Ischemic Preconditioning in Isolated Cells

Michael S. Marber

Ischemic preconditioning describes the increased resistance to myocardial infarction that follows short sublethal periods of ischemia. After the ischemia that triggers preconditioning, there are 2 phases of protection: an early (short-lived) phase termed early or classic preconditioning and a late (more prolonged) phase termed late preconditioning or the second window of protection. The purpose of the present minireview is to highlight the contributions being made to our current understanding of early preconditioning by models based on isolated cardiac cells.

Within the preconditioning literature are disparate findings usually explained by variations in species, maturity, preconditioning trigger, anesthetic, and/or choice of end point. This lack of generality is a particular problem with cell-based models. Variability in the circumstances of the trigger, simulation of ischemia, in vitro maintenance conditions, cell type, and species of origin result in innumerable combinations and permutations (see online data supplement at http://www.circresaha.org), making findings difficult enough to compare between cell models let alone between these models and preconditioning in the whole heart. Given these drawbacks, why are an increasing number of preconditioning investigators adopting a cell-based approach?

Relative Merits of Cell-Based Models of Preconditioning

Little controversy surrounds the surface receptors able to trigger preconditioning. In the whole heart, their successful pharmacological manipulation can be verified by alterations in vascular resistance, rate and strength of contraction, and atrioventricular conduction. However, attention has now shifted to intracellular signaling pathways, and the specificity of pharmacological agents is diminished, their effects on physiology are less certain, and their costs are greatly increased. The cell-based models overcome these disadvantages through a small volume of distribution, through an ability to manipulate signaling proteins by the introduction of cDNAs, antisense RNA, recombinant protein, and interfering peptides, and through the interrogation of altered signaling cascades and their consequences within a homogeneous cell type. However, these advantages are at the expense of a cell phenotype that differs from the intact heart and cannot be subjected to true ischemia/reperfusion. Thus, mechanisms may not reflect those in vivo. The advantages and disadvantages are listed in an online data supplement (see http://www.circresaha.org). The remainder of the present review focuses on the different cell-based models and their contributions to the preconditioning field.

Cell-Based Models of Preconditioning Using Immature Cardiomyocytes

Mature (adult) cardiac myocytes die or dedifferentiate in long-term culture and are resistant to classic transfection. Thus, preconditioning models based on immature or dedifferentiated cardiocytes were the first described and are still favored by many investigators (see Table 1). The cells used fall into 3 types: (1) embryonic, usually chick myocytes; (2) neonatal, usually rat myocytes; and (3) adult cells, usually human, that have been “dedifferentiated,” resulting in lost rod-shaped morphology and recrudescence of mitosis.

The popularity of immature cardiocytes is based on their familiarity as a paradigm of hypertrophy and the similarities that exist between the signaling processes of hypertrophy and preconditioning. However, documented differences between

Received January 18, 2000; accepted March 29, 2000.
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(Circ Res. 2000;86:926-931.)
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**TABLE 1. Models of Preconditioning in Immature Cardiocytes**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>PC Stimulus*</th>
<th>Lethal Injury†</th>
<th>End Point‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick embryonic cardiomyocyte</td>
<td>10 min of 80% N₂, 20% CO₂, 20 mmol/L FDG, 8.0 mmol/L K⁺, pH 6.8, 10-min recovery</td>
<td>60 min of 20% CO₂, 80% N₂, 20 mmol/L FDG, 8.0 mmol/L K⁺, pH 6.8</td>
<td>Propidium iodide nuclear staining</td>
</tr>
<tr>
<td>Chick embryonic cardiomyocyte</td>
<td>5 min of no glucose, &gt;=99% N₂, 10-min recovery</td>
<td>90 min of no glucose, &gt;=99% N₂</td>
<td>CK and LDH release, live cells (by resedimentation after trypsinization), [ATP], [Ca²⁺]²⁺ uptake</td>
</tr>
<tr>
<td>Neonatal rat cardiomyocyte</td>
<td>25 min of 95% N₂, 5% CO₂ with glucose, 30-min recovery</td>
<td>4–6 h of 95% N₂, 5% CO₂ with glucose</td>
<td>[³H]Arachidonic acid release, failure of spontaneous contraction</td>
</tr>
<tr>
<td>Neonatal rat cardiomyocyte</td>
<td>60 min of 100% Ar, 3-h recovery</td>
<td>3 h of 100% Ar</td>
<td>LDH release</td>
</tr>
<tr>
<td>Neonatal rat cardiomyocyte</td>
<td>4×90 min of 99% N₂, 1% CO₂, 60-min recovery or 1×30 min of no glucose, 99% N₂, 1% CO₂</td>
<td>7–9 h of no glucose, 99% N₂, 1% CO₂</td>
<td>LDH release, viability with Eukolight kit (Molecular Probes)</td>
</tr>
<tr>
<td>Neonatal rat cardiomyocyte</td>
<td>90 min of 95% Ar, 5% CO₂, 16 mmol/L K⁺, pH 6.2, 30-min recovery</td>
<td>4–8 h of 95% Ar, 5% CO₂, no glucose, 20 mmol/L lactate, 16 mmol/L K⁺, pH 6.2</td>
<td>Trypan blue uptake, LDH release, β-galactosidase activity</td>
</tr>
<tr>
<td>Pediatric human cardiomyocyte</td>
<td>20 min of 100% N₂, 20 min recovery</td>
<td>90 min of 100% N₂</td>
<td>Trypan blue uptake, [ATP], LDH release, lactate release, adenosine release, H⁺ release</td>
</tr>
<tr>
<td>Fetal human cardiomyocyte</td>
<td>15–60 min of 95% N₂, 5% CO₂, 11 mmol/L 2-DOG, 30- to 75-min recovery</td>
<td>15–24 h of 95% N₂, 5% CO₂</td>
<td>Trypan blue uptake</td>
</tr>
</tbody>
</table>

PC indicates preconditioning; FDG, F-2-deoxyglucose; 2-DOG, 2-deoxyglucose; and CK, creatine kinase.

*Pharmacological agents that initiate PC have been omitted.
†In the studies examining various durations of injury, only those during which injury was significantly diminished by the PC stimulus are stated.
‡Not all end points were measured in every study.

mature and immature myocardies require proposed cell-based models to faithfully recapitulate key features of ischemic preconditioning. These features include initiation by simulated ischemia, the temporal relationship between initiating and lethal simulated ischemia, the involvement of ligands to G-protein–coupled receptors, and protein kinase C (PKC) dependence. Despite wide variations in species of origin and experimental detail, models based on immature cells fulfill these criteria. In common with ischemic preconditioning in the whole heart are temporal associations between sublethal and lethal ischemia, ligands able to trigger protection, PKC inhibitors able to block protection, and end points such as intracellular protein release and trypan blue uptake, which are more indicative of cell death by necrosis than apoptosis.

### Triggers for Preconditioning

Immature cardiocytes provide confirmatory evidence that early,3,8–10 and late3,8,10 preconditioning exists in human cardiomyocytes but not in human endothelial cells.10 Furthermore, there is sufficient adenosine release to confer protection to “naïve” cells, an effect mimicked by a nonselective adenosine agonist and blocked by a nonselective adenosine antagonist or PKC inhibitor.5 This pattern of adenosine-triggered hypoxic preconditioning is identical to that seen in cultured chick cardiomyocytes.11 However, this model has the advantage of permissive transfusion with efficiencies of 40% with calcium phosphate.12 Indirect evidence suggests that protection triggered by hypoxic preconditioning is A₁,11,13 and A₃13,14 adenosine receptor dependent. In contrast, an A₂a-selective agonist during brief hypoxia aggravates injury, whereas an antagonist is protective on its own and augments the protection seen with the nonselective adenosine agonist R-phenylisopropyladenosine,14 suggesting that preconditioning could be even more protective with concomitant blockade of the adenosine A₃ receptor. This observation is further reinforced by transient transfection of the cDNAs of the human A₁ and A₃ receptors. Monolayers expressing these receptors are more resistant to lethal hypoxia but more sensitive to the protective effects of sublethal/preconditioning hypoxia.12 This suggests that adenosine receptor occupancy is protective during both lethal and sublethal hypoxia, reflecting findings in the intact heart as well as findings for other G-protein–coupled receptor agonists in isolated immature cardiocytes.15 Another interesting aspect of these studies is that atrial myocytes, deficient in endogenous A₁ receptors, can be rendered A₁ responsive and resistant to simulated ischemia by forced expression of the human A₁ receptor.13 Moreover, protection initiated by the A₁ receptor seems longer lasting than that initiated by the A₃ receptor,13 and this may in part be explained by differential coupling of A₁ to phospholipase C and A₃ to phospholipase D.16

Evidence in these models favors endogenous adenosine as the initiator of preconditioning, but protection can also be triggered “directly” by morphine through opioid receptors.7 Under this circumstance, protection is prevented by ATP-sensitive K⁺ (K₄ATP) channel blockade before, but not necessarily during, lethal hypoxia.7 Although it is apparently controversial, there is similar evidence in the intact heart and emerging evidence in isolated adult cardiocytes indicating that the mitochondrial K₄ATP channel may not be the
end effector of protection. This would be in keeping with observations in embryonic myocytes, in which the trigger for preconditioning during sublethal hypoxia involves the mitochondrial export of superoxide generated at cytochrome b-c1 of complex III of the electron transport chain. Acetylcholine-triggered preconditioning in this model also requires mitochondria-derived superoxide, and this is also dependent on the opening of mitochondrial K\textsubscript{ATP} channels. The hypothesis that the opening of mitochondrial K\textsubscript{ATP} channels causes partial collapse of the mitochondrial potential and therefore “functional” uncoupling of electron transport with increased superoxide generation is attractive, potentially unifying and merits further attention.

**Signaling Pathways Leading to Protection**

The experiments above confirm and extend the knowledge gained in the intact heart of the ligands that can lead to preconditioning. However, the pathways that lie distal to adenosine (or similar) receptors are more controversial. Because hypoxic preconditioning in immature cardiomyocyte-based models of preconditioning, in common with preconditioning in the intact heart, is blocked by pharmacological inhibition of PKC, these models have been used to further explore the importance of individual PKC isotypes.

PKCs constitute a catalytic subunit linked by a flexible hinge region to a regulatory domain containing an amino acid sequence nearly identical to that used to recognize substrate. In the model proposed for PKC regulation, this pseudosubstrate site allosterically prevents the binding, and therefore phosphorylation, of target proteins. On activation, a conformational change is envisaged that opens up the hinge region and dissociates the pseudosubstrate domain, freeing the substrate binding site and also exposing residues that bind to specific receptors for activated C kinases (RACKs). The RACKs, in turn, are thought to traffic activated PKC isotypes to their correct subcellular location (Figure). Recent evidence is also emerging that other events may modulate PKC function through key phosphorylation events within an activation loop, which may allow activation in the absence of translocation.

In a model of hypoxic preconditioning of neonatal rat cardiomyocytes, PKC\textsubscript{e} and PKC\textsubscript{\(\delta\)} translocate in response to preconditioning, and translocation is associated with protection from subsequent more prolonged hypoxia. Johnson et al. have previously demonstrated that a peptide corresponding to residues 14 to 21 (V1-V2) of PKC\textsubscript{e} is capable of inhibiting the translocation of PKC\textsubscript{e}. It is thought that a RACK-binding domain exists in the V1-V2 region so that the peptide saturates the appropriate RACK, preventing the translocation of PKC\textsubscript{e} and protection.

We have adopted a complementary approach by expressing mutant PKC isotypes rendered constitutively active by deletions within the pseudosubstrate domain, which prevent the autoinhibition seen in the wild-type molecule (see above). We have shown that active PKC\textsubscript{\(\delta\)} consistently reduces hypoxic injury, an effect not seen with the expression of wild-type PKC\textsubscript{\(\delta\)}. These experiments demonstrate that active PKC\textsubscript{\(\delta\)} is able to trigger protection but do not indicate that PKC\textsubscript{\(\delta\)} is the endogenous isotype responsible for protection. The rat neonatal cardiocyte model has also been used to investigate the more distal mitogen-activated protein kinase (MAPK) pathways involved in protection. These experiments are controversial and heavily reliant on the nonspecific p38-MAPK inhibitor SB203580. Data from Mackay and Mochly-Rosen and preliminary data from our group demonstrate that p38-MAPK undergoes a period of prolonged activation during lethal hypoxia and that if this kinase is inhibited by SB203580, then injury is reduced. Moreover, the p38-MAPK isotype preferentially activated by simulated ischemia is
TABLE 2. Models of Preconditioning in Mature Cardiocytes

<table>
<thead>
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<th>Cell Type</th>
<th>PC Stimulus*</th>
<th>Lethal Injury†</th>
<th>End Point‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult rabbit cardiomyocyte</td>
<td>5–15 min of no glucose at 30°C, 30-min recovery</td>
<td>90–180 min of pelleting under mineral oil before resuspension in 85 mOsm buffer</td>
<td>Trypan blue uptake, morphology</td>
</tr>
<tr>
<td>Adult rabbit cardiomyocyte</td>
<td>10–15 min of pelleting at 37°C–38°C, 15–30 min of resuspension</td>
<td>60–180 min of pelleting under mineral oil before resuspension in 85 mOsm buffer</td>
<td>Trypan blue uptake, morphology</td>
</tr>
<tr>
<td>Adult rabbit cardiomyocytes</td>
<td>10 min of 80 mOsm, 20-min recovery</td>
<td>60–120 min of pelleting under mineral oil before resuspension in 85 mOsm buffer</td>
<td>Trypan blue uptake, morphology</td>
</tr>
<tr>
<td>Adult porcine cardiomyocytes</td>
<td>20 min of 100% N₂, 20-min recovery</td>
<td>120 min of 24 mmol/L K⁺ at 4°C</td>
<td>Velocity of shortening</td>
</tr>
</tbody>
</table>

All the models described use freshly isolated cardiomyocytes.  
*Pharmacological agents that initiate PC have been omitted.  
†In the studies examining various durations of injury, only those during which injury was significantly diminished by the PC stimulus are stated.  
‡Not all end points were measured in every study.

p38α,32 reinforcing the known role of this isotype in mediating cell death within this model.33

Cell-Based Models of Preconditioning Using Mature Cardiomyocytes

Isolated adult cardiomyocytes have been used for almost 2 decades to study cell injury,34 despite a rate of attrition as great as 30% in the first 24 hours after isolation.35 Therefore, models largely rely on suspensions of freshly isolated cells. The first, and most widely adopted, model is that described by Armstrong et al36 of lethal simulated ischemia achieved by overlaying rabbit adult cardiomyocytes compacted into a cell pellet with mineral oil (see Table 2). Preconditioning initiated by either a short period of cell pelleting or by suspension in glucose-free buffer shares many features with preconditioning in the intact heart, including the adenosine,36,37 PKC,36,38 and K_ATP20,35,39 dependence of protection.

Triggers for Preconditioning

Preconditioning is initiated in the intact rabbit heart by A1-selective agonists.40 However, in isolated rabbit cardiomyocytes, the relationship is more complex because an A1/A2-selective agonist does not substitute for 15 minutes of glucose-free preconditioning, but a nonselective adenosine receptor antagonist during the glucose-free period prevents preconditioning.36 This apparent paradox was resolved in a subsequent study in which 5 minutes of glucose-free incubation, with or without pyruvate, initiated preconditioning, which could be blocked by an A2- but not A1-specific antagonist.37 Exclusive initiation through the A1 receptor was confirmed by triggering preconditioning with a mixed A1 and A2 agonist alone or together with an A1, but not an A2, antagonist.37 The finding of A1-initiated protection is consistent with immature cardiocytes and isolated adult porcine cardiocytes.12,14,41 Protection can also be initiated through opioid receptors in the rabbit cardiomyocyte pelleting model,42 metabolic inhibition in adult rat cardiocytes,35 and chloride channels during cell swelling–induced preconditioning in adult rabbit cardiocytes.43

Signaling Pathways Leading to Protection

The PKC isotype dependence and MAPK pathways leading to preconditioning have also been examined in adult cardio-
adult rabbit cardiomyocytes and differentiated C2C12 (mouse skeletal muscle–derived) cells precondition, but HEK293 (human embryonic kidney–derived) cells, HIT-TIG (hamster pancreatic islet–derived) cells, and undifferentiated C2C12 cells do not.47 Similarly, although the supernatant from endothelial cells exposed to brief hypoxia is able to precondition ventricular myocytes,10,41 the endothelial cells themselves are not protected10 but have attenuated posthypoxic intercellular adhesion molecule-1 induction.48 Despite an inability to initiate preconditioning with simulated ischemia in undifferentiated cell lines, these cells still seem to be protected by the introduction of components of the preconditioning pathway.49,50 Therefore, undifferentiated cell lines may still be of use in the molecular dissection of pathways leading to protection.

Future Directions and Summary

To an outside observer, it must seem that the preconditioning field has stagnated in KATP channels and kinase pathways. The weight of evidence is overwhelming that both contribute to protection, but what is lacking is the detail. The manipulations required to determine this detail are most easily achieved in isolated cells. The work summarized in the present review has begun to examine this detail and to link sarcolemmal receptors with PKC, KATP channels, p42/44 MAPK, and p38-MAPK (see Figure). What is now required is further investment in models and tools to complete the task. In particular, a combination of techniques is required to confirm that the mechanistic insights derived from isolated cells reflect the mechanisms in vivo.51

The concern that the mechanisms of preconditioning may vary with the detail of the model has made many wary of extrapolating findings from cell-based models. However, within the present review, there is a high degree of concordance between the mechanisms underlying preconditioning in different cellular models and those found in the intact animal heart. Ultimately, irrespective of the model, insights into how preconditioning will need to be tested in the only circumstance that counts, true myocardial ischemia in humans.

References


Key Words: ischemic preconditioning ■ signaling ■ cytoprotection ■ isolated cardiomyocytes
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Circ Res. 2000;86:926-931
doi: 10.1161/01.RES.86.9.926

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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