Remote ischemic preconditioning elaborates a transferable blood-borne effector that protects mitochondrial structure and function and preserves myocardial performance after neonatal cardioplegic arrest

Lixing Wang, MD, PhD,a Norihiko Oka, MD,a Michael Tropak, PhD,b John Callahan, PhD,b,c John Lee, MD,a Greg Wilson, MD,d Andrew Redington, MD,b and Christopher A. Caldaronea,c

Objective: Remote ischemic preconditioning is known to elicit production of a blood-borne cardioprotective factor that is infarct sparing in models of ischemia–reperfusion injury and myocardial damage reducing after cardiopulmonary bypass in human subjects. The mechanism of protection remains incompletely understood. In this study, we examined effects on mitochondrial structure and function in a noninfarct model of cardioplegic arrest.

Methods: Explanted neonatal rabbit hearts were mounted in a Langendorff preparation and perfused with dialysate of blood taken from sham-treated or remotely preconditioned rabbits. Each heart was subsequently subjected to 1-hour cardioplegic arrest and 30-minute reperfusion periods, during which hemodynamic responses were measured. Mitochondria were isolated for structural and functional measurements.

Results: Relative to hearts with sham-treated dialysate, myocardial performance (systolic pressure, maximum positive and negative first derivatives of left ventricular pressure, and left ventricular end-diastolic pressure) was better preserved with dialysate from preconditioned rabbits. Similarly, mitochondria isolated from hearts with dialysate from preconditioned rabbits showed preserved respiration at complex I and IV in the electron transport chain (P < .01 and P < .05, respectively). Mitochondrial outer membrane integrity was also preserved, with diminished sensitivity of mitochondrial respiration to exogenous cytochrome c (P < .01) and less cytosolic diffusion of cytochrome c (P < .01). Mitochondrial resistance to calcium-mediated mitochondrial permeability transition pore opening was not affected.

Conclusion: The cardioprotective factor in plasma dialysate after remote preconditioning preserves mitochondrial structure and function in a noninfarct cardioplegic arrest model. This protection is associated with preservation of global myocardial performance.

Ischemic preconditioning is a phenomenon in which brief antecedent ischemic periods render tissue more resistant to subsequent prolonged and potentially lethal ischemic insults. First described by Murry and colleagues,1 preconditioning is now recognized as one of the most potent innate protective mechanisms against ischemia–reperfusion injury. Although preconditioning-mediated myocardial protection is well documented in preclinical experimental models, there have been relatively few descriptions of its clinical use, reflecting the practical difficulties in inducing local tissue ischemia before an ischemic insult.2–4

Unlike classic ischemic preconditioning, which requires ischemia of the target organ, remote ischemic preconditioning (rIPC) uses ischemia of a distant organ to
confer the protection against myocardial infarction.\(^5\)\(^{–}\)\(^9\) Although the mechanisms of rIPC-derived cardioprotection have not yet been determined,\(^10\)\(^,\)\(^11\) recent studies by our group and others have suggested that a transferable blood-borne factor confers rIPC-induced cardioprotection.\(^12\)\(^–\)\(^14\) Furthermore, we have recently shown that rIPC reduces markers of myocardial damage in infants and children undergoing cardiac surgery with cardiopulmonary arrest.\(^14\)

Myocardial infarction is uncommon after cardiopulmonary arrest in the neonate. Nonetheless, cardiopulmonary arrest is associated with a constellation of structural and functional alterations in mitochondria\(^15\)\(^,\)\(^16\) that may play an important role in global cardiac performance.\(^17\) In addition to energy production, the mitochondria also play a central role in modulating the myocardial response to ischemic injury, such as either temporary cellular dysfunction or cell death.\(^18\) Mitochondria are therefore likely to be downstream participants in the protective effects of rIPC.

Numerous factors have been shown to be cardioprotective, including eicosatetraenoic acids, adipokines, cytokines, bradykinins, and enkephalins. It is not clear which (if any) of these factors are responsible for the infarct-sparing effects seen after rIPC. We have shown, however, that dialysate from plasma contains one or more cardioprotective factors with similar potency to plasma from remotely preconditioned rabbits.\(^19\) In the absence of a clearly identified protective factor, the dialysate from plasma of preconditioned animals thus represents a convenient source of the cardioprotective factor associated with remote preconditioning. Our study provides evidence that a blood-borne cardioprotective factor present in dialysate of plasma from remotely preconditioned rabbits preserves mitochondrial structure and function after cardiopulmonary arrest. Furthermore, this mitochondrial protection is associated with preservation of myocardial performance.

### Materials and Methods
#### Plasma Dialysate Preparation

The rIPC-treated dialysate was isolated according to the following protocol. New Zealand adult rabbits (3.5–4.0 kg) were anesthetized with pentobarbital. The left internal carotid artery was cannulated with a blood collection tube. The rIPC was induced with four cycles of hind limb ischemia (by cuff) and reperfusion (5 minutes each). Absence of distal pulse in the limb during ischemia was confirmed by pulse oximetry. At the end of the experiment, rabbits were heparinized (150 units/kg intravenously) and blood (approximately 100 mL) was withdrawn. After centrifugation of the blood at 3000 rpm for 20 minutes, the supernatant (approximately 50 mL plasma) was transferred into a dialysis tubing, which was submerged in 1 L of Krebs–Henseleit buffer (KHB: 118-mmol/L sodium chloride, 25-mmol/L sodium hydrogen carbonate, 1.2-mmol/L monobasic potassium phosphate, 4.7-mmol/L potassium chloride, 1.2-mmol/L magnesium sulfate, 1.8-mmol/L calcium chloride, and 11-mmol/L glucose) with mild stirring overnight. The dialysate blended with KHB was used for Langendorff perfusion buffer. Sham-treated dialysate was created according to an identical protocol in rabbits that did not undergo the repetitive limb ischemia.

### Experimental Model

Neonatal New Zealand white rabbits (age 6 days, weight 150–200 g) were anesthetized with pentobarbital (50 mg/kg intraperitoneally), anticoagulated with heparin (1000 units/kg intraperitoneally), and mechanically ventilated. The aorta was cannulated, and the heart was retrogradely perfused in situ to avoid ischemia. The heart was then excised, mounted on a Langendorff apparatus, and perfused with KHB with either sham-treated or rIPC-treated dialysate at a perfusion pressure of 75 mm Hg. Perfusion was filtered with a 2-µm filter (Invitrogen Corporation, Carlsbad, Calif) and equilibrated with 95% oxygen and 5% carbon dioxide, adjusted to a pH of 7.35 to 7.4.

All animals received humane care and treatment in accordance with the “Guide for the Care and Use of Laboratory Animals” (www.nap.edu/catalog/5140.html).

### Experimental Protocol

After being perfused for 20 minutes with KHB with plasma dialysate from rIPC-treated rabbits (n = 6) or from sham-treated rabbits (n = 6), hearts were subjected to cardioplegic arrest for 1 hour at 37°C (70-mL/kg Plegisol; Hospira, Inc, Lake Forest, Ill). The hearts were then reperfused with the respective dialysate-blended KHB for another 30 minutes and then removed.

For mitochondrial oxygen consumption measurements, myocardium was immediately fractionated as previously described.\(^17\) Fresh mitochondrial fractions were used for oxygen consumption measurements. Mitochondrial purity was confirmed by inspection of randomly chosen electron micrographs. In a second set of animals (n = 4 per group), left ventricles (LVs) were imbedded in optimal cutting temperature compound, frozen in liquid nitrogen, and stored at −80°C for apoptosis assessment and fluorescence immunohistochemical imaging.

### LV Functional Assessment

Isovolumetric LV performance was evaluated with a water-filled balloon connected to a force transducer (MLT844; ADInstruments, Inc, Colorado Springs, Colo) inserted in the LV across the mitral valve. The volume of the water-filled balloon was determined at a constant physiologic end-diastolic pressure in a range of 0 to 5 mm Hg, and its volume was kept consistent throughout the entire experiment. LV performance was assessed by measurements of LV systolic pressure, LV end-diastolic pressure, and positive and negative first derivatives of LV pressure. Heart rate was detected with a small disk electron probe (Harvard Apparatus, Inc, Holliston, Mass) connected to an electrocardiographic amplifier (ML136; ADInstruments). Analog data were digitized and analyzed with software (chart IV; ADInstruments).
Mitochondrial Isolation

The LV was used for mitochondrial isolation by differential centrifugation as described. Briefly, the LV was finely minced in 5 mL ice-cold mitochondrial isolation buffer (5-mmol/L 3-[N-morpholino]propanesulfonic acid, 2-mol/L ethyleneglycol bis-2-aminoethyl ether-N, N', N”, N”-tetraacetic acid, 70-mmol/L sucrose, 220-mmol/L mannitol, 1-mmol/L dithiothreitol, 17-μg/mL phenylmethylsulfonyl fluoride, 8-μg/mL aprotinin, and 2-μg/mL leupeptin, pH 7.2) with 0.1% bovine serum albumin and was homogenized on ice with a blade homogenizer. After 5 cycles (5 minutes each) of low spin (700g), the supernatant was transferred to a new tube and centrifuged at 8000g for 10 minutes. The pellet was resuspended in 10 mL mitochondrial isolation buffer with 0.1% bovine serum albumin and centrifuged at 8000g for another 10 minutes. The final pellet was suspended in mitochondrial isolation buffer without bovine serum albumin for mitochondrial oxygen consumption measurements.

Clark-Type Electrode Oxygen Consumption Measurement

Mitochondrial complex I, II, and IV respiration was measured by the method of Ricci and associates with a Clark-type oxygen electrode (Instech Laboratories, Inc, Plymouth Meeting, Pa). Oxygen consumption was measured in the presence of sequential administration of substrates and inhibitors (glutamate and malate for complex I, rotenone and succinate for complex II, and antimycin, N, N, N’N’-tetramethyl-p-phenylenediamine, and ascorbate for complex IV) added in the following order and final concentrations: 2.5-mmol/L glutamate, 2.5-mmol/L malate, 2-mmol/L adenosine diphosphate, 2-mmol/L rotenone, 5-mmol/L succinate, 1-μmol/L antimycin A, 1-mmol/L ascorbate, and 0.4-mmol/L N, N, N’N’-tetramethyl-p-phenylenediamine. Respiration rates are expressed as micromoles of oxygen per minute per milligram of mitochondrial protein. All the substrates and inhibitors were purchased from Sigma-Aldrich (St Louis, Mo). The voltage signal was amplified and digitized by a computer-supported PowerLab ADInstruments System (ADInstruments Pty Ltd, Castle Hill, Australia).

Outer Mitochondrial Membrane Permeability

The integrity of the outer membrane was assessed as previously described by measuring isolated mitochondrial oxygen consumption after administration of exogenous cytochrome C (10 μmol/L) into the respiratory chamber during the measurement of ascorbate-driven mitochondrial respiration (complex IV). The subsequent increase in complex IV activity reflects permeabilization of the outer mitochondrial membrane.

Fluorescent Immunohistochemical and Confocal Imaging

Optimal cutting temperature compound–embedded transverse ventricular slices were cut into 5-μm serial sections and fixed in acetone; after blocking, rabbit monoclonal anti–cytochrome c oxidase IV (1:250; Cell Signaling Technology, Inc, Beverly, Mass) was used. Sections were incubated with secondary antibody (Cy 2 reactive dye (red)°-conjugated donkey anti–mouse Ig G, 1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). Mouse monoclonal anti–cytochrome c (1:200; BD Pharmingen, San Diego, Calif) was used as the primary antibody for cytochrome c staining. Sections were immersed in secondary antibody (Cy 3 reactive dye (red)°-conjugated donkey anti–mouse Ig G, 1:200; Jackson ImmunoResearch Laboratories). The images from at least three different sections were acquired at 64× with a Zeiss LSM510 Multiphoton Laser Scanning Confocal Microscope (Carl Zeiss, Oberkochen, Germany) with the same pinhole setting, pixel format (1024×1024), and scanning data depth (0.8 μm). Double fluorescence for green and red channels was imaged with excitation of Argon-HeNe1 at the wavelengths 488 and 530 μm. Fifty high-power fields from each animal were analyzed with imaging processing software (Volocity 3.0; Improvision Inc, Waltham, Mass). High-intensity red and green (>551 voxels) were each regarded as specific immunoreactive signals. The red signal (cytochrome c oxidase IV) was set as a reference, and the contribution of the green signal (cytochrome c) to the colocalization of both signals was quantified as the overlap coefficient and used to compare the diffusion of cytochrome c staining between groups.

Mitochondrial Calcium Tolerance: Mitochondrial Permeability Transition Pore Opening Threshold Experiments

Mitochondrial calcium tolerance was determined by calcium-induced swelling of isolated cardiac mitochondria. Administration of exogenous calcium results in mitochondrial swelling, which is measured spectrophotometrically as a reduction in absorbance at 520 nm. Isolated cardiac mitochondria were resuspended in a swelling buffer to a final protein concentration of 0.22 mg/mL. Calcium chloride (100 μmol/L) was added, and the percentage decline of absorbance at 520 nm was continuously recorded. Cyclosporine (INN cyclosporin), an inhibitor of mitochondrial permeability transition (MPT) pore opening, was used as a control. We have previously demonstrated that cyclosporine is a potent inhibitor of MPT pore opening after cardioplegic arrest.

Statistics

Data are expressed as mean ± SEM. Group comparisons were made with the Fisher least significant difference analysis of variance. A Tukey test was used for post hoc comparison.

Results

Hemodynamic Responses

There was no significant difference in heart rate between groups with sham-treated dialysate and those with rIPC-treated dialysate throughout the reperfusion period (Table 1). The LV end-diastolic pressure was significantly greater at 5, 15, and 30 minutes after reperfusion in hearts with sham-treated dialysate relative to rIPC-treated dialysate. LV systolic pressure and positive and negative maximum first derivatives of LV pressure were significantly better preserved in hearts with rIPC-treated dialysate relative to those with sham-treated dialysate.

Mitochondrial Oxygen Consumption

Deficits in state 3 mitochondrial respirations at complex I and IV were greater in mitochondria from animals with...
sham-treated dialysate than those with rIPC-treated dialysate. These differences remained significant after normalization of state 3 to state 2 mitochondrial respiration rates (Table 2 and Figure 1). Complex II oxygen consumption rates were not different. Baseline isolated mitochondrial oxygen consumption was assessed during basal state 2 respiration for mitochondrial complexes I, II, and IV and was not different between groups, suggesting that there was no significant mitochondrial injury after fractionation.25

**Mitochondrial Outer Membrane Integrity**

Administration of exogenous cytochrome c both measured the oxygen consumption of isolated mitochondria and also assessed the integrity of the outer mitochondrial membrane. Cardioplegic arrest resulted in a deficit in complex IV state 3 respiration in mitochondria with sham-treated dialysate. This deficit was minimized with rIPC-treated dialysate (Figure 2, B). Administration of exogenous cytochrome c resulted in a more than 3-fold increase in complex IV state 3 respiration in mitochondria with sham-treated dialysate (indicating permeabilization of the outer mitochondrial membrane), whereas there was a minimal increase in the mitochondria with rIPC-treated dialysate (indicating maintenance of outer mitochondrial membrane integrity, 3.46 ± 0.33 vs 1.17 ± 0.22, P < .01, Figure 2, C). The deficit in complex IV activity in mitochondria with sham-treated dialysate was fully reversible with addition of exogenous cytochrome c, suggesting that permeabilization and loss of cytochrome c may have important consequences in terms of mitochondrial performance.

**Mitochondrial Cytochrome c Release**

Merged images of cytochrome c oxidase IV and cytochrome c staining of tissue sections from hearts perfused with sham-treated dialysate show subjectively decreased colocalization relative to myocardium perfused with rIPC-treated dialysate (Figure 3, A). Decreased colocalization in the myocardium with sham-treated dialysate is suggestive of mitochondrial release of cytochrome c into the cytoplasm, whereas preservation of the colocalization is consistent with maintenance of outer mitochondrial membrane integrity. The overlap quantification coefficient was greater in the myocardium with rIPC-treated dialysate than in the myocardium with sham-treated dialysate (1.46 ± 0.03 vs 1.21 ± 0.05 arbitrary units, P < .01), providing objective evidence of rIPC-treated dialysate’s association with preservation of outer mitochondrial membrane integrity.

**MPT Pore Opening**

The amount of calcium ion required to trigger calcium-induced MPT pore opening was not different between mitochondria isolated from myocardium perfused with sham-treated dialysate versus rIPC-treated dialysate, suggesting that the rIPC-treated dialysate did not directly promote

### TABLE 1. Hemodynamics in hearts perfused with sham-treated dialysate and remote ischemic preconditioning–treated dialysate

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline 5 min</th>
<th>Baseline 15 min</th>
<th>Baseline 30 min</th>
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<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>216 ± 8</td>
<td>203 ± 10.9</td>
<td>206 ± 8.6</td>
</tr>
<tr>
<td>rIPC</td>
<td>210 ± 4.5</td>
<td>210 ± 4.7</td>
<td>215 ± 4.5</td>
</tr>
<tr>
<td>LVP</td>
<td>88.1 ± 1.9</td>
<td>93.8 ± 1.1</td>
<td>88.1 ± 1.5</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>2.1</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>+dP/dt (% positive)</td>
<td>12.6 ± 1.7</td>
<td>8.9 ± 0.7</td>
<td>7.03 ± 0.26</td>
</tr>
<tr>
<td>−dP/dt (% negative)</td>
<td>62.2 ± 3.7</td>
<td>76.1 ± 2.7</td>
<td>82.4 ± 2.1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 6 per group. rIPC, Remote ischemic preconditioning; LVP, left ventricle pressure; LVEDP, left ventricle end diastolic pressure; +dP/dt, positive first derivative of left ventricular pressure; −dP/dt, negative first derivative of left ventricular pressure. *P < .01 versus sham-treated dialysate.

### TABLE 2. Isolated mitochondrial oxygen consumption

<table>
<thead>
<tr>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex IV</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>rIPC</td>
<td>Sham</td>
</tr>
<tr>
<td>State 2</td>
<td>0.585 ± 0.018</td>
<td>0.585 ± 0.022</td>
</tr>
<tr>
<td>State 3</td>
<td>3.343 ± 0.018</td>
<td>6.133 ± 0.701*</td>
</tr>
<tr>
<td>Ratio 3/2</td>
<td>5.669 ± 0.6</td>
<td>10.452 ± 1.024*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM in micromoles oxygen per minute per milligram mitochondrial protein, n = 6 per group. rIPC, Remote ischemic preconditioning. *P < .01 versus sham-treated dialysate. †P < .05 versus sham-treated dialysate.
MPT pore opening (Figure 4). Calcium ion–mediated MPT pore opening in both groups, however, could be blocked with cyclosporine, an inhibitor of MPT pore opening.

Discussion
We have recently reported a clinical benefit of rIPC in children subjected to cardioplegic arrest during cardiac surgery. The cellular mechanisms of this benefit remain incompletely understood. Nonetheless, it is clear that transient ischemia liberates at least one transferable blood-borne factor that confers remote protection against myocardial infarction, and dialysis of plasma from preconditioned animals isolates an equipotent dialysate with infarct-sparing effects in isolated heart preparations.

In contrast to myocardial infarction, typical pathologic changes after cardioplegic arrest include a constellation of apoptosis-related alterations in mitochondrial structure and function, such as the permeabilization of the outer mitochondrial membrane, cytochrome c release, and deficiency of electron transport. Because mitochondria are thought to play crucial roles in mediating both cardiac function and cardiomyocyte cell death, and rIPC is a reliable method for protecting myocardium against ischemia and reperfusion–induced cell death, we hypothesized that rIPC prevents...
postcardioplegia myocardial dysfunction through preservation of mitochondrial structure and function. This study demonstrates that rIPC-treated dialysate protects against loss of outer mitochondrial membrane integrity and subsequent deficits in electron transport and that this protection is correlated with preservation of global myocardial performance.

Laboratory reports evaluating preconditioning typically use reduction in infarct size as a primary end point. Although a reduction in infarct size is of potential clinical importance in coronary revascularization, it does not necessarily confer a clinical benefit in settings where ischemia–reperfusion injury is not associated with infarction (e.g., protected ischemia during cardioplegic arrest). This study demonstrates that the rIPC-related cardioprotective factor confers benefits extending beyond reduction of infarct size and including mitochondrial preservation and augmentation of postcardioplegic myocardial performance.

Our group previously demonstrated dependence of the rIPC cardioprotective factor on mitochondrial adenosine triphosphate–dependent potassium (KATP) channels in an infarct-reduction model. With a model of cardioplegic arrest, we have also demonstrated that diazoxide, a mitochondrial

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Figure 3. A, Immunofluorescence of cytochrome c oxidase IV (COX IV) and cytochrome c in myocardium from myocardium with sham-treated dialysate and with remote ischemic preconditioning (rIPC)-treated dialysate. In each panel, cytochrome c oxidase IV (mitochondria) is stained red, cytochrome c is stained green, and merged images are shown. Superimposition of red and green staining results in brownish color that suggests retention of cytochrome c in remote ischemic preconditioning–treated dialysate. Fine, diffuse green staining can be seen in merged images of myocardium with sham-treated dialysate, suggesting mitochondrial release of cytochrome c. Scatter plots display intensity range of red and green pixels in merged images of both myocardium groups, as well as various degrees of colocalization, as shown in orange and yellow. B, Quantification of overlap coefficient (Kx-green) in merged images. Double asterisk indicates $P < .01$ versus sham-treated dialysate. rIPC, Remote ischemic preconditioning.
KATP opener, preserves mitochondrial structure and function after cardioplegic arrest,\(^\text{15}\) with a pattern of protection (preservation of mitochondrial integrity and function of complex I) that is strikingly similar to that seen in this study. Consequently, this study is consistent with a role for the mitochondrial KATP channel as a mediator of rIPC protection.

It is important to note, however, that the MPT pore is also an important mediator of myocardial ischemic injury and initiation of apoptosis,\(^\text{27,28}\) and pharmacologic inhibition of MPT pore opening is associated with myocardial protection against ischemia–reperfusion injury.\(^\text{29,30}\) We have previously demonstrated that cyclosporine, which blocks MPT pore opening, is a secondary amplification loop of a mitochondrial KATP channel–mediated cardioprotection in the model used in this study; calcium-induced MPT pore opening was not altered with rIPC-treated dialysate but could be blocked with cyclosporine, suggesting that the mechanism of rIPC-mediated cardioprotection in the model used in this study is not mediated directly through MPT pore opening. The MPT pore and the mitochondrial KATP channel thus may be important but distinct sequential mediators in mitochondrial preservation after cardioplegic arrest. The MPT pore may be involved in a secondary amplification loop of a mitochondrial KATP channel–mediated signal that participates in subsequent permeabilization not evident in the current in vitro assessment of calcium-induced mitochondrial permeabilization.\(^\text{30}\)

At present, the identity of the cardioprotective factor remains elusive. Lang and coworkers\(^\text{5}\) used a proteomics approach and were unable to identify a cardioprotective factor larger than 8 kDa. Interestingly, albumin fragments and liver regeneration–related protein (LRRG03) were noted to be upregulated, and the potential for a small protein (<8 kDa) was not excluded. Other characteristics of the cardioprotective factor include hydrophobicity and activity through a protein kinase C–related pathway.\(^\text{31}\) Other groups have demonstrated that the protective factor is dependent on KATP channels\(^\text{32}\) or opioid receptors.\(^\text{33}\) Ongoing efforts to identify and characterize the cardioprotective factor will be required to clarify these protective mechanisms.

Conclusions

In addition to infarct-sparing effects, rIPC is associated with release of a blood-borne cardioprotective factor that maintains mitochondrial structure and function and preserves global myocardial performance after neonatal cardioplegic arrest. Characterization and identification of the rIPC cardioprotective factor will foster recruitment of innate protective mechanisms to improve myocardial preservation.

References


