Preconditioning by Mitochondrial ATP-Sensitive Potassium Channel Openers
An Effective Approach for Improving the Preservation of Heart Transplants

Egidijus Kevelaitis, MD, PhD; Abdeslam Oubénaissa, MD, PhD; Jacqueline Peynet, MD; Christian Mouas; Philippe Menasché, MD, PhD

Background—Recent studies have implicated mitochondrial ATP-sensitive potassium (K\textsubscript{ATP}) channels in the cardioprotective effects of ischemic preconditioning. The present study used a model of prolonged cold heart storage to assess whether the mitochondrial K\textsubscript{ATP} opener diazoxide could reproduce the protection conferred by ischemic preconditioning.

Methods and Results—Fifty-four isolated rat hearts were arrested with and stored in Celsior at 4°C for 10 hours before a 2-hour reperfusion. They were divided into 5 groups. Group 1 hearts served as controls. In group 2, hearts were preconditioned by two 5-minute episodes of global ischemia, each separated by 5 minutes of reperfusion before arrest. In group 3, hearts received a 15-minute infusion of the mitochondrial K\textsubscript{ATP} opener diazoxide (30 \textmu mol/L) followed by 5 minutes of washout before arrest. In groups 4 and 5, hearts underwent a protocol similar to that used in groups 2 and 3, respectively, except that the preconditioning was preceded by a 10-minute infusion of the mitochondrial K\textsubscript{ATP} blocker 5-hydroxydecanoate (5-HD, 100 \textmu mol/L). Both ischemic and diazoxide preconditioning provided a similar degree of cardioprotection demonstrated by a significantly better preservation of left ventricular compliance, reduced leakage of creatine kinase, and smaller degree of myocardial edema compared with control hearts. These beneficial effects were abolished by 5-HD pretreatment. Postischemic left ventricular contractility and endothelium-dependent coronary response to 5-hydroxytryptamine and acetylcholine were not different among groups. However, the endothelium-independent vasodilatory postischemic response to papaverine was better preserved after ischemic and diazoxide preconditioning than in the other groups.

Conclusions—These data support the concept that the cardioprotective effects of ischemic preconditioning can be duplicated by a mitochondrial K\textsubscript{ATP} opener and suggest that activation of these channels could be an effective means of improving the preservation of globally ischemic cold-stored hearts, as occurs during cardiac transplantation. (Circulation. 1999;100[suppl II]:II-345–II-350.)

Key Words: ischemia ■ reperfusion ■ diastole ■ systole ■ vasodilation

Ischemic preconditioning has recently emerged as a new strategy for improving the preservation of heart transplants.1–3 However, because the induction of preconditioning by an ischemia-type stimulus is rather unappealing,4 there is a continuing effort to identify the endogenous mediators of the preconditioning-induced signaling pathway in an attempt to use some of them therapeutically.5,6 In this setting, extensive pharmacological evidence has implicated sarcolemmal ATP-sensitive potassium (K\textsubscript{ATP}) channels as the end effectors of this pathway with the underlying assumption that shortening of the action potential duration would reduce calcium influx, energy consumption, and ultimately ischemia-induced tissue injury.7 Recently, however, this hypothesis has been challenged by 2 major observations: (1) the protection provided by either classic ischemic preconditioning8 or K\textsubscript{ATP} openers9 does not necessarily require an abbreviation of the action potential duration, and (2) this protection can still be demonstrated in unstimulated cardiac myocytes.10 These considerations have raised the hypothesis that mitochondrial rather than sarcolemmal K\textsubscript{ATP} channels, which are also activated by ATP depletion,6 could mediate preconditioning-induced cardioprotection. This hypothesis is indeed supported by several studies that have documented the preconditioning-like cardioprotective effects of the selective mitochondrial K\textsubscript{ATP} opener diazoxide in various experimental settings, including a cellular model of simulated ischemia,12 normothermic global ischemia of Langendorff-perfused hearts,13 and in vivo regional ischemia.14 The present study was therefore

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designed to assess whether the conclusions of these studies also applied to the more surgically relevant setting of prolonged global hypothermic ischemic arrest, as occurs during the ex vivo storage period inherent in heart transplantation.

Methods

Experimental Preparation

Male Wistar rats weighing 300 to 350 g were anesthetized with pentobarbital sodium (60 mg/kg IP). The rats were given 0.2 mL IV (200 U) heparin. The beating heart was excised and rapidly mounted on a nonrecirculating Langendorff perfusion column. All rats were cared for in compliance with Principles of Laboratory Animal Care formulated by the National Society of Medical Research and Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Retrograde aortic perfusion was instituted at a constant pressure of 100-cm H$_2$O with ultrafiltered (5-μm-pore filter), oxygenated (95% oxygen and 5% carbon dioxide), normothermic (37°C) Krebs-Henseleit solution. The solution had a pH of 7.3 to 7.4 when gassed. The pulmonary outflow tract was incised to allow drainage of the coronary effluent. A catheter was placed through the apex of the left ventricle to drain the thebesian flow. The left atrium was opened, and a latex balloon was inserted into the left ventricle. Left ventricular pressure was continuously recorded on line with a pressure transducer (TSD104, BIOPAC Systems, Inc) connected via the universal interface module (UIM100A) to the actual data acquisition unit (MP100A), which converted analog into digital signals. Data were then processed with a Power Macintosh 6100/66 computer (Apple Computer) with AcqKnowledge III software. Left ventricular developed pressure (LVDP) was measured as the difference between peak systolic pressure and left ventricular end-diastolic pressure (LVEDP). The first derivative (dP/dt) was calculated with the software. Coronary flow was measured by timed collection of the coronary venous effluent. Left ventricular pacing was maintained at a rate of 320 bpm throughout the control and reperfusion periods.

Experimental Protocol

The hearts were initially allowed to equilibrate for 15 minutes after being instrumented and stable recordings were established. The left ventricular balloon was inflated to the volume that gave an LVEDP of ~8 mm Hg. After stabilization, systolic and diastolic functions and coronary flow were measured in triplicate under these isovolumic conditions. In addition, the left ventricular balloon was inflated in 0.02-mL increments to construct pressure-volume curves. Two sets of pressure-volume measurements were generated, the first of which was discarded because of small balloon shifts. Zero volume was defined at the point at which LVEDP was zero. On completion of the pressure-volume curve, the left ventricular balloon was deflated to set the LVEDP back to its baseline value of 8 mm Hg. The endothelium-dependent coronary flow response was then tested by a 5-minute perfusion with 5-hydroxytryptamine (5-HT, 10$^{-7}$ mol/L). Coronary flow was measured during the last 4 minutes of 5-HT administration. This was followed by a 12-minute washout perfusion with 10$^{-6}$ mol/L acetylcholine. Results are expressed as international units per gram of dry weight.

At the end of reperfusion, hearts were removed from the Langendorff column, and the ventricles were weighted. Wet weights were measured after both ventricles were incised and the excess fluid was blotted. Dry weights were measured after drying for 24 hours at 80°C. Water content was computed from this formula: 100×(wet weight−dry weight)/wet weight.

Experimental Groups

The hearts were divided into 5 groups, which differed only by the treatment administered before cardioplegic arrest. Group 1 hearts (n = 12) had no prearrest intervention and served as controls. In group 2 (n = 12), hearts were preconditioned by two 5-minute episodes of total (no-flow) global ischemia, each separated by 5 minutes of reperfusion before arrest. In group 3 (n = 12), hearts received a 15-minute infusion of the mitochondrial K$_{ATP}$ opener diazoxide (30 μmol/L) followed by 5 minutes of washout buffer perfusion before arrest. In groups 4 (n = 10) and 5 (n = 8), hearts underwent a protocol similar to that used in groups 2 and 3, respectively, except that the preconditioning intervention was preceded by a 10-minute infusion of the mitochondrial K$_{ATP}$ blocker 5-hydroxydecanoate (5-HD, 100 μmol/L).

Solutions and Drugs

The Krebs-Henseleit buffer was prepared fresh the day of use and contained (in mmol/L) NaCl 118, KCl 4.7, MgSO$_4$ 1.2, NaHCO$_3$ 25, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, and glucose 11. The Celsior solution, provided by Immix-Sangstat, had the following composition (in mmol/L): potassium 15, sodium 100, magnesium 13, calcium 0.26, chloride 41.5, histidine 30, glutamate 20, lactobionate 80, mannitol 60, and reduced glutathione 3. We purchased 5-HD sodium salt, 5-HT, papaverine hydrochloride, prostaglandin F$_2$ alpha, Tris salt, and acetylcholine hydrochloride from Sigma Chemical Co. Diazoxide (Hyperstat) was obtained from Schering-Plough. All drugs were dissolved in Krebs-Henseleit solution immediately before use.

Statistical Analysis

Functional data were compared by 2-factor ANOVA with repeated measures, with treatment as 1 factor and time as the second one.
Intergroup differences were specified by use of post hoc Student's *t* test with Bonferroni's correction for multiple comparisons. Left ventricular compliance curves were assessed by linear regression analysis of LVEDP data to calculate slope. Coronary flow and resistance, total creatine kinase leakage, and myocardial water content were compared between groups by unpaired 2-tailed *t* tests. Preischemic and postischemic coronary flow responses to 5-HT and papaverine within the same group were compared by paired 2-tailed *t* tests. A value of *P*<0.05 was considered significant. Data were reported as mean±SEM.

### Results

#### Left Ventricular Diastolic and Systolic Functions

Baseline functional data were not significantly different among the 5 groups (Table 1). After reperfusion, both ischemic and diazoxide preconditioning resulted in a similar improvement of left ventricular diastolic function compared with control hearts. Thus, the postischemic time-averaged value of LVEDP was significantly (*P*<0.001) lower in ischemically and pharmacologically preconditioned hearts than in controls. These protective effects were abolished by the prior administration of 5-HD (Table 1). Similar patterns were seen when LVEDP was analyzed in relation to balloon volume. Thus, the baseline (prestorage) slopes of the pressure-volume curves were not significantly different between the 5 groups: 416±6 mm Hg/mL in control hearts, 374±1.3 mm Hg/mL in diazoxide-preconditioned, 5-HD-ischemic preconditioning, 5-HD+ischemic preconditioning, and 5-HD+diazoxide preconditioning groups, respectively. After storage, all groups demonstrated an increase in LVEDP measured at a given balloon volume compared with the corresponding preischemic values. However, the upward shift of the postarrest LVEDP-volume curve was significantly less pronounced in hearts preconditioned with ischemia or diazoxide compared with control hearts or those receiving 5-HD before preconditioning (Figure 1). Thus, the slopes of reperfusion pressure-volume curves were increased to 1212±32 mm Hg/mL in control hearts, 1207±57 mm Hg/mL in hearts receiving 5-HD before ischemic preconditioning, and 1132±30 mm Hg/mL in hearts receiving 5-HD before diazoxide preconditioning, whereas they were reduced to 825±37 mm Hg/mL in ischemically preconditioned hearts (*P*<0.0001 versus control and 5-HD+ischemic preconditioning groups) and 943±55 mm Hg/mL in diazoxide-preconditioned hearts (*P*<0.05 versus control and 5-HD+diazoxide preconditioning groups).

In contrast, neither ischemic nor diazoxide preconditioning improved postarrest systolic function over that of control or 5-HD–pretreated hearts (Table 1). The recovery of dP/dt grossly paralleled that of LVDP in all groups.

#### Coronary Vascular Responsiveness

During the preischemic period, administration of 5-HT significantly increased coronary flow above baseline values (*P*<0.001 in all groups). During reperfusion at constant pressure, the endothelium-dependent vasodilatory response to 5-HT was equally lost in all groups. Likewise, in the constant-flow experiments, the endothelium-dependent vasodilation to acetylcholine was not significantly different among groups (Table 2).

Before storage, the endothelium-independent vasodilation to papaverine was similar in all groups (*P*<0.001 versus baseline). After storage, papaverine significantly increased coronary flow only in ischemically and diazoxide-preconditioned hearts (*P*<0.02 and *P*<0.05 versus reperfusion baseline flow, respectively). In contrast, papaverine

<table>
<thead>
<tr>
<th>Group</th>
<th>LVEDP, mm Hg</th>
<th>LVDP, mm Hg</th>
<th>LV dP/dt max, mm Hg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=12)</td>
<td>8.9±0.2</td>
<td>61.7±1.6</td>
<td>3905±97</td>
</tr>
<tr>
<td>Ischemic PC (n=12)</td>
<td>8.5±0.2</td>
<td>46.0±1.5*</td>
<td>3962±68</td>
</tr>
<tr>
<td>Diazoxide PC (n=12)</td>
<td>8.7±0.2</td>
<td>46.3±1.1*</td>
<td>3934±145</td>
</tr>
<tr>
<td>5-HD+ischemic PC (n=10)</td>
<td>8.2±0.2</td>
<td>56.9±1.3</td>
<td>3894±48</td>
</tr>
<tr>
<td>5-HD+diazoxide PC (n=8)</td>
<td>8.5±0.2</td>
<td>62.3±1.1</td>
<td>3867±80</td>
</tr>
</tbody>
</table>

LV indicates left ventricular; PC, preconditioning. Reperfusion values represent the average of values measured at the different postischemic study points (30, 45, and 60 minutes).

*P*<0.001 vs control, 5-HD+diazoxide PC, and 5-HD+ischemic PC groups. All values are mean±SEM.
Figure 2. Endothelium-independent coronary flow response to papaverine (5×10⁻⁶ mol/L). All values are mean±SEM. PC indicates preconditioning. *P<0.001 vs corresponding preischemic baseline value; †P<0.02 vs corresponding reperfusion baseline value; ‡P<0.05 vs corresponding reperfusion baseline value.

Myocardial Water Content
Percent tissue water was significantly smaller in hearts preconditioned (613±59 IU/g dry weight) than in controls (1013±138 IU/g dry weight, P<0.02). Administration of 5-HD before ischemic preconditioning blunted this protective effect because in this group creatine kinase leakage increased to 817±76 IU/g dry weight (P<0.05 versus ischemic preconditioning). Diazoxide preconditioning also reduced the postischemic creatine kinase release (782±55 IU/g dry weight), but the difference with the control group failed to reach statistical significance (P=0.13). Pretreatment with 5-HD before diazoxide preconditioning increased the total creatine kinase leakage to 996±98 IU/g dry weight, but the difference with diazoxide preconditioning alone still remained slightly above the threshold of statistical significance (P=0.062).

Discussion
The major finding of the present study is that the cardioprotective effects of ischemic preconditioning can be duplicated by a selective pharmacological opening of mitochondrial K<sub>ATP</sub> channels. The selectivity of this opening is based on the fact that cardiac mitochondrial K<sub>ATP</sub> channels are 2000 times more sensitive to diazoxide than sarcolemmal K<sub>ATP</sub> channels. Additional evidence for the involvement of mitochondrial K<sub>ATP</sub> channels in mediating preconditioning-induced cardioprotection comes from our findings that protection was lost in hearts pretreated with 5-HD, which selectively blocks these channels. Of note, use of the fluorescence of FAD-linked enzymes as an index of the mitochondrial redox state has allowed Liu et al to show that the EC<sub>50</sub> for diazoxide to induce oxidation, which results from opening of the mitochondrial K<sub>ATP</sub> channels, was 0.16 mol/L. This concentration is almost equivalent to that (30 μmol/L) used in the present experiments.

The cardioprotective effects of mitochondrial K<sub>ATP</sub> channels have previously been established in studies that have reported that diazoxide enhanced survival of isolated ventricular myocytes in a pelleting model of ischemia, improved functional recovery in rat and rabbit hearts undergoing 20- to 50-minute periods of normothermic global ischemia, and reduced infarct size in a rabbit model of regional ischemia. The present results extend these conclusions to the more surgically relevant setting of prolonged hypothermic ischemic arrest, as occurs during cold storage of cardiac allografts, and consequently suggest that the “memory” of cardiac cells to diazoxide exposure is abrogated neither by lengthening of the occlusion period nor by the hypothermic conditions prevailing during this ischemic interval.

The mechanism by which diazoxide elicits cardioprotection is not yet conclusively established. It is known, however, that opening of the mitochondrial K<sub>ATP</sub> channels dissipates the inner mitochondrial membrane potential created by the pro-
ton pump. Dissipation of this potential might have at least 2 major consequences: (1) a reduction in calcium influx and consequently mitochondrial calcium overload, which has been correlated with improved functional recovery after an ischemic insult, and (2) enhancement of the inhibition of mitochondrial ATP-synthase by the regulatory protein IF$_1$ and the subsequent better sparing of ATP. Regardless of the precise mechanism, these hypotheses are consistent with previous observations that preconditioning delays both calcium overload and ATP depletion during the period of sustained ischemia. In turn, one would expect from these metabolic events a reduction in the degree of postischemic myocardial stunning.

In contrast, postischemic values of developed pressure and dP/dt were not significantly different between the 5 groups. Indeed, previous studies have shown that ischemic preconditioning was protective primarily on the diastolic (as opposed to systolic) function of rat hearts subjected to prolonged cardioplegic arrest and hypothermic arrest. It is noteworthy, however, that both ischemically and diazoxide-preconditioned hearts were able to yield values of systolic indexes similar to those of control or 5-HD–pretreated hearts at the cost of significantly lower LVDPs, which may provide indirect evidence of a better preservation of contractility after either form of preconditioning. A greater inotropic reserve in the 2 preconditioned groups can also be marshaled from the reduced postischemic creatine kinase leakage seen in these hearts. This suggests a decrease in the amount of necrotic tissue; indeed, it is through this mechanism that ischemic preconditioning has been reported to improve postischemic function. However, an alternate hypothesis for explaining the failure of our preconditioning protocols to have improved systolic function could be that postischemic stunning seems to be related to decreased myofilament responsiveness to calcium rather than to shortage of energy supply. This could explain why the presumed ATP-sparing effect of preconditioning did not translate into better recovery of contractile function. Furthermore, a possible mechanism of this desensitization of myofilaments to calcium seems to be the production of oxygen-derived free radicals. In this study, all hearts were arrested with and stored in Celsior, a new preservation solution with antioxidant properties that have been directly demonstrated by electron spin resonance spectroscopy studies. It can thus be reasonably postulated that the limitation of oxidative damage was similar in the 5 experimental groups, which would prevent us from demonstrating free radical–mediated differences in the degree of postischemic myocardial stunning.

Likewise, both ischemic and diazoxide preconditioning failed to improve preservation of endothelium-dependent coronary responsiveness in our experiments. This result is consistent with experimental data obtained in a canine model of coronary artery occlusion, isolated buffer-perfused rat hearts, and human cell cultures showing that ischemic preconditioning does not preserve function of endothelial cells. With regard to endothelium-independent function, it is likely that the greater postsischemic coronary flow response to papaverine in the ischemic and diazoxide preconditioning groups resulted only from the reduced postsischemic myocardial contracture seen in these 2 groups.

In conclusion, these data strongly support the possibility of pharmacologically duplicating the cardioprotective effects of ischemic preconditioning by a selective opening of mitochondrial K$_{ATP}$ channels. The recent demonstration that the activity of these channels can be modulated by protein kinase C fits well within the currently accepted paradigm that attributes a central role to this protein (and other kinases) for linking the preconditioning-induced activation of various membrane receptors to the downstream effectors of the pathway, accounting for cardioprotection. Identification of mitochondrial K$_{ATP}$ channels as these effectors opens interesting therapeutic perspectives, because diazoxide is a drug available for human use and might consequently find a place within our armamentarium of strategies designed to improve myocardial preservation during cardiac transplantation and, perhaps more generally, open-heart operations involving a period of global ischemia.

Acknowledgments

This study was supported in part by a grant from the European Commission, Biomed-2 Concerted Action No. 95-0838, The New Ischemic Syndromes. Dr Kevelaitis is a recipient of a research fellowship from the European Society of Cardiology.

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_Circulation_. 1999;100:II-345-II-350
doi: 10.1161/01.CIR.100.suppl_2.II-345
_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

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