Cardioprotection
Nitric Oxide, Protein Kinases, and Mitochondria

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Over the past 2 to 3 decades, several phenomena have been identified that provide powerful protection against myocardial infarction and other sequelae of ischemia/reperfusion: myocardial hibernation that is related to stunning, ischemic preconditioning, delayed or second-window ischemic preconditioning, ischemic postconditioning, and their pharmacological recruitment. Stunning and hibernation share contractile function as an end point. In stunning, reduced postischemic contractile function is viewed as reversible injury, whereas in hibernation dysfunction is viewed as an adaptive response. Ischemic preconditioning is characterized by infarct size reduction as its most robust end point but shares with hibernation the underlying idea of a regulated protective response. These phenomena have been confirmed in patients with coronary artery disease, Nitroglycerin induces delayed protection against perinterventional ischemic ECG alterations, contractile dysfunction, and pain sensation, and cyclosporin A, which inhibits opening of the mitochondrial permeability transition pore (MPTP), attenuates reperfusion injury in patients with acute myocardial infarction.

Articles pp 1961 and 1970

Stunning Versus Hibernation Versus Preconditioning: What Is the Difference?
The “new ischemic syndromes” have intrigued scientists and clinicians trying to understand their underlying pathophysiology and to recruit their cardioprotective potential. This initial enthusiasm has not materialized into translational medicine. Why is that? There has been a long but ultimately fruitless debate as to whether the phenotype of hibernating myocardium is the result of ongoing ischemia with reduced baseline coronary blood flow or repetitive cycles of ischemia/reperfusion and consequent stunning without reduced baseline flow. Clearly, perfusion-contraction matching cannot be maintained for >12 hours. Conversely, repetitive cycles of ischemia/reperfusion with subsequent stunning finally result in hibernating myocardium with reduced contractile function and baseline blood flow. Thus, the cumulative effect of stunning is a progression to hibernation. True stunning, if seen at all, is rare, modest, and, apart from its paradigmatic value, important only in its cumulative manifestation as hibernation. Chronic, reversible contractile dysfunction also results from coronary microembolization in which small microinfarcts initiate an inflammatory process, including increased expression of tumor necrosis factor-α. Upregulated cardioprotective gene and protein programs were identified in preconditioned and hibernating myocardium of animals and humans, thus making the distinction between hibernation and preconditioning more ambiguous.

The report by Shen et al in this issue of Circulation further undermines the artificial distinction between stunning, hibernation, and preconditioning. In chronically instrumented pigs, 6 cycles of severe coronary stenosis with subsequent reperfusion did not induce necrosis but did result in profound contractile dysfunction. Repetitive coronary stenosis also resulted in the upregulation of potentially protective proteins and reduced infarct size from complete coronary occlusion and reperfusion. In other words, a protocol of cumulative stunning induced the phenotype of hibernating myocardium that was preconditioned. The transcripts of genes encoding proteins involved in cell cycle control and autophagy were upregulated in the myocardium at risk with this repeated coronary stenosis but not with a delayed preconditioning protocol. Protein kinase C (PKC)-ε was not translocated to the particulate fraction, and NO synthase (NOS) activity was not increased, which again was different from delayed preconditioning. In pigs, however, the α isoform of PKC is more important in preconditioning than the ε isoform, and the lack of effect of NOS blockade does not exclude an important role for NO. A causal role for the differentially expressed gene and protein program was not established, largely because of the only retrospective tissue sampling and the non–hypothesis-driven nature of such a genomic approach. Unfortunately, the study did not address inflammatory signals such as tumor necrosis factor-α, which protects from infarction several hours after coronary microembolization.

Cardioprotective Signaling
In this issue of Circulation, West et al address the interaction of NO with mitochondria in effecting cardioprotection. Using mice with cardiomyocyte-specific constitutive overexpression of inducible NOS (iNOS), they confirm the NO hypothesis of delayed preconditioning as originally proposed by Bolli et al and extend it by demonstrating sustained protection by iNOS overexpression. They attribute protection by iNOS overexpression or pretreatment with an NO donor to inhibition of MPTP opening. Multiple end points of protec-
tion, notably infarct size in situ and ex vivo, functional recovery, and reactive oxygen species (ROS) formation ex vivo, were measured. Three different methods were used to determine MPTP opening in isolated mitochondria, and the results were confirmed by nicotinamide dinucleotide (NADH) loss in ex vivo hearts. The MPTP opener atractyloside did not abolish protection in iNOS-overexpressing hearts. The classic MPTP inhibitor cyclosporin A induced disparate effects; it increased ROS formation on reperfusion but improved functional recovery. Cyclosporin A also decreased cytochrome c release into the cytosol but did not affect mitochondrial deoxyglucose retention. The atractyloside and cyclosporin A data are somewhat difficult to reconcile, but the authors' explanation of an NO action somewhere upstream of MPTP appears plausible. The mechanistic strength of the present study notwithstanding, important questions remain: What is the role of the other enzymes that are upregulated in delayed preconditioning (cyclooxygenase 2, manganese superoxide dismutase, aldose reductase), and how do they relate to MPTP opening? Which other proteins are upregulated or downregulated in cardiomyocytes with constitutive iNOS overexpression, and what is their role in cardioprotection? The phenotype of the iNOS-overexpressing mice is not entirely innocuous; they have mild left ventricular hypertrophy, contractile dysfunction, and alterations in respiratory chain complexes.

**NO: Trigger, Mediator, Potential Effector of Cardioprotection**

NO at low concentration increases ventricular function and matches myocardial oxygen consumption to contractile function in short-term hibernating myocardium; NO at high concentration triggers inflammation and suppresses contractile function. Exogenous NO triggers preconditioning, whereas NOS-derived endogenous NO is not important for classic ischemic preconditioning. Endogenous NO is both a trigger (through endothelial NOS) and a mediator (through iNOS) of delayed ischemic preconditioning. In addition, ischemic postconditioning uses NO as a signal. Apart from the specific NOS isoform that generates NO and its cellular (endothelium, cardiomyocyte, leukocyte, etc) and subcellular (cytosol, caveolae, etc) localization, significant amounts of NO are formed nonenzymatically during myocardial ischemia, so negative findings using pharmacological NOS blockade do not rule out an important role for endogenous NO.

**Protein Kinase Activation in Cardioprotection**

A variety of protein kinases are activated and interact at multiple levels and at various time points during ischemia/reperfusion. Three different cardioprotective protein kinase programs have been proposed (Figure). The first program is recruited during the trigger and mediator phase of acute ischemia.
ischemic preconditioning and is shown in Figure, A. After activation of G-protein–coupled receptors by adenosine, bradykinin, opioids, urocortins, etc., or of receptors for peptides such as natriuretic peptides, PKC is directly activated (only through adenosine) or phosphoinositide 3 kinase (PI3K)/Akt is activated with further downstream activation of endothelial NOS and NO formation, guanylate cyclase activation, protein kinase G (PKG) and PKC activation; this leads to the activation of the mitochondrial ATP-dependent K channel (KATP) and ROS formation, resulting in p38 mitogen-activated kinase and PKC activation23,25 and priming of MPTP for opening.26 The second program is recruited during early reperfusion with the execution phase of acute ischemic preconditioning and with ischemic postconditioning and is shown in Figure, B. Activation of sarcoclemmal G-protein–coupled receptors or of receptors for growth factors results in activation of the reperfusion injury salvage kinase (RISK) program. RISK involves the parallel activation of the PI3K/Akt and the extracellular regulated kinase system (ERK) and for NO (the Table).33

### Table. Mitochondrial Target Molecules/Structures in Cardioprotection That Have Interaction With Protein Kinases and NO

<table>
<thead>
<tr>
<th>Target Molecule/Structure</th>
<th>OM</th>
<th>IMS</th>
<th>IM</th>
<th>Matrix</th>
<th>Target of Kinases</th>
<th>Function</th>
<th>Interaction</th>
<th>Effect of NO on Expression/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory chain complexes</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Respiration ↑, ROS ↑</td>
<td>↓</td>
<td></td>
<td></td>
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<tr>
<td>Uncoupling proteins</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Proton leakage ↑, respiration ↑, ROS ↓</td>
<td>↑</td>
<td></td>
<td></td>
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<tr>
<td>KATP/Cx43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PKG, PKCe</td>
<td>ROS ↑, matrix volume ↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Cx43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GSK3β</td>
<td>ROS ↑</td>
<td>↑</td>
<td></td>
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<tr>
<td>MPTP</td>
<td>(VDAC)</td>
<td>(ANT)</td>
<td>(Cyp D)</td>
<td>GSK3β</td>
<td>Matrix volume ↑, OM rupture ↑</td>
<td>↑, ↓ Dose dependently</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antioxidant defense</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PKC</td>
<td>Aldehyde metabolism ↑</td>
<td>↑</td>
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<tr>
<td>MnSOD</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>ROS ↓</td>
<td></td>
<td>↓</td>
<td></td>
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<tr>
<td>Glutathione</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>ROS ↓</td>
<td></td>
<td>↓</td>
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</tr>
</tbody>
</table>

OM indicates outer membrane; IMS, intermembrane space; IM, inner membrane; Cx43, connexin 43; PKG, protein kinase G; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide transporter; Cyp D, cyclophilin D; MnSOD, manganese superoxide dismutase; ↑, increased formation/activity; ↓, decreased formation/activity. (): dispensable; and ?, still under debate.

Mitochondrial Targets of Cardioprotection and Their Interaction With Protein Kinases and NO

Mitochondria are the major site of cellular energy/ATP production. The respiratory chain accepts electrons from NADH/H and flavine adenine dinucleotide (FADH)/H and transports them over 4 complexes ultimately onto oxygen, creating a proton gradient that then drives ATP production. Apart from ATP production and its role in cell function and survival, mitochondria are decisive elements for cell death by apoptosis, autophagy, and necrosis and, conversely, are targets for protection from cell death by several of the above kinases (PKC, PKG, GSK3β) and for NO (the Table).33

Respiratory Chain

Respiratory chain proteins are downregulated in repetitively14 or chronically13 poststenotic myocardium. ADP-stimulated respiration is reduced by ischemia. Ischemic preconditioning does not prevent this reduction.34 The respiratory chain releases small amounts of ROS predominantly by complex I under physiological conditions.35 Partial uncoupling of the respiratory chain protects against ischemia/reperfusion injury similar to preconditioning, supporting the importance of mitochondrial ROS for cardioprotection. During early reperfusion, ROS formation from various sources, including the respiratory chain, is largely augmented. Reversible inhibition of complex I by S-nitrosation (NO-mediated modification of thiols) is cardioprotective by limiting excess ROS formation during reperfusion.36
Uncoupling proteins in the inner membrane mediate a leakage of protons back into the matrix and thereby accelerate oxygen consumption. Delayed ischemic preconditioning is associated with upregulation of uncoupling proteins, and mitochondria with upregulated uncoupling proteins have diminished ROS formation.37

Mitochondrial KATP Channel
The K_{ATP} channel in the inner membrane is inhibited by ATP and activated by PKCε and PKG. The exact molecular composition of the K_{ATP} channel and the participation of the sulfonylurea receptor subunit SUR2A and the potassium channel proteins Kir6.1 and 6.2 remain elusive. A purified inner membrane fraction, including the adenine nucleotide transporter and succinate dehydrogenase, confers K_{ATP} channel activity and is targeted by K_{ATP} agonist/antagonist drugs.38 Mitochondrial K_{ATP} channels are causally involved in ischemic preconditioning and postconditioning.39,40 However, insight into their exact function in cardioprotection is difficult because their molecular structure is still unclear, they have not yet been identified in cardiomyocytes by patch-clamp techniques, and pharmacological agents such as diazoxide (agonist) and 5-hydroxydecanoate (antagonist) are not specific.41 Mitochondrial K_{ATP} channel opening induces potassium and accompanying water flux along the electrochemical gradient, resulting in matrix swelling. The influx of potassium also triggers modest ROS formation by respiratory gradient, resulting in matrix swelling. The influx of potassium and accompanying water flux along the electrochemical gradient, resulting in matrix swelling. This leads to rupture of the outer membrane results in depolarization of the membrane potential and all of these conditions occur during ischemia and reperfusion. The opening of the MPTP results in depolarization of the membrane potential and matrix swelling. This leads to rupture of the outer membrane and release of proteins such as cytochrome c from the intermembrane space into the cytosol. Mitochondria and sarcoplasmic reticulum interact, and the mitochondrial Ca^{2+} concentration during early reperfusion is determined by sarcoplasmic reticulum–dependent Ca^{2+} oscillations in the cytosol.47 Cyclosporin A blocks the enzymatic activity of cyclophilin D, inhibits MPTP opening, and attenuates reperfusion injury.9,35

Antioxidant Defense
Aldose reductase metabolizes toxic aldehydes, which are generated by lipid peroxidation and contribute to cell death. It is localized in the cytosol; however, it translocates in a PKC-dependent mechanism into mitochondria. Delayed ischemic preconditioning is known to increase aldose reductase protein and activity, and inhibition of its activity abolishes protection by delayed preconditioning.48 Manganese superoxide dismutase in the matrix catalyzes the reaction of superoxide to H_{2}O_{2} and oxygen. Delayed ischemic preconditioning upregulates manganese superoxide dismutase and decreases ROS.48 Glutathione peroxidase, another ROS detoxifying enzyme, consumes glutathione to form hydrogen peroxide. Glutathione, however, reacts with NO to form S-nitrosoglutathione, which, during its degradation, may form glutathionyl radicals and subsequently ROS.49

The apparent paradox that characterizes both ischemic preconditioning and postconditioning, ie, a little ischemia/reperfusion before or after sustained ischemia/reperfusion protects, also is found at the signaling level for ROS, MPTP, and NO. Whereas high levels of ROS are detrimental for cardiomyocytes, moderate levels of ROS function as signaling molecules for cardioprotection by activating protein kinases. Likewise, inhibition of MPTP opening is cardioprotective, but transient opening of MPTP is required for cardioprotection.50 MPTP opening depends on NO. Low levels of NO prevent MPTP opening, whereas high NO levels accelerate MPTP opening and cytochrome c release.36 Part of the apparent paradox may be methodological in nature. Subsarcolemmal and interfibrillar mitochondria differ in their morphology and function. It is possible that sarcolemmal mitochondria serve a signaling function, whereas interfibrillar mitochondria are targets of damage and protection from it.

Apart from the translational gap from mice to humans, we need to know more about fundamental cardioprotective signaling. Do not take NO for the answer (yet).

Disclosures
None.

References
Heusch et al Cardioprotection and Its Signaling 1919


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Circulation. 2008;118:1915-1919
doi: 10.1161/CIRCULATIONAHA.108.805242
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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