Cardioprotection From Ischemia/Reperfusion Injury
– Basic and Translational Research –

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Because ischemic heart diseases (IHDs) are a major cause of mortality and heart failure in western countries and Japan. Thus, developing novel drugs or interventions to improve the clinical outcomes of patients with IHDs is a world-wide unmet medical need. Because myocardial infarct size is recognized as a determinant of acute and long-term prognosis in patients with acute myocardial infarction (AMI), reducing the size of the infarct is a therapeutic goal. Early reperfusion can prevent the myocardial damage due to ischemia and reduce infarct size. This concept was quickly introduced for patients with AMI by the use of primary percutaneous coronary intervention (PCI) and thrombolytic therapy. Although reperfusion can salvage myocardium after sustained ischemia, the reperfusion itself paradoxically induces myocardial injury named “reperfusion injury”, which attenuates the benefits of myocardial reperfusion.

Over 20 years ago, Murry et al first demonstrated that brief episodes of nonlethal ischemia and reperfusion before sustained ischemia reduce myocardial infarct size, and it was termed “ischemic preconditioning”. The infarct-size limiting effects of ischemic preconditioning have been consistently confirmed in many species and different models of ischemia-reperfusion (IR) injury. Brief episodes of nonlethal IR at the onset of reperfusion also reduce myocardial infarct size, known as “ischemic postconditioning”. The therapeutic goal of ischemic postconditioning is to attenuate “reperfusion injury” (Figure 1). After these landmark studies, extensive basic investigation has elucidated the underlying mechanisms of ischemic conditioning and led to their translation into the clinical setting by pharmacological agents.

Here, I will review the potential mechanisms of ischemic conditioning and the “proof-of-concept” translational studies.

Ischemic Preconditioning

Ischemic preconditioning confers different forms of cardioprotection and can reduce infarct size, lethal arrhythmia and contractile dysfunction. Originally, Murry et al hypothesized that ATP preservation during ischemia is the major cardioprotective mechanism underlying ischemic preconditioning, but this hypothesis is not sufficient to explain its cardioprotection. Currently, the major effects of ischemic preconditioning are assumed to prevent cell death due to reperfusion injury. Different factors such as autacoids (eg, adenosine, bradykinin, opioids), their respective receptors, kinase signaling pathways, and mitochondrial modulation are involved in ischemic conditioning. Modification of these factors by pharmacological agents mimics the cardioprotection by ischemic preconditioning and provides a novel therapeutic intervention for IHDs. Here, the potential mechanisms of ischemic conditioning and its “proof-of-concept” translational studies are reviewed. In the near future, large, multicenter, randomized, placebo-controlled, clinical trials will be required to determine whether pharmacological and ischemic preconditioning can improve the clinical outcomes of patients with IHDs.

Key Words: Pharmacological conditioning; Postconditioning; Preconditioning; Proof-of-concept clinical studies; Remote conditioning
pathways and mitochondria modulation are implicated in the cardioprotective effects of ischemic preconditioning (Figure 2). Nonlethal ischemia results in the production of endogenous autacoids such as adenosine, opioids, bradykinin. These autacoids initiate numerous signaling pathways that activate protein kinases through their respective receptors (Figure 2). These cardioprotective signaling pathways, including extracellular-regulated kinase (ERK)/p38, phosphatidylinositol 3 kinase (PI3K)/Akt, protein kinase C and protein kinase G, lead to the inactivation of mitochondrial glycerol synthase kinase-3β (GSK-3β). The inactivation of GSK-3β inhibits the opening of the mitochondrial permeability transition pore (mPTP), which plays a crucial role in myocardial necrosis. Reactive oxygen species (ROS) production in mitochondria, where the mitochondrial ATP-dependent potassium channels play an essential role, is also involved in the cardioprotective mechanisms of ischemic preconditioning. Although these findings are consistently observed in experimental models, applying ischemic preconditioning in the clinical setting is restricted to scheduled cardiac operation and elective PCI. A meta-analysis showed that ischemic preconditioning may provide additional myocardial protection over cardioplegia alone. However, cardiovascular surgeons do not like to repeatedly clamp and unclamp the aorta in patients with advanced atherosclerosis.

The cardioprotective effects of ischemic preconditioning disappear 2–3 h after the onset of the preconditioning insult, but reappear 24 h later. This phenomenon is recognized as “delayed” ischemic preconditioning. A major difference in the cardioprotective mechanisms of early and delayed preconditioning is that early ischemic preconditioning results in the modification or turnover/translocation of existing molecules, whereas delayed ischemic preconditioning is exerted by newly synthesized cardioprotective proteins. The triggers and mediators of early and delayed ischemic preconditioning are largely common and lead to the activation of transcriptional factors (Figure 2). They transcribe the de novo synthesized proteins involved in delayed ischemic preconditioning, including manganese superoxide dismutase, heat stress proteins and inducible nitric oxide synthase. A potential clinical example of delayed ischemic preconditioning is “pre-infarct angina” by which patients who have suffered from repeated episodes of angina can preserve postischemic left ventricular function. However, the clinical application of delayed ischemic preconditioning has not been fully investigated.

**Ischemic Postconditioning**

In 2003, Zhao et al demonstrated that brief episodes of coronary occlusion and reperfusion at the onset of reperfusion following 60 min of coronary occlusion reduced myocardial infarct size by 40% in canine hearts. The protocols for ischemic postconditioning have been extensively investigated and the cardioprotective effects afforded by ischemic postconditioning have been confirmed in many species, including humans. At the same time, the existence of reperfusion injury is strongly supported by the cardioprotection afforded by the intervention during reperfusion. One proposed mechanism through which ischemic postconditioning attenuates reperfusion injury is the prevention of rapid changes in intracellular pH and robust ROS generation. In the ischemic/reperfused myocardium, the ionic environment dramatically changes. Within a few minutes of myocardial ischemia, the interstitial and intracellular pH values rapidly decrease due to the accumulation of protons. Upon reperfusion, these interstitial protons are promptly washed out and intracellular low pH is corrected through the sarcolemmal Na+/H+ exchanger, which results in a massive Na+ influx. Intracellular Na+ accumulation stimulates the passive, inverted action of the sarcolemmal Na+/Ca2+ exchanger and in turn allows intracellular Ca2+ overload, which causes myocardial cell death or myocardial contractile dysfunction. Therefore, the rapid normalization of intracellular pH enhances myocardial damage in the early stage of reperfusion and a gradual correction of low intracellular pH by acidic reperfusion would be cardioprotective through inhibition of the opening of mPTP, preventing the activation of Ca2+-dependent protease and reducing the gap junction communication involved in spreading cell death. The cardioprotective effects of ischemic postconditioning are associated with the maintenance of low intracellular pH during reperfusion and are comparable to the effects of acidic reperfusion. Furthermore, during the early stage of reperfusion, there is robust ROS production in vascular endothelium, cardiomyocytes and mito-
chondria. ROS generation is suppressed in the postconditioned heart.\textsuperscript{33,34}

In addition to the effects of ischemic postconditioning on ionic changes and ROS production, ischemic postconditioning activates intracellular signal transduction in a way that is analogous to ischemic preconditioning. Autacoids (eg, adenosine, bradykinin and opioids), natriuretic peptides (atricial and brain natriuretic peptides) and cytokines play a crucial role in postconditioning\textsuperscript{35} (Figure 2). These autacoids activate a kinase signaling pathway known as the reperfusion injury risk kinases (RISK) pathway, which consists of the PI3K/Akt and ERK1/2 pathways.\textsuperscript{36} The activation of RISK pathway inactivates GSK3β, which inhibits mPTP opening at reperfusion. The inhibition of mPTP opening is the final common target

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**Figure 2.** Signaling pathways involved in ischemic preconditioning and postconditioning. Several autacoids play an essential role in “early” ischemic preconditioning. Upon binding to their respective receptors, autacoids activate intracellular signaling pathways. They then modulate mitochondrial components. Ischemic postconditioning activates intracellular signal pathway in a way that is analogous to ischemic preconditioning. Delayed ischemic preconditioning recruits transcriptional factors that transcribe de novo proteins, which afford cardioprotection against ischemia/reperfusion injury. See text for abbreviations.

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**Table 1. Clinical Trials of Ischemic/Pharmacological Conditioning in Patients With STEMI**

<table>
<thead>
<tr>
<th>Conditioning</th>
<th>Outcome</th>
<th>Reference</th>
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<tr>
<td>Postconditioning</td>
<td>Decrease (IS), improved LVEF at 12 months</td>
<td>25, 39</td>
</tr>
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<td>Remote conditioning</td>
<td>Decrease (IS)</td>
<td>86</td>
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<td><strong>Pharmacological agents</strong></td>
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<td>Adenosine</td>
<td>Decrease (IS)</td>
<td>44</td>
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<tr>
<td>Atrial natriuretic peptide</td>
<td>Decrease (IS), improved LVEF at 6–12 months</td>
<td>49</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Decrease (IS), improved LVEF at 6 months</td>
<td>62</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High dose</td>
<td>No change (IS, LVEF)</td>
<td>52, 53, 53</td>
</tr>
<tr>
<td>Low dose</td>
<td>Improved LVEF at 6 months</td>
<td>55, 56</td>
</tr>
<tr>
<td>Nicorandil</td>
<td>No change (IS)</td>
<td>49</td>
</tr>
<tr>
<td>Statin</td>
<td>No change (IS)</td>
<td>67</td>
</tr>
<tr>
<td>Protein kinase C inhibitor</td>
<td>No change (IS, LVEF)</td>
<td>68</td>
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STEMI, ST-elevation myocardial infarction; IS, infarct size; LVEF, left ventricular ejection fraction.
Cardioprotection From IR Injury

through which the signaling pathways can protect against necrosis. Activation of the JAK-STAT pathway by cytokines has also been implicated in the cardioprotective effects induced by ischemic preconditioning. This pathway is named the “survivor activating factor enhancement (SAFE)” pathway; however, it is not fully understood how SAFE pathway is involved in the cardioprotection afforded by ischemic postconditioning. In contrast to ischemic preconditioning, ischemic postconditioning can be easily applied in patients with AMI undergoing primary PCI. A small number of “proof-of-concept” studies have showed that a postconditioning procedure reduced myocardial infarct size and improved left ventricular ejection fraction (LVEF) at 1 year. Prospective and randomized studies are now ongoing to evaluate the infarct-size-limiting effects of ischemic postconditioning in patients with ST-segment elevation myocardial infarction (STEMI) who are undergoing primary PCI.

Coronary blood flow must be interrupted in order to apply ischemic postconditioning, which increases the time required for the procedure and could potentially cause atherosclerotic emboli. Pharmacological manipulation of autacoids, their receptors, kinase signaling pathways and modulation of the mPTP opening, all of which are involved in ischemic postconditioning, could be easily utilized to treat patients with AMI undergoing primary PCI (Table 1). Adenosine is a representative autacoid that is involved in both ischemic preconditioning and postconditioning, and its administration at the onset of reperfusion provides myocardial protection from IR injury in animal models. The results of a randomized, double-blinded, placebo-controlled multicenter trial of a 3-h adenosine infusion as an adjunct to thrombolytic reperfusion in the treatment of anterior wall STEMI (AMISTAD-II) have been reported. Clinical outcomes, including new congestive heart failure, first re-hospitalization for chronic heart failure and death, were not significantly improved with adenosine administration, although infarct size was reduced in response to a high-dose infusion. Post-hoc analysis revealed that adenosine infusion within the first 3.17 h after the onset of anterior wall STEMI enhanced early and late survival, and reduced the composite clinical endpoints of death or chronic heart failure at 6 months. In the J-WIND study, a multicenter, randomized clinical trial was conducted to test the acute effect of either the sarcolemmal KATP channel opener, nicorandil, or the recombinant human atrial natriuretic peptide (ANP), carperitide, as an adjunct to successful PCI. The administration of carperitide, but not nicorandil, produced a small but significant 15% reduction in myocardial infarct size and an improvement in LVEF.

Experimental studies showed that erythropoietin, a hematopoietic cytokine, reduces myocardial infarct size and prevents cardiac remodeling in the chronic stage. In patients with STEMI, the administration of a high dose of erythropoietin did not improve LVEF or reduce infarct size; however, the use of erythropoietin was related to fewer major adverse cardiovascular events in 1 study. In contrast, a low dose of erythropoietin appears to be cardioprotective. Platelet activation by a high-dose of erythropoietin and the existence of an optimal dose for limiting infarct size will explain the dose-dependent discrepancy of erythropoietin-induced cardioprotection. Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models. Recently, Piot et al demonstrated that the mPTP inhibitor, cyclosporine A, administered as an intravenous bolus immediately before coronary artery reperfusion by PCI, resulted in a 40% reduction in enzyme release and prevented cardiac remodeling. The data are promising and large, multicenter, randomized, placebo-controlled, clinical trials are ongoing to clarify the effects of a low dose of erythropoietin on cardiac function after 6 months in patients with AMI who received successful PCI in Japan (UMIN000005721). Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models. Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models. Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models. Pharmacological inhibitors of mPTP reduce myocardial infarct size in experimental models.
required to elucidate the improvement in clinical outcomes.

To date, most clinically tested agents that induce cardioprotection, except adenosine and cyclosporine A, have failed to reduce infarct size in the clinical setting\textsuperscript{[63-65]} (Table 1). These negative results of "proof-of-concept" studies can be attributed to multiple factors.\textsuperscript{[66-68]} Pharmacological intervention as an adjunct to primary PCI is estimated to be effective for only 25% of AMI patients with an infarct size larger than 20% of the left ventricle and who have adverse symptoms.\textsuperscript{[68,69]} Proper patient selection is required to evaluate the benefit of pharmacological conditioning. In addition to the ischemic risk zone, infarct size is also determined by the duration of ischemia. If the duration of ischemia exceeds beyond 60 min, the infarct-size limiting effects of ischemic postconditioning are largely attenuated in experimental models.\textsuperscript{24} Thus, some proportion of patients in the study may have already been beyond the appropriate time-window within which myocardial salvage can be achieved. Another important point is the timing of drug administration. Reperfusion injuries such as robust ROS production, $\text{Ca}^{2+}$ overload and mPTP opening occur within the first few minutes of myocardial reperfusion.\textsuperscript{70} In the cyclosporine A study, this compound was administered just before coronary artery reperfusion by PCI, whereas most drugs were administered after successful reperfusion therapy. Finally, we need to consider confounders such as sex and age and comorbidities such as hypercholesterolemia, diabetes and hypertension, which are not present in animal studies as compared with clinical reality.\textsuperscript{71} For example, pharmacological postconditioning with cyclosporine A failed to provide cardioprotection in the prediabetic but normoglycemic heart of Zucker obese rats.\textsuperscript{72} Erythropoietin fails to exert infarct-size limiting effects in hypertensive hypertrophied hearts.\textsuperscript{73} Thus, both appropriate study design and execution are required to translate future novel cardioprotective agents into the clinical setting.\textsuperscript{66,67}

**Remote Ischemic Conditioning**

Brief episodes of nonlethal ischemia and reperfusion applied to the organ or tissue distal to the heart reduce myocardial infarct size, which is known as "remote ischemic conditioning".\textsuperscript{74,75} Transient upper or lower limb ischemia is a simple noninvasive stimulus with important potential clinical applications and high-cost performance. Furthermore, the remote ischemic conditioning procedure can be applied before and during sustained ischemia\textsuperscript{76} and at the onset of reperfusion.\textsuperscript{77} An experimental study showed that the infarct-size-limiting effects

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**Figure 4.** Detection of fluorescent-labeled nano-sized particles in ischemic/reperfused myocardium. Representative photographs at 3 h after myocardial infarction of hearts from rats that received fluorescent-labeled nano-sized liposomes. (Upper panels) Short axis cross-section of each heart (bright field). (Middle panels) Same sections analyzed under a fluorescence microscope. (Lower panels) Same sections stained with triphenyltetrazolium chloride. Note that high fluorescent areas correspond to the infarcted and border areas. Scale bar=5 mm. (Adapted from Takahama and Minamino\textsuperscript{90} with permission.)
of remote conditioning are comparable to the effects of ischemic postconditioning. It remains unclear how remote ischemic conditioning exerts cardioprotection; however, 2 major hypotheses have been proposed (Figure 3). The neural hypothesis states that autacoids released from the ischemic remote organ affect the local afferent neural pathway, which in turn, activates the efferent neural pathways to trigger end-organ protection. The humoral hypothesis states that autacoids released from the ischemic remote organ are transported to the end organ, resulting in the activation of kinase signaling pathways in the end organ. Remote ischemic preconditioning is associated with the activation of PI3K/Akt or STAT5 in the heart. The clinical application of remote ischemic conditioning was tested in patients undergoing CABG, but the results were inconsistent. Multicenter randomized double-blinded controlled clinical trials to clarify the effects of remote conditioning on clinical outcomes and the incidence of atrial fibrillation in patients with CABG are now ongoing. Recently, remote ischemic conditioning before hospital admission was shown to increase myocardial salvage measured by myocardial perfusion imaging and have a favorable safety profile in patients with AMI.

**Future Directions**

Recent advances in nanotechnology open up new possibilities in the development of drug delivery systems (DDS) for the treatment of patients with IHDs. DDS enhance the therapeutic concentrations of the drugs in diseased tissues and reduce the side effects. Nano-sized particles can passively accumulate in tissues where vascular permeability is enhanced. This concept is particularly applicable for developing anti-cancer and anti-inflammatory drugs, because vascular permeability is enhanced in tumors and inflamed tissues. In the rat IR model, the intravenous administration of nano-sized liposomes containing adenosine, but not free adenosine, at the onset of reperfusion significantly reduced myocardial infarct size and lethal arrhythmia during reperfusion. Encapsulated adenosine in nano-sized liposomes enhances the cardioprotective effects of adenosine and attenuates the hypotension induced by the systemic administration of adenosine. Targeting cardioprotective agents to ischemic/reperfused tissues using nano-sized liposomes may maximize the effect of the drug and minimize its side effects. Liposomes are a promising DDS for developing new treatments for patients with AMI who have undergone successful PCI.

MicroRNAs have emerged as important regulators of gene expression that affects cardiovascular function. MicroRNAs regulate gene expression through the degradation and translational inhibition of target messenger RNAs. IR stimuli alter the expression of microRNAs. Recent studies revealed that microRNAs are implicated in cardiac pathology including hypertrophy and failure and IR injury (Table 2). Therefore, microRNAs are novel promising therapeutic targets for IHDs. Cheng et al demonstrated that ischemic preconditioning up-regulates microRNA 21, which protects the heart against IR injury. Yin et al showed that an injection of microRNAs induced by ischemic preconditioning in the heart exerted cardioprotective effects against IR injury, which is comparable to that induced by the late phase of ischemic preconditioning. With advances in nanotechnology, microRNAs are potentially good candidates for targeting ischemic/reperfused myocardium with nano-sized liposomes.

**Perspectives**

Basic and translational research examining the therapeutic potential of ischemic conditioning are now actively ongoing. We need to continue to investigate the molecular mechanisms of ischemic conditioning, improve DDS, design study protocols to consider the timing and dose of drug administration and select patients who can benefit from pharmacological intervention. These efforts will lead to solving the unmet medical need for therapeutic drugs and interventions that improve the clinical outcomes of patients with IHD.

### Table 2. MicroRNAs Involved in Hypertrophy/Failure and Ischemia/Reperfusion

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<tr>
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<th>Hypertrophy/failure&lt;sup&gt;94&lt;/sup&gt;</th>
<th>Ischemia&lt;sup&gt;95&lt;/sup&gt;</th>
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NC, not changed; ND, not determined.
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