Opening of Potassium Channels
The Common Cardioprotective Link Between Preconditioning and Natural Hibernation?
Egidijus Kevelaitis, MD, PhD; Jacqueline Peynet, MD; Christian Mouas; Jean-Marie Launay, MD; Philippe Menasché, MD, PhD

Background—The tolerance of hibernating mammals to cold hypoxia is related to a factor similar to agonists of δ-opioid receptors. This study was designed to assess whether activation of these receptors could reproduce the protection conferred by ischemic preconditioning and whether such cardioprotection was similarly mediated by an opening of ATP-sensitive potassium (KATP) channels.

Methods and Results—Thirty-two isolated rat hearts were arrested with and stored in Celsior at 4°C for 5 hours before being reperfused for 2 hours. They were divided into 4 equal groups. Group 1 hearts served as controls. In group 2, ischemic preconditioning was elicited by two 5-minute global ischemia periods interspersed with 5 minutes of reperfusion before arrest. In group 3, hearts were pharmacologically preconditioned with a 15-minute infusion of the δ-opioid receptor agonist D-Ala2-D-Leu5-enkephalin (DADLE; 200 μmol/L). In group 4, the protocol was similar to group 3 except that infusion of DADLE was preceded by infusion of the KATP blocker glibenclamide (50 μmol/L). The salutary effects of both forms of preconditioning were primarily manifest as a better preservation of diastolic function, a reduced myocardial edema, and reduced creatine kinase leakage. This protection was abolished by administration of glibenclamide before DADLE.

Conclusions—These data suggest that activation of δ-opioid receptors improves recovery of cold-stored hearts to a similar extent as ischemic preconditioning, most likely through an opening of KATP channels. This provides a rationale for improving the preservation of hearts for transplantation by pharmacologically duplicating the common pathway to natural hibernation and preconditioning. (Circulation. 1999;99:3079-3085.)

Key Words: transplantation ■ ischemia ■ potassium ■ receptors

A
cute graft failure still accounts for ≈20% of early deaths after heart transplantation.1 Although its origin is multifactorial, an important role is likely to be played by inadequate myocardial preservation throughout the arrest/storage/implantation/reperfusion sequence to which the allograft is exposed. Among the recent strategies that have been proposed to enhance donor heart preservation, ischemic preconditioning is generating a great deal of interest because of the consistent cardioprotective effects associated with its use across a wide variety of experimental models and animal species.2

However, because the induction of preconditioning by an ischemic-type stimulus is rather unappealing,3 several studies have tried to identify the endogenous mediators of this adaptive pathway in an attempt to use them as pharmacological alternates. Although much interest has been paid to adenosine4 and α1-adrenoceptor5 agonists, opioid receptors have also been reported to trigger the preconditioning pathway.6,8 Interestingly, opioid receptor stimulation also seems to be involved in the enhancement of tissue survival inherent in natural hibernation of mammals9,10 a situation that closely parallels that of cardiac allografts during the cold-storage interval. The present study, performed in a rat model of prolonged cold heart storage, was therefore designed to address the following 3 questions: (1) Can activation of δ-opioid receptors improve the functional preservation of cold-stored hearts? (2) To what extent does this protection compare with that conferred by classic ischemic preconditioning? and (3) Is the opioid-linked cardioprotection similarly mediated by an opening of ATP-sensitive potassium (KATP) channels, which are currently considered the main effectors of the ischemic preconditioning pathway?

Methods

Experimental Preparation
Thirty-two isolated rat heart preparations were used. Male Wistar rats weighing 300 to 330 g were intravenously heparinized (0.2 mL)
Experimental Time Course and Measurements

The hearts were initially allowed to equilibrate for 15 minutes. The left ventricular balloon was then inflated to the volume that gave an end-diastolic pressure of ~8 mm Hg. Contractile function and coronary flow were measured in triplicate under these isovolumic conditions. In addition, a left ventricular pressure-volume curve was constructed by incremental inflation of the balloon volume by 0.02-mL aliquots. Two sets of pressure-volume measurements were generated, the first of which was discarded because of small balloon shifts. Zero volume was defined as the point at which the left ventricular end-diastolic pressure was zero. On completion of the pressure-volume curve, the left ventricular balloon was deflated to set the end-diastolic pressure back to the baseline value of 8 mm Hg. The endothelium-dependent vasodilatory response was then tested by a 5-minute perfusion of 5-hydroxytryptamine (5-HT, 10^{-6} mol/L), which yields a stable level of vasoconstriction. The endo-thelium-dependent vasorelaxation to acetylcholine (10^{-6} mol/L) was then tested. At the end of the 2-hour reperfusion interval, hearts were removed from the Langendorff column and the ventricles weighed. Wet weights were determined after excess fluid was blotted from specimens. Dry weights were determined after the specimens were dried for 24 hours at 80°C. Water content (%) was then computed as 100×(wet weight−dry weight)/wet weight.

In addition to measurements of function, flow, and edema, creatine kinase leakage in the collected coronary effluent was measured over the initial 45 minutes of reperfusion. Total creatine kinase activity was assessed enzymatically with an automatic analyzer (Olympus). The results are expressed as international units (IU) per gram of dry weight.

Statistical Analysis

Functional data were compared by 2-factor ANOVA with repeated measures, with treatment group as 1 factor and time as the second. When calculated values of F exceeded tabular values of the 5% level, intergroup differences were specified by use of a post hoc Student t test with Bonferroni correction for multiple comparisons. Compliance curves were assessed by linear regression analysis of the end-diastolic pressure data to calculate a slope. Linear regression provided a reasonable model for the diastolic function curves (R^2 of 0.88 to 0.98). Coronary flows, creatine kinase leakage, and myocardial water content were compared among the 4 groups by unpaired t tests. A P value <0.05 was considered significant. Data are reported as mean±SEM.
value of diastolic pressure (ie, the average of values measured at the different time points) was significantly lower in ischmically and pharmacologically preconditioned hearts than in controls. However, the protective effect of DADLE preconditioning was abolished by the prior administration of glibenclamide. Similar patterns were seen when left ventricular end-diastolic pressures were analyzed in relation to balloon volume. Thus, whereas slopes of the pressure-volume curves were not significantly different between the 4 groups before storage, their postischemic leftward shift was significantly less pronounced in hearts preconditioned with ischemia or DADLE than in control hearts or in those receiving glibenclamide before DADLE preconditioning (Figure 1).

In contrast, neither form of preconditioning improved postarrest systolic function over that of control or glibenclamide. Similar patterns were seen when left ventricular end-diastolic pressures were analyzed in relation to balloon volume. Thus, whereas slopes of the pressure-volume curves were not significantly different between the 4 groups before storage, their postischemic leftward shift was significantly less pronounced in hearts preconditioned with ischemia or DADLE than in control hearts or in those receiving glibenclamide before DADLE preconditioning (Figure 1).

Coronary Vascular Responsiveness
Before storage, administration of 5-HT significantly increased coronary flow above baseline values ($P<0.001$ in all groups). During reperfusion, 5-HT still elicited a significant increase in coronary flow, but this endothelium-dependent response was smaller than before storage ($P<0.05$ versus baseline values). There was no significant difference in the poststorage response to 5-HT among the 4 groups (Figure 2). Within-group changes in the endothelium-independent response to papaverine featured slightly different patterns (Table 3). Thus, before storage, papaverine caused an increase in coronary flow that was of similar extent in all groups ($P<0.001$ versus baseline). Conversely, after storage, papaverine-induced vasodilation was of greater magnitude in the 2 preconditioned groups ($P<0.02$ versus baseline) than in control hearts or hearts pretreated with glibenclamide before DADLE infusion ($P<0.05$ versus baseline). In the constant-flow experiments, the endothelium-dependent vasodilatory response to acetylcholine was not significantly different among the 4 groups (Table 4).

Myocardial Water Content
Percent tissue water was significantly greater in control hearts (81.23±0.23%) and in hearts pretreated by glibenclamide before DADLE infusion (80.90±0.13%) than in hearts preconditioned by either ischemia (79.88±0.22%) or DADLE-induced activation of δ-opioid receptors (79.46±0.37%; for these 2 preconditioning groups, $P<0.001$ versus controls and $P<0.005$ versus glibenclamide plus DADLE).

Creatine Kinase Leakage
In keeping with functional data, both forms of preconditioning presumably resulted in infarct limitation, because creatine kinase leakage during the initial 45 minutes of reperfusion was significantly lower in ischmically preconditioned hearts (345±54 IU/g dry weight) and DADLE-preconditioned hearts (379±62 IU/g dry weight) than in controls (548±79 IU/g dry weight; $P<0.001$ versus the 2 preconditioned groups). Administration of glibenclamide before DADLE blunted this protective effect, because in this group, postischemic enzyme release increased to 453±43 IU/g dry weight. This difference compared with the 2 preconditioned groups failed, however, to reach the level of statistical significance ($P=0.18$).

Discussion
Three major results emerge from the present study: (1) A pharmacological activation of δ-opioid receptors improves the functional preservation of cold-stored hearts (as occurs

### TABLE 1. Composition of the Preservation Solution Celsior

<table>
<thead>
<tr>
<th>Additives</th>
<th>Concentration, mmol/L</th>
</tr>
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<tbody>
<tr>
<td>Impermeants</td>
<td></td>
</tr>
<tr>
<td>Lactobionate</td>
<td>80</td>
</tr>
<tr>
<td>Mannitol</td>
<td>60</td>
</tr>
<tr>
<td>Antioxidants</td>
<td></td>
</tr>
<tr>
<td>Reduced glutathione</td>
<td>3</td>
</tr>
<tr>
<td>Metabolic substrates</td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>20</td>
</tr>
<tr>
<td>Buffers</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>30</td>
</tr>
<tr>
<td>Electrolytes</td>
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</tr>
<tr>
<td>Potassium</td>
<td>15</td>
</tr>
<tr>
<td>Sodium</td>
<td>100</td>
</tr>
<tr>
<td>Magnesium</td>
<td>13</td>
</tr>
<tr>
<td>Calcium</td>
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</tr>
<tr>
<td>Chloride</td>
<td>41.5</td>
</tr>
<tr>
<td>pH (at 20°C)</td>
<td>7.3</td>
</tr>
<tr>
<td>Osmolarity, mOsm/L</td>
<td>360</td>
</tr>
</tbody>
</table>

### TABLE 2. Effects of Preconditioning on Diastolic and Systolic Function

<table>
<thead>
<tr>
<th>Group (n=8)</th>
<th>LV End-Diastolic Pressure, mm Hg</th>
<th>LV Developed Pressure, mm Hg</th>
<th>LV+dP/dt max, mm Hg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Reperfusion</td>
<td>Baseline</td>
</tr>
<tr>
<td>Control</td>
<td>9±1</td>
<td>38±3</td>
<td>136±4</td>
</tr>
<tr>
<td>Ischemic PC</td>
<td>8±1</td>
<td>25±2*</td>
<td>141±6</td>
</tr>
<tr>
<td>DADLE PC</td>
<td>8±1</td>
<td>24±2*</td>
<td>139±5</td>
</tr>
<tr>
<td>Glib+DADLE PC</td>
<td>9±1</td>
<td>40±3</td>
<td>136±4</td>
</tr>
</tbody>
</table>

LV indicates left ventricular; PC, preconditioning; and Glib, glibenclamide.

Reperfusion values represent the average of values measured at the different postischemic study points.

*P<0.001 vs control and Glib+DADLE PC groups. All values are mean±SEM.
during transplantation. (2) This improvement is of similar magnitude as that conferred by 2 brief cycles of classic ischemic preconditioning. (3) The opioid-linked cardioprotective pathway is likely to involve an opening of KATP channels, because it is abolished by glibenclamide. Because this receptor-mediated mechanism displays close similarities with the one involved in natural hibernation, a closer look at the biology of this unique phenomenon might provide useful clues for improving the preservation of heart transplants.

**Endogenous Mediators of Natural Hibernation**

The tolerance of hibernating animals to extended periods of cold hypoxia is attributed to a plasma factor (hibernation-induction trigger), the opiate nature of which is supported by 2 major lines of evidence: (1) the behavioral and physiological changes induced in primates by intracerebroventricular infusion of the hibernation-induction trigger present in the blood of hibernating woodchucks is reversed or delayed by opiate antagonists, and (2) the ability of the hibernation-induction trigger to induce hibernation can be mimicked by the d-opioid receptor agonist DADLE, whereas μ- and κ-opioids are ineffective. Likewise, both hibernation-induction trigger and DADLE have been shown to similarly improve protection in animal models of multiorgan block autoperfusion or cardioplegic arrest.

**Endogenous Mediators of Preconditioning**

The cardioprotective effects of preconditioning are well established and are primarily manifest as a reduction in infarct size and, through this mechanism, an improvement in postischemic function. That prolonged storage of heart transplants could represent an elective indication of preconditioning interventions is supported by the study of Karck and coworkers, who have shown, in rat hearts cold-stored for 10 hours, that a 5-minute preconditioning episode before arrest significantly improved recovery of function and concomitantly reduced creatine kinase leakage. The results yielded by our group of ischemically preconditioned hearts support this hypothesis. However, safety concerns about inflicting an ischemic insult to the heart justify the ongoing search for pharmacological mimetics.

According to the current scheme, the preconditioning signal activates various membrane receptors, which trigger an intracellular signaling pathway leading to the activation of several kinases, in particular protein kinase C (PKC). This, in
turn, causes opening of the K<sub>ATP</sub> channels, possibly at the mitochondrial level, with subsequent protection through limitation of calcium overload and/or better control of cellular volume. Recent studies have highlighted that in addition to adenosine and α<sub>1</sub>-agonists, opioids also play an important role in mediating preconditioning-induced cardioprotection.

Cardioprotective Effects of Opioid Receptor Activation

The role of opioids in preconditioning is based on the observation that their infarct-limiting effect can be mimicked by an infusion of morphine, a nonspecific agonist of μ-receptors, in both rat and rabbit hearts. It was demonstrated that ischemic preconditioning in the intact rat heart is mediated by δ- (probably the δ<sub>1</sub>-subtype) but not μ- or κ-opioid receptors. The present study shows that these observations, made in models of regional ischemia, can be extended to the setting of global ischemic arrest, because the functional preservation conferred to cold-stored rat hearts by ischemic preconditioning could be reproduced by a selective DADLE-induced activation of δ-opioid receptors. Equally successful results with DADLE pretreatment have been reported in rabbit hearts subjected to a similar protocol of prolonged cold storage.

Interestingly, in the latter experiments, DADLE was ineffective when given only as an additive to the arrest solution, which supports the concept that opioid receptor activation acts by preconditioning the heart before the onset of the ischemic interval.

Opioid receptors are indeed present in cardiac myocytes, and at least 3 mechanisms can account for their cardioprotective effects: (1) an increased production of inositol 1,4,5-triphosphate (IP<sub>3</sub>) and a subsequent depletion of the sarcoplasmic reticulum from its calcium stores, which might then reduce cytosolic calcium accumulation during sustained ischemia; (2) an activation of PKC by diacylglycerol, a compound formed in addition to IP<sub>3</sub> from the hydrolysis of membrane inositol-containing phospholipids and the subsequent PKC-mediated opening of K<sub>ATP</sub> channels; and (3) a direct, PKC-independent opening of these channels. Thus, regardless of the mechanism, activation of K<sub>ATP</sub> channels appears to be the final effector of the opioid-coupled cardioprotective transduction pathway. Additional evidence for their involvement is provided by the ability of glibenclamide to antagonize the cardioprotection elicited by morphine or, as in the present study, by DADLE preconditioning. We acknowledge that because glibenclamide (in contrast to 5-hydroxydecanoate) is a nonspecific blocker of K<sub>ATP</sub> channels, our data do not enable us to determine which fraction (sarcolemmal or mitochondrial) of these channels was involved. This, however, does not alter the conclusion pertaining to their role in mediating the opioid-triggered cardioprotective pathway. The reduction in calcium overload expected from such an opening of K<sub>ATP</sub> channels (regardless of their cellular location) is indeed consistent with our finding of an improved preservation of diastolic function in DADLE-preconditioned hearts.

**TABLE 3. Endothelium-Independent Vasorelaxation in Response to Papaverine (5×10<sup>-6</sup> mol/L)**

<table>
<thead>
<tr>
<th>Coronary Flow, mL·min&lt;sup&gt;-1&lt;/sup&gt;·g dry wt&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Preischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After Papaverine</td>
</tr>
<tr>
<td>Control</td>
<td>86.9±4.4</td>
<td>114.5±3.6*</td>
</tr>
<tr>
<td>Ischemic PC</td>
<td>86.9±3.9</td>
<td>110.0±3.9*</td>
</tr>
<tr>
<td>DADLE PC</td>
<td>79.4±2.8</td>
<td>104.9±3.2*</td>
</tr>
<tr>
<td>Glib+DADLE PC</td>
<td>80.4±3.3</td>
<td>108.0±3.6*</td>
</tr>
</tbody>
</table>

PC indicates preconditioning and Glib, glibenclamide.
All values are mean±SEM.
*P<0.001 vs corresponding baseline preischemia value; †P<0.05 vs corresponding baseline reperfusion value; ‡P<0.02 vs corresponding baseline reperfusion value.

**TABLE 4. Endothelium-Dependent Relaxation to Acetylcholine (10<sup>-6</sup> mol/L) After Precontraction With Prostaglandin F<sub>2a</sub> (10<sup>-5</sup> mol/L)**

<table>
<thead>
<tr>
<th>Coronary Flow, mL·min&lt;sup&gt;-1&lt;/sup&gt;·g dry wt&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Coronary Vascular Resistance, mm Hg·mL&lt;sup&gt;-1&lt;/sup&gt;·min&lt;sup&gt;-1&lt;/sup&gt;·g dry wt&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Relaxation to ACh, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (n=8)</td>
<td>Baseline</td>
<td>After PGF&lt;sub&gt;2a&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>38.6±4.0</td>
<td>1.43±0.22</td>
</tr>
<tr>
<td>Ischemic PC</td>
<td>37.5±2.8</td>
<td>1.28±0.16</td>
</tr>
<tr>
<td>DADLE PC</td>
<td>42.6±3.3</td>
<td>1.13±0.12</td>
</tr>
<tr>
<td>Glib+DADLE PC</td>
<td>40.3±3.4</td>
<td>1.19±0.13</td>
</tr>
</tbody>
</table>

ACh indicates acetylcholine; PGF<sub>2a</sub>, prostaglandin F<sub>2a</sub>; PC, preconditioning; and Glib, glibenclamide.
All experiments were performed after 90 minutes of reperfusion under conditions of constant coronary flow. All values are mean±SEM.
The expected reduction of calcium overload associated with DADLE preconditioning should in turn limit the breakdown of ATP. Indeed, both hibernation-induction trigger and DADLE preserve myocardial levels of ATP. This should result in better functioning of energy-driven ion pumps, particularly sarcoplasmatic sodium/potassium ATPase activity, and could thus account for the lesser degree of myocardial edema seen in the DADLE-preconditioning group.

Conversely, opioid receptor stimulation failed to improve preservation of endothelium-dependent coronary responsiveness beyond that seen in the control group. This result is consistent with the data of Bauer and coworkers showing that ischemic preconditioning was unable to prevent the deterioration of myocardial blood flow and the loss in the endothelium-dependent vasodilation seen during reperfusion. Likewise, Shirai and associates recently reported that preconditioning reduced injury in cardiomyocytes but not endothelial cells. It is noteworthy that the endothelium-dependent response to 5-HT, although of smaller magnitude than before ischemia, was still present during reperfusion in all groups, which is consistent with the ability of the antioxidant content of Celsior to preserve endothelial function after prolonged cold heart storage. Furthermore, the trend toward better preservation of the endothelium-independent vasodilatory response in hearts preconditioned by ischemia or DADLE might be related to the lesser degree of posts ischemic myocardial contracture seen in these 2 groups.

Clinical Implications and Limitations
From a practical standpoint, the opioid-linked transduction pathway could be therapeutically exploited by acting on its trigger (the opioid receptor) or its putative effector (the KATP channel). In surgical practice, opioid receptor stimulation implemented shortly before the ischemic interval may be less effective than expected because of the almost universal use of fentanyl-based anesthesia. Fentanyl is a μ-opioid agonist that, on the basis of right atrial biopsy samples taken from patients at the onset of cardiopulmonary bypass, results in full opioid receptor occupancy and thus leaves little, if any, room available for further activation by a δ-agonist (J.-M. Launay, MD, unpublished data, 1998). Furthermore, opioid-coupled transduction pathways can be fundamentally different depending on the type of agonist, and fentanyl, in contrast to morphine, does not seem to result in KATP channel opening. It is thus possible that interventions directly targeted at opening these channels might be more promising, whether based on the currently available drug nicorandil, the mitochondrial KATP channel opener diazoxide, or anesthetics like isoflurane. We acknowledge the limitations of our model, in particular the use of an isolated heart preparation, the nonheme nature of the perfusate, and the relatively brief period of posts ischemic observation. Nevertheless, these models have proven to be fairly reliable for screening myocardial preservative strategies. Thus, the present results strongly suggest that opioid receptor stimulation and opening of KATP channels are effective approaches for pharmacologically duplicating the cardioprotective effects of ischemic preconditioning. It is tempting to speculate that their use in heart transplantation would be nothing but the logical exploitation of the mechanisms accounting for maintenance of tissue survival during natural hibernation.

Acknowledgments
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