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THE LATE PHASE OF ISCHEMIC PRECONDITIONING INDUCES A PROSURVIVAL GENETIC PROGRAM THAT RESULTS IN MARKED ATTENUATION OF APOPTOSIS

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Abstract

Although the cardioprotection afforded by the late phase of ischemic preconditioning (PC) in ischemia/reperfusion (I/R) injury has been well studied, it is unknown whether this beneficial effect can be attributed to inhibition of apoptosis. We hypothesized that ischemic PC affords protection by suppressing apoptosis and examined the underlying mechanisms.

Myocardial infarction was produced in mice (30-min coronary occlusion). In animals preconditioned 24 h earlier with six 4-min coronary occlusion/4-min reperfusion (O/R) cycles, there was a marked decrease in apoptosis as assessed by three different parameters: hairpin-1 assay, caspase-3 activity, and immunohistochemical analysis of active caspase-3 and cleaved poly (ADP-ribose) polymerase-1 (PARP-1). This protective effect was accompanied by increased expression of multiple antiapoptotic proteins that regulate both the mitochondria-mediated (Bcl-x_L and Mcl-1) and the death receptor-mediated (c-FLIP_L and c-FLIP_S) pathway of apoptosis and by decreased expression of the proapoptotic protein Bad.

This is the first demonstration that the late phase of ischemic PC attenuates cardiac apoptosis after ischemia/reperfusion injury and that this salubrious effect is associated with a complex genetic prosurvival program that results in modulation of several key proteins involved in both the mitochondrial and the death receptor pathways of apoptosis.

Keywords

Apoptosis; antiapoptotic protein; myocardial ischemia; preconditioning; ischemia/reperfusion

INTRODUCTION

The late phase of ischemic preconditioning (PC) is a delayed protective adaptation whereby brief episodes of ischemia enhance the resistance of the heart to ischemia/reperfusion injury 12 to 72 hours later. The development of late PC after the ischemic PC challenge (on day 1) occurs via the coordinated activation of various signaling molecules, including protein kinases and transcription factors, that control the transcriptional activation of genes and the expression

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of cardioprotective proteins [1]. However, the mechanism of the delayed cardioprotective effects of ischemic PC remains unclear. In particular, it is unknown whether these actions are due to inhibition of cardiac apoptosis and, if so, which molecular pathways underlie this protection. Many of the transcription factors that play a role in late PC (e.g., NF- κ B and STAT1/3) [2,3] bind to the promoter region of several major antiapoptotic genes and/or play a role in the regulation of cell death via apoptosis [4,5].

Previous studies have shown that apoptosis contributes to cardiomyocyte cell death in both human and animal models of ischemia/reperfusion injury [6,7] and that it is possible to attenuate ischemia/reperfusion injury by modifying apoptosis [8-11]. Although previous studies have shown that early PC induced by ischemia or pharmacological agents such as sildenafil can attenuate apoptosis [12-15], the role of late PC in modulating cardiomyocyte apoptosis and the signaling mechanism involved remain unknown. Late PC has potential clinical relevance because its cardioprotective effects are sustained (~30 times longer than those of early PC) and because it affords a broader range of protection (against infarction as well as stunning) [1].

The mechanism of apoptosis can be broadly divided into the mitochondria-mediated and the death receptor-mediated pathways [16]. Although the details of these pathways are not completely understood, it is clear that there exists a balance between pro- and antiapoptotic mechanisms in the cell, such that manipulations which shift this balance one way or the other may have profound consequences on cell survival [17,18]. The mitochondria-mediated (intrinsic) pathway of apoptosis regulates cell death via the Bcl-2 family of proteins. This pathway influences apoptosis by regulating mitochondrial permeability, the release of cytochrome *c*, and the subsequent activation of procaspase-9 [17-19]. Important members of the Bcl-2 family include the antiapoptotic proteins Bcl-x_L and Mcl-1 and the proapoptotic proteins Bax and Bad [16,20].

The death receptor-mediated (extrinsic) pathway of apoptosis is initiated by the binding of ligands, including tumor necrosis factor- α (TNF- α), FasL and TRAIL (TNF-related apoptosis-inducing ligand), to membrane-bound receptors that belong to the TNF superfamily [16]. This pathway ultimately results in the activation of procaspase-8, which leads to cell death. Caspase-8 activation can be blocked by antiapoptotic proteins known as FLICE-like inhibitor proteins (c-FLIP_L and c-FLIP_S) [21,22]. Although members of both the Bcl-2 family and the c-FLIP family have been identified as endogenous natural inhibitors of apoptosis [16,17,20, 22], their role in ischemic PC-induced delayed cardioprotection remains unknown.

Caspase-3 is the final common executioner of both death receptor- and mitochondria-mediated apoptosis and is ultimately responsible for the majority of the apoptotic effects when either pathway is activated [16,23]. Poly (ADP-ribose) polymerase-1 (PARP-1), in its uncleaved state, plays a crucial role in myocardial ischemia/reperfusion injury and in DNA repair, replication, transcription, and cell death. When caspase-3 is cleaved, it becomes active and cleaves PARP-1 [16,23,24]. Cleavage of PARP-1 by caspase-3 or other caspases yields a 24 kDa and an 89 kDa fragment. The 24 kDa fragment facilitates apoptosis by blocking access of PARP-1, which can mend damaged DNA, to the fragmented chromatin. Thus, both active caspase-3 and cleaved PARP-1 represent general markers of apoptosis that are common to the intrinsic as well as the extrinsic pathways. Although both caspase-3 and PARP-1 play an important role in apoptotic cell death, it is unknown whether late PC modulates the activation of caspase-3 or the cleavage of PARP-1.

The purpose of this study was to investigate the role of apoptosis in the late phase of ischemic PC. Specifically, we hypothesized that the cardioprotective effects of late PC are due, at least in part, to suppression of apoptosis, and that PC induces a prosurvival genetic program by

upregulating antiapoptotic proteins that inhibit both the death receptor-(extrinsic) and the mitochondria-dependent (intrinsic) pathways of apoptosis. In the first series of studies (Phase A), we determined whether the cardioprotective effects of late PC are due to inhibition of apoptosis, as determined by multiple parameters (*in situ* hairpin-1 ligation assays, by enzymatic analysis of caspase-3 activity, and by immunohistochemical analysis of active caspase-3 and cleaved PARP-1). In Phase B, we investigated the underlying phenotype responsible for the antiapoptotic properties of PC by determining whether late PC increases the expression of the antiapoptotic proteins Bcl-x_L and Mcl-1 (mitochondria-mediated pathway) and c-FLIP_S and c-FLIP_L (death receptor-mediated pathway) and whether late PC alters the expression of the proapoptotic proteins Bad and Bax and/or the post-translational modification of Bad (phosphorylation). All studies were conducted in a well-established murine model of myocardial infarction [25].

MATERIALS AND METHODS

Animal care

Male ICR mice (body wt. ~30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine (Louisville, KY) and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, National Institutes of Health, Publication No. 86-23).

Experimental preparation

The murine model of late PC has been previously described in detail [25]. In brief, the chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid-left anterior descending coronary artery by using an 8-0 nylon suture. Ischemic PC was elicited by a sequence of six 4-min coronary occlusion/4-min reperfusion (O/R) cycles.

Phase A

To determine whether ischemic PC attenuates apoptosis, mice were divided into eight groups (Fig. 1). Tissue from mice in groups I-V was used for *in situ* hairpin-1 ligation assays (n=5/ group), for enzymatic activity of caspase-3 (n=5/ group), and for immunohistochemistry for active caspase-3 (n=5/ group). Tissue from mice in groups VI-IX was utilized for immunoblot analysis of active caspase-3 (n=5/ group) and cleaved PARP-1 (both the 89 kDa and 24 kDa fragments; n=5/ group), and for immunohistochemistry of cleaved PARP-1 (the 89 kDa fragment; n=5/ group).

Phase B

In the second part of this study, we investigated the impact of ischemic PC on the expression of major antiapoptotic proteins (Mcl-1, Bcl-x_L, c-FLIP_S, and c-FLIP_L) and proapoptotic proteins (Bad, p-Bad, and Bax). As shown in Fig. 1, mice were subjected to sham PC (60min of open-chest state) (group X, n=5) or ischemic PC (group XI, n=5) on day 1 and the hearts were harvested 24 h later for immunoblotting.

Determination of apoptosis by *in situ* hairpin-1 ligation assay

Apoptosis was determined in heart sections with *in situ* hairpin-1 ligation (hairpin-1-biotin probe: 5'-GCGCTAGACCT*GGTCTAGCGCA-3'; T* represents biotinylated deoxythymidine [dT]). Briefly, fixed sections (4-μm) were first digested with Proteinase K (25 μg/ml) for 15 min, then incubated with 50 μl of T4 ligation buffer (T4 DNA ligase, 1 mM EDTA, 15% polyethylene glycol) and a biotin labeled hairpin-1 probe for 16 h, and then

incubated with streptavidin-conjugated Texas-Red. Cardiomyocytes were stained with monoclonal anti-troponin-T antibodies followed by FITC-conjugated donkey anti-mouse secondary antibodies. The slide was counterstained with DAPI and visualized using a fluorescent microscope. The number of apoptotic cardiomyocytes was determined by the *in situ* hairpin-1 ligation assay and expressed as a percent of the total cardiomyocyte nuclei that were counted. A total of 2,000 cardiomyocyte nuclei were counted in each section after co-staining with troponin-T, DAPI, and hairpin-1.

Caspase-3 activity

Caspase-3 activity was determined by measuring the cleavage of a caspase-3 specific substrate (acetyl-Asp-Glu-Val-Asp(DEVD)-p-nitroanilide (pNA) (DEVD-pNA)) using a caspase-3 activity assay kit according to the manufacturer's instructions (R&D Systems, MN).

Immunohistochemical analysis of active caspase-3 and cleaved PARP-1

Slide sections were incubated with a FITC-conjugated rabbit polyclonal anti-active caspase-3 antibody (Cell Signaling) or anti-cleaved PARP-1 (the 89 kDa fragment) (BioSource International, Inc., USA) for 16 h at 4°C. Cardiomyocytes were stained with monoclonal anti-troponin-T antibodies followed by incubation with FITC-conjugated donkey anti-mouse secondary antibodies. The slide was counterstained with DAPI and visualized under a fluorescent microscope.

Preparation of cytosolic, membranous, nuclear, and mitochondria fractions

Cytosolic, membranous, and nuclear fractions were prepared from heart samples as previously described [2,3,26]. The mitochondrial fraction was isolated according to previously published methods [27].

Western immunoblotting

Western immunoblot analysis was performed with standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting techniques as previously described [2,3, 26]. Equal loading was confirmed by staining with Ponceau-S.

Statistical analysis

Data are reported as means±SEM. Measurements were analyzed by ANOVA followed by unpaired Student *t* tests with the Bonferroni correction. In all Western blot analyses, the content of the specific protein of interest was expressed as a percentage of the corresponding protein in the anterior left ventricular wall of control mice.

RESULTS

Effects of ischemic PC on apoptosis, caspase-3 activation, and PARP-1 cleavage

As shown in Figs. 2A-2C, at 3 h after a 30-min coronary occlusion (group II), there was an increase in apoptotic DNA breaks as evidenced by hairpin-1 labeling (red fluorescence) (Fig. 2A). These positive hairpin-1 signals were localized to the nuclei (blue fluorescence, Fig. 2B) of cardiomyocytes (green fluorescence, Fig. 2C). Apoptotic bodies and/or chromatin margination were also apparent in these apoptotic nuclei. Sham operation was not associated with apoptotic cell death (Fig. 2D). These data demonstrate that a 30-min coronary occlusion followed by 3 h of reperfusion induces cardiomyocyte apoptosis in mice. As shown in Fig. 2E, mice preconditioned 24 h earlier (group V) exhibited a dramatic reduction in cardiomyocyte apoptosis (PC, 3.7±1.2% vs. I/R [group II]; 21.0±5.1%, $P<0.05$) compared with nonpreconditioned controls (group II). Sham operation 24 h prior to ischemia/reperfusion injury (group III) did not significantly change apoptosis compared with group II. There was

no detectable apoptosis in preconditioned hearts not subjected to a 30-min occlusion (group IV) (data not shown). Collectively, these results demonstrate that ischemic PC elicits powerful delayed cardioprotective actions against apoptosis.

As shown in Fig. 3, at 3 h of reperfusion after a 30-min coronary occlusion (group II) there was a marked increase in caspase-3 activity ($+194 \pm 20\%$, $P < 0.05$) compared with non-preconditioned sham controls (group I), indicating activation of caspase-3. This increase in caspase-3 activity was blunted in preconditioned mice (group V). To verify whether caspase-3 activation occurs in cardiomyocytes, immunohistochemical analysis of active caspase-3 was performed in tissue sections. Immunohistochemistry confirmed that a 30-min coronary occlusion followed by 3 h of reperfusion (group II) resulted in an increase in the cleaved (active) form of caspase-3 (Fig. 4A). The active caspase-3 signals were localized to the nuclei of cardiomyocytes, as shown by co-staining with DAPI (Fig. 4B) and troponin-T (Fig. 4C). There was no detectable active caspase-3 in tissue sections taken from sham control mice (group I; Fig. 4D). Next, we quantified active caspase-3 content in the nuclear fraction following ischemia/reperfusion injury with or without ischemic PC. As illustrated in Fig. 5A, a 30-min coronary occlusion followed by 3 h of reperfusion (group VII) resulted in an increase in the cleaved (active) form of caspase-3 in the nuclear fraction. This increase in cleaved caspase-3 was almost completely abrogated in mice preconditioned 24 h prior to the 30-min occlusion (group IX, Fig. 5A). PC itself had no effect on caspase-3 activation (group VIII, Fig. 5A).

Since PARP-1 is a major substrate of active caspase-3 in the apoptotic signaling cascade, we next determined whether PARP-1 is cleaved during myocardial ischemia/reperfusion. Immunohistochemical analysis revealed that at 3 h after a 30-min coronary occlusion (group II) there was formation of the 89 kDa fragment of PARP-1 (Fig. 4E). As was the case for caspase-3, the cleaved 89 kDa fragment was localized to the nuclei of cardiomyocytes, as shown by co-staining with DAPI (Fig. 4F) and troponin-T (Fig. 4G). Immunoblotting of the nuclear fraction revealed that after a 30-min coronary occlusion and 3 h of reperfusion (group VII) there was a striking increase in the cleaved 89 kDa fragment (6.2-fold) (Fig. 5B) and in the cleaved 24 kDa fragment (3-fold) (Fig. 5C) as compared with control (group VI) mice. This increase in the cleaved 89 kDa and 24 kDa fragments of PARP-1 was markedly inhibited by preconditioning mice 24 h prior to the 30-min occlusion (group IX). Ischemic PC (group VIII) itself had no effect on the cleavage of PARP-1 (Figs. 5B and 5C).

Taken together, these results provide conclusive evidence that the late phase of ischemic PC protects against cardiac apoptosis, as determined by a decrease in hairpin-1 positive nuclei, and that this attenuation of apoptosis is associated with a decrease in caspase-3 activity, active caspase-3, and cleaved PARP-1.

Ischemic PC upregulates antiapoptotic proteins involved in both the mitochondria- and the death receptor-mediated pathways of apoptosis

Next, we studied the impact of PC on antiapoptotic proteins. The PC protocol (group XI) resulted in a significant increase in Bcl-x_L and Mcl-1 in the cytosolic and mitochondrial fractions (Figs. 6A and 6B) and in c-FLIP_L and c-FLIP_S in total cell homogenates (Fig. 6C) compared with nonpreconditioned mice (group X). These results demonstrate that ischemic PC upregulates antiapoptotic proteins that modulate both the mitochondria-mediated (Bcl-x_L and Mcl-1) and the death receptor-mediated pathway of apoptosis (c-FLIP_L and c-FLIP_S).

Ischemic PC does not alter Bax or p-Bad expression and minimally attenuates the expression of Bad

We also examined the effect of PC on the proapoptotic proteins Bax, Bad, and p-Bad in the isolated mitochondrial fraction. As shown in Figs. 6D-6F, ischemic PC (group XI) induced a

small albeit significant decrease in the expression of Bad ($75 \pm 3.3\%$ vs group X, $P < 0.05$) but had no significant effect on the expression of Bax and on the phosphorylated form of Bad (p-Bad).

DISCUSSION

Despite the fact that the late phase of ischemic PC affords long-last protection against myocardial cell death and that apoptosis is an important cause of cell death after ischemia/reperfusion injury, the effect of late PC on apoptosis and the signaling mechanisms underlying the protective effects of PC remain unknown. The goal of this study was to bridge these gaps of knowledge. Our results demonstrate for the first time that: (i) ischemic PC decreases apoptosis after ischemia/reperfusion injury as demonstrated by three different assays (hairpin-1 staining, caspase-3 activation and cleavage, and PARP-1 cleavage); and (ii) the cardioprotective phenotype induced by ischemic PC is associated with marked upregulation of antiapoptotic proteins involved in both the mitochondria-mediated (Mcl-1 and Bcl-x_L) and the death receptor-mediated (c-FLIP_L and c-FLIP_S) pathways of apoptosis.

Although previous studies have suggested that the early phase of ischemic PC protects against apoptosis [12, 14, 15], to our knowledge this study is the first to provide definitive evidence that late PC protects against apoptotic cell death during ischemia and reperfusion injury. We utilized state-of-the-art techniques to draw this conclusion. All studies were conducted *in vivo* utilizing a well-characterized murine model in which fundamental physiologic variables that modulate ischemia/reperfusion injury are carefully monitored and controlled [25]. Apoptotic cell death was determined by an *in situ* dual-labeling assay for hairpin-1 and troponin-T. Previous studies have generally utilized TUNEL staining [12,14] as a marker for apoptosis. It has been shown that apoptotic cell death can be specifically differentiated from non-apoptotic cell death by utilizing techniques that detect single base 3' overhangs [28,29]. Although TUNEL staining does detect apoptotic cell death, it does not exclusively detect apoptotic nuclei. TUNEL detects 1-4 base 3' overhangs produced by both DNase I and DNase II and thus is a marker of both apoptotic and necrotic cell death, whereas hairpin-1 ligation assays detect only double strand apoptotic DNA breaks with single base 3' overhangs produced by DNase I and thus is specific for apoptosis [6,7,30]. We chose to utilize *in situ* hairpin-1 ligation assays to accurately detect and quantify only apoptotic cell death. In this study, the *in situ* hairpin-1 ligation assay definitively demonstrates that ischemia/reperfusion induces apoptosis of cardiomyocytes and that ischemic PC attenuates the apoptotic death of these cells. Importantly, the results obtained with hairpin-1 staining correlate well with the other variables analyzed to detect apoptosis, namely, the activity of caspase-3 and the expression and location of cleaved caspase-3 and cleaved PARP-1. We found that caspase-3 activity, as measured by enzymatic cleavage, was attenuated by a PC stimulus applied 24 h earlier. In addition, immunohistochemical analysis revealed that the cleaved (active) form of caspase-3 and cleaved PARP-1 were increased at 3 h after the 30-min coronary occlusion. Immunohistochemistry showed that both of these cleaved proteins were localized to the nuclei of cardiomyocytes, and that this increase was diminished in preconditioned hearts. We then performed immunoblotting of the nuclear fraction in order to quantify the expression of cleaved caspase-3 and cleaved PARP-1 (24 and 89 kDa fragments). The results confirmed that late PC abrogates the nuclear accumulation of both of these proteins.

Taken together, these results provide compelling evidence, utilizing several different lines of support, that the late phase of ischemic PC inhibits ischemia/reperfusion-induced apoptosis. In addition, these observations have important practical implications. Previous studies have demonstrated that inhibition of PARP-1 cleavage with PARP inhibitors limits infarct size in an *in vivo* rat model [11]. Our data on the antiapoptotic effects of late PC support the notion

that inhibiting apoptosis is a potentially important therapeutic strategy for minimizing infarct size in patients with an acute myocardial infarction.

Having established that ischemic PC attenuates cardiomyocyte apoptosis, we next sought to investigate the mechanisms that underlie this protection. Since apoptosis can occur by either mitochondria- or death receptor-mediated pathways, we examined major antiapoptotic proteins involved in the mitochondria-mediated pathway (Mcl-1 and Bcl-x_L) and in the death receptor-mediated pathway (c-FLIP_L and c-FLIP_S). These proteins were selected not only because they modulate the two apoptotic pathways, but also because they are regulated by transcription factors (STAT1/3 and NF-κB) that are known to play an essential role in the late phase of ischemic PC [1-3,26]. Since apoptosis is governed by the balance between anti- and proapoptotic signals, we also examined the effect of ischemic PC on the proapoptotic proteins Bad, p-Bad, and Bax. Our results demonstrate that ischemic PC upregulates Bcl-x_L and Mcl-1 expression, suggesting inhibition of mitochondria-mediated apoptosis, and c-FLIP_L and c-FLIP_S expression, suggesting inhibition of death receptor-mediated apoptosis as well. Ischemic PC did not have a significant impact on expression of Bax but did decrease the expression of the proapoptotic protein Bad (although the amount of phosphorylated Bad did not change). Taken together, these results demonstrate that ischemic PC induces a phenotypic switch in the heart that protects against apoptosis by upregulating antiapoptotic proteins involved in both the mitochondrial and the death receptor pathways and by downregulating the proapoptotic protein Bad. Our data warrant further investigation of the transcriptional regulation of these antiapoptotic proteins. NF-κB and the STAT1/3 are known to play an important role in upregulating the cardioprotective proteins iNOS and COX-2 during the late phase of ischemic PC [1-3,26]. It is possible that these transcription factors are also critical in controlling the transcriptional regulation of Bcl-x_L, Mcl-1, c-FLIP_L, and c-FLIP_S after a PC stimulus. Although it would be of interest to examine the individual roles of each of these antiapoptotic proteins using genetic manipulations that result in gain or loss of function, the modulation of multiple proteins makes it unlikely that one protein could be responsible for the salutary effects of late PC. Furthermore, genetic knockout of Bcl-x_L, Mcl-1, c-FLIP_L, c-FLIP_S and Bad is embryonically lethal and deletion of Bad results in neoplasms.

Our observations provide the first demonstration that c-FLIP_L and c-FLIP_S are upregulated by ischemic PC. One of the first steps in death-receptor mediated apoptosis is the formation of a death-inducing signaling complex (DISC). At this step, procaspase-8 is cleaved to caspase-8, which subsequently mediates the execution phase of apoptosis [31]. Although the mechanism by which c-FLIP protects from apoptosis is still unclear, it is postulated that it prevents the proper cleavage of procaspase-8. Previous studies have shown that c-FLIP is highly expressed in cardiac tissue including human hearts, and that it plays a critical role in cardiac development [21,22,31,32]. c-FLIP expression has been found to be attenuated in infarcted cardiac tissue [32] it has also been reported that in ischemia/reperfusion injury, cardiac myocytes that underwent apoptosis lacked c-FLIP expression whereas surrounding viable myocytes expressed c-FLIP [21,32,33], supporting an important role of this protein as an arbiter of cell fate. This prior work is consistent with our results suggesting that c-FLIP may play an important role as an endogenous signaling mechanism whereby ischemic PC inhibits apoptosis.

We also found that late PC favorably alters the expression of members of the Bcl-2 family of proteins, including upregulation of the antiapoptotic proteins Bcl-x_L and Mcl-1 and downregulation of the proapoptotic protein Bad. The cell's fate depends upon the balance between anti- and proapoptotic proteins (such as the Bcl-2 family) in the mitochondria. Although the exact mechanisms by which these proteins inhibit apoptosis remain unclear, mitochondrial preservation is known to be crucial to cardioprotection [16-20,23]. The endpoint of the mitochondrial pathway is the release of cytochrome *c* and the opening of the mitochondrial permeability transition pore (mPTP). Previous work has shown that cardiac

Bcl-2 overexpression alleviates ischemia/reperfusion injury [34], and that Bcl-x_L gene transfer protects cardiomyocytes from ischemia/reperfusion injury [35]. Mcl-1 has been reported to be antiapoptotic in noncardiac cells [36]. On the other hand, Bad promotes apoptosis by binding to Bcl-2 and Bcl-x_L, thereby inhibiting the protective functions of these proteins [37]. Our findings of an increase in the antiapoptotic proteins Bcl-x_L and Mcl-1 and a decrease in total expression of Bad in the mitochondria suggest that late PC induces a shift of the cell's machinery to a phenotype that favors survival.

The survival signaling mechanism of late PC against apoptosis is poorly understood. However, it is becoming increasingly apparent that the ability of the cell to prevent the inappropriate activation of apoptosis, and thus to ensure survival, is mainly regulated by the expression of endogenous natural inhibitors of apoptosis-antiapoptotic proteins [16,17,20]. Our results demonstrate that ischemic PC stimuli upregulate several major antiapoptotic proteins including Bcl-x_L, Mcl-1, c-FLIP_L and c-FLIP_S, indicating the involvement of transcription factors and the transcriptional activation of these antiapoptotic genes by ischemic PC. This concept is supported by our previous studies showing that activation of the transcription factors NF-κB and STAT1/3 is necessary for the delayed protection against myocardial infarction [2,3,26]. In comparison, the time course for early PC dictates that the protective phenotype is likely induced by the posttranslational modification and protein-protein interaction of constitutively expressed proteins. For example, previous work on the antiapoptotic signaling mechanism involved in early PC has focused on the phosphorylation of prosurvival kinases and the phosphorylation of Bad [38]. Thus, although both types of PC attenuate apoptosis, the upregulation of the antiapoptotic proteins that we have demonstrated is unique to the late phase of preconditioning.

In conclusion, using a well-characterized and physiologically relevant murine model of myocardial infarction, this study demonstrates that the late phase of ischemic PC induces a genetic prosurvival reprogramming of cardiac myocytes that is associated with profound inhibition of apoptosis after ischemia/reperfusion injury. Our observations further demonstrate that the antiapoptotic actions of late PC are complex, resulting in modulation of several key proteins involved in both the mitochondrial and the death receptor pathways. Thus, it seems unlikely that any individual protein, in itself, could account for the antiapoptotic effects of late PC. Rather, it appears more plausible that this adaptation of the heart to stress is underlain by the concerted modulation of multiple pathways that ultimately converge and suppress programmed cell death. The present results expand our understanding of the molecular mechanisms that underlie the late phase of ischemic PC. They also lay the groundwork for further studies examining the specific role of individual proteins in late PC and in myocardial ischemia/reperfusion injury in general. Finally, the observations reported herein provide a rationale for novel therapeutic strategies aimed at inhibiting cardiomyocyte apoptosis with pharmacologic or genetic approaches.

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Figure 1. Experimental protocol

O indicates 4-min coronary occlusion; R, 4-min reperfusion. IHC, immunohistochemistry; PARP-1, poly (ADP-ribose) polymerase-1.

A-C. Hairpin-1 positive DNA breaks

D. Control

Figure 2. Apoptosis after ischemia/reperfusion injury. Panels A-C shows identification of apoptosis by *in situ* hairpin-1 ligation assays in a heart section (4- μ m) prepared from mice that underwent a 30-min coronary occlusion and 3 h of reperfusion. **Panel A** shows positive hairpin-1 labeling (red fluorescence); hairpin-1 identifies apoptotic cells. **Panel B:** the positive hairpin-1 red signals are localized to the nuclei, as shown by DAPI (blue fluorescence). **Panel C** shows nuclear localization of the hairpin-1 signals in cardiomyocytes (arrows) and non-myocytes (arrowhead). Myocyte cytoplasm was stained by troponin-T (green fluorescence) (x 600). **Panel D:** no hairpin-1 positive nuclei were detected in control mice (group I). **Panel E:** ischemic PC inhibits ischemia/reperfusion-induced apoptosis. On day 1, mice underwent no intervention (I/R, group II), six cycles of 4-min coronary O/R (PC+I/R, group V), or 1 h of open-chest state (sham+I/R, group III); on day 2, all mice underwent a 30-min occlusion and 3 h of reperfusion. Cardiomyocyte apoptosis was determined by *in situ* hairpin-1 ligation assay coupled with troponin-T and DAPI staining. Data are means $\pm$ SEM.

Figure 3. Ischemic PC inhibits caspase-3 activity

On day 1, mice underwent 30 min of open-chest state (sham, group I), six cycles of 4-min coronary O/R (PC+I/R, group V), or 1 h of open-chest state (sham +I/R group III); on day 2, mice in groups III and V underwent a 30-min occlusion and 3 h of reperfusion. Cardiac samples were prepared for measurement of caspase-3 activity. Ischemic PC (group V) blunted the increase in caspase-3 activity seen after I/R (I/R, group III). Data are means $\pm$ SEM.

A-C. Active caspase-3

D. Control

E-G. Cleaved PARP-1

H. Control

Figure 4. Immunohistochemical identification and localization of active caspase-3

Immunohistochemical analysis of active caspase-3 (**Panels A-C**) and cleaved 89 kDa fragment of PARP-1 (**Panels E-G**) in heart sections prepared from mice that underwent a 30-min coronary occlusion and 3 h of reperfusion. **Panel A**: green fluorescent labeling of active caspase-3. **Panels B and C**: the caspase-3 signals are localized to cardiomyocyte nuclei (arrows), as shown by co-staining with α -sarcomeric actin antibodies (red fluorescence) and DAPI (blue fluorescence). **Panel D**: no active caspase-3 signal is observed in control mice (group I). **Panel E**: green fluorescent labeling of the cleaved 89 kDa fragment of PARP-1. **Panels F and G**: the 89 kDa fragment signals are localized to cardiomyocyte nuclei, as shown by co-staining with α -sarcomeric actin antibodies (red fluorescence) and DAPI (blue fluorescence). **Panel H**: no cleaved PARP-1 is observed in control mice (group I).

Figure 5. Ischemic PC inhibits the activation of caspase-3 and the cleavage of PARP-1 in the nuclear compartment induced by ischemia and reperfusion

Mice in groups VI (sham control) and VII (Sham+I/R) underwent 1 h of open-chest state, whereas mice in groups VIII (PC) and IX (PC+I/R) were subjected to a sequence of six 4-min coronary O/R cycles on day 1. On day 2, mice in groups VII and IX underwent a 30-min coronary occlusion followed by 3 h of reperfusion. Cardiac samples were taken and nuclear fractions were prepared for immunoblotting of active caspase-3 (**Panel A**) and cleaved 89 kDa (**Panel B**) and 24 kDa (**Panel C**) fragments of PARP-1. A 30-min coronary occlusion (group VII) resulted in activation of caspase-3 and cleavage of PARP-1 3 h later; these effects were inhibited by preconditioning mice with six 4-min coronary O/R cycles 24 h prior to the 30-min occlusion (group IX). Data are mean±SEM.

Figure 6. Ischemic PC upregulates Bcl-x_L, Mcl-1, c-FLIP_L, and c-FLIP_S protein expression and downregulates Bad expression

Mice were subjected to a sequence of six 4-min coronary O/R cycles (PC, group XI) or 1 h of open-chest state (sham control, group X); myocardial samples were taken 24 later and homogenates, cytosolic fractions, and mitochondrial fractions were prepared for immunoblotting of Bcl-x_L, Mcl-1, c-FLIP_L, and c-FLIP_S, Bad, p-Bad, and Bax. Ischemic PC

(group XI) resulted in a significant increase in the expression of Bcl-x_L (**A**) and Mcl-1 (**B**) in both cytosolic and mitochondrial fractions and of c-FLIP_L and c-FLIP_S in total homogenates (**C**) 24 h later. Ischemic PC resulted in a small but significant decrease in the expression of total Bad in the mitochondrial fraction (**D**), but had no effect on the phosphorylated form of Bad (p-Bad; **E**) or on Bax expression (**F**) in the mitochondrial fraction 24 h later. Data are means±SEM.