Death and Survival of Cardiomyocytes in Acute Ischemia

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ABSTRACT

Ischemia is the most common and important cause of injury to cardiomyocytes. Acute ischemia causes profound derangement of the cellular energetics and metabolism, and this ultimately leads to cell death. Experimental studies have demonstrated the presence of an endogenous protective mechanism that can diminish or delay cell death from ischemic insult; this is known as ischemic preconditioning. In this review, we summarize the recent knowledge of the cellular biology of acute ischemic injury and also signaling mechanisms of cardioprotection that are involved in preconditioning. Further, we briefly discuss the clinical implications. (**Korean Circulation J 2006;36:165−177**)

KEY WORDS : Ischemia ; Myocytes, cardiac ; Cell death ; Ischemic preconditioning.

Introduction

Ischemia is by far the commonest form of injury inflicted upon the myocardium. The clinical presentation of myocardial ischemia encompasses a broad spectrum of anginal syndromes, myocardial infarction and congestive heart failure. Because of the widespread and increasing prevalence of coronary atherosclerosis, ischemic heart disease is currently the single most important identifiable cause of cardiovascular morbidity and mortality throughout the world.[1]

During acute ischemia, the ensuing derangement of intracellular metabolism and energetics leads to the rapid demise of the myocardial cells if the cause of ischemia is not promptly removed. Actually, despite the progress in reperfusion therapy that has been made during the last decades, many patients with acute myocardial infarction do not get the optimal therapy due to delays in their arrival to the hospital or the lack of medical resources.[2] Therefore, a strategy that takes advantage of cells' ability to prolong their survival under ischemic conditions can be an alternative way to protect the myocardium.

This review is intended to describe the intracellular biology involved in acute ischemic injury, and to discuss the potential protective mechanisms inherent in cardio-myocytes, which can promote the survival of cells under ischemic conditions. This review will not cover reperfusion injury per se.

Cellular Injury in Acute Ischemic Stress

The mammalian heart is an obligatory aerobic organ; it consumes more oxygen per minute than any other organ in the body.[3] It cannot produce enough energy under anaerobic condition to maintain its essential cellular processes. Thus, a constant supply of oxygen is indispensable to sustain cardiac function and viability. Any abrupt cessation of the blood supply causes dramatic intracellular changes in the cardiomyocytes, and this ultimately leads to cell death.

Metabolic changes

The oxygen supply that's available to the myocardium is depleted within 10 second after the interruption of blood flow. In the absence of an oxygen supply, oxidative metabolism ceases, and the rate of anaerobic glycolysis increases dramatically to compensate for the loss of high-energy phosphate production.[4] The source of glucose is entirely from glycogenolyis,[5] although exogenous glucose can be used during less severe ischemia.[6] Because even the maximum glycolytic rate cannot adequately compensate for the loss of oxidative adenosine triphosphate(ATP) generation,[7] the creatine phosphate(CP), which is the stored energy, is rapidly consumed. Consumption of this stored energy can save intracellular ATP during the first several minutes of

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ischemia. However, once the CP stores are depleted, ATP levels decline precipitously.[8] As the high-energy phosphate bonds are hydrolyzed, inorganic phosphate (Pi) and protons (H^{+}) accumulate.

In the continued absence of oxygen, anaerobic glycolysis produces lactate rather than acetyl-CoA. Restricted washout in myocardial tissue allows for the accumulation of products of anaerobic metabolism and the nucleotide catabolites pool in the tissue. The build-up of lactate, H^{+} and the reduced form of the coenzyme nicotinamide adenine dinucleotide (NADH) progressively inhibits glycolysis at the level of glyceraldehyde phosphate dehydrogenase (GPD) (Fig. 1).[9] The rate of anaerobic glycolysis markedly slows within 60 seconds. The later cessation of glycolysis in ischemia is attributed to the very low sarcoplasmic ATP concentration.[10] In the absence of ATP, glycolysis is inhibited at the level of fructose-6-phosphate (F6P) because ATP is required to phosphorylate F6P to fructose-1, 6-diphosphate (F1, 6P) via phosphofructokinase, which is the key rate-limiting enzyme in the glycolytic pathway. The accumulation of intracellular calcium ([Ca^{2+}]i) also inhibits glycolysis by facilitating binding of calmodulin to phosphofructokinase.[11] The end-result is a complete shutdown of the myocardial metabolism, and the myocyte is left with no means for maintaining its survival.

Intracellular ion changes

The intracellular ion homeostasis is profoundly disturbed during ischemia, although the cause-and-effect relationship with ischemic injury is often difficult to ascertain. 1) Over the course of several minutes of ischemia, intracellular Na$^+$ ([Na$^+$]i) increases up to several times the normal level, in part through the entry of Na$^+$ via the voltage-dependent channels,[12] or through the action of Na$^+$/H$^+$ and Na$^+$/Ca^{2+} exchange.[13] Later during ischemia, inhibition of the Na$^+$ pump that results from ATP depletion may cause accumulation of [Na$^+$]i.[14] Elevated [Na+]i may be a critical factor that contributes to other functional abnormalities such as an overload of intracellular Ca^{2+} or an K$^+$ efflux. 2) A net cellular K$^+$ loss occurs within 15-30 seconds after the onset of myocardial ischemia.[15] This is predominantly due to an increase of the K$^+$ efflux rather than a decrease of the K$^+$ influx due to decreased activity of the Na$^+$ pump.[15] The con-sequent extracellular K$^+$ accumulation predispose the heart to the development of arrhythmia. 3) Because numerous factors regulate intracellular Ca^{2+} ([Ca^{2+}]i) during ischemia, it has been difficult to determine [Ca^{2+}]i levels in the ischemic heart in vivo. Although intracellular Ca^{2+} ([Ca^{2+}]i) overload has been implicated in the pathogenesis of reperfusion injury, recent studies have indicated that [Ca^{2+}]i rises after the onset of ischemia.[16] This may be related to the increased [Na$^+$]i,[14] which served to drive Ca^{2+} intracelluarly via Na$^+$/Ca$^+$ exchange, or it may be secondary to the alterations in the calcium cycling of the sarcoplasmic reticulum.[17] Elevated levels of [Ca^{2+}]i lead to activation of the calcium-dependent enzymes, including proteases and phospholipases, which these can degrade various cellular structures.[18][19] Furthermore, calcium overload in the mitochondria can induce opening of the permeability transition pore (PTP), allowing the release of calcium, cytochrome *c*, nicotinamide adenine dinucleotide (NAD) and apoptogenic factor into the cytosol.[20]
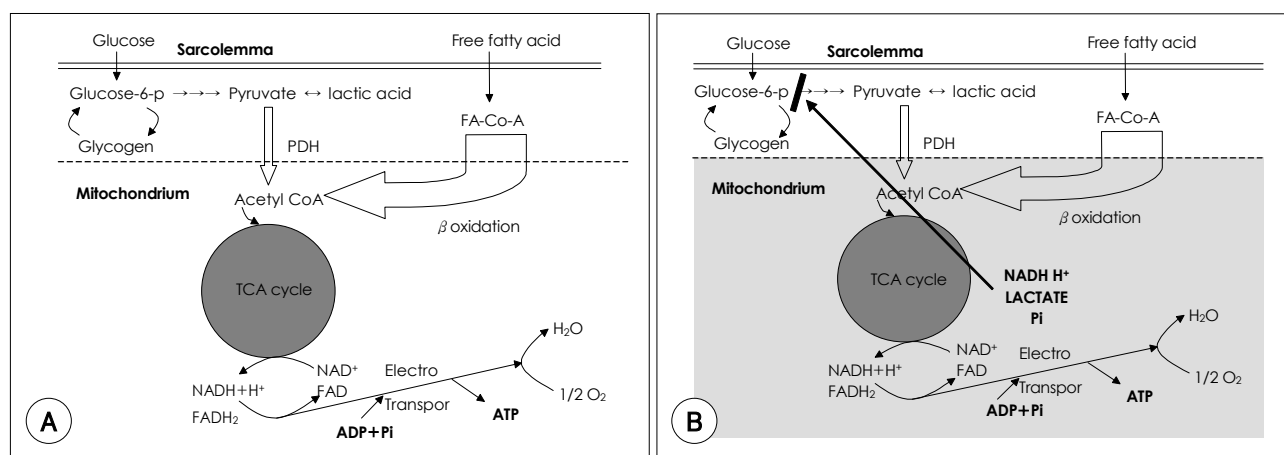
Fig. 1. Energy metabolism of cardiomyocytes in the normal condition (A) and during ischemia (B). A: the normal myocardium generates most of its ATP by oxidative metabolism and it generates less than 10% of its energy by anaerobic glycolysis. Fatty acid, in the form of fatty (acyl) esters containing acyl-CoA (FA-Co-A), is the preferred metabolic substrate, with glucose accounting for the remaining part. B: in acute ischemia, oxidative metabolism ceases almost immediately (shaded area), and the rate of anaerobic glycolytic metabolism dramatically increases to compensate. Restricted washout and accumulation of ions and metabolites such as the hydrogenion (H$^+$) and inorganic phosphate (Pi) suppress the glycolytic metabolism (arrow), leading to a complete standstill of the energy generating machinery. ADP: adenosine diphosphate, ATP: adenosine triphosphate, FAD: flavin adenine dinucleotide, FADH: the reduced form of the coenzyme flavin adenine dinucleotide, NAD: nicotinamide adenine dinucleotide, NADH: the reduced form of the coenzyme nicotinamide adenine dinucleotide.

Structural changes

Few changes occur in the ultrastructure of the myocyte during the early phase of ischemic injury.[21] The myofibrils of the myocytes are generally stretched, and glycogen deposits are diminished, reflecting the presence of anaerobic glycolysis. The chromatin of the nucleus is aggregated peripherally and some of the mitochondria become swollen.

After 30-40 minutes of severe ischemia, the injury becomes biologically irreversible. This transition to irreversibility is associated with new ultrastructural changes.[21] These include diffuse mitochondrial swelling, the appearance of amorphous densities in the matrix space of the mitochondria, virtual absence of glycogen, marked peripheral aggregation of nuclear protein and the appearance of discontinuities in the cell membrane.

Cell Death in Acute Ischemia

Apoptosis, oncosis and necrosis

Cell death is an ultimate result of severe ischemia unless the ischemia is interrupted during its course. Conventionally, cardiomyocyte death following ischemia was considered to involve necrosis or 'accidental cell death'. However, collective evidence over the last decade from several experimental and clinical studies have indicated that early cell death after myocardial ischemia predominantly involves apoptosis followed by necrosis[22] and/or 'oncosis'.[23] Apoptosis is a highly regulated, energy-dependent, sequential form of cell death that is characterized by such morphological features as membrane blebbing, chromatin condensation, nuclear condensation and cell shrinkage.[24] These changes are direct consequence of the activation of preexisting protease called caspases, which executes the cleavage of substrate such as cytoskeletal proteins, DNA repair enzymes and protein kinase.[25] Oncosis is cell death that is characterized by swelling, disruption of the sarcolemmma and the mitochondria, chromatin clumping and removal

of debris by the inflammation process.[24] Necrosis is the final, common process of cellular degradation, and this is regardless of the initial mode of cell death, be it apoptosis or oncosis.

Mode of death in the ischemic model

Since its first discovery, apoptosis has been implicated in numerous cardiovascular diseases, including myocardial infarction, reperfusion and heart failure.[26] Nevertheless, the relative contributions of apoptosis and oncosis during ischemia/reperfusion are a subject of fierce debate.[27] Apoptosis might be directly initiated by the sub-lethal injury of cardiomyocytes in an energy-depleted state. For example, hypoxia without deprivation of the glucose in media induces apoptosis in cultured rat cardiomyocytes.[28] However, the role of apoptosis does not appear important in the severe form of myocardial ischemia. A canine experiment showed that during global ischemia, apoptosis affects only a small fraction of myocytes while the majority of cells die by oncosis.[29] An in vitro study with isolated adult rat cardiomyocytes showed that simulated ischemia mostly caused necrosis of cells.[30] In our laboratory, we employed cultured neonatal rat ventricular myocytes (NRVMs) to test whether a simulated model of severe ischemia induces apoptosis.[31] The ischemic condition was created by incubating cells with 95% N_2 and 5% CO_2 gas and depleting the glucose energy source of the media. Biochemical analysis and morphological examination revealed that under such conditions, most cells die by onconsis/necrosis (Fig. 2). Because apoptosis is energy-dependent process, the decision between oncosis and apoptosis may depend on the ATP concentrations.[32] An ATP loss of more than 70%, as is present in severe ischemia, is not compatible with apoptosis and so it causes oncosis.[32] Studies on human infarcts point to the fact that apoptosis in the center of the infarcted area is negligible while more cells are apoptotic in the border zone.[33][34] Thus, the overall results from both experimental and clinical studies sup-
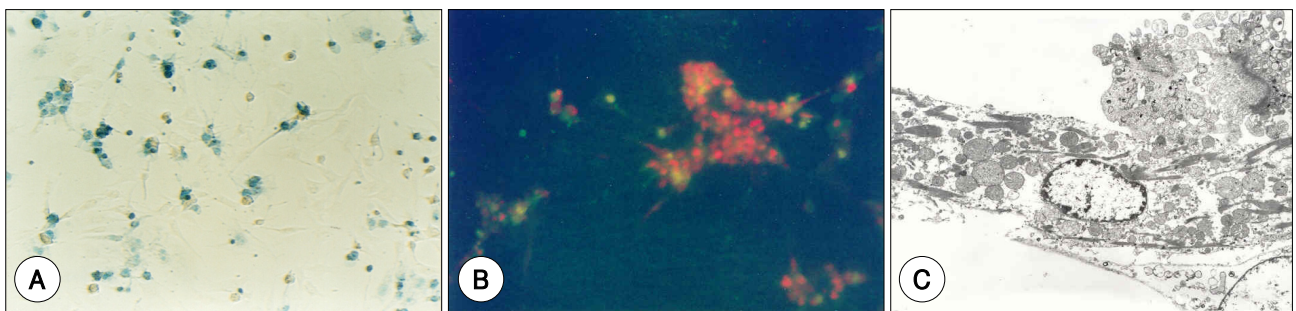
Fig. 2. Death of neonatal rat ventricular myocytes cultured under simulated acute ischemia. A: trypan blue staining 12 hours after ischemic culture shows the dead cardiomyocytes, which failed to exclude the dye. B: annexin V/propodium iodide (PI) staining shows the uptake of red propodium dye in the dead cardiomyocytes; this signified cell membrane disruption and oncotic cell death. C: the electron microscopic findings of dead cardiomyocytes in simulated acute ischemia illustrate the features of onconotic cell death, including nuclei with serrated clumping of the peripheral chromatin and diffuse mitochondrial swelling. No signs of apoptosis were observed, such as nuclear condensation or apoptotic bodies.

port that oncosis is the predominant mode of cell death in acute, severe ischemia. Apoptosis may be more responsible for the cell death occurring in reperfusion injury where large amounts of reactive oxygen species (ROS) are generated.[35)36)] There still exist unresolved issues regarding apoptosis in the pathogenesis of ischemic injury, especially as to the methods for quantifying and recognizing it.[37)] For example, 'apoptotic' myocytes in the infarct area appeared to be oncotic cells with DNA fragmentation when this was analyzed with a newer technique.[23)] Further studies are necessary to establish the role of apoptosis in myocardial ischemia. Identification of the mode of cell death does matter because understanding the mechanisms might allow for potential future therapeutic interventions to treat ischemia.

Role of mitochondria

The mitochondria play a key role in determining the

cells' fate during exposure to stress. Their role during ischemia/reperfusion is particularly important, not only because they are critically involved in the process of both apoptosis and oncosis, but also because they are associated with a cardioprotective mechanism. Breakdown of the mitochondrial machinery for energy generation quickly leads to oncosis of the myocytes during ischemia.

In apoptosis, the mitochondria participate in one of major signaling pathways (the intrinsic/mitochondrial pathway), leading to final activation of the caspase cascade (Fig. 3). Another pathway involves the external death receptors that are present on the cell surface (the extrinsic/death receptor pathway). In the extrinsic pathway, binding of the ligand to the receptor promotes receptor trimerization and recruitment of such adaptor proteins as the TNF-R1 associated death domain (TR-ADD) or the Fas associated death domain (FADD), which form a death-inducing signaling complex (DISC).[38)] Multiple procaspase-8 molecules bind to these adaptor
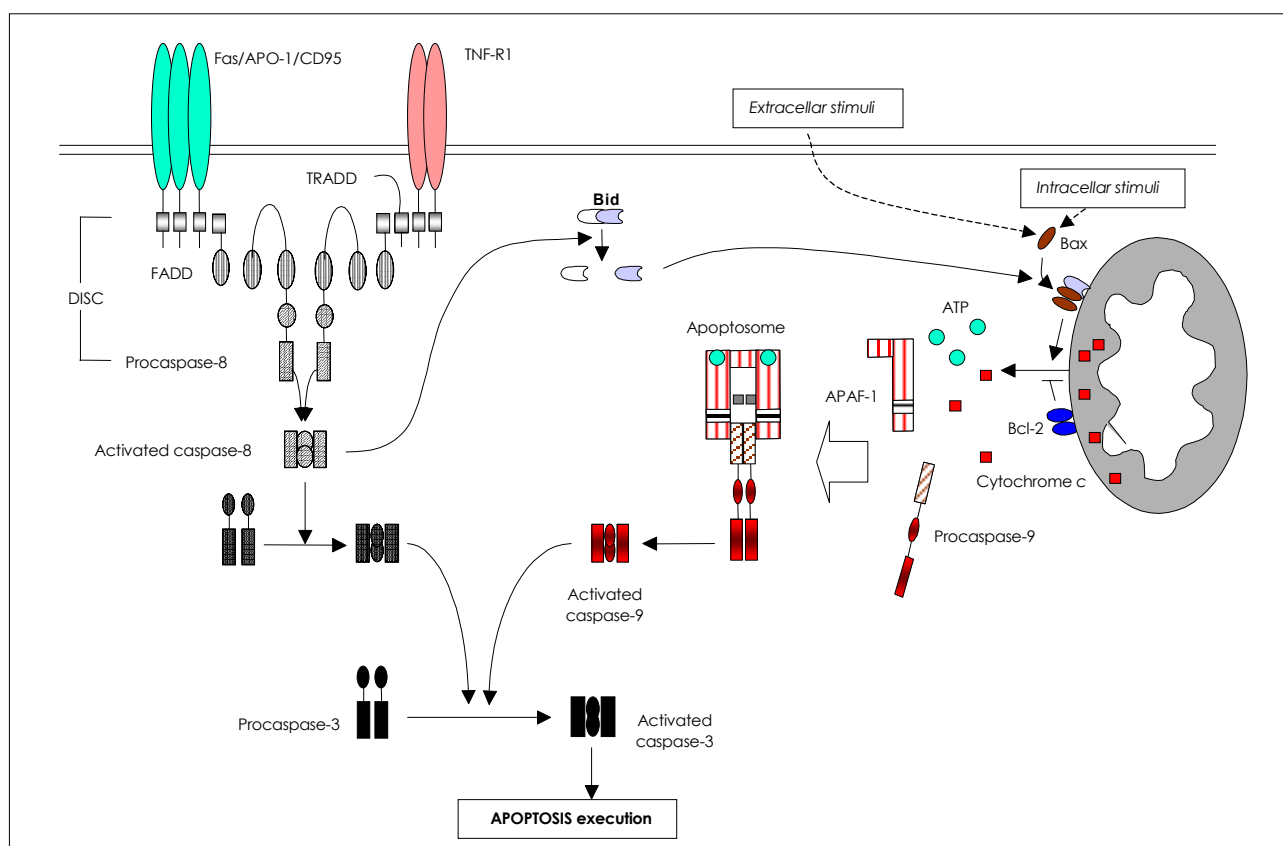
Fig. 3. The major apoptotic pathways. In the extrinsic (death receptor) pathway, binding of the oligomerized ligand to its cognite cell surface receptor initiates the formation of a multi-protein complex termed the death-inducing signal complex (DISC). This includes the recruitment of adaptor proteins such as FADD: Fas associated death domain, TRADD: TNF-R1-associated death domain and procaspase-8, leading to dimerization and the activation of procaspase-8. Activated caspase-8 cleaves and activates downstream procaspase-3. Once activated, caspase-3 executes the final process of apoptosis. Caspase-8 also activates Bid (BH3 [B cell leukemia/lymphom-2 {Bcl-2} homology domain 3] interacting domain), a proapoptotic protein, and Bid links the extrinsic and intrinsic pathways. In the intrinsic (mitochondrial) pathway, intracellular and extracellular death signals are transmitted to the mitochondria via BH3-only protein (eg. Bid) and Bax (Bcl-2-associated X protein). Bax and Bak (Bcl-2 antagonist/killer, not shown) stimulate the release of cytochrome c (red), and the other apoptogens. This process is opposed by Bcl-2. Cytochrome c, ATP (green), Apaf-1: apoptosis proteinase activating factor-1 and procaspase-9 assemble into the apoptosome, leading to activation of procaspase-9, which subsequently activates procaspase-3. TNF-R1: tumor necrosis factor type 1 receptor.

proteins via the death effector domains, resulting in proximity-induced cleavage and activation of caspase-8.[39] Active caspase-8 acts on a downstream target such as procaspase-3 to initiate a caspase cascade.

In the intrinsic/mitochondrial pathway, members of the B cell leukemia/lymphom-2 gene (Bcl-2) family are important for the transduction of an internal apoptotic signal. Bcl-2 protein shares homology in several domains; this is termed Bcl-2 homology (BH domains). The anti-apoptotic members of the Bcl-2 family include Bcl-2 and Bcl-X_L and they share homology in the BH domains 1-4. The pro-apoptotic family members generally lack the BH4 domain.[40] The pro-apoptotic family can be further subdivided into those members that share homology in domains 1, 2 and 3 (Bax and Bak), and the "BH-3 only" subset (Bid, Bad, Bnip/Nix and others) that share homology only in domain 3. Anti-apoptotic members such as Bcl-2 are localized on the cytoplasmic surface of the outer mitochondrial membrane where they sequester pro-apoptotic BH-3 only protein, and so they prevent the activation of Bax and Bak.[41] Apoptotic signaling initiates the movement of BH-3-only protein to the outer mitochondrial surface where it triggers Bax or Bak to undergo a conformational change; this is followed by oligomerization and insertion into the outer membrane. This is then followed by alterations of the mitochondrial structure that causes dissociation of cytochrome c and permeabilization of the outer mitochondrial membrane, leading to release of apoptogenic factors such as cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G.[42] cytochrome c, dATP, Apaf-1 and procaspase-9 assemble into the apoptosome leading to procaspase-9, which subsequently activates procaspase-3.[43] The death receptor pathway and the mitochondrial pathway appear distinct, but cross-talk between the two pathways is possible: Bid is a substrate of caspase-8; thus, this is the connection between the extrinsic and intrinsic pathways.[44] With regard to the upstream events, the intrinsic pathway integrates a broad spectrum of extracellular and intracellular stresses, in contrast to the extrinsic pathway that transduces a specialized set of death stimuli. The apoptotic stimulus for the intrinsic pathway during ischemia has not been clearly defined, but acidosis and intracellular Ca^{2+} have been shown to activate the mitochondrial pathway.[45]

Mitochondria use electron transport to generate a large electrochemical gradient across the inner membrane, which consists of the membrane potential ($\triangle \Psi$m® -200 mV, the major component under aerobic conditions) and a proton gradient ($\triangle$pH). This electrochemical gradient is then used by ATP synthase (F1-F0 ATPase) to phosphorylate ADP to ATP. To sustain the $\triangle \Psi$m requires that the inner membrane (IM) remains relatively impermeable to ions, which is regulated by a multiprotein complex called the mitochondrial permeability transition (MPT) pore.[46] The MPT pore is composed of adenine nucleotide translocator (ANT) at the inner membrane and the voltage-dependent anion channel (VDAC, also called porin) at the outer membrane and cyclophilin D (CyP-D) in the matrix (Fig. 4).[46] Mitochondrial permeability transition triggered by the opening of the MPT pore immediately depolarizes the $\triangle \Psi$m; this causes ATP synthase to operate in reverse consuming ATP in a futile attempt to restore the proton gradient.[47] This accelerates the depletion of cellular energy and hastens cell death. Mitochondrial permeability transition contributes to apoptosis by releasing apoptogenic proteins such as cytochrome c into the cytosol, although a non MTP-mediated release model has been recently suggested.[48] Mitochondrial permeability transition can also cause oncosis/necrosis in the ATP depleted state (Fig. 5).[49] Thus, MTP-mediated cell death is the main process of apoptosis in reperfusion injury, whereas oncosis is the dominant mode in acute ischemia without reperfusion.[49]

Protection of Myocytes from Ischemic Injury

Ischemic preconditioning

Jennings and Reimer in 1986 discovered that myocytes have an endogenous mechanism to protect themselves from lethal ischemic injury.[50] They showed in an anesthetized dog experiment that repetitive episodes of brief ischemia markedly decreased the extent of myocardial damage from a subsequent ischemic insult.[50]
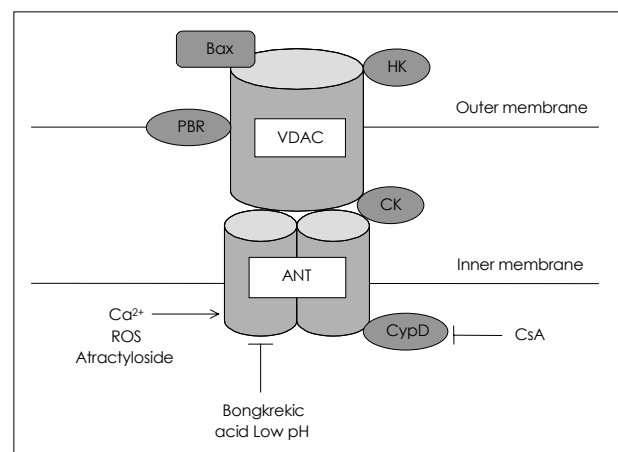
Fig. 4. Proposed model for the structure of the mitochondrial permeability transition (MPT) pore. The consensus model of the MPT pore is comprised of a voltage-dependent anion channel (VDAC) from the outer membrane, adenine nucleotide transporter (ANT) from the inner membrane, cyclphilin D (CypD) from the matrix and other proteins such as peripheral benzodiazepine receptor (PBR), hexo-kinase (HK), and creatine kinase (CK). Atractyloside, Ca^{2+} and reactive oxygen species (ROS) induce the mitochondrial permeability transition, whereas bongkrekic acid blocks the mitochondrial permeability transition.
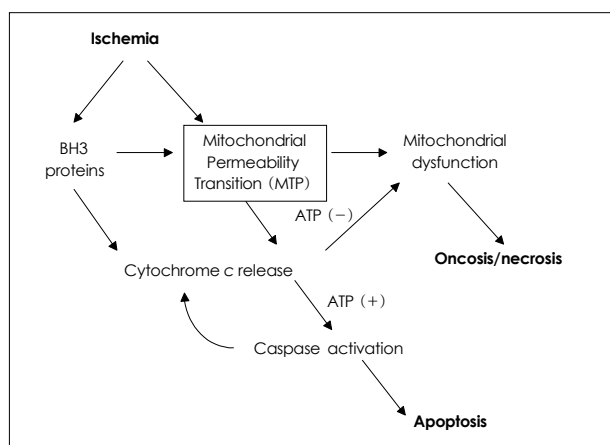
Fig. 5. Mitochondrial mechanisms of cell dysfunction and death in ischemic myocardium. Ischemia causes the release of mitochondrial perameability transition (MPT)-related cytochrome c, which then induces caspase activation and apoptosis. Caspase activation feedback may induce further release of cytochrome c, and the release of cytochrome c is partially responsible for mitochondrial dysfunction; this might lead to contractile dysfunction or oncosis/necrosis at the time of reperfusion.

The concept of endogenous myocardial adaptation to sublethal ischemia that results in protection against subsequent ischemia has been termed ischemic preconditioning. Most of the benefit from ischemic preconditioning is observed within the time period of 1 to 3 hours after treatment (classic or early preconditioning), but a lesser protective effect can be seen again in 24 to 96 hours (delayed or late preconditioning).[51)52)] Besides reducing the infarct size, preconditioning has been shown to protect against other effect of ischemia/reperfusion injury such as arrhythmia.[53)54)] Nonetheless, preconditioning delays but does not prevent myocyte death during the test episode of ischemia; if reperfusion of the ischemic myocardium does not follow, then myocardial necrosis is inevitable.

Mechanisms of preconditioning

The effect of preconditioning has been observed in all the mammalian hearts that have been tested thus far. Preconditioned myocardium exhibits metabolic
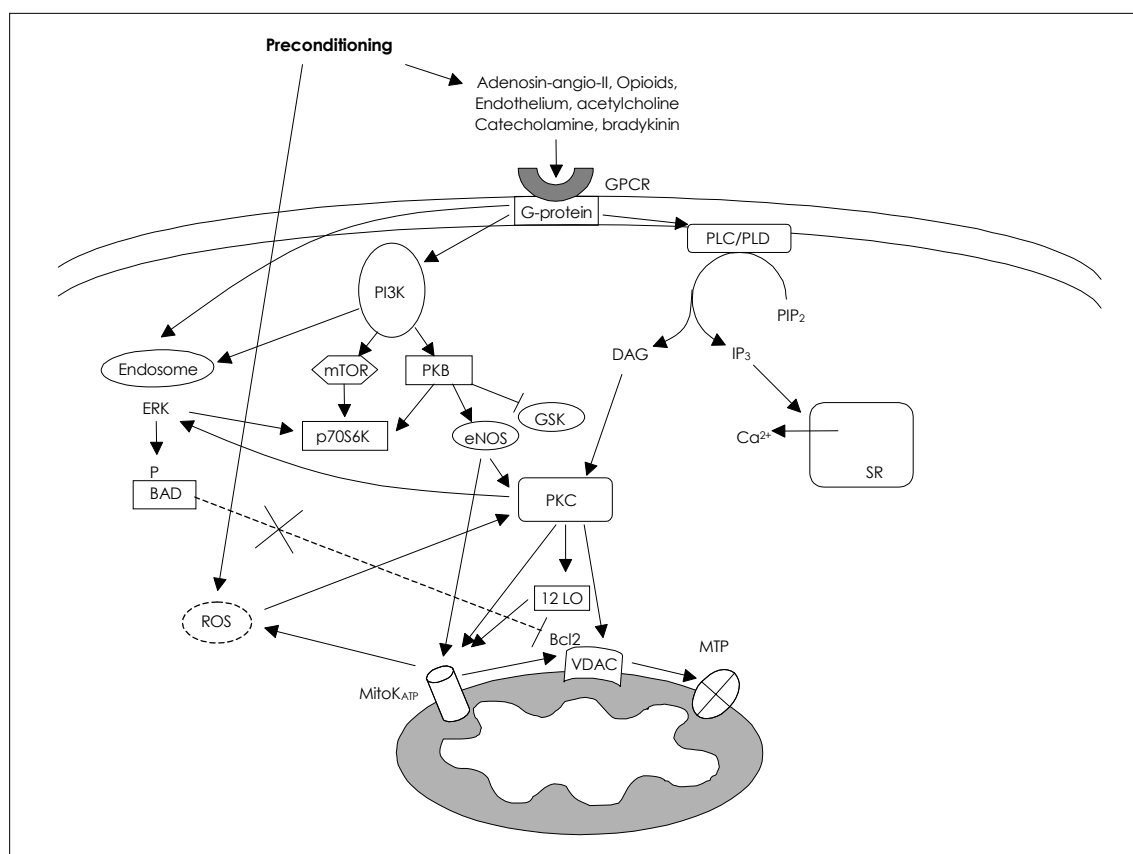
Fig. 6. Overview of the preconditioning signaling. Primary signaling pathways: preconditioning leads to the release of adenosine, opioids and bradykinin, which bind to the G protein coupled receptor (GPCR), and this initiates a signaling cascade that involves activation of phosphoinositide-3-kinase (PI3K) and a variety of downstream kinases. Preconditioning also activate phosholipase (PLC and PLD) via GPCR, causing production of diacylglycerol (DAG), which then activates protein kinase C (PKC). Preconditioning also induce generation of reactive oxygen species (ROS) that may play a role in the activation of PKC and the other signaling events. Secondary signaling: a variety of primary signaling pathways converge on a few mitochondrial proteins such as mitochondrial ATP-sensitive K⁺ channel (mitoK$_{ATP}$), the mitochondrial permeability transition (MTP) pore and the bcl-2 family members. Modulation of these mitochondrial proteins results in altered metabolism and the inhibition of cell death, thus resulting in cardioprotection. BAD: Bcl-2-antagonist of cell death, Bcl-2: B cell leukemia/lymphom-2, eNOS: endothelial nitric oxide synthase, ERK: extracellular-regulated kinase, GSK: glycogen synthase kinase, IP3: inositoltrisphosphate, mTOR: mammalian target of rapamycin, p70S6K: p70S6-kinase, PIP2: phosphatidylinositol bisphosphate, 12-LO: 12-lipoxygenase.

changes that are similar to those observed in reversible ischemia, but the preconditioned myocardium reacts to a second episode of ischemia differently than does the naive myocardium in that preconditioned myocardium utilizes ATP and accumulates lactate and H^+ much more slowly.[55-57] Because lower levels of intracellular ATP and higher levels of tissue lactate and H^+ are strongly associated with ischemic cell death, it has been postulated that the preconditioned tissue dies more slowly because of this reduced energy demand.[56][57]

Signaling pathways in preconditioning

The signaling pathways involved in cardioprotection are complex and recounting all of them is beyond the scope of this review. Acute preconditioning, which does not require new transcription, mediates cardioprotection by modulating cell metabolism or the signaling pathways directly or by posttranslational modification of protein. Acute preconditioning consists of two part: the primary and secondary signaling pathways (Fig. 6).[58] The primary signaling pathways involve the release of substance such as adenosine, opiods or bradykinins, which bind to the G protein-coupled receptors (GPCR) and initiate a signaling cascade that involves activation of phosphoinositide-3-kinase (PI3K), protein kinase B (PKB, also known as Akt), endothelial NO synthase (eNOS), protein kinase C (PKC), glycogen synthase kinase 3β (GSK3β), mammalian target of rapamycin (mTOR) and p70S6-kinase (p70-S6K). GPCR activation also causes the release of G$\beta\gamma$, and this leads to activation of the extracellular-regulated kinase (ERK) pathways via an endosomal-signaling pathway.[58] These diverse signals converge on a few final common pathways, which are the secondary signal pathways, leading to the ultimate amelioration of cell death. Most of secondary pathways act on key mitochondrial proteins such as the mitochondrial ATP-sensitive K^+ channels (mitoK$_{ATP}$), the mitochondrial permeability transition pore and the bcl-2 family members.[58]

Primary signaling: role of protein kinase C(PKC)

Numerous studies have demonstrated the role of PKC for cardioprotection in preconditioning. GPCR activation leads to activation of phospholipases (PLC and PLD) and liberation of the second messenger dia-
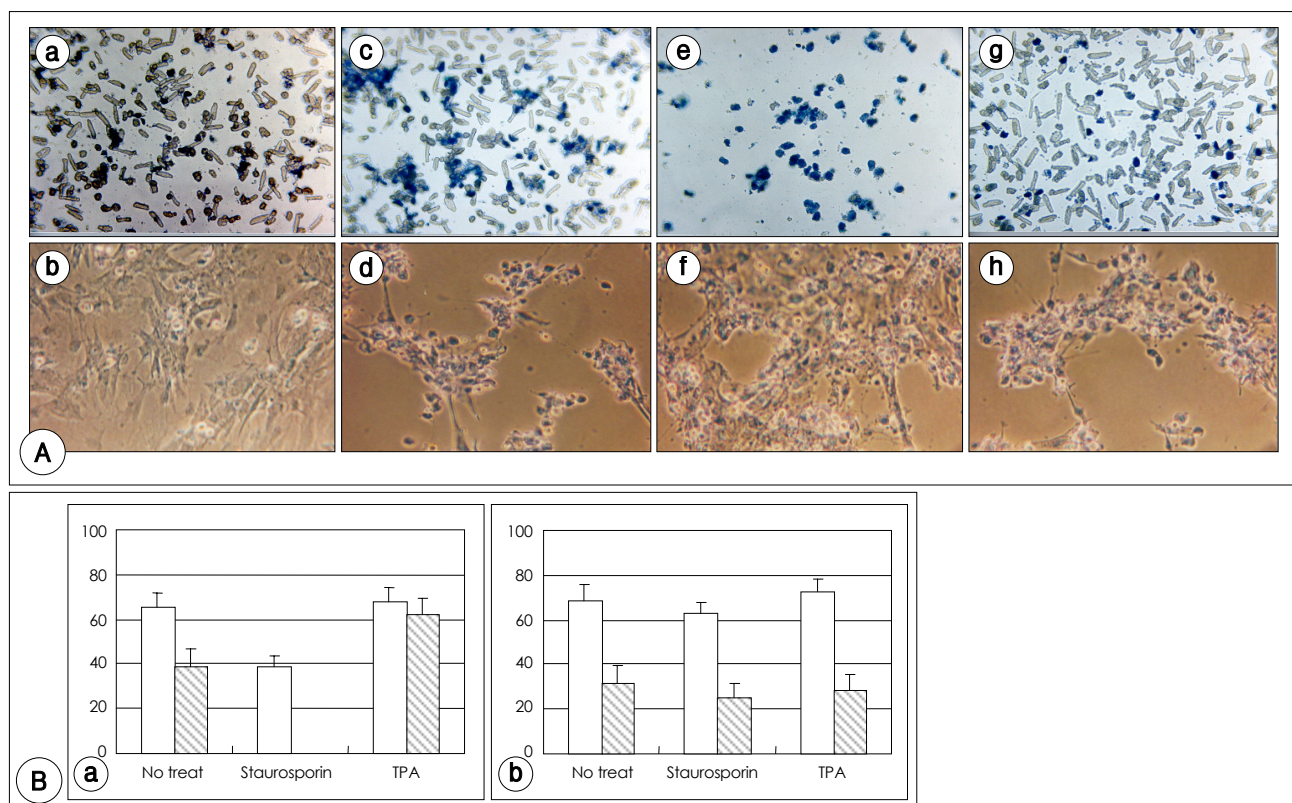
Fig. 7. The different effects of protein kinase C (PKC) on myocardial protection from ischemic injury between neonatal and adult rats. A: trypan blue staining of cultured adult rat ventricular myocytes (ARVM; a, c, e, g) and neonatal rat ventricular myocytes (NRVM; b, d, f, h). Dark colored cells represent dead cells. a and b: cells cultured under a normal condition (95% O_2+5% CO_2, glucose substrate in media). c and d: cells cultured under an ischemic conditon (95% N_2+5% CO_2, deprived of glucose in the culture media), e and f: cells under ischemic condition, treated with PKC inhibitor staurosporin 100 mM. g and h: cells under the ischemic condition, treated with PKC activator 12-0-tetradecanoyl-phorbol 13-acetate (TPA) 100 mM. B: the percentage of viable myocytes under an ischemia condition with the designated treatment. a: ARVM, b: NRVM. Activation of PKC with TPA markedly enhanced the survival of the ARVMs in the ischemic condition, while the effect of TPA on the survival of NRVMs was minimal or nonexistent.

cylglycerol (DAG) for directly targeting the PKC.[59)60] Reactive oxygen species (ROS), which are generated by preconditioning, have also been reported to be involved in the activation of PKC.[61] Inhibition of PKC has been shown to block the protection afforded by preconditioning, and the pharmacological activators of PKC have been shown to be cardioprotective.[59)62] PKC exists in a variety of isoforms that exhibit structural and functional specificities.[63] The amount of the cellular PKC isoform in the myocardium can change by as much as several times during development from the neonate to the adult.[64] Such a difference in the PKC isoform during development can translate into different capacities to protect myocytes from acute severe ischemia, which we have demonstrated in in vitro experiments (Fig. 7).[65] Among the many PKC isoforms, PKC ε has been suggested to play a important role for cardio-

protection. Most studies have used specific inhibitors/activators or they have observed the patterns of translocation of PKC ε to indirectly demonstrate its role.[66-68] We have recently conducted an experiment to directly show the protective effect of PKC ε by expressing PKC ε in cardiomyocytes using the lenti viral vector. The cultured neonatal rat ventricular myocytes, which have a low level of intracellular PKC ε, were subjected to simulated ischemia. For in vivo study, left coronary artery ligation model was used in the adult rat heart. The expression of PKC ε in cultured neonatal cardiomyocytes markedly reduced the cell death during the simulated ischemia, and it reduced the infarct size by 49% in the adult rat heart (Fig. 8). Experiments on transgenic mice having the cardiacspecific overexpression of PKC ε have also shown the endogenous protection.[69] Taken together, these findings strongly support a role for PKC ε in pre-
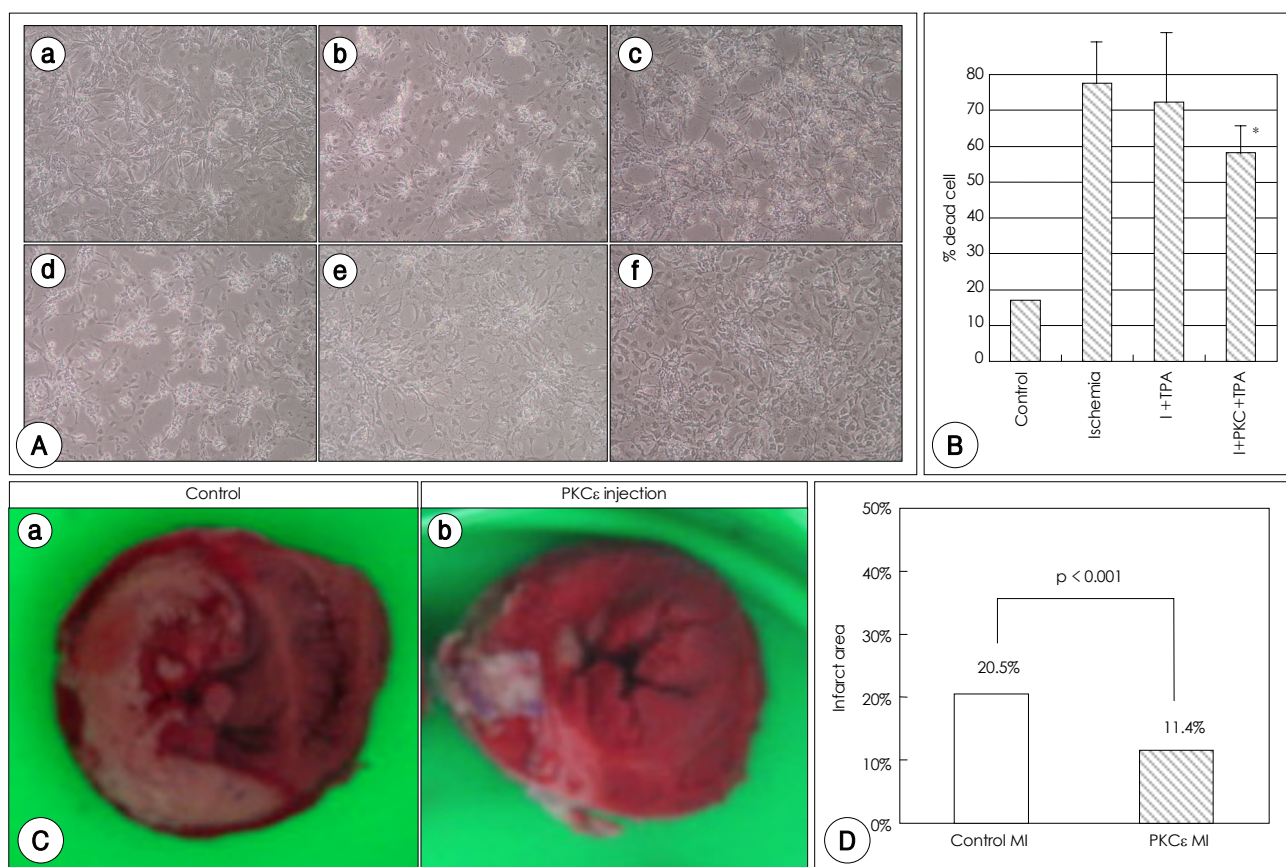
Fig. 8. Protective effect of the protein kinase Cε isoform (PKCε) expression via the lentiviural vector. The experiment was performed in cultured neonatal rat ventricular myocytes (A and B) under simulated ischemia conditions, and the coronary ligation model of the adult rat (C and D). A: microscopic findings of NRVMs cultured under the designated conditions. a; NRVM cultured under a normal condition (95% O_2 + 5% CO_2 and glucose substrate in media), b; NRVM cultured under an ischemic condition (95% N_2 + 5% CO_2 and deprived of glucose in the culture media), c; NRVM under the ischemic condition treated with an empty vector, d; NRVM under the ischemic condition and treated with PKC activator 12-0-tetradecanoylphorbol 13-acetate (TPA), e; NRVM under the ischemic condition treated with an empty vector and TPA, f; NRVM under the ischemic condition and treated with PKCε vector and TPA. B: percentage of cell death of the NRVMs treated with the designated condition. The PKCε expression and TPA treatment (I+PKC+TPA) significantly reduced the death of the NRVMs in a simulated acute ischemic condition (Ischemia) (58.1 ± 7.2% vs 77.6 ± 12.8%, p<0.05). C: representative samples stained by phthalocyanin blue and triphenyltetrazolium chloride (TTC) from the removed section of rat myocardium that underwent left coronary ligation. The perfused myocardium is stained blue by phthalocyanin blue. Ischemic, but viable myocardium is stained red by TTC. The nonviable, infracted area is not stained by either dye, and it remains white in color. Left panel; myocardium injected with control buffer before coronary artery ligation (control), Right panel; myocardium injected with PKCε vector before coronary ligation. D: the quantitative data shows that compared with the control (control MI), injection of the PKCε vector into the myocardium (PKCε MI) reduced the area of infarction by 49%, which was statistically significant (p<0.01).

conditioning.

The downstream target of PKC in cardioprotection needs to be elucidated. PKC, via phosphorlyation, may activate the mitochondrial K_{ATP} channel[70] and transcription factors, including the nuclear factor κB (NF-κ B),[71] and PKC may modify other myofibrillar and cytoskeletal proteins.[72] In addition, several types of non-receptor tyrosine kinase (particularly Src and Lck) may be linked in the signaling cascades either upstream and/or downstream of PKC and their targets.[73] A recent study has reported that ERK and PKCε are contained in a multimeric mitochondrial signaling complex, and PKC might lead to activation of ERK.[74] Whether activated ERK leads to activation of the mitoK$_{ATP}$ channel remains to be seen.

### Secondary signaling pathway: role of mitochondrial proteins

The diverse pathways initiated during preconditioning seem to converge on secondary signals that integrate the signals from different pathways. To achieve cardioprotection, these signaling pathway or end effectors must reduce cell death, be it from oncosis or apoptosis. Much data has suggested that mitochondrial proteins are the possible candidates as end effectors. Because mitochondria are the primary organelle involved in ATP production, which is essential for maintaining cell integrity, and as mitochondria are also key players in the process of apoptosis, mitochondrial components such as bcl-2 can inhibit both oncosis and apoptosis.[75] Other mitochondrial proteins like the mitochondrial K_{ATP} (mitoK$_{ATP}$) channel, the apoptotic proteins such as BAD and the mitochondrial permeability transition (MPT) pore, are suggested as possible end effectors. However, it is still possible that a non-mitochondrial component, i.e., the cytoskeleton or membrane proteins, can be targets in the secondary pathways for cardioprotection.[76]

A wealth of recent evidence has indicated that activation of the mitoK$_{ATP}$ channel is important in cardioprotection.[77-79] Selective activators of the mitoK$_{ATP}$ channel, which do not activate the sarcolemmal K_{ATP} channel, have been shown to be cardio-protective, and selective mitoK$_{ATP}$ channel inhibitors block the protection afforded by preconditioning.[77)78)80] The mechanisms by which activation of mitoK$_{ATP}$ channel can reduce cell death are not well understood. Three mechanisms have been suggested. These include inhibition of mitochondrial calcium uptake, modulation of ROS and modulating the mitochondrial permeability of the MPT pore.[58)79] Activation of mitoK$_{ATP}$ channel induces $\triangle \Psi m$ depolarization, and this reduces the driving force for Ca^{2+} uptake by mitochondria and thereby prevents mitochondrial matrix Ca^{2+} overload, which is a major trigger for MPT.[81] MitoK$_{ATP}$ activation also causes mild mitochondrial swelling, and this is proposed to protect the mitochondrial intermembrane contact site and thereby limit the depletion of adenine nucleotide from the matrix,[82] although there is conflicting data reporting that the changes in the mitochondrial volume do not correlate with protection.[83] Finally, it has been shown that preconditioning or mitoK$_{ATP}$ channel activation causes the increased generation of mitochondrial ROS, which can trigger the cardioprotective action of preconditioning.[84] Much of data have been collected from studies on isolated cells or from mitochondrial studies, making it difficult to determine whether the conclusions are relevant in the context of the whole heart. However, overall, these studies lend strong support that there are robust links between mitoK$_{ATP}$ and cardioprotection.

Clinical Implications

Understanding the mechanisms underlying cell death and protection during acute ischemic insult might help researchers find ways to develop novel therapeutic measures. Earlier efforts have attempted to enhance metabolic tolerance during ischemia. Experimental studies have suggested that an increased level of glycolytic substrate enhances ATP synthesis in hypoxic tissue.[85] Clinically, the efficacy of supplying glucose has been tested by infusing a glucose-insulin-potassium (GIK) solution to the patients with acute myocardial infarction (AMI). In the era of reperfusion, some studies have suggested that GIK infusion may reduce the mortality rate in certain groups of patient,[86] but other studies have failed to show clear benefit.[87] Thus, the role of GIK infusion in AMI remains to be clarified. Inhibition of the Na^+/H^+ exchanger (NHE) attenuates the Ca^{2+} overload and following pathologic consequences in cardiomyocytes during ischemia, exerting a cardioprotective effect.[88] A number of animal studies have indicated that inhibition of NHE, especially the sarcolemmal isofom NHE-1, can reduce the infarct size, remodeling and heart failure in postinfarction myocardium.[89)90] In one clinical study, administration of cariporide, a selective inhibitor or NHE-1, in the patients with AMI after percutaneous coronary intervention resulted in increased ejection fraction, reduced infarct size and reduced end-systolic volume.[91] In the Guard During Ischemia Against Necrosis (GUARDIAN) trial,[92] cariporide reduced the incidence of periorperative myocardial infarction in the patients undergoing coronary artery bypass surgery. However, in other studies, the effect of NHE inhibitor on the infarct size or postoperartive cardiac events was not positive.[93] Therefore, although some of the collected data appears to be promising, more research is needed to establish the clinical benefits of administering NHE inhibitors.
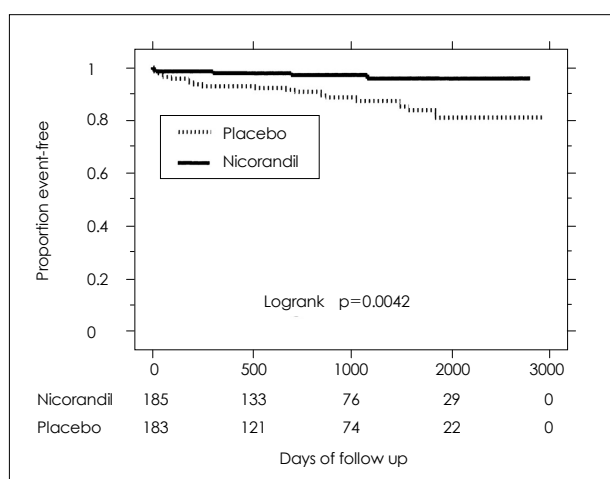
Fig. 9. Kaplan-Meier estimates for the patients with acute ST-elevation myocardial infarction who were randomly assigned to intravenous nicorandil (nicorandil, n=185) or placebo (placebo, n=183) before reperfusion treatment. The patients were followed-up for 5 years. Estimates are the survival free from events (coronary death and hospital administration for congestive heart failure).[98]

A number of efforts to utilize the component of pre-conditioning have centered on the pharmacological manipulation of opening the K_{ATP} channel, which is a central mechanism for cardioprotective signaling. In animal studies, the K_{ATP} channel openers (KOC) such as bimakalim, nicorandil and diazoxide exhibited pre-conditioning mimetic properties,[77)94)95] while K_{ATP} channel blockers can actually block ischemic preconditioning.[96] The protective effects of nicorandil have been also shown in human in the setting of acute myocardial infarction. Intravenous nicorandil in conjunction with coronary angioplasty was associated with improved left ventricular function, improved wall motion score and better clinical outcomes, including reduced frequency of heart failure and malignant arrhythmia, compared to angioplasty alone in the patients suffering with anterior AMI.[97] The salutary effect of nicorandil has been highlighted in a recent study showing that a single administration of nicorandil before reperfusion in ST-segment-elevation myocardial infarction reduced the post-myocardial infarction death (Fig. 9).[98] Thus, although these study didn't have sufficient statistical power, they did imply that the effect of pharmacological preconditioning, via the use of K_{ATP} channel openers, can actually translate into clinical benefit. Further studies that will have adequate statistical power will give a definite solution with respect to the therapeutic potential of using pharmacological preconditioning with K_{ATP} channel openers in the setting of acute ischemic heart disease.

Conclusion

Cardiomyocytes require a constant flow of substrate and oxygen for maintaining their activity and vitality, and they are vulnerable to ischemic insult. Acute ischemia causes profound disturbance in the cellular energetics and metabolism. Cell death is inevitable if the interrupted blood flow is not promptly restored. Evidence from different experiments have suggested that most cardiomyocytes under the condition of acute, severe ischemia die via oncosis rather than from apoptosis. The discovery of ischemic preconditioning has provided a novel strategy for salvaging the cardiomyocytes from ischemic injury. Preconditioning activates diverse upstream pathways (the primary signaling) that seem to converge on the final effectors (the secondary signaling). Experimental studies have indicated that some molecules such as PKCε are central coordinators in the primary signaling. As for the secondary signaling, there is abundant evidence linking the opening of the mitoK_{ATP} channels and protection against ischemic injury. Mitochondria play an important role in acute ischemia because they are critically involved both in the process of cell death and protection. Clinical studies have suggested that pharmacological preconditioning with K_{ATP} channel openers can alleviate the injury from acute myocardial infarction when they are used in conjunction with reperfusion therapy. Further studies are needed to definitely assess the clinical benefit of pharmacological preconditioning.

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