Ischemic but Not Pharmacological Preconditioning Requires Protein Synthesis

Nanritsu Matsuyama, MD; John E. Leavens, BS; David McKinnon, PhD; Glenn R. Gaudette, MSc; Tunc O. Aksehirli, BS; Irvin B. Krukenkamp, MD

**Background**—Ischemic preconditioning (IPC) and pharmacological preconditioning (PPC) have both been shown to confer cardioprotective effects. However, the role of protein synthesis in preconditioning is unclear.

**Methods and Results**—Isolated rabbit hearts were treated with cycloheximide (CHx, 10 μmol/L), a protein synthesis inhibitor at the translational level, before 2 cycles of IPC (5 minutes of global ischemia/5 minutes of reperfusion, n=6) or PPC by pinacidil (PIN, 10 μmol/L; n=6), an ATP-sensitive potassium channel opener. Six rabbit hearts received actinomycin D (Act D, 20 μmol/L; n=6), a protein synthesis inhibitor at the transcriptional level, before IPC. The left anterior descending coronary artery was then occluded for 60 minutes and reperfused for 120 minutes. Control hearts received no treatment before prolonged ischemia (n=6). Left ventricular pressure, action potential duration, and coronary flow were measured. Infarct size is expressed as a percentage of the area at risk. IPC (n=6) and PIN (n=8) hearts experienced reduced infarct size compared with control hearts (22 ± 3% and 27 ± 2% versus 46 ± 3%, IPC and PIN versus control; P<0.01). Translational blockade (CHx) reversed the IPC infarct size reduction effect (22 ± 3% versus 48 ± 4%, IPC versus CHx+IPC; P<0.01) but not the effects of pinacidil (27 ± 2% versus 29 ± 3%, PIN versus CHx+PIN; P=NS). Transcriptional blockade (Act D) did not abolish the IPC effect (23 ± 5% versus 22 ± 3%, Act D+IPC versus PCI; P=NS). There were no significant differences in electromechanical function consequent to CHx and Act D treatment.

**Conclusions**—These findings suggest an important role for protein synthesis in the mechanism for IPC-mediated protection at the translational level, which may be different from PPC. (Circulation. 2000;102[suppl III]:III-312-III-318.)

**Key Words:** ischemia ■ proteins ■ myocardial infarction

---

The preconditioning phenomenon involves a multitude of cellular events that render myocardium resistant to a subsequent, more prolonged ischemic insult.1 Ischemic preconditioning (IPC) has classically been generated with repetitive episodes of transient global or regional ischemia and reperfusion, and its protective effects include a decrease in reperfusion arrhythmias and a reduction in postischemic left ventricular infarction.2 IPC has been demonstrated in dogs,1 rabbits,3 rats,4 and pigs.5 Although many investigators have been able to reproduce the preconditioning response, the intracellular signaling pathways have yet to be fully understood. Several investigators have suggested a role for adenine,3,6,7 protein kinase C,8,9 and/or the opening of ATP-sensitive potassium (K+_ATP) channels in the IPC response.7,10 Early studies involving IPC as a means of achieving myocardial ischemic tolerance have indicated the expression of several myocardial stress-related genes and proteins.11 These phenotypic changes appear to result in the development of an adaptive tolerance to ischemia and reperfusion injury. Such de novo proteins could be antioxidant enzymes,12 heat shock proteins (HSPs),13 or some currently unknown proteins. However, whether the cardioprotective characteristic of IPC can be attenuated by protein synthesis inhibition remains controversial.

In 1990, Thornton et al14 suggested that de novo protein synthesis was not involved in the IPC response in the open-chest anesthetized rabbit, which perhaps blunted research investigating the relationship between protein synthesis and preconditioning. Until recently, there was little work in this area. In 1997, Rowland et al15 showed that de novo protein synthesis is necessary for the protective effects of IPC in rat hearts.

IPC can be mimicked pharmacologically with adenosine administration,3,6 β₁-adrenergic agonists,16 K+_ATP channel openers,10 phorbol esters,8 bradykinin,17 and NO.18,19 Pharmacological preconditioning (PPC) refers to the ability of pharmacological agents given before coronary occlusion to reduce myocardial infarct size (IS) by stimulating the second-messenger pathways thought to be involved in preconditioning.2 PPC may be advantageous because it is accomplished...
Inc). The aorta was then perfused with oxygenated (95% O₂ /5% CO₂) cannula within a heated glass chamber (Radnoti Glass Technology stainless-steel 8F cannula, and the heart was suspended from the pyruvate 4.9, and fumarate 5.4). The aorta was cannulated with a back bleeding cannula. The right ventricle was excised, and the left ventricle was attached and perfused with 1000 U sodium heparin via an ear vein. Once the coronary reflex was abolished, the rabbits were placed in the supine position, and the chest was entered through a bilateral thoracotomy. The heart was rapidly excised and placed in an iced bath of Krebs-Henseleit solution (in mmol/L: Na⁺ 154.0, K⁺ 4.7, Ca²⁺ 1.7, PO₄³⁻ 1.1, Mg²⁺ 1.2, HCO₃⁻ 25, glucose 11.5, pyruvate 4.9, and fumarate 5.4). The aorta was cannulated with a stainless-steel 8F cannula, and the heart was suspended from the cannula within a heated glass chamber (Radnoti Glass Technology Inc). The aorta was then perfused with oxygenated (95% O₂/5% CO₂) Krebs' solution at 37°C and 75 mm Hg root pressure.

The heart was permitted to equilibrate for 15 minutes. During that period, both atria were excised, and a small balloon was placed through the mitral valve into the left ventricle. Balloon pressure was monitored continuously with an indwelling catheter probe (Millar Instruments Inc); initial end-diastolic pressure (EDP) was set to 5 to 10 mm Hg by water inflation, and the volume remained constant throughout the experiment. End-systolic pressure and EDP were measured directly from the balloon pressure tracings, and peak developed pressure was calculated as the difference between end-systolic pressure and EDP for each beat. The heart was paced at 150 bpm with an asynchronous pacemaker (model 5880A, Medtronic Inc). Monophasic action potentials were recorded from the left ventricular epicardium using the distribution of left anterior descending coronary artery (LAD) with an 8F spring-loaded epicardial probe (model 200, EP Technologies Inc). Coronary flow (CF) was measured directly by timed collection of Krebs effluent.

Ischemia was induced by encircling the LAD close to its origin with 3-0 silk suture and snaring of that suture. At the end of 60 minutes of ischemia, reperfusion (120 minutes) was achieved by releasing the ligature and briefly massaging the LAD with a moistened cotton swab.

### Experimental Protocol

#### IPC and Protein Synthesis Inhibitor

Control hearts (n=6) received no treatment before LAD ischemia. IPC hearts (n=6) were exposed to 2 cycles of 5-minute global ischemia, followed by 5 minutes of reperfusion. To inhibit protein translational synthesis, a separate group was infused with cycloheximide (CHx, 10 μmol/L) for 15 minutes before IPC (CHx+IPC group, n=6). Furthermore, a fourth group was infused with actinomycin D (Act D, 20 μmol/L), a protein transcriptional inhibitor, before IPC (Act D+IPC, n=6). Neither CHx nor Act D was administered during the reperfusion phase of IPC.

#### PIN Preconditioning and Protein Synthesis Inhibitor

Pinacidil (PIN)-preconditioned hearts (n=8) received a 5-minute infusion of PIN (10 μmol/L), a K⁺ /Ca²⁺ channel opener, followed by a 5-minute drug-free washout period before LAD ischemia. To inhibit protein translational synthesis, a separate group was infused with CHx (10 μmol/L) for 15 minutes before PPC (CHx+PIN group, n=6).

CHx was administered at a standard dose that causes a 95% inhibition of amino acid incorporation into protein. Act D was administered at a slightly higher than standard dose that causes an 85% inhibition of total protein synthesis. CHx was not administered during or after PIN infusion.

### IS Measurements

After 2 hours of reperfusion, the heart was removed from the perfusion apparatus, the ligature was resnared, and 2 mL of phthalylcyanine blue (Engelhard Corp) was infused through the aortic cannula. The right ventricle was excised, and the left ventricle was sectioned horizontally at 2-mm intervals into 5 to 7 slices. Both sides of the unstained area were scanned (SigmaScan Pro, version 4.01, Jandel Scientific) into an IBM-compatible personal computer (Dell Corp) and represented the area at risk. The slices were then incubated in triphenyltetrazolium chloride (Sigma Chemical Co) at 37°C for 20 minutes, and the unstained white area was defined as the infarct region. This area was determined for both sides of each slice. Overall...
infarct area was computed by the following formula and expressed as a percentage of the area at risk:

\[
\text{IS} = \left( \frac{\sum \text{Slices} \left( \frac{(\text{IR}_{\text{SideA}} + \text{IR}_{\text{SideB}})}{\text{Total Area}_{\text{SideA}} + \text{Total Area}_{\text{SideB}}} \times \text{Weight}_{\text{Section}} \right)}{\sum \text{Slices} \left( \frac{(\text{AR}_{\text{SideA}} + \text{AR}_{\text{SideB}})}{\text{Total Area}_{\text{SideA}} + \text{Total Area}_{\text{SideB}}} \times \text{Weight}_{\text{Section}} \right)} \times 100 \right)
\]

where AR is the area at risk, and IR is the infarct region.

**Statistical Analysis**

All data are presented as mean±SEM. Comparisons between groups were made with ANOVA for repeated measures (Systat version 5.02, Systat, Inc). As indicated, within-group multiple comparisons were made with the Tukey post hoc test. Changes were considered significant at the \(P<0.05\) level.

**Results**

**IPC and Protein Synthesis**

**Mechanical Function**

The effects of pretreatment, regional ischemia, and reperfusion on developed pressures are shown in Figure 2A. CHx and Act D treatment did not affect developed pressure. Hearts subjected to IPC entered the prolonged ischemia with a lower developed pressure than did the control hearts (72±5 mm Hg versus 115±5 mm Hg, \(P<0.01\)). There were no significant differences among the groups during regional ischemia and reperfusion.

Also, there were no significant differences in EDP among the groups during regional ischemia and reperfusion. (Figure 2B).

**APD at 50% Repolarization**

During regional ischemia, action potential duration (APD) at 50% repolarization (APD_{50}) was shortened. However, there were no significant differences during reperfusion (Figure 2C).

**Coronary Flow**

The mean CF for all groups is shown in Figure 2D. CHx and Act D did not affect CF. CF was significantly decreased in all groups during regional ischemia. There were no significant differences among the groups during the reperfusion period.

**Infarct Size**

IS for all experiments is expressed as a percentage of the area at risk (Figure 3). All hearts had similar left ventricular weight and risk area weight (Table). Control hearts had an IS of 46±3%. IPC significantly reduced IS to 22±3% (\(P<0.01\) versus control). CHx pretreatment with IPC abolished this reduction in IS to 48±4% (\(P<0.01\) versus IPC). Act D did...
not abolish the protective effect of IPC (23±5% versus 22±3%, P=NS).

PIN Preconditioning and Protein Synthesis Inhibitor

Mechanical Function
The effects of pretreatment, regional ischemia, and reperfusion on developed pressures are shown in Figure 4A. There was a significant difference in developed pressure after infusion of PIN (105±8 versus 90±9 mm Hg, pre-PIN versus post-PIN infusion; P<0.05) CHx did not abolish the vasodilatory effect of PIN. There were no significant differences among the groups during the prolonged ischemia and reperfusion period.

There were no significant differences in EDP among all groups throughout the experiments (Figure 4B).

APD at 50% Repolarization
During regional ischemia, APD∞ shortened. However, there were no significant differences during the reperfusion period. Infusion of PIN did not affect APD∞ (Figure 4C).

Coronary Flow
The mean CF for all groups is shown in Figure 4D. PIN increased CF compared with the baseline value (77±3.0 versus 54±3 mL/min, P<0.01). CHx did not abolish the vasodilatory effect of PIN. There were no significant differences among the groups during the reperfusion period.

Infarct Size
IS is shown in Figure 5. PIN significantly reduced IS to 27±2% (P<0.01 versus control). CHx pretreatment with PIN did not abolish this reduction (29±3% versus 27.0±1.7%, CHx+PIN versus PIN; P=NS).

Discussion
The principal aim of the present study was to assess whether protein synthesis is involved in IPC in a Krebs-Henseleit–perfused rabbit heart model. The present study confirms (1) the beneficial effects of acute IPC, which have been extensively studied by multiple investigators with respect to IS, and (2) that protein synthesis–dependent ischemic myocardial protection appears to be regulated at the translational level, without the contributing effects of gene transcription. We have no ready explanation for the discrepancy between the present data and the previously mentioned study. It might be due to the different model and experimental protocol.

The proposed mechanisms responsible for acute IPC,3,6–10 although not completely understood, are multiple and complex. Attempts to further dissect these mechanisms have questioned the role of myocellular protective proteins and peptide synthesis–dependent ischemic cardioprotection. Das et al11 have demonstrated that the stress induced by repeated IPC results in the expression of 15 to 20 new proteins, revealed by 2D gel electrophoresis. However, it remains unclear whether such new proteins are necessary for the protection to develop or are merely an epiphenomenon. HSPs would be logical candidates as mediators of preconditioning. Heat shock and ischemia are known to cause the rapid induction of stress proteins in the heart.22 Benjamin et al23 have reported that myocyte cultures show elevated mRNA for HSP70 when exposed to a hypoxic challenge. Mehta et al24 have reported that myocyte cultures show elevated mRNA coding for stress proteins within 20 minutes after the onset of global ischemia in the isolated heart. The results of their study neither prove nor disprove the possibility that the expression of stress protein is in any way related to the protective phenomenon of IPC. HSP70 mRNA, which may be involved in ischemia, induced by ischemic or thermal preconditioning has been shown to be inversely correlated with IS in animal models.25 However, the role of HSP proteins may be more important in the “second window of protection” (a period of protection that begins ∼24 hours after the stimulus), inasmuch as Heads et al25 found a significant increase in these proteins 24 hours after the IPC stimulus, which was not present during the initial IPC period.
It is important to note that the present study addresses only the early phase of preconditioning and not the second window of protection.

The small molecular weight HSPs, such as HSP27 and αβ-crystallin, protect ischemic injury in rat cardiomyocytes. These chaperones seem likely to be candidates for the “first line of defense” against nonlethal stress.26

In the present study, we demonstrated that the IPC-induced myocardial protection was lost after protein synthesis inhibition with CHx, suggesting that de novo protein synthesis is indeed involved. CHx alone did not affect developed pressure, APD, CF, or IS (data not shown). CHx is a powerful inhibitor of protein synthesis and binds to ribosomes within the cell cytosol to inhibit peptide elongation during translation. To separate 2 of the steps involved in protein synthesis, 2 inhibitors were used to delineate protein synthesis dependence at the transcriptional (DNA→RNA) and/or translational (RNA→proteins) level. Act D binds to DNA, blocks the movement of RNA polymerase, and prevents RNA synthesis. These findings indicate that the myocellular proteins that may be responsible for protection afforded by IPC are controlled at the level of translation rather than at gene transcription. This mechanism is not uncommon. In other organs, such as the liver, hepatic ferritin turnover also appears to be regulated at the level of mRNA translation and not gene transcription.27 Inactive mRNA in the cytoplasm can readily respond to the need for de novo protein synthesis by translating these mRNAs into protective proteins.28

By concluding the involvement of peptide production, the present study does not propose that new protein synthesis is the only mechanism responsible for the myoprotective effects of IPC. Rather, de novo protein production likely represents one of many cardioprotective mediators involved. Many other possibilities unrelated to
protein synthesis also exist for how preconditioning could protect the heart. These include inhibition of mitochondrial adenosine triphosphatase, attenuation of leukocyte function, or stimulation of adenosine receptors.

There is little work investigating the relationship between protein synthesis and PPC. Meldrum et al. suggested that adenosine-induced and $\beta_1$-adrenergic agonist-induced preconditioning was not blocked by CHx, nor were the effects on mechanical function and creatine kinase loss. We chose PIN as a PPC-mimicking agent because the $K^{+}_{ATP}$ channel may be the end effector in preconditioning. Our data suggest that PIN preconditioning is independent of protein synthesis, similar to other PPC agents. Compared with IPC, PIN preconditioning may confer cardioprotection in more direct or different pathways. Recently, it has been shown that the mitochondrial $K^{+}_{ATP}$ channel, but not the sarcolemmal $K^{+}_{ATP}$ channel, may confer an important role in preconditioning. It is hoped that future experiments will clarify these hypotheses.

**Study Limitations**

We have demonstrated that protein synthesis plays an important role in IPC at the translational level. The literature strongly suggests that the dose of CHx used is effective at blocking protein translation; however, we do not have any direct evidence of its effectiveness in translational blockade in these experiments. Act D was used at a higher dose than the standard in vivo dose, which has been shown to inhibit adenine incorporation into RNA by 80%; however, direct RNA synthesis was not measured in these experiments. Therefore, we cannot rule out the possibility that we may have missed a critical dose in these specific experiments. Nonetheless, the same dose of CHx was able to abolish the protective effects of IPC but not PIN preconditioning. Therefore, either the 2 methods of preconditioning accomplish their protective effects via separate pathways, or compared with IPC, PIN preconditioning provides superior protection.

We applied PIN, an agent that stimulated PPC. Although our results are in agreement with other investigators, there are many methods of pharmacologically preconditioning the heart. The protective effects of L-arginine (NO precursor) were not abolished by CHx (data not shown), which is also in agreement with our observations that PPC is mediated partially by a mechanism different from that involved in IPC. We have no data indicating whether CHx blocks other agents responsible for PPC.

Our model did not show the protective effects of preconditioning on postischemic function. Lasley et al. reported that IPC in the rat heart greatly improved postischemic function. Jenkins et al. reported that postischemic function in preconditioned hearts was not significantly improved in the rabbit heart, despite the limiting effect of IS. In addition to species-dependent differences, there appear to be model- or preparation-dependent differences within the same species.

The reperfusion time used in the present study was 120 minutes, which may have an effect on IS measurement with triphenyltetrazolium chloride. Brinbaum et al. suggest 180 minutes of reperfusion, although many investigators use various reperfusion times. In a separate set of experiments, control and IPC hearts were subjected to 180 minutes of reperfusion, and there was no significant difference in IS between hearts reperfused for 120 and hearts reperfused for 180 minutes. In any event, all hearts in the present study were subjected to the same reperfusion period; therefore, any possible systematic error would theoretically be canceled out.

**Conclusions**

In the present study, IPC and PPC by PIN confer an IS-limiting effect. Protein synthesis plays an important role in IPC-mediated protection at the translational level. On the other hand, PIN, a $K^{+}_{ATP}$ channel opener that mediates PPC, may occur via a more direct pathway independent of protein synthesis.

Because preconditioning has several clinical applications in cardiac surgery (including beating heart surgery and the use of preconditioning before cardioplegic arrest or in conjunction with cardioplegia or cardiac transplantation), more investigation is needed to determine whether PPC or PPC coupled with IPC may provide optimal protection.

**Acknowledgment**

We would like to thank Siddharth Agarwal for his technical assistance.

**References**


34. Brinbaum Y, Hale SL, Kloner RA. Differences in perfusion length following 30 minutes of ischemia in the rabbit influence infarct size, as measured by triphenyltetrazolium chloride staining. J Mol Cell Cardiol. 1997;29:657–666.
Ischemic but Not Pharmacological Preconditioning Requires Protein Synthesis
Nanritsu Matsuyama, John E. Leavens, David McKinnon, Glenn R. Gaudette, Tunc O. Aksehirli
and Irvin B. Krukenkamp

Circulation. 2000;102:i-312-i-318
doi: 10.1161/01.CIR.102.suppl_3.III-312

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/102/suppl_3/i-312

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/