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Forum Review

Reactive Oxygen Species as Mediators of Signal Transduction in Ischemic Preconditioning

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ABSTRACT

Ischemic preconditioning (IPC) is a most powerful endogenous mechanism for myocardial protection against ischemia/reperfusion injury. It is now apparent that reactive oxygen species (ROS) generated in the mitochondrial respiratory chain act as a trigger of IPC. ROS mediate signal transduction in the early phase of IPC through the posttranslational modification of redox-sensitive proteins. ROS-mediated activation of Src tyrosine kinases serves a scaffold for interaction of proteins recruited by G protein-coupled receptors and growth factor receptors that is necessary for amplification of cardioprotective signal transduction. Protein kinase C (PKC) plays a central role in this signaling cascade. A crucial target of PKC is the mitochondrial ATP-sensitive potassium channel, which acts as a trigger and a mediator of IPC. Mitogen-activated protein (MAP) kinases (extracellular signal-regulated kinase, p38 MAP kinase, and c-Jun NH_2-terminal kinase) are thought to exist downstream of the Src-PKC signaling module, although the role of MAP kinases in IPC remains undetermined. The late phase of IPC is mediated by cardioprotective gene expression. This mechanism involves redox-sensitive activation of transcription factors through PKC and tyrosine kinase signal transduction pathways that are in common with the early phase of IPC. The effector proteins then act against myocardial necrosis and stunning presumably through alleviation of oxidative stress and Ca^{2+} overload. Elucidation of IPC-mediated complex signaling processes will help in the development of more effective pharmacological approaches for prevention of myocardial ischemia/reperfusion injury. *Antioxid. Redox Signal.* 6, 449–469.

INTRODUCTION

OXIDATIVE STRESS and the production of intracellular reactive oxygen species (ROS) have been implicated in the pathogenesis of a variety of diseases, including ischemia/ reperfusion injury (133, 237). In excess, ROS and their by-products that are capable of causing oxidative damage may be cytotoxic to cells. However, it is now well established that sublethal amounts of ROS play a crucial role in signal transduction processes involved in the acquisition of tolerance against lethal cytotoxic stress.

Many years ago, Murry *et al.* (182) demonstrated that repeated brief periods of ischemia significantly reduced infarct size following a prolonged period of coronary artery occlusion. This phenomenon has been termed ischemic preconditioning (IPC). Now, it is apparent that IPC is a most powerful endogenous mechanism for myocardial protection against ischemia/reperfusion injury. Better understanding of the molecular biology involved in IPC confers an enormous benefit in developing a novel pharmacological approach to myocardial protection. Numerous investigators have attempted to elucidate signal transduction pathways and effector molecules involved in IPC. Despite extensive investigation, there is considerable debate for or against the role of several established signaling pathways in mediating IPC. Among the candidates of signaling molecules, ROS generated during brief ischemia and reperfusion cycles have been consistently implicated in the trigger of IPC (13, 44, 259, 293, 295). However, the exact roles of ROS in IPC remain poorly understood. Accordingly, the following major aspects have been examined in this review: (a) how excess production of ROS triggers cardiomyocyte cell death, (b) how a modest amount of ROS triggers cardioprotection afforded by IPC; (c) what is the source of ROS in-

volved in the trigger of IPC; and (d) how signal transduction pathways are modulated by ROS in early as well as late IPC.

ROS AS A TRIGGER OF CELL DEATH AND MYOCARDIAL STUNNING

ROS possess a wide variety of functions in cell physiology and biochemistry. Burst generation of ROS is dangerous for survival of cells. A number of studies (8, 53, 114, 177, 192, 193) have implicated ROS as a potential mediator of myocardial reperfusion injury following a certain period of ischemia. The mechanism by which ROS exert detrimental effects on living cells, including cardiomyocytes, has been extensively reviewed (89, 169, 279). The net results of ROS-induced cell damage appear to be altered membrane structure due to peroxidation of phospholipids containing unsaturated free fatty acids and impaired enzymatic activities due to oxidation of sulfhydryl proteins. Eventually, abnormal handling of Ca^{2+} in plasma membranes, mitochondria, and sarcoplasmic reticulum results in intracellular Ca^{2+} overload, the major pathogenesis of myocardial reperfusion injury (186). Besides this direct peroxidative damage on the cell structural proteins, enzymes, and phospholipids, recent studies emphasize the importance of specific cell death pathways arising from mitochondria upon exposure to excessive ROS. There is a large conductance channel in the inner membrane of mitochondria named the mitochondrial permeability transition (MPT) pore (100), which is formed by a Ca^{2+}-triggered conformational change of the adenine nucleotide translocase (ANT) that is facilitated by the binding of cyclophilin D (105). As MPT opening requires not only Ca^{2+} but also oxidative stress, it is assumed that MPT has been implicated in myocardial reperfusion injury (62, 90). In this context, Grijalba *et al.* (92) have proposed interplay between Ca^{2+} and ROS in promoting MPT. According to their hypothesis, Ca^{2+} alters the lipid organization of the inner mitochondrial membrane by interacting with the anionic head of cardiolipin, an abundant component of this membrane. These alterations in membrane organization may affect respiratory chain function, including coenzyme Q (CoQ) mobility, and favor monoelectric oxygen reduction to form superoxide anion (O_2^-) at an intermediate step of the respiratory chain. In addition, the antioxidant system of mitochondria is impaired by MPT accompanied with extensive depletion of NAD (160). NADPH is known to maintain the antioxidant function of the glutathione reductase/peroxidase and thioredoxin reductase/peroxidase systems. Generated ROS in turn potentiate MPT by inducing thiol oxidation of crucial membrane proteins such as ANT (135). Furthermore, ROS generation promotes Ca^{2+} release from nearby sarcoplasmic reticulum, the predominant Ca^{2+} store site in cardiomyocytes, leading to mitochondrial Ca^{2+} accumulation that further increases the rate of ROS production (121). Thus, MPT is a self-amplifying process unless the vicious cycle is interrupted by preventing the accumulation of intramitochondrial Ca^{2+}, the increase in the production of ROS, and depletion of the antioxidant system.

Although the exact nature of MPT remains undetermined, circumstantial evidence suggests that MPT may be an initial event in the process of cell death that occurs in a variety of pathological states that cause oxidative stress and Ca^{2+} overload (209, 238, 291). MPT causes release of cytochrome *c,* which in the presence of Apaf (apoptotic protease activating factor) and dATP (17, 294) activates caspases that culminate in the degradation phase of apoptosis. MPT, on the other hand, dissipates the H^+ gradient across the inner membrane. Subsequent abrogation of ATP synthesis and ATP hydrolysis via F_0-F_1 ATPase leads to necrosis.

ROS are also a cause of reversible myocardial contractile dysfunction termed "myocardial stunning" (26, 28, 185). There is evidence, however, that the amount of hydroxyl radical ($^{\bullet}OH$), which is a most reactive ROS generated during postischemic reperfusion, did not correlate with the severity of myocardial dysfunction after 10 minutes of ischemia, and attenuation of $^{\bullet}OH$ production by deferoxamine failed to reduce myocardial injury in the isolated and buffer-perfused rat heart (251). Such a discrepant observation may be due in part to the property of deferoxamine to inhibit the production of only $^{\bullet}OH$. It is possible that other available ROS are involved in the occurrence of myocardial dysfunction and necrosis.

The molecular mechanism underlying myocardial stunning mediated by ROS is an enigma. Changes in contractile force at the cellular level can be affected by modulation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and modulation of the contractile protein response to $[Ca^{2+}]_i$. In cardiomyocytes, cyclic oscillations of $[Ca^{2+}]_i$ are generated by excitation–contraction coupling that stimulates Ca^{2+} influx through the voltage-dependent Ca^{2+} channels at the plateau of the action potential and through the Na^+-Ca^{2+} exchanger during the systolic phase. Subsequent Ca^{2+} release from the sarcoplasmic reticulum is a predominant source of activator Ca^{2+} for contraction. During the diastolic phase, cytosolic Ca^{2+} is taken up by the sarcoplasmic reticulum, extruded via a reversed mode of the Na^+-Ca^{2+} exchanger, or pumped out by the sarcolemmal Ca^{2+}-ATPase. ROS have been shown to interfere with Ca^{2+} transport systems across the sarcolemmal membrane (124, 125). ROS have also been shown to interfere with the Na^+-Ca^{2+} exchanger and to inhibit Na^+,K^+-ATPase activity (109, 216). Impairment of Na^+,K^+-ATPase activity results in Na^+ overload with consequent activation of the Na^+-Ca^{2+} exchanger that could lead to intracellular Ca^{2+} overload (109, 131, 217). Intracellular Ca^{2+} overload provokes random Ca^{2+} release from the sarcoplasmic reticulum during the diastolic phase that reduces the amplitude of Ca^{2+} transients and contraction development. It is also plausible that ROS cause decreased responsiveness of myofilaments to Ca^{2+} by producing selective damage to contractile proteins, for example, by oxidation of critical thiol groups (247). In this regard, exposure of myofilaments to O_2^- has been shown to result in a dose-dependent reduction in maximal Ca^{2+}-activated force (with no alteration in Ca^{2+} sensitivity) (158, 219). Finally, ROS have been shown to impair sarcoplasmic reticulum function (109, 219). Indeed, a recent study points to the role of ROS in impaired Ca^{2+} handling by the sarcoplasmic reticulum as a critical mechanism for cardiac contractile dysfunction (70).

Alternatively, intracellular Ca^{2+} overload leads to activation of proteases such as calpain that hydrolyze contractile apparatus, *e.g.,* troponin I. Selective degradation of troponin I

and resultant alteration of Ca^{2+} responsiveness have been implicated in myocardial stunning (84, 170, 268). However, there is argument against the role of troponin I degradation in the pathogenesis of myocardial stunning. Accumulating evidence demonstrates that myocardial stunning could be found in the absence of significant degradation of troponin I after a relatively brief period of ischemia (132, 154, 256). In view of this, modification of protein function rather than degradation of contractile proteins has been paid attention as a potential mechanism of myocardial stunning.

Besides direct effects of ROS 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, as will be discussed later, and this process is pivotal in regulating the function of contractile machinery. Particularly, protein kinase C (PKC) and p38 mitogen-activated protein (MAP) kinase have been implicated in the pathophysiology of ischemc contractile dysfunction (147, 278), although the precise mechanism of regulation of contractile function by these kinases has been poorly understood.

Myocardial stunning is not merely a consequence of ROS-mediated contractile dysfunction, but may also be a manifestation of the self-defense mechanism against mechanical force-induced myocardial injury. Restoration of contractile force upon reoxygenation or reperfusion results in cardiomyocyte cell death associated with massive Ca^{2+} influx through the disrupted sarcolemma, a phenomenon known as contraction band necrosis (227, 252, 267). A preliminary study from our laboratory (T. Sumida and H. Otani, unpublished observations) demonstrated that myocardial stunning after temporary ischemia prevented necrosis of cardiomyocytes that were depleted with dystrophin, an integral membrane protein involved in the stability of the sarcolemmal membrane (82, 204, 221).

In summary, ROS play a crucial role in two distinct forms of cell death, *i.e.,* apoptosis and necrosis, as well as in myocardial stunning. Whether or not oxidative stress-induced myocardial stunning represents a self-defense mechanism against mechanical force-induced injury warrants further study.

ROS AS A TRIGGER OF CARDIOPROTECTION AFFORDED BY IPC

In contrast to detrimental effects by massive generation of ROS, sublethal amounts of ROS could serve as a trigger of IPC. Because IPC is implemented by pretreatment with single or multiple brief periods (<10 min) of ischemia and reperfusion prior to a more prolonged and potentially lethal period of ischemia, it is conceivable that reperfusion during IPC procedures generates relatively small amounts of ROS compared with a lethal period of ischemia followed by reperfusion. Such ROS production during IPC could function as a messenger of signaling cascades to protect against lethal oxidative stress induced by a subsequent prolonged period of ischemia and reperfusion. Several independent investigators have indeed suggested the involvement of ROS or redox modulation in activating cardioprotective signal transduction pathways in IPC (13, 44, 259, 293, 295). In addition, ROS signaling may be a universal feature of cardiomyocyte responses to all forms of stress and a mechanism for acquisition of ischemic tolerance, because hyperthermic preconditioning has also been shown to be dependent on ROS generation (284).

SOURCES OF ROS PRODUCTION DURING IPC

Mitochondrial respiratory chain

Accumulation of reducing equivalents during hypoxia or ischemia promotes H^+ leakage from the mitochondrial electron transport chain at the ubisemiquinone site (37, 65), which allows transfer of an electron to any available oxygen leading to generation of O_2^-. O_2^- is then converted to hydrogen peroxide (H_2O_2) and to $^{\bullet}OH$ by an action of catalase and iron-catalyzed Fenton reaction, respectively. There are two sites of O_2^- production in the mitochondrial electron transport chain: complex I and III CoQ-cytochrome *c* reductase) (37). The relative importance of these two sites seems to vary with experimental conditions and between tissues and species (18). Complex I has been considered as a potential source of ROS production in the electron transport chain (261), although the precise nature of the site and its contribution to mitochondrial ROS production remain obscure. Staniek and Nohl (243) reported that mitochondria respiring on complex I substrates do not generate H_2O_2 except in the presence of the complex III inhibitor antimycin A, arguing against the role of complex I as a physiological source of ROS production in mitochondria. However, a recent study (142) demonstrated that ROS production was enhanced at complex I when cytochrome *c* was depleted from the mitochondria. Because cytochrome *c* release could be induced by various noxious stimuli associated with MPT (17, 294), this observation implies that under certain pathological conditions complex I plays an important role in ROS production in mitochondria.

Ever since the pioneering work by Boveris and Chance (34), the role of complex III in mitochondrial production of ROS has been supported by a number of investigators (35, 140, 261). They proposed pinpointed autooxidation of the ubisemiquinone radical of CoQ located in the o-center of complex III as the mechanism of O_2^- generation in mitochondria oxidizing succinate and treated with antimycin A. However, the role of CoQ in O_2^- production remains a controversial issue. Lines of evidence that support redox cycling of CoQ as an alternative site of direct oxygen interaction during respiration were derived from the facts that H_2O_2 release from decomposing O_2^- was inhibited after removal of CoQ from mitochondria, but was reestablished after reincorporation of CoQ (261). In addition, myxothiazol, which prevents the existence of ubisemiquinone at its outer binding center to the bc1 complex, inhibited mitochondrial O_2^- production (188). On the other hand, argument against the role of CoQ in the source of O_2^- has also been provided by Nohl and Stolze (189), who reported that O_2^- formation did not occur through redox cycling of CoQ in a water-free nonpolar reaction system that resembles

the lipophilic character of the inner mitochondrial membrane, but became significant when the membrane was permeable to protons by toluene pretreatment. This observation suggests that CoQ does not play a major role in O_2^- formation in intact mitochondria, but may become an important source of O_2^- under certain pathological conditions in which the inner mitochondrial membrane is protonated (190). Consistent with this hypothesis is the fact that addition of CoQ_{10} to the isolated rat heart mitochondria increased antimycin A-induced and Ca^{2+}-induced H_2O_2 production, but paradoxically inhibited peroxidation of mitochondrial proteins by acting as an antioxidant (283), suggesting that CoQ_{10} plays dual roles in generating redox signaling and inhibiting death signaling. Ischemia and reperfusion may underlie favorable conditions for O_2^- production in the inner mitochondrial membrane. It has been suggested that there is a time window of ischemia in ROS generation by mitochondria (192). The magnitude of ROS generation upon reperfusion is determined by the balance between the amount of reducing equivalents and the activity of the mitochondrial respiratory chain. The shorter the ischemic period the smaller the reducing equivalents, while mitochondrial respiratory function is fully active. Vice versa occurs when the ischemic period is prolonged. Maximum ROS generation could be induced by reperfusion after a moderate ischemic period of ~30–40 min, which corresponds to the time frame when myocardial reperfusion injury is most prominent. Longer periods of myocardial ischemia simply lead to ischemic cell death resulting from ATP deprivation. In contrast, temporary ischemia and reperfusion produce sublethal amounts of ROS, which may act as messengers to generate signals that feed back to suppress a catastrophic increase in ROS induced by a more prolonged period of ischemia. Small amounts of ROS may also be utilized as a sensor to stimulate signal transduction pathways involved in cardiac myocyte responses to hypoxia. Recent studies (36, 64, 265) have raised the hypothesis that hypoxia could generate ROS signaling in cardiac myocytes that provokes adaptive mechanisms against chronic deprivation of oxygen. These studies also suggest that mitochondria are the source of ROS production within the cardiac myocytes, when the cells are exposed to hypoxia.

Xanthine oxidase system

As proposed by McCord (169), the mechanism of ROS production through the xanthine oxidase system involves enhanced degradation of adenosine to hypoxanthine during ischemia, as well as the conversion of the cytosolic enzyme xanthine dehydrogenase, which uses NAD^+ as electron acceptor, to xanthine oxidase, which uses molecular oxygen. When oxygen is reintroduced into the system during reperfusion of ischemic tissue, the oxidation of the accumulated hypoxanthine by xanthine oxidase produces O_2^-. Not all the mammalian species contain xanthine oxidase in the heart cells (59). However, ROS generation through this system appears to be involved in reperfusion injury in the human heart (145, 159).

Neutrophil NADPH oxidase

Myocardial infarction triggers infiltration of circulating neutrophils within the tissue. Neutrophils possess NADPH oxidase on the plasma membrane that reduces molecular oxygen to O_2^- at the expense of NADPH upon activation (12), which further initiates a series of reactions that produce toxic oxidizing agents. O_2^- rapidly dismutates to yield H_2O_2 and neutrophils that contain myeloperoxidase, which catalyzes the oxidation of chloride, generating highly reactive hypochlorous acid (255). Neutrophil-derived ROS have been implicated in reperfusion injury for many years (153, 220). Clinical trials using a leukocyte removal filter during reperfusion in patients undergoing open heart surgery have confirmed the participation of neutrophil-derived ROS in the occurrence of reperfusion injury in human subjects (224, 225).

Nonmitochondrial NADH oxidase

Nonmitochondrial NADH oxidase has recently emerged as a fourth mechanism of ROS generation in the heart. Mohazzab *et al.* (179) have proposed that membrane-bound NADH oxidase activity linked to cytosolic NADH redox is a major source of O_2^- production especially under the condition where lactate concentrations and oxygen tensions are high. This hypothesis has led to the assumption that increased levels of lactate and cytosolic NADH that accumulate during hypoxia or ischemia are likely to contribute to the transient overproduction of O_2^- during reoxygenation or reperfusion.

Endothelial nitric oxide synthase

Nitric oxide (NO) is produced by the oxidation of L-arginine by a family of NO synthase (NOS) that includes two constitutive isoforms, *i.e.,* endothelial NOS (eNOS) and neuronal (or brain) NOS (nNOS), and an inducible isoform (iNOS) (95, 130). Of these isoforms, the source of increased NO formation during IPC is likely to be eNOS (30). eNOS has been identified not only in endothelial cells, but also in cardiomyocytes (95, 130). eNOS produces NO via a complex reaction that is stimulated by Ca^{2+} and requires NADPH, along with other cofactors (95). Reperfusion following transient ischemia could stimulate rapid NO synthesis by providing the oxygen needed to produce NO, because Ca^{2+} and NADPH have already been made available by the ischemic insult of endothelial cells. At the same time, production of O_2^- is also accelerated in the early phase of reperfusion (296). O_2^- and NO react rapidly to form the peroxynitrite anion ($ONOO^-$), which then protonates and decomposes to generate $^{\bullet}OH$ or some other potent oxidant with similar reactivity (19).

Currently, no concrete evidence is available as to which mechanisms of ROS production play a crucial role in triggering IPC. It is unlikely that ROS are produced through the xanthine oxidase system and neutrophils responsible for IPC, because IPC could be induced in animals that are devoid of the xanthine oxidase system and in isolated buffer-perfused hearts that are virtually absent of neutrophils. As available information is limited, the relative contribution of these systems to the net production of ROS during reperfusion remains to be investigated. The role of NOS in the occurrence of early IPC is also controversial (150, 151, 206, 269, 280). This is in contrast with a well established role of NO in late IPC (29, 31). Thus, mitochondria are the most likely source of ROS responsible for triggering IPC, because it is the mitochondria that

decide cell death or survival, and a predominant part of cardioprotective signaling cascades provoked by IPC seems to converge on this organelle.

ROLE OF MITOCHONDRIAL K_{ATP} CHANNELS AS A TRIGGER OF ROS SIGNALING AND AS A MEDIATOR OF PROTECTION

In the last decade, many laboratories have shown that openers of ATP-sensitive K^+ (K_{ATP}) channels protect the heart against ischemia/reperfusion injury (50, 75, 93, 96). The question has arisen as to which K_{ATP} channels play a role in cardioprotection afforded by IPC: the sarcolemmal (surface) or mitochondrial K_{ATP} (mitoK_{ATP}) channels. It has been suggested that surface K_{ATP} channels may not be involved in cardioprotection because there was a lack of correlation between the extent of action potential shortening and the reduction of infarct size by K_{ATP} channel openers bimakalim (286), cromakalim (98), or BMS-180448 (97). Furthermore, preventing ischemic action potential shortening by concomitant treatment with dofetilide did not eliminate protection (99). Finally, in simulated ischemia models of isolated cardiomyocytes, protection was conferred by K_{ATP} channel openers, even though the cells were quiescent and no action potentials were being generated (10). Although the possibility that surface K_{ATP} channels participate in IPC cannot be eliminated (94, 148, 253), recent studies have suggested that K_{ATP} channels in the mitochondrial inner membrane play a predominant role over sarcolemmal K_{ATP} channels in cardioprotection conferred by IPC (86, 94, 149).

It is increasingly clear that mitoK_{ATP} channels act as a trigger of IPC or pharmacological preconditioning with a mitoK_{ATP} channel opener diazoxide (40, 72, 171, 198). The trigger role of mitoK_{ATP} channels appears to be mediated by production of ROS. Obata and Yamanaka (191) demonstrated that treatment of the perfused rat heart with several classes of mitoK_{ATP} channel openers increased generation of $^{\bullet}OH$ in a manner sensitive to mitoK_{ATP} channel inhibitors. Direct evidence that mitoK_{ATP} channels induce ROS production was provided by Krenz *et al.* (137), who demonstrated that K^+ movement through mitoK_{ATP} channels leads directly to ROS production by the mitochondrial electron transport chain, although the site of ROS production in the mitochondrial electron transport chain during activation of mitoK_{ATP} channels has not been identified.

The mediator role of mitoK_{ATP} channels in cardioprotection afforded by IPC has been proposed by Fryer and associates (76). They demonstrated that 5-hydroxydecanoate (5-HD) was effective in inhibiting cardioprotection afforded by IPC in *in situ* rat hearts subjected to 30 min of regional ischemia and reperfusion even when 5-HD was administered after preconditioning ischemia. Dual roles of mitoK_{ATP} channels in cardioprotection have been proposed by Wang and associates (273), who demonstrated that a mitoK_{ATP} channel opener diazoxide conferred cardioprotection when administered for 5 min followed by a 10-min washout, and this cardioprotection was blocked by 5-HD administered before and during diazoxide treatment or 5 min before and throughout 30 min of ischemia in the isolated and perfused rabbit heart. Collectively, these observations suggest that mitoK_{ATP} channels act as both a trigger and a mediator of cardioprotection afforded by IPC. However, care must be taken to interpret the data obtained by the studies utilizing diazoxide and 5-HD to activate and inhibit the mitoK_{ATP} channel, respectively, because serious questions have been asked about the specificity of these agents (106, 107).

The mediator role of mitoK_{ATP} channels has been attributed to preservation of mitochondrial integrity induced by oxidative stress at a step proximal to the MPT (5). This protective action appears to be mediated by prevention of mitochondrial Ca^{2+} overload during ischemia and reperfusion. Recent studies have raised several potential mechanisms for this protective action. First, mitoK_{ATP} channel activation may improve mitochondrial bioenergetics (136). Activation of mitoK_{ATP} channel results in K^+ influx and expansion of mitochondrial matrix volume that has been shown to activate electron transport and stimulate flavoprotein oxidation (104). Maintenance of mitochondrial matrix volume during ischemia through activation of the mitoK_{ATP} channel may represent a potential mechanism of cardioprotection. Theoretically, maintenance of mitochondrial matrix volume preserves intermembrane space architecture during ischemia with consequent slowing of ATP hydrolysis and preserving of the mitochondrial ability to use creatine efficiently as substrate on reperfusion (136). Second, Holmuhamedov and associates (113) reported that diazoxide induced membrane depolarization and decreased Ca^{2+} uptake in isolated rat cardiac mitochondria. Murata and associates (181) showed that diazoxide prevented mitochondrial matrix Ca^{2+} accumulation during simulated ischemia and reperfusion in isolated adult rabbit cardiomyocytes, and they attributed this effect to depolarization of mitochondrial membrane. Whether or not mitoK_{ATP} channel activation causes significant mitochondrial membrane depolarization is a matter of debate (85); however, it seems likely that this could occur in the deenergized mitochondria and may represent a crucial mechanism for prevention of Ca^{2+} uptake at the early stage of ischemia (181).

A third potential mechanism of cardioprotection conferred by mitoK_{ATP} channel activation is the prevention of lethal oxidative stress. It has been shown that either hypoxic preconditioning or the mitoK_{ATP} channel opener pinacidil applied only at reperfusion after simulated ischemia attenuates oxidative stress and protects chick cardiomyocytes (266). In addition, diazoxide inhibited reoxygenation-induced ROS production in isolated rat heart mitochondria associated with preservation of oxidative phosphorylation and mitochondrial membrane integrity (196). Similarly, activation of mitoK_{ATP} channels renders cardiomyocytes tolerant to oxidative stress-induced apoptosis (4). Taking into account the notion that ROS act as a trigger of cardioprotection, these findings suggest that a small amount of ROS generated in mitochondria through the activation of mitoK_{ATP} channels promotes signaling cascades that feedback to inhibit burst generation of ROS upon reoxygenation by this organelle. Moreover, activity of mitoK_{ATP} channels itself is the subject of redox regulation. It has been demonstrated that oxidation of thiol groups in the channel-forming protein causes channel closure (91), suggesting that mitoK_{ATP} channel activity undergoes redox-sensitive feedback regulation.

ROS IN SIGNAL TRANSDUCTION MEDIATED BY IPC

ROS modulation of redox-sensitive proteins

In the early phase of IPC, which develops within minutes from the initial ischemic insult and lasts 2–3 hours (25, 60), cardioprotective signal transduction is acutely evoked by post-translational modification of proteins. Certainly, the most thoroughly studied intracellular transduction mechanisms entail cascades of protein phosphorylation and dephosphorylation involving the interplay of a broad repertoire of kinases and phosphatases. In this critically important mode of intracellular signaling, specificity comes from the residues being phosphorylated and dephosphorylated (Ser, Thr, Tyr) and their positions in the relevant proteins, the tissue distribution and developmental diversity of the participating kinases and phosphatases, and the molecular events that trigger their activity. It is now apparent that many signaling molecules, including PKC, nonreceptor tyrosine kinases, MAP kinases, and protein kinase A (PKA), are involved in IPC. These kinase activities are under the control of redox-sensitive signaling (3). The nature of ROS-induced alteration of protein kinases can be divided into two major categories: (a) the direct effect of ROS on the kinase, which can alter conformation and activity; and (b) the effect of cysteine-rich, redox-sensitive proteins, which have been shown to play an important role in the regulation of stress-responsive proteins exemplified by thioredoxin and glutathione *S*-transferase. ROS causes the formation of disulfide bonds between these cysteine-rich proteins that create dimers and multimers potentially influencing association with other cellular proteins. In most cases, dissociation of the redox-sensitive proteins from the stress-responsive proteins results in their activation.

ROS involvement in signal transduction in early phase of IPC

It has long been known that myocardial reperfusion enhances phosphatidylinositol (PI) lipid turnover including phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) associated with generation of inositol 1,4,5-trisphosphate in the isolated rat heart (194). The PI response was abrogated by hypoxic reperfusion, as well as by inhibition of Ca^{2+} influx. Those previous observations suggest that myocardial reperfusion stimulates PI-specific phospholipase C (PLC) activity along with activation of PI kinases in a redox-sensitive and a Ca^{2+}-dependent manner. The PLC family of enzymes consists of three isoforms (β, γ, and δ) that are differentially regulated and expressed. PLC-β has been demonstrated to be regulated by heterotrimeric G proteins of the Gq family or by $\beta\gamma$ subunits (38, 254), whereas PLC-γ is tyrosine-phosphorylated by growth factors or by transactivation of growth factor receptors, as will be described later. Although the target molecule of PLC-β and PLC-γ is PI-4,5-P_2, these two phospholipases appear to provoke a distinct signaling cascade downstream of PI-4,5-P_2 breakdown (69). The regulation of PLC-δ isoforms remains unknown, although there is evidence that suggests the involvement of GTP-binding proteins (15, 68). PLC-γ, which is the most abundant PLC isoform in the heart (57), has been found to be positively regulated by ROS in concanavalin-induced protein-tyrosine kinase signaling pathways in THP-1 cells (218). However, because recent studies have demonstrated that sulfhydryl oxidation promoted by oxidative stress had either no or an inhibitory effect on the catalytic activity of PLC (126, 174), ROS activation of PLC-γ should occur through the modulation of the upstream regulatory mechanism.

ROS is known to be involved in the mechanism of transactivation in which G protein-coupled receptor stimulation causes activation of receptor tyrosine kinases that leads to activation of downstream proteins such as PLC-γ. For example, Gi protein-coupled receptors induce transactivation of epidermal growth factor receptors (EGFR), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) receptors in various cell types via production of ROS (156, 264, 272). This mechanism may also be effective in the heart undergoing preconditioning. Krieg *et al.* (138) reported that preconditioning of the isolated rabbit heart with acetylcholine and adenosine afforded cardioprotection via transactivation of EGFR.

The Src family of tyrosine kinases appears to play a crucial role in the ROS-mediated transactivation of receptor tyrosine kinases (1, 138). The Src family of tyrosine kinases is known to be activated by oxidative stress (184, 230). Severalfold increase in activation of Src family kinases has been reported during hypoxia/reoxygenation and ischemia/reperfusion (108, 230) or in response to activation of a variety of G protein-coupled receptors with their respective ligands (45, 55, 155). Src kinases are known to interact with many signaling proteins, including focal adhesion kinases, PKC, and phosphatidylinositol 3-kinase (PI 3-kinase) (1, 240). Such a protein–protein interaction forms a signaling module that may be important in integrating cardioprotective signal transduction in IPC (108, 271).

Growth factor receptor hypothesis in IPC is not novel. Several independent groups of investigators have demonstrated that treatment of the heart with IGF-II and acidic as well as basic fibroblast growth factors mimics the cardioprotective effect of IPC (116, 197, 270). Thus, circumstantial evidence lends strong support to the idea that the redox-linked pathway of growth factor receptor activation is an upstream signaling event in IPC. Growth factor receptor activation is coupled to diverse signaling pathways involved in myocardial protection. Insulin, IGF, PDGF, and possibly EGFR activation produces not only PLC-γ-mediated signaling, *i.e.,* PKC activation, but also PI 3-kinase- and MAP kinase-mediated signaling (67, 127, 201). One of the earliest steps in signal transduction by IGF-I receptor is the extensive phosphorylation of insulin receptor substrate-1 (IRS-1), a 185-kDa protein. Tyrosil-phosphorylated IRS-1 then interacts with numerous Src homology 2 domain-containing proteins, including PLC-γ, PI 3-kinase, and the guanine-nucleotide exchange factor Grb2/Sos. Whereas PI 3-kinase initiates PI turnover, Grb2/Sos1 activation results in initiation of the MAP kinase signal transduction cascade by sequential phosphorylation and activation of protooncogenes Ras and Raf, and MAP kinase kinase (MEK).

The consequence of PLC activation is the production of diacylglycerol (DAG), which activates the classical and the novel isoforms of PKC in Ca^{2+}-dependent and Ca^{2+}-independent manners, respectively, although the atypical isoform does not require Ca^{2+} or DAG for activation (23). In the rat heart, the classical isoform PKC-α and the novel isoforms PKC-δ and ε

play a crucial role during the development of IPC (6, 128, 289). These isoforms are translocated to the membrane and activated at different stages of myocardial ischemia and on Ca^{2+} loading or administration of chemical agonists, such as adenosine and phorbol esters (23, 175, 176, 288). Moreover, it is increasingly clear that different preconditioning stimuli provoke the activation of distinct PKC isoforms, which play a distinct role in cardioprotection (176, 207). For example, PKC-α was activated by potassium cardioplegia and low-grade IPC, which was produced by five cycles of 1-min-ischemia and 5-min reperfusion, whereas PKC-δ and ε were activated by high-grade IPC, which was produced by three cycles of 5 min ischemia and 5-min reperfusion, and this activation of novel isoforms was associated with greater cardioprotection (152). Although it has been hypothesized that PKC-ε activation mediates cardioprotection, whereas PKC-δ activation leads to cell death (43, 110), there is a contradictory report showing that PKC-δ is positively involved in opioid-initiated cardioprotection (79). Thus, the role of PKC-δ activation in IPC remains controversial.

Although there is general agreement that PKC plays a crucial role in cardioprotection afforded by IPC (235), timing of PKC activation is important in determining efficacy of protection. Yang *et al.* (285) demonstrated that addition of the PKC inhibitor staurosporine around preconditioning ischemia failed to block the infarct size-limiting effect of IPC, whereas addition of the PKC inhibitor just prior to and continued 10 min into the prolonged ischemia abolished cardioprotection afforded by IPC. These data indicate that PKC acts as a mediator and not a trigger of IPC. However, an argument for the trigger role of PKC in preconditioning has been raised by Wang *et al.* (276), who demonstrated that the PKC inhibitor chelerythrine abolished the beneficial effects of diazoxide on functional, biochemical, and pathological changes induced by Ca^{2+} overload injury. This study has provided evidence that PKC exists upstream of activation of mitoK_{ATP} channels, and thus plays a trigger role in pharmacological preconditioning with diazoxide. In addition, it has been shown that PKC activation itself is not sufficient to open mitoK_{ATP} channels, but it primes mitoK_{ATP} channels to open earlier and more intensely (223). More recently, it has been demonstrated that PKC plays a crucial role in both the trigger and the mediator phase of mitoK_{ATP} channel activation in IPC and proposed that the interplay between PKC and mitoK_{ATP} channels is responsible for amplification of cardioprotective signaling (262, 263).

In contrast to PKC, the role of MAP kinases in cardioprotection against ischemia and reperfusion is controversial. MAP kinase comprises a superfamily of serine/threonine protein kinases: extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun NH_2-terminal kinase (JNK). ERKs are activated by G protein-coupled and growth factor receptors through a cascade of phosphorylation events, including Raf and Ras, which exist downstream of PKC and Grb2 as described above. p38 MAP kinases and JNKs are generally associated with cellular response to diverse stresses, including oxidative stress (24, 245). As is the case for ERK activation, Src may exist upstream of these MAP kinases (129, 250). The activation of p38 MAP kinase requires phosphorylation of Thr180 and Tyr182 within a TGY motif (213). Phosphorylation of both of these residues is carried out by dual-specificity MAP kinase kinases (MKKs), and MKK3 and MKK6 are the physiological activators of p38 MAP kinase (214). JNKs are activated by the upstream MKK4 and MKK7 (56, 71). MKK4 is unique in that it is capable of activating p38 MAP kinase, as well as JNK *in vitro* (56), whereas MKK7 is specific for JNKs (71). Although exact cellular processes downstream of activation of these MAP kinases remain poorly understood, accumulating evidence suggests that ERKs are part of the "survival" pathway (78, 208, 290), although little is known about the involvement of ERKs in IPC. The role of p38 MAP kinase and JNK in cardioprotection mediated by IPC is a matter of debate. It has been proposed that activation of p38 MAP kinase during hypoxia and ischemia promotes a signaling cascade leading to cardiomyocyte death (157, 161). Consistent with this hypothesis is the fact that treatment of the isolated and perfused rat heart with p38 MAP kinase inhibitor SB202190 reduced infarct size after 40 min of ischemia (229). In addition, no correlation was found between p38 MAP kinase activation and cardioprotection afforded by IPC (11, 103, 163). Conversely, p38 MAP kinase activation has been considered as a mediator of cardioprotection afforded by IPC (178, 183). These conflicting observations are attributed to the differences in species and experimental models, as well as the selectivity of different inhibitors in a given dose range, but more importantly, could arise from dual involvement of p38 MAP kinase in cell survival and cell death pathways. p38 MAP kinase phosphorylates MAP kinase-activated protein kinase 2 (MAPKAPK2), which in turn phosphorylates heat shock protein (HSP) 27, a member of a small HSP family (74). Activation of this pathway was thought to be cytoprotective, because overexpression of HSP27 conferred protection against ischemia in myocytes (118, 165). In addition, αB crystalline, which is another member of the small HSP family, is phosphorylated at Ser59 through the p38 MAP kinase signal transduction pathway and inhibits apoptosis by inactivating caspase-3 (180). On the other hand, p38 MAP kinase was shown to promote an apoptotic cascade upstream of cytochrome *c* release and caspase activation (46). As apoptotic and necrotic cardiomyocyte cell death are independent contributors in myocardial infarction (123), it is possible that the balance between inhibition of necrosis and promotion of apoptosis, both of which are regulated by p38 MAP kinase activation, determines ultimate infarct size. In this context, different p38 MAP kinase isoforms may play distinct roles in cardiomyocyte survival and death (275). Much less is known about the role of JNK activation in cardioprotection afforded by IPC. JNK has been shown to participate directly or indirectly in cardiomyocyte cell death during oxidative stress and simulated ischemia/reperfusion (9, 81). However, activation of JNK could promote survival of cardiomyocytes after oxidative stress induced by hypoxia and reoxygenation (58). Consistent with the cardioprotective role of JNK in IPC is the fact that administration of JNK inhibitors curcumin and SB203580 attenuates cardioprotection afforded by IPC (77, 222). However, because these inhibitors are not specific for JNK, further studies are needed to determine the exact role of JNK in IPC.

PI 3-kinase-mediated signaling pathways have gained increasing interest in cardiovascular research as a survival pathway (14, 83, 173). PI 3-kinase activation has been shown to be a part of protective signaling mediated by preconditioning against

myocardial ischemia/reperfusion injury (138, 212, 258). One of the important downstream targets of PI 3-kinase pathways is Akt/protein kinase B (PKB). Akt/PKB is homologous to the PKA and PKC families of protein kinases. *In vivo,* the activity of Akt/PKB is regulated by serum and growth factors that activate PI 3-kinase. Activation of PI 3-kinase results in the production of PI-3,4-P_2 at the membrane. Akt/PKB binds to this lipid, dimerizes, and is stabilized in a partially active state. The location at the membrane and/or the dimerization then enhances the ability of Akt/PKB to be phosphorylated. Overexpression of Akt/PKB prevents apoptosis in primary cultures of cerebellar neurons that are induced by survival factor withdrawal or inhibition of PI 3-kinase (63). The expression of dominant negative forms of Akt/PKB interferes with growth factor-mediated survival in these cells, indicating that Akt/PKB is necessary and sufficient for neuronal survival. In addition, although Ras is involved in activation of Akt/PKB, Akt/PKB does not appear to be involved in the pathway leading to the activation of MAP kinase (73). Therefore, IGF-I and certain other growth factors stimulate a cell survival pathway that involves Ras-dependent stimulation of PI 3-kinase, leading to activation of Akt/PKB. This pathway appears to be independent of MAP kinase and $p70^{S6kinase}$ and to prevent cell death induced by a variety of cellular challenges. The exact mechanism for Akt/PKB-mediated prevention of cell death remains elusive. One possible target of Akt/PKB action for cytoprotection is the proapoptotic Bcl-2 family proteins Bad and Bax. Translocation of these proteins to mitochondria participates in the formation of ion channels that trigger MPT and induce programmed cell death (139, 215, 292). Akt/PKB appears to inhibit translocation of Bad and Bax to mitochondria and promotes cell survival (112, 260).

PI 3-kinase may also participate in cardioprotective signal transduction upstream of PKC. Robust activation of PKC-ε requires interaction not only with Src, but also with PI 3-kinase. G protein-coupled receptor activation leads to membrane translocation of PKC-ε by generating the lipid second messenger DAG as described before. In addition to binding to lipids in the particulate fraction, specific anchoring proteins collectively termed receptors for activated C-kinase (RACK) participate in binding PKC-ε (241). However, binding of PKC-ε to its RACK may not be sufficient to fully activate the enzyme. It has been shown that membrane translocation and phosphorylation act cooperatively to increase novel PKC activity (199). There is evidence that a 12-O-tetradecanoyl13-acetate-induced fast migrating (dephosphorylated) form of PKC-α was inactive (33) and, more directly, that the purified protein could be inactivated following phosphatase treatment (202). By comparison, phosphorylation of particular sites of PKC leads to a conformational change of the enzyme that renders it more active and resistant to phosphatase action, thereby allowing long-lasting activation of PKC (32). Recent studies have led to the conclusion that phosphorylation of PKC-ε in their activation loop sites is under the control of phosphoinositide-dependent kinase 1 (PDK1) or closely related kinase (42, 200). PDK1 displays a requirement for phosphatidylinositol 3,4,5-trisphosphate, which is generated by PI 3-kinase. Consistent with the role of PI 3-kinase in PKC activation loop phosphorylation is the fact that IPC-induced activation of PKC-ε was blocked by the PI 3-kinase inhibitor wortmannin (258). In addition, PI 3-kinase promotes recruitment of RhoA, the small G protein, to the membrane upon activation by G protein-coupled receptor agonists of distinct receptors that are not coupled with pertussis toxin-sensitive G proteins (146). RhoA and PKC are known to play an essential role in activating phospholipase D (PLD) (61, 146, 162), which generates a greater amount of DAG than does PLC in a delayed but a long-lasting fashion through the hydrolysis of phosphatidylcholine, the most abundant membrane phospholipid (49). The PKC isoform responsible for PLD activation in cardiomyocytes has been found to be PKC-ε (66), suggesting that PKC-ε and PLD activities are regulated in a positive-feedback manner in G protein-coupled receptor-mediated signal transduction.

The protective effect of IPC on myocardial stunning is a controversial issue. This is partly because of the inherent difficulty in eliminating the contribution of irreversibly damaged myocardium to the loss of contractile function. Indeed, improvement of cardiac function by IPC after a lethal period of ischemia has been exclusively attributed to reduction of infarct size (48, 122, 195). Nevertheless, subsequent reports kept the question alive whether improved contractile recovery by IPC is based solely on the limitation of the infarct size. Cave (41) has raised a possibility that attenuation of myocardial stunning contributes to functional protection afforded by IPC, because he found that IPC-induced recovery of left ventricular function was not always proportional to the reduction of cardiomyocyte necrosis. Moreover, Perez *et al.* (203) provided evidence that IPC could reverse contractile dysfunction in the isolated and perfused rat heart model with 20 min of global ischemia, which showed no sign of irreversible injury based on intact cell–cell coupling, unaffected Ca^{2+} transients, absence of contracture, maintained response to inotropic stimulation, and no difference in triphenyltetrazolium chloride staining between control and IPC hearts. Another report (87) demonstrated that IPC was capable of improving developed pressure after 30 min of ischemia, and at least 50% of this recovery of left ventricular function in preconditioned hearts was attributed to amelioration of ROS-induced myocardial stunning. Interestingly, however, the protective effect of IPC on myocardial stunning was observed in the rat heart, which contains xanthine oxidase as a source of ROS, but not in the rabbit heart deficient in xanthine oxidase, suggesting that xanthine oxidase-derived ROS plays a crucial role in myocardial stunning in the rat heart. The question as to whether or not xanthine oxidase-derived ROS is a universal mechanism of myocardial stunning seen in other species remains to be investigated. The ROS hypothesis and the Ca^{2+} hypothesis are not mutually exclusive and, in fact, may represent different steps of the same pathophysiological cascade (28). In light of the fact that IPC attenuates ROS production presumably by enhancing the antioxidative defense system (26, 51, 54, 185, 266) and prevents intracellular Ca^{2+} overload (274), it seems reasonable to conclude that functional protection afforded by IPC is at least in part due to amelioration of myocardial stunning. It will also be intriguing to investigate whether reversal of myocardial stunning by IPC is related to inhibition of specific signaling cascades, particularly with respect to PKC and p38 MAP kinase.

In summary, ROS-modulated signal transduction during IPC converges on the protection of mitochondria against ROS- and Ca^{2+}-mediated dysfunction. This mechanism involves ac-

tivation of mitoK_{ATP} channels and displacement of death-promoting proteins from mitochondria, both of which could prevent MPT and subsequent cell death in the form of apoptosis and necrosis. It is conceivable, however, that other yet unidentified mechanisms may also be involved in protection of mitochondria in the early phase of IPC. The protective effect of the early phase of IPC on myocardial stunning requires further proof.

ROS involvement in signal transduction in late phase of IPC

Late IPC develops 12–24 h after the IPC stimulus and lasts for ~48 h (143, 164). Unlike the early phase, there is unequivocal evidence that the late IPC protects against myocardial stunning, in addition to preventing myocardial necrosis (25, 29, 30, 246). Because of sustained duration, efforts have been made to exploit the mechanism of this adaptive metamorphosis to protect the ischemic myocardium in patients (27). Similar to the mechanism of early IPC, late IPC is also tightly related to the production of ROS that trigger the synthesis of intrinsic reactive proteins after initial ischemic stress (115). The synthesis of cardioprotective proteins is under the regulation of a broad array of transcription factors, including nuclear factor-κB (NFκB), activating protein-1 (AP-1), and members of the signal transducers and activators of transcription (STAT) family. Their activation is mediated by recruitment of the same signaling cascades as are used to induce the early phase of IPC. Indeed, NFκB activation is dependent on the oxidant-sensitive recruitment of PKC and Src kinase (16). The activation of PKC and Src in turn provokes activation of the serine-threonine kinases IKK (IκB kinase) that phosphorylate IκB. Phosphorylation of IκB results in dissociation from NFκB, which enters the nucleus to exert its transcriptional activity (102, 117). Regulation of AP-1 activity seems to utilize MAP kinases. p38 MAP kinase and JNK are known to induce the immediate early genes c-fos and c-jun mRNAs, the protein products of which heterodimerization constitute AP-1 (257). Moreover, MAP kinases phosphorylate and activate c-Fos and c-Jun (7, 88). In addition, O_2^- or H_2O_2 induces STAT activation in a manner dependent on activation of janus kinases (JAK) or Src kinases (39, 226, 236). Conversely, NO radicals increase the transcription of IκB and cause retention of NFκB in a cytoplasmic, inactive form (242).

NFκB is the major transcription factor involved in the synthesis of cardioprotective proteins in late IPC (281). NFκB is known to be involved in induction of gene expression of free radical scavengers in mitochondria (47, 166). Manganese superoxide dismutase (Mn SOD), which is a scavenger of O_2^- generated by the electron transport system in mitochondria, has been shown to play a crucial role in cardioprotection during late IPC (115). An increase in the activity of various antioxidant enzymes, including Mn SOD, Cu-Zn SOD, catalase, and/or glutathione peroxidase, has also been reported 24–72 h after pharmacological preconditioning with interleukin-1 (167) and endotoxin (168), concomitant with increased myocardial resistance to ischemia/reperfusion injury. Although a cause-and-effect relationship remains unclear and not all studies have found up-regulation of antioxidant enzymes in late IPC, reinforcement of the antioxidant defense system is a paradigm of molecular adaptation to oxidative stress that is induced by preconditioning.

Another mechanism that controls the expression of stress-responsive genes is the activation of STAT. These proteins are tyrosine-phosphorylated by JAKs in a redox-sensitive manner following the binding of cytokine to its receptor and by a variety of receptor and nonreceptor protein tyrosine kinases (52, 120, 226). Activation of STAT requires phosphorylation of tyrosine residues in the Src homology 2 domain (52, 111, 119). Once they are phosphorylated, STAT proteins homodimerize or heterodimerize and translocate to the nucleus, where they transactivate STAT-responsive genes. The JAK-STAT pathway has been implicated in cardiac hypertrophy (141), apoptosis (231, 244), and inflammation (80, 172). Activation of STAT3 has been reported to limit apoptosis in rat models of myocardial infarction (187). STAT1 and STAT3 have also been found to exert proapoptotic and antiapoptotic effects, respectively, in cultured neonatal cardiomyocytes subjected to anoxia, metabolic inhibition, and acidosis (244). More recently, Xuan and associates (282) have demonstrated that IPC induces isoform-selective activation of JAK1, JAK2, STAT1, and STAT3, and that activation of the JAK-STAT pathway is responsible for up-regulation of iNOS, which plays an essential role in the development of late IPC (27, 30).

The first demonstration that the cardioprotective effects of the late phase of IPC are mediated by NOS was provided by two studies in conscious rabbits, in which the delayed protection against both myocardial stunning and myocardial infarction was found to be completely abrogated when preconditioned animals were given a NOS inhibitor N^{ω}-nitro-L-arginine 24 h after IPC, just before the second ischemic challenge (30, 248). The same effects were observed with the relatively selective iNOS inhibitors aminoguanidine and *S*-methylisothiourea, implicating iNOS as the specific NOS isoform involved in mediating the protective effects of late IPC (30, 248). Because of the limited selectivity and possible nonspecific effects of iNOS inhibitors, conclusive identification of the NOS isoform responsible for enhancing tolerance to ischemia during late IPC cannot be attained pharmacologically. Using an *in vivo* murine model of myocardial infarction, Guo *et al.* (101) were the first to demonstrate that the late phase of IPC is associated with up-regulation of myocardial iNOS and that targeted disruption of the iNOS gene completely abrogates the delayed infarct-sparing effect, providing unequivocal molecular genetic evidence for an obligatory role of iNOS in the cardioprotection afforded by the late phase of IPC. Immunohistochemical and *in situ* hybridization studies have identified cardiomyocytes as the specific cell type that expresses iNOS during late IPC (277). Besides ischemia-induced preconditioning, iNOS may serve as an obligatory mediator of NO donor-induced, δ-opioid receptor-induced, and exercise-induced late preconditioning against infarction (27). However, Bell and Yellon (20) have demonstrated that significant cardioprotection occurs in iNOS knockout mice 24 h after treatment with an adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine. Because this delayed cardioprotection in iNOS knockout mice was associated with up-regulation of eNOS, unlike the delayed phase of IPC, eNOS plays a crucial role in delayed adenosine A_1-triggered preconditioning. Thus, the NOS isoforms responsible for mediating late preconditioning appear to depend on the preceding stimulus.

Mediators of cardioprotection downstream of iNOS have been extensively investigated by Bolli and collaborators. K_{ATP} channels have been proposed as a distal effector to iNOS in cardioprotection afforded by late IPC (21, 249). However, K_{ATP} channel activation participates only in the infarct size-limiting effect of late IPC and not myocardial stunning (249), indicating that K_{ATP} channels are not the sole effector of cardioprotection afforded by late IPC.

The recent study investigating comediators of late IPC have identified inducible cyclooxygenase-2 (COX-2) as an obligatory enzyme to protect against myocardial stunning and infarction (232). This observation also strongly points to prostaglandin (PG) E_2 and/or PGI_2 as the likely effectors of COX-2-dependent protection. The induction of iNOS in response to stresses such as cytokines, hypoxia, and ischemia is associated with simultaneous induction of COX-2 in various cell types, including cardiomyocytes (144). COX is the rate-limiting enzyme in PG synthesis, catalyzing the conversion of arachidonic acid to PGH_2. The signaling elements that control the expression of COX-2 after stress appear to be similar to those controlling the induction of iNOS; the sequence of events includes the production of ROS that trigger activation of PKC and tyrosine kinases, leading to the recruitment of redox-sensitive transcription factors such as NFκB (2, 228, 239). Furthermore, there is interaction between iNOS and COX-2 in the heart. Shinmura *et al.* (234) have demonstrated that COX is located downstream of iNOS and COX-2 activity is increased by iNOS-derived NO.

Aldose reductase and HSPs have emerged as other candidates for comediators of late IPC. Aldose reductase is a member of the aldo-keto reductase superfamily that metabolizes toxic aldehydes generated by lipid peroxidation, suggesting that this enzyme may represent an important defense system against lethal oxidative stress. Shinmura *et al.* (233) have found that protein expression of aldose reductase is up-regulated 24 h after IPC in conscious rabbits and that inhibition of this enzyme abrogates the infarct-limiting effects observed in untreated animals. Thus, in addition to iNOS and COX-2, aldose reductase is a third necessary mediator of the cardioprotective actions in the late phase of IPC. In contrast to aldose reductase, the role of HSPs in cardioprotection afforded by late IPC is equivocal. This is because up-regulation of HSP72, HSP70, and HSP27 during late IPC or pharmacological preconditioning was not consistently recognized (22, 134, 164, 287) and the changes in myocardial content of these HSPs by whole body hyperthermia or by ischemia do not correlate with protection against infarction (210, 211). Therefore, the significance of HSP up-regulation in the late phase of IPC remains undetermined.

CONCLUSIONS

IPC is emerging as an adaptive mechanism for cardiomyocyte protection against lethal ischemia/reperfusion injury. Figure 1 depicts the proposed mechanism as to how IPC uses redox signaling to mobilize a complex sequence of cellular events. The initial event immediately followed by preconditioning ischemia and reperfusion is the occupation of G protein-coupled receptors. Stimulation of G protein-coupled receptors provokes βγ subunits-induced activation of PLC-β, leading to activation of PKC, which primes mitoK_{ATP} channels to open. Opening of mitoK_{ATP} channels produce ROS that trigger both the early and the late phases of IPC. ROS are involved in transactivation of growth factor receptors in a manner dependent on Src tyrosine kinase. Src kinase activation is necessary to amplify cardioprotective signaling by acting as a scaffold for interaction with many signaling proteins. PLC-γ contributes to further activation of PKC, which then activates MEK and ERK via the Ras/Raf signal transduction cascade. Grb2/Sos1 activation also results in initiation of the MAP kinase signal transduction cascade by sequential phosphorylation and activation of Ras, Raf, and MEK. PI 3-kinase promotes an alternative pathway for PKC activation via the action of PDK1 and recruitment of RhoA to the membrane. PDK1 is necessary for PKC-ε activation loop phosphorylation. RhoA and PKC-ε are involved in activation of PLD, which hydrolyzes phosphatidylcholine and generates more DAG in a long-lasting fashion. These positive feedback mechanisms play a crucial role in maintaining robust activation of PKC that may be necessary for "memory" of cardioprotection conferred by IPC.

PKC plays not only a trigger but also a mediator role in IPC by participating in activation of mitoK_{ATP} channels that protect mitochondria from oxidative- and Ca^{2+} overload-induced opening of MPT. Inhibition of MPT prevents the sequence of events eventually leading to both apoptosis and necrosis.

Activation of PI 3-kinase, on the other hand, is involved in activation of Akt. Akt phosphorylates the downstream target proteins Bad and Bax and inhibits their translocation to mitochondria that may contribute to the prevention of apoptosis by inhibiting MPT.

ROS play an important role in activation of p38 MAP kinase and JNK through the activation of upstream signaling MKK. Despite extensive research for MAP kinases-mediated intracellular events, their roles in IPC remain largely unknown.

In contrast to a well established cardioprotective effect of early IPC against cardiomyocyte cell death, the protective effect against myocardial stunning requires further proof, although circumstantial evidence points to the conclusion that mitigation of myocardial stunning contributes at least in part to functional protection afforded by the early phase of IPC.

Late IPC is mediated by ROS-dependent modulation of cardioprotective gene expression. This mechanism involves redox-sensitive activation of transcription factors through PKC and tyrosine kinase signal transduction pathways that are in common with the early phase of IPC. PKC and Src signaling modules activate NFκB through serine and tyrosine phosphorylation of IκB that facilitates translocation of NFκB into the nucleus. p38 MAP kinase and JNK activation induces expression of the immediate early genes c-fos and c-jun mRNAs, and phosphorylates these protein products to constitute AP-1. JAK provokes tyrosine phosphorylation and activation of STAT, leading to up-regulation of cardioprotective genes. So far identified cardioprotective genes induced by transcription factors include Mn SOD, iNOS, COX, and aldose reductase. These proteins themselves or in concert with others confer cardioprotection against both myocardial stunning and infarction. Although mitoK_{ATP} channels act as an end-effector of late IPC, as in the case of the early phase of IPC, other effectors

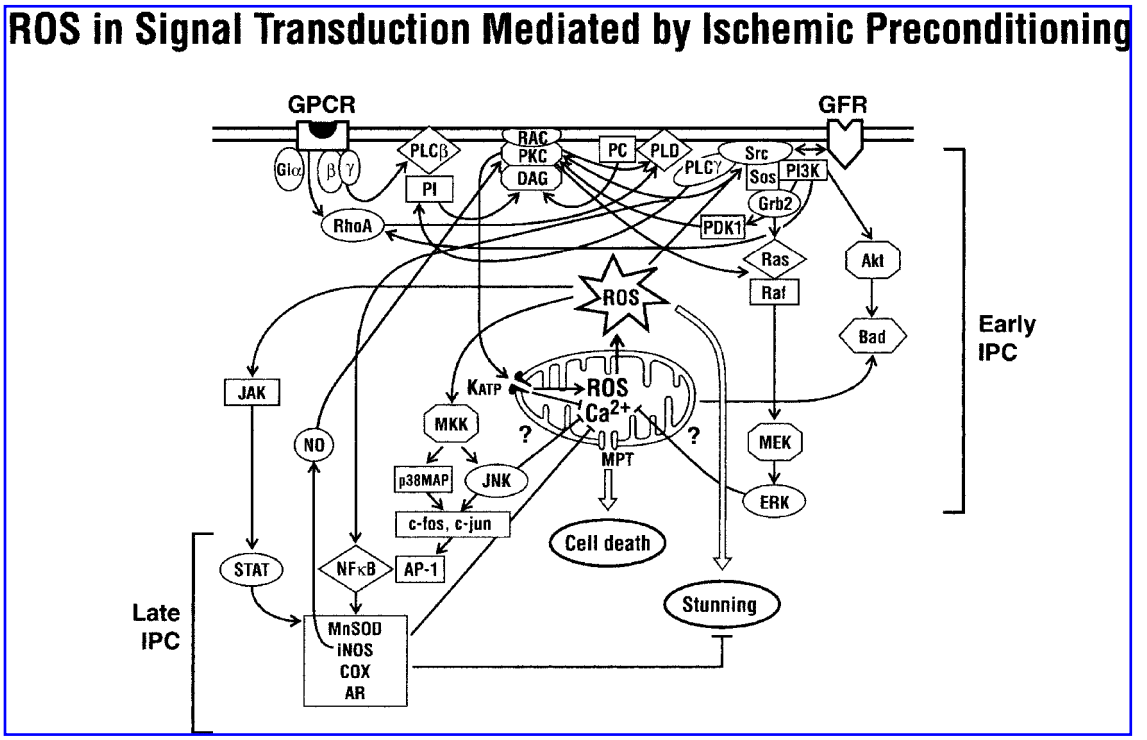

FIG. 1. Diagram of the hypothetical role of ROS in signal transduction mediated by IPC. GPCR, G protein-coupled receptor; GFR, growth factor receptor; PLC, phospholipase C; PLD, phospholipase D; PI, phosphatidylinositol; PC, phosphatidylcholine; PKC, protein kinase C; DAG, diacylglycerol; RAC, receptors for activated C-kinase; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide-dependent kinase 1; K_{ATP}, ATP-sensitive K^+ channel; MPT, mitochondrial permeability transition; NO, nitric oxide; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; MKK or MEK, MAP kinase kinase; JAK, janus kinases; STAT, signal transducers and activators of transcription; NFκB, nuclear factor-κB; AP-1, activating protein-1; HSP, heat shock protein; MnSOD, manganese superoxide dismutase; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; AR, aldose reductase; →, stimulation; −|, inhibition.

of late IPC that are responsible for antinecrotic and antistunning effects remain to be identified.

Although this review has delineated ROS-mediated signaling cascades involved in cardioprotection afforded by IPC, the diagram presented here is perhaps too simplified to express all the putative signal transduction pathways (Fig. 1). Recently emerging proteomic analysis (205) supports the idea that signaling events provoked by IPC would be much more complex than has been thought. Many yet unidentified signaling modules may be recruited in a redox-sensitive manner to integrate cardioprotection in IPC. Elucidation of these signaling processes will help to develop optimal approaches in pharmacological cardioprotection.

ABBREVIATIONS

ANT, adenine nucleotide translocase; AP-1, activating protein-1; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CoQ, coenzyme Q; COX, cyclooxygenase; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; 5-HD, 5-hydroxydecanoate; H_2O_2, hydrogen peroxide; HSP, heat shock protein; IGF, insulin-like growth factor; iNOS, inducible nitric oxide synthase; IPC, ischemic preconditioning; IRS-1, insulin receptor substrate-1; JAK, janus kinases; JNK, c-Jun NH_2-terminal kinase; K_{ATP}, ATP-sensitive potassium channel; MAP, mitogen-activated protein; MEK and MKK, mitogen-activated protein kinase kinase; mitoK_{ATP}, mitochondrial K_{ATP} channel; Mn SOD, manganese superoxide dismutase; MPT, mitochondrial permeability transition; NFκB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide anion; $^{\bullet}OH$, hydroxyl radical; PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent kinase 1; PG, prostaglandin; PI, phosphatidylinositol; PI 3-kinase, phosphatidylinositol 3-kinase; PI-4,5-P_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RACK, receptors for activated C kinase; ROS, reactive oxygen species; STAT, signal transducers and activators of transcription family.

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