNA expression and endothelin synthesis and blood pressure lowering with HMG-CoA reductase inhibitors occur independent of the lipid-lowering effect. In addition, HMG-CoA reductase inhibitors increased endothelial cell–derived NO levels (*i.e.*, upregulated eNOS expression *via* posttranscriptional mechanisms and prevented its downregulation by oxidized LDL-cholesterol). Finally, HMG-CoA reductase inhibitors have been shown to modulate the immune response by inhibiting activation of immune-competent cells, such as macrophages, and antigen presentation to macrophages by T cells. Treatment with HMG-CoA reductase inhibitors can reduce expression, production, and circulating levels of chemokines, such as MCP-1, and proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β, thereby inhibiting inflammation on endothelial cells. Although the exact mechanism of this pleiotropic action mediated by HMG-CoA reductase inhibitors remains to be investigated, removal of oxidative stress from endothelial cells appears to be involved in their cardioprotective effects.

Administration of AT1-receptor blockers, TDZs, and statins is only an example of causal therapy against oxidative stress. Many more pharmacologic tools will emerge as "preventive antioxidants" that eliminate ROS but preserve redox signaling for cardioprotection against I/R injury.

X. CONCLUDING REMARKS

Cardiomyocyte apoptosis and oncosis are the major manifestations of myocardial injury associated with I/R. These two forms of cardiomyocyte death are not mutually exclusive but are linked together at a mitochondrial level. In line with this concept, the combination of biochemical interventions that protect mitochondria with mechanical interventions that prevent disruption of the sarcolemma until regaining stability of the sarcolemma may represent a novel paradigm of cardioprotection against myocyte apoptosis and oncosis during the early phase of reperfusion.

Recent advances in preconditioning research at molecular and cellular levels suggest that cardioprotective signal transduction proceeds through a self-perpetuating cycle of redox signaling, including activation of PKC-ε and PI3K, which culminates in protection of mitochondria through activation of mitoK$_{ATP}$ channels and inhibition of GSK-3β, which prevent ROS- and Ca^{2+}-induced opening of mPTP. Positive feedback and feed-forward amplification of redox signaling induced by the activation of mitoK$_{ATP}$ channels plays a crucial role in developing the memory of cardioprotection mediated by IPC. Complete reproduction of IPC by PPC may require the combined addition of pharmacologic tools that include GPCR agonists, mitoK$_{ATP}$ channel openers, and NO donors.

Postconditioning has recently emerged as a realistic means for cardioprotection against reperfusion injury in the clinical arena. Activation of the RISK pathway is presumed to prevent mPTP opening for cardiomyocyte apoptosis and oncosis, as is the case for IPC.

Transient or temporary exposure to oxidative stress under the various physiologic and pathologic conditions renders the heart tolerant to lethal I/R injury akin to late preconditioning. Regular exercise and regular consumption of red wine may represent practical means to induce cardioprotective effects mimicking late preconditioning. Diseased hearts in the absence of overt heart failure may also be equipped with machinery for cardioprotection against I/R injury through the activation of redox signaling. However, prolonged exposure to oxidative stress and long-term activation of redox signaling in the failing heart precipitate a defect in cardioprotective signal transduction, presumably at a site between RTK and PI3K that renders the heart susceptible to I/R injury and refractory to preconditioning and possibly postconditioning. Elucidation of such a defect is particularly important, because any future preconditioning therapies would inevitably be effective in treating the failing heart.

Causal therapy against oxidative stress in hypertension, diabetes, hyperlipidemia, and aging helps to restore the responsiveness to preconditioning while preserving the tolerance to I/R injury. Nitrate tolerance may also be reversed by causal therapy for oxidative stress without compromising the ability of NO to induce preconditioning. In line with this notion, antioxidant medicine must be more site specific, being targeted to a specific ROS or cellular compartment, without a deleterious effect on other redox-sensitive signaling pathways. As classic antioxidants such as vitamins E and C have failed to show beneficial effects, the use of ACE inhibitors, AT1-receptor block-

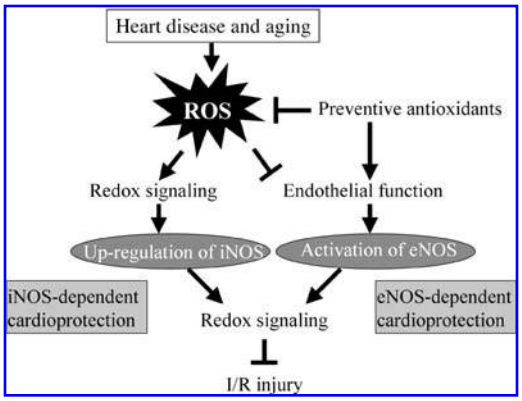

FIG. 18. Model of cardioprotection against ischemia/reperfusion injury in diseased hearts by "preventive antioxidants." Diseased and aging hearts are exposed to reactive oxygen species (ROS) and activated by redox signaling, which is coupled with activation of transcriptional factors responsible for upregulation of iNOS. Upregulation of iNOS renders diseased hearts potentially tolerant to ischemia/reperfusion injury through downstream activation of redox signaling, which is coupled with cardioprotective signal transduction. The use of "preventive antioxidants" can eliminate only injurious ROS upstream of iNOS in a site-specific manner and preserve endothelial function and redox signaling responsible for eNOS-dependent cardioprotection.

ers, TZDs, and statins, even while we await specific trials, should be encouraged for their effectiveness as "preventive antioxidants." Some of these newly emerging cardioprotective drugs have already been proven to be effective to reduce not only the incidence of major cardiovascular events including stroke and MI, but also heart failure and cardiac-related death in many randomized controlled clinical trials. Because "preventive antioxidants" can protect endothelial cells and preserve the activity of eNOS, it is tempting to hypothesize that "preventive antioxidants" can convert the mechanism of cardioprotection from iNOS dependent to eNOS dependent in diseased and aging hearts, as illustrated in Fig. 18. Diseased and aging hearts are exposed to ROS and activated by redox signaling, which is coupled with activation of transcriptional factors responsible for upregulation of iNOS. Upregulation of iNOS renders diseased and aging hearts potentially tolerant to I/R injury through downstream activation of redox signaling, which is coupled with cardioprotective signal transduction, including PKC-ε/PI3K/Akt. Although genuine antioxidants eliminate both injurious ROS upstream of iNOS and protective ROS downstream of iNOS, the use of "preventive antioxidants" can eliminate only injurious ROS upstream of iNOS in a site-specific manner and preserve endothelial function and redox signaling responsible for eNOS-dependent cardioprotection. Site-specific elimination of ROS is also beneficial in improving responsiveness to preconditioning and possibly postconditioning by preserving redox-sensitive cardioprotective signal transduction, which may become defective with prolonged exposure to ROS.

In conclusion, although we are still far from developing optimal cardioprotective approaches against I/R injury, better understanding of the pathophysiology of I/R injury and redox signaling in normal as well as in diseased hearts will pave the way for establishing strategies that can reduce mortality and morbidity after MI by minimizing I/R injury and expansion of infarction in the face of coronary artery occlusion and recanalization.

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ABBREVIATIONS

$\Delta\Psi$m, Mitochondrial membrane potential; ACE, angiotensin-converting enzyme; ADP, adenosine diphosphate; AMP, adenosine monophosphate; Ang, angiopoietin; AP-1, activating protein-1; ASK, apoptosis signal–regulating kinase; AT1, angiotensin II type 1; ATP, adenosine triphosphate; BDM, butanedione monoxime; BH_4, tetrahydrobiopterin; CNS, cardiac sympathetic nerve; COX, cyclooxygenase; Cx43, connexin43; Cu/ZnSOD, copper/zinc superoxide dismutase; DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; DNP, dinitrophenol; DOCA, deoxycorticosterone acetate; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; GFR, growth factor receptor; GPCR, G protein–coupled receptor; GSH, reduced glutathione; GSK-3β, glycogen synthase kinase-3β; GSSG, oxidized glutathione; GTP, guanosine triphosphate; H_2O_2, hydrogen peroxide; HD, hydroxydecanoate; HIF, hypoxia-inducible factor; HMG-CoA, hydroxymethylglutaryl coenzyme A; HSP, heat-shock protein; IL, interleukin; iNOS, inducible nitric oxide synthase; IPC, ischemic preconditioning; I/R, ischemia/reperfusion; IRS, insulin-receptor substrate; JAK, Janus kinase; JNK, c-Jun-NH_2-terminal kinase; LDL, low-density lipoprotein; LV, left ventricle; LVH, left ventricular hypertrophy; MAPK, mitogen-activated protein kinase; MAPKAP, mitogen-activated protein kinase-activated protein; MAPKKK, mitogen-activated protein kinase kinase kinase; MCP, monocyte-chemoattractant protein; mETC, mitochondrial electron-transport chain; MI, myocardial infarction; MK2, mitogen-activated protein kinase-activated protein kinase 2; mitoK_{ATP}, mitochondrial K_{ATP}; MKK, mitogen-activated protein kinase kinase; MnSOD, manganese superoxide dismutase; MPG, 2-mercaptopropionyl glycine; mRNA, messenger ribonucleic acid; mPTP, mitochondrial permeability transition pore; NADH, diphosphopyridine nucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappa B; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NT, nitrotyrosine; $OH^{\cdot}$, hydroxyl radical; PCI, percutaneous intervention; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PMN, polymorphonuclear leukocyte; PPAR, peroxisome proliferator-activated receptor; PPC, pharmacologic preconditioning; PTEN (phosphatase and tensin homologue deleted on chromosome 10); PTX, pertussis toxin; RISK, reperfusion injury salvage kinase; RNA, ribonucleic acid; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; STAT, signal transducers and activators of transcription; TIMI, thrombolysis in myocardial infarction; TNF, tumor necrosis factor; Trx-1, thioredoxin-1; TZD, thiazolidinedione; UCP, uncoupling protein; VEGF, vascular endothelial growth factor.

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