Role of Akt Signaling in Mitochondrial Survival Pathway Triggered by Hypoxic Preconditioning

Takamichi Uchiyama, MD; Richard M. Engelman, MD; Nilanjana Maulik, PhD; Dipak K. Das, PhD

Background—The signaling pathways that control ischemia/reperfusion-induced cardiomyocyte apoptosis in heart have not been fully defined. In this study, we investigated whether Akt signaling has a role in the antiapoptotic pathways of preconditioning against hypoxia/reoxygenation (H/R).

Methods and Results—Primary cultures of adult rat ventricular myocytes (ARVMs) were subjected to preconditioning (PC) by exposing the cells to 10 minutes of hypoxia followed by 30 minutes of reoxygenation. Non-PC and PC myocytes were subjected to 90 minutes of hypoxia followed by 120 minutes of reoxygenation. Hypoxic-PC protected the myocytes from subsequent H/R injury, as evidenced by decreased apoptosis and LDH release and increased cell viability. H/R-induced cytochrome c release and activation of caspase-3 and -9 were blocked by PC. This protective effect was inhibited by treating the cells with LY294002 (50 μmol/L), a PI3 kinase inhibitor, for 10 minutes before and during PC. PC also induced phosphorylation of Akt and BAD. Protein levels of Bcl-2 in mitochondria were maintained in PC. ARVMs were infected with either a control adenovirus (Adeno lac-Z), an adenovirus expressing dominant-negative Akt, or an adenovirus expressing constitutively active Akt. Ectopic overexpression of constitutively active Akt protected ARVMs from apoptosis induced by hypoxia/reoxygenation compared with Adeno lac-Z. In contrast, dominant negative Akt overexpression abolished the antiapoptotic effect of PC.

Conclusions—Our data demonstrated that in adult cardiomyocytes, the antiapoptotic effect of PC against H/R requires Akt signaling leading to phosphorylation of BAD, inhibition of cytochrome c release, and prevention of caspase activation. (Circulation. 2004;109:3042-3049.)

Key Words: hypoxia n kinases n caspases n apoptosis

Myocardial cell death caused by necrosis and apoptosis is the main feature of pathological conditions associated with ischemia and reperfusion. Adult cardiomyocytes possess minimal capacity to reenter the cell cycle; the control of myocyte loss through suppression of cell death pathways represents a logical strategy to prevent heart disease. The induction of apoptosis in myocardium triggered during hypoxia and reperfusion may contribute to cell death in which mitochondria play a key role. In the maintenance of mitochondrial function, membrane potential may have a dramatic influence on cardiomyocyte energy production and ultimately the survival of an individual cell. Cellular injury or stress stimulation directly elicits alterations in mitochondrial architecture, membrane potential, and oxidative capacity, which are associated with an irreversible loss of mitochondrial matrix contents and integral membrane protein constituents such as cytochrome c oxidase. The release of cytochrome c or mitochondrial permeability transition directly mediates cellular apoptosis through coupling proteins that coordinate the activation of caspase and DNA fragmentation.

In contrast, ischemic preconditioning (PC) potentiates a mitochondrial signaling pathway that promotes cell survival rather than cell death. This endogenous process, well documented to be operative in vivo, refers to the paradoxical protection against lethal ischemia. PC leads to the activation of multiple kinases, including protein kinase C (PKC), MAP kinases, and tyrosine kinases. Downstream targets of kinases include activation of mitochondrial KATP channels, which may aid to restore mitochondrial bioenergetics and inhibit mitochondrial Ca2+ overload. Although the mechanism by which PC suppresses apoptosis pathways remains controversial, mitochondria appear to play a crucial role.

Recent investigation has suggested an emerging paradigm whereby stress-responsive intracellular signaling pathways influence mitochondrial membrane potential, oxidative capacity, and coupling of apoptosis-initiating factor. Akt is a critical regulator of PI3 kinase–mediated cell survival. Constitutive activation of Akt signaling is sufficient to block cell death induced by a variety of apoptotic stimuli. Several downstream targets of Akt have been recognized as apoptosis-regulatory molecules, including the bcl-2-family member BAD, procaspase-9, cAMP-responsive element–binding protein (CREB), and Forkhead family of transcription factors. Insulin-like growth factor-1 (IGF-1) also regulates...
PI3 kinase and Akt is activated by PC as a result of PI3 kinase activation, leading to the stimulation of PKC and endothelial NO synthase (eNOS). Akt is activated by PC as a result of PI3 kinase activity, which is required for Akt phosphorylation and downstream targets of Akt. Attempts were made to study the ability of somatic gene transfer to manipulate these pathways in a model of hypoxia/reoxygenation (H/R) of adult rat ventricular myocytes (ARVMs).

**Methods**

**Materials**

Primary antibodies raised against Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology; raised against BAD, phospho-BAD, and Bcl-2 were purchased from Santa Cruz Biotechnology; and raised against cytochrome c was purchased from Clontech Laboratories. Secondary antibodies horseradish peroxidase–conjugated anti-rabbit and anti-goat IgG were purchased from Amersham Life Science. LY294002 (LY) was purchased from Amersham.

**Isolation of ARVMs and Experimental Protocol**

ARVMs were isolated from hearts of male Sprague-Dawley rats (300–400 g) by standard techniques. Cells were plated in 10-mm dishes precoated with laminin (1 μg/cm²) at a density of 1×10⁶ cells per dish in DMEM with 5% FBS. Cells were maintained in tissue culture incubators at 37°C under 5% CO₂ atmosphere.

Preconditioning was carried out as described previously. Briefly, before PC, the medium was changed in a HEPES-buffered medium that contained (in mmol/L) 139 NaCl, 4.7 KCl, 0.5 MgCl₂, 0.9 CaCl₂, 5 HEPES, and 5% FBS, pH 7.4, at 37°C. Hypoxic PC was induced by incubating the cells in an airtight chamber in which O₂ was replaced by N₂. PC was induced by exposing ARVMs to 10 minutes of hypoxia and 30 minutes of reoxygenation before second 90-minute hypoxic periods followed by 120 minutes of reoxygenation. Control, non-PC cells were treated identically except that they were not exposed to the 10 minutes of hypoxia. LY294002 (50 μmol/L), a PI-3 kinase inhibitor, was treated for 10 minutes before and during PC.

**Cell Viability Assessment**

The amount of dead cells was assessed by staining trypsinized cells after treatment with trypan blue, and the fraction of blue cells was quantified by counting under a microscope. In each dish, a total of at least 200 cells were counted. The extent of cellular injury was monitored by determining lactate dehydrogenase (LDH) release in culture medium using an LDH kit (Sigma).

**In Situ Labeling of DNA Fragments**

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) was performed in cells plated on glass coverslips with the CardioTACS in situ Apoptosis Detection kit (R&D Systems) according to the manufacturer’s instructions. In each group, at least 200 cells were counted. DNA fragmentation was measured quantitatively with a Cell Death Detection ELISA kit (Roche).

**Caspase-3 and Caspase-9 Measurement**

Caspase activity was evaluated by use of caspase-3 and caspase-9 colorimetric assay (R&D Systems).

**Western Blot Analysis**

For immunodetection, ARVMs were harvested in lysis buffer (1.0% Igepal CA.630, 0.5% sodium deoxycholate, 0.1% SDS, 100 mmol/L PMSE, aprotinin, 100 mmol/L sodium orthovanadate). For detection of mitochondrial cytochrome c and Bcl-2, cardiac cells were fractionated into mitochondrial and cytoplasmic compartments with an ApoAlert cell fractionation Kit (Clontech). Total protein was electrohoresed on SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) with a semidry transfer system (Bio-Rad). The blotted membranes were incubated with antibodies and treated with enhanced chemiluminescence reagent (Amersham).

**Generation of Recombinant Adenovirus and Adenovirus Infection**

The recombinant adenovirus expressing a dominant-negative Akt (dnAkt) and constitutively activated Akt (caAkt), which were kindly provided by Dr Junichi Sadoshima (University of Medicine and Dentistry of New Jersey, Newark), and prepared as described previously. Adenovirus vector expressing β-galactosidase (Adeno-lacZ) was used as a control. ARVMs were infected with adenovirus diluted in DMEM with 5% FBS at a 100 multiplicity of infection (MOI) and incubated for 2 hours. The viral suspension was removed, and ARVMs were cultured with DMEM with 5% FBS for 24 hours. The efficiency of the expression examined by the lac-Z gene expression is >65% to 70% by this method.

**Statistical Analysis**

All data are expressed as mean±SEM. Statistical analysis was performed with 1-way ANOVA followed by Bonferroni multiple-comparison test. Student’s t test was undertaken to compare the results between the 2 groups. The results were considered significant at a value of P<0.05.

![Figure 1](https://example.com/figure1.jpg)

**Figure 1.** Preconditioning-induced increase in cellular viability against hypoxia and H/R-induced cell death. A. Quantification of trypan blue exclusion; n=3, †P<0.01 vs control, ‡P<0.001 vs control, ††P<0.01 vs hypoxia, †††P<0.001 vs H/R. B. Amount of LDH release in medium; n=3, *P<0.05 vs control, †P<0.001 vs hypoxia, ††P<0.05 vs H/R.
Results

PC Prevents H/R-Induced Cell Death and Increases Cell Viability

Exposure of ARVMs to hypoxia and H/R led to increased cell death, as assessed by trypan blue exclusion staining (hypoxia, 10.7±2.1%, P<0.01 versus control; H/R, 16.2±2.6%, P<0.001 versus control) (Figure 1A). PC decreased cell injury (PC/hypoxia, 6.1±1.5%, P<0.01 versus hypoxia; PC+H/R, 12.1±1.2%, P<0.01 versus H/R) (Figure 1A). Characterization of the distinct mode of cell death revealed significant activation of apoptosis, which was demonstrated by several specific biochemical markers of apoptosis. In the TUNEL assay, hypoxia, especially H/R, significantly increased TUNEL-positive ARVMs compared with control cells (control, 4.6±1.1%; hypoxia, 7.7±1.4%, P<0.01 versus control; H/R, 13.2±1.5%, P<0.001 versus control). PC blocked hypoxia and H/R-induced apoptosis (PC/hypoxia, 5.6±1.1%, P<0.01 versus hypoxia; PC+H/R, 9.1±1.5%, P<0.01 versus H/R) (Figure 2B). Pretreatment with LY abolished PC effects (LY+PC/hypoxia, 7.4±1.4%, P=NS versus hypoxia; LY+PC+H/R, 12.1±1.6%, P=NS versus H/R) (Figure 2B). An apoptosis-specific cell death ELISA demonstrated that increased DNA fragmentation compared with control (hypoxia, 2.1±0.2-fold over control, P<0.01 versus control; H/R, 2.7±0.3-fold over control, P<0.001 versus control) and H/R-induced DNA fragmentation were completely blocked by hypoxic preconditioning (PC+hypoxia, 1.3±0.1-fold over control, P<0.01 versus hypoxia; PC+H/R, 1.4±0.2-fold over control, P<0.01 versus H/R). LY abolished PC effects and increased DNA fragmentation (LY+PC/hypoxia, 1.9±0.4-fold over control, P=NS versus hypoxia LY+PC+H/R, 2.4±0.1-fold over control, P=NS versus H/R) (Figure 2C).

We measured LDH in the media to determine the extent of the leakage of the cytosolic components during oxidative insult. Hypoxia and H/R resulted in a significant increase in LDH level compared with control (control, 0.003±0.001 U/mL; hypoxia, 0.014±0.003 U/mL, P<0.01 versus control; H/R, 0.019±0.004 U/mL, P<0.001 versus control) (Figure 1B). PC blocked hypoxia and H/R-induced LDH release (PC+hypoxia, 0.004±0.002 U/mL, P<0.01 versus hypoxia; PC+H/R, 0.014±0.002 U/mL, P<0.05 versus H/R). LY did not affect the effect of PC on LDH release (LY+PC/hypoxia, 0.004±0.003 U/mL, P<0.01 versus hypoxia; LY+PC+H/R, 0.014±0.002 U/mL, P<0.05 versus H/R) (Figure 1B). These data suggest that PI-3 kinase contributes to apoptotic cell death rather than necrotic cell death in ARVMs.

Preconditioning Prevents Cytochrome c Release and Caspase Activation

To determine whether the mitochondria-mediated apoptosis pathway is active during H/R-induced apoptosis, immuno-
blots of cytochrome c were studied. There was a significant increase in cytosolic cytochrome c in H/R. PC blocked H/R-induced cytochrome c release, which was prevented by LY (Figure 3A).

Caspases were measured using the synthetic caspase substrates DEVD-pNA and LEHD-pNA. Both caspase-3 and -9 increased during H/R (caspase-3, H/R, 1.29±0.028-fold over control, P<0.05; caspase-9, H/R, 1.19±0.04-fold over control P<0.05). PC blocked the increased caspase activities (caspase-3, PC+H/R, P<0.05 versus H/R; caspase-9, PC+H/R, P<0.05 versus H/R), whereas LY abolished the effects of PC (caspase-3, LY+PC+H/R, 1.26±0.2-fold over control, P=NS versus H/R; caspase-9, LY+PC+H/R, 1.11±0.06-fold over control, P=NS versus H/R) (Figure 3B). These data suggest the role of PI-3 kinase in the mitochondrial apoptosis pathway involving cytochrome c and caspase-3 and -9.

**Antipoptotic Effect of PC Requires Phosphorylation of Akt and Bad**

To investigate whether Akt was involved in PC signaling, we examined the role of LY in cardiomyocyte apoptosis. As shown in Figures 1 and 2, LY blocked preconditioning-induced improvement in cell viability, TUNEL-positive ARVMs, DNA fragmentation, and LDH release. Western blot revealed that preconditioning induces phosphorylation of Akt and BAD in ARVMs during hypoxia and H/R (PC+hypoxia, 1.55±0.23-fold over control, P<0.05 versus control; PC+H/R, 1.77±0.41-fold over control, P<0.01) (Figure 4A). Inhibition of Akt using LY prevents PC-induced Akt phosphorylation. For investigation of downstream signaling molecules of Akt, we immunoprecipitated BAD, followed by the analysis of its phosphorylation status of serine residues 136 (S136) using phospho-specific antibodies. In hypoxia and H/R, preconditioning caused phosphorylation at S136 (PC+hypoxia, 3.43±0.32-fold over control, P<0.001 versus control; PC+H/R, 3.97±0.32-fold over control, P<0.001 versus control) (Figure 4B). The effect of preconditioning on the phosphorylation of BAD was dependent on Akt-mediated signaling, because LY abrogated PC-dependent phosphorylation.

**Effects of Preconditioning on Bcl-2**

To determine the effect of PC on antideath protein, we analyzed mitochondrial Bcl-2. An induction of Bcl-2 occurred by PC. H/R and inhibition of PI-3 kinase using LY induced the loss of Bcl-2 from the mitochondria (Figure 5). These data indicate that PC preserves H/R-induced loss of Bcl-2 from mitochondria via Akt pathway.
Akt Overexpression Prevents H/R-Induced Apoptosis, Cytochrome c Release, and Caspase Activation

To confirm the effect of Akt during H/R, we overexpressed Akt in ARVMs using recombinant adenovirus. Adenovirus expressing nuclear lacZ was used as control. Recombinant adenovirus infected ARVMs in a titer-dependent manner (Figure 6B). The titer of 100 MOI, resulting in >65% to 70% infection efficiency (Figure 6A) and significantly increasing Akt expression (Figure 6B), was used for experiments.

Akt overexpression significantly inhibited H/R-induced apoptosis. dnAkt abolished the effects of preconditioning (AdlacZ+H/R, 14.1±2.1%; caAkt+H/R, 8.9±1.1%, \(P<0.01\) versus AdlacZ+H/R; dnAkt+PC+H/R, 13.6±1.7%, \(P=\text{NS}\) versus AdlacZ+H/R) (Figure 6C). Akt overexpression also inhibited H/R-induced DNA fragmentation, and dnAkt abolished the effect of PC (caAkt+H/R, \(P<0.01\) versus AdlacZ+H/R; dnAkt+PC+H/R, \(P=\text{NS}\) versus AdlacZ+H/R) (Figure 6D).

To elucidate the molecular mechanisms involved in the antiapoptotic effects Akt, we measured cytosolic cytochrome
c and the activities of caspase-3 and caspase-9. H/R-induced cytochrome c release was inhibited by Akt overexpression (Figure 7A). Akt overexpression also resulted in inhibition of H/R-induced caspase-3 and -9 activation (caspase-3, caAkt, \( P < 0.05 \) versus AdlacZ + H/R; caspase-9, caAkt, \( P < 0.05 \) versus AdlacZ + H/R). dnAkt abolished the effects of PC (dnAkt + PC + H/R, \( P = \text{NS} \) versus AdlacZ + H/R) (Figure 7, B and C), confirming the crucial role of Akt in antiapoptotic effects of PC.

Effects of Akt Overexpression on Bcl-2
Mitochondrial Bcl-2 levels were maintained by Akt overexpression as PC. dnAkt completely blocked Bcl-2 expression (Figure 8). These data indicate that Akt overexpression inhibits H/R-induced the loss of Bcl-2 as PC.

Discussion
In this study, we demonstrated that PC potentiated activation of survival pathways through PI-3 kinase signaling. Inhibition of PI-3 kinase with LY294002 abolished the protective effect of PC by stimulating apoptosis and DNA fragmentation without affecting LDH release from the myocytes. These data suggest that PI-3 kinase has an important role in antiapoptotic effects of PC. Hypoxia followed by reoxygenation (H/R) was associated with both apoptotic and necrotic cell death, whereas hypoxia contributed to only a minimal amount of cell death. Therapeutic reperfusion is currently performed without any measure to protect myocardium from apoptosis during the treatment of myocardial infarction.23 Our results indicate that it may be possible to further limit the infarct size by inhibition of apoptosis, and PI-3 kinase might play a key role in this process. PC also stimulates phosphorylation of Akt, a kinase directly downstream of PI-3 kinase. Pretreatment with LY294002 blocks the PC-induced increase in Akt phosphorylation. Akt inhibits caspase-mediated cell death through the phosphorylation of the death agonist Bcl-xl/Bcl-2-associated death promoter (BAD) releasing Bcl-2 family members13 and direct phosphorylation of caspase-9.14 In the present study, PC caused phosphorylation of BAD, and LY294002 blocked PC-induced increase in BAD phosphorylation. The increase of cytosolic cytochrome c and the activation of caspase-3 and -9 by H/R were blocked by PC, and dnAkt prevented these PC effects. Akt overexpression with caAkt and PC also inhibited H/R-induced loss of Bcl-2.
from mitochondria, but pretreatment with LY294002 and dnAkt induced the loss of Bcl-2. These results indicate that PC inhibits translocation of BAD in response to H/R-induced loss of Bcl-2 from the mitochondria by phosphorylated BAD via Akt. These findings are consistent with the previous findings in other cell lines, in which Akt acts at the level of mitochondria to release cytochrome c via Bcl-2 in adult cardiomyocyte. PI-3 kinase and Akt signaling pathways were also found to play a critical role in the prevention of apoptotic cell death by PC. It should be noted, however, that Akt could not completely abolish apoptotic myocyte death in our study.

This may be because more than 1 survival pathways are triggered by PC.

PC is likely to manipulate multiple stress-responsive signaling pathways that contribute to the regulation of cellular apoptosis. Several studies have examined the potential role of the MAPK family, such as the ERKs (p42/p44), JNKs, and p38-MAPK, in PC. In particular, activation of p38-MAPK, which appears to be extremely sensitive to stress signals, has been studied intensively as a possible mediator for the generation of a protective protein during PC. However, the MAP kinase family is also implicated in the initiation of apoptosis in pathological states like ischemia and heart failure. A candidate pathway not investigated in the present study is the Janus kinase (Jak) signal transduction and activation of transcription (STAT) cascade, a pathway found to be upregulated in parallel to PI-3 kinase. Relatively underexplored in the context of PC, recent evidence suggests that the JAK/STAT pathways are activated by PC. STATs play an important role in controlling cell cycle progression and apoptosis.

Adult cardiomyocytes are thought to be largely refractory to cell cycle reentry and cytokinesis. Thus, apoptotic events...
in the myocardium most likely result in a cumulative decrease in cell number, which is thought to be a contributing factor in heart failure. The description of apoptosis in the failing myocardium and ischemia/reperfusion injury was initially controversial, in part because of unusually high approximation of overall cell death. More recent studies in several animal models of cardiomyopathy as well as failed human hearts have confirmed the increased occurrence of cardiomyocyte apoptosis as a potential contributing factor in the progressive loss of pump function. Cumulative levels of apoptosis identified in the failing heart suggest that acute myocardial infarction promotes massive apoptosis of cardiomyocytes because of a primary lack of oxygen and/or a reperfusion-induced hyperemia that is associated with free radical production. We conclude that in the isolated adult rat cardiomyocyte model of hypoxic PC, PI-3 kinase and Akt signaling pathways are required for the maintenance of mitochondria-stabilizing Bcl-2 protein, which subsequently antagonize mitochondrial dysfunction of cytochrome c release and caspase activation, thereby antagonizing apoptosis.

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References

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