Both Metabolic Inhibition and Mitochondrial K\textsubscript{ATP} Channel Opening Are Myoprotective and Initiate a Compensatory Sarcolemmal Outward Membrane Current

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Background—Blockade of oxidative phosphorylation may activate ATP sensitive mitochondrial potassium (mitoK\textsubscript{ATP}) channels. We examined whether both metabolic inhibition and mitoK\textsubscript{ATP} channel openers protect both the whole organ and isolated cells from ischemia.

Methods and Results—Using a Langendorff preparation, one group of isolated rabbit hearts were exposed to ischemic preconditioning (IPC) via 2 episodes of flow interruption. The second group of hearts was preconditioned with 2 episodes of either the metabolic inhibitor, sodium cyanide (NaCN), or the mitoK\textsubscript{ATP} channel opener, diazoxide. The third group of hearts was exposed to the mitoK\textsubscript{ATP} channel inhibitor, 5-hydroxydecanoic acid (5-HD) prior to preconditioning with NaCN, diazoxide or IPC. Controls had no drug infused. Then, ischemia was induced in all hearts by left anterior descending coronary artery occlusion and infarct size was determined. Compared with controls (40±3%), infarct size was significantly reduced in hearts preconditioned with NaCN, diazoxide or IPC (18±3%, 26±3%, 21±2%, respectively; P<0.05 versus control). These reductions were reversed by 5-HD (36±3%, 33±2%, 37±2%; NaCN, diazoxide, IPC, respectively). Secondly, whole cell patch clamped isolated guinea pig ventricular myocytes were preconditioned with 2 episodes of either NaCN or diazoxide followed by Tyrodes perfusion with membrane potential set to −70 mV. Control cells were exposed to Tyrodes solution. All cells were then clamped to −20 mV and exposed to NaCN, which caused induction of an outward potassium current. Compared with controls, the average time to induction of the outward current was significantly reduced in cells preconditioned with either brief application of NaCN (11.6±1.8 versus 5.1±1.0 minutes, control versus NaCN, P<0.05) or diazoxide (5.5±1.4 versus 2.0±0.8 minutes, control versus diazoxide, P<0.05).

Conclusion—Preconditioning protects the heart through mitoK\textsubscript{ATP}. This protection also alters a surface membrane current, which may be important in myocardial protection. (*Circulation.* 2003;108[suppl II]:II-341-II-347.)

Key Words: metabolic inhibition ■ mitochondrial K\textsubscript{ATP} channel ■ ischemia

The beneficial effects of ischemic preconditioning include preservation of left ventricular systolic function, decreased myocardial enzyme release and increased high-energy phosphate stores. These beneficial effects have been elicited by prior transient reductions of coronary flow, which are thought to be essential for ischemic preconditioning. Although, this protection can be obtained from a reduction of coronary flow, a principal effect of transient ischemia is altered myocardial metabolism. Myocardial metabolism can also be altered pharmacologically by blockade of oxidative phosphorylation with the use of sodium cyanide (NaCN). A decrease in the ATP concentration will open the ATP sensitive potassium (K\textsubscript{ATP}) channels. These channels have been proposed to be the end-effector of preconditioning, and are found in both the sarcolemmal and mitochondrial membrane. Furthermore, a recent study suggests “cross talk” between mitochondrial and sarcolemmal channels.

The blockade of oxidative phosphorylation yields decreased ATP production and thereby may activate the mitochondrial K\textsubscript{ATP} current. This current is also increased by mitochondrial K\textsubscript{ATP} channel openers. Opening of mitochondrial K\textsubscript{ATP} channels may play a key role in myocardial protection, as opening of these channels during ischemia is a turning point of self-protection against ischemic insult in myocytes. Based on these observations, we hypothesized that both metabolic inhibition and mitochondrial K\textsubscript{ATP} channel openers protect both the whole organ and the isolated cell from ischemia.

This study consisted of 2 approaches: a whole organ model and an isolated cell model. The isolated cell model has the

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advantage that the simulated ischemia occurs after cell isolation, and so changes inherent to the cell isolation precede the ischemic insult. This model also allows for determination of changes that occur during ischemia. The whole organ model, on the other hand, allows for conventional mechanical function and infarct size measurements (which require a reperfusion phase) in response to clinically relevant ischemia and reperfusion conditions. The use of both models facilitates confirmation of findings and bridges the gap to future large animal and clinical investigations.

Materials and Methods

Animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 85 to 23, revised 1985). All studies were performed in accordance with institutional guidelines as approved by the IACUC committee at SUNY-Stony Brook.

Chemicals

Diazoxide was dissolved in DMSO before addition to experimental solutions with a final concentration of DMSO less than 0.1%. Sodium cyanide (NaCN), diazoxide, 5-hydroxydecanoic acid (5-HD) solutions are bubbled with 100% O₂. The isolated cells were stored in KB solution at 4°C. The dissociated cells are then kept in KB solution containing (in mM, KCl 140; NaCl 5; MgCl₂ 1; CaCl₂ 1; HEPES 10; pH 7.4). Under these conditions, the L-type Ca²⁺-current, the Na/K⁺ exchange current, and the Na/K⁺ pump current are blocked.

Single Guinea Pig Cell Method

Single cardiac myocytes were enzymatically isolated from adult male guinea pig hearts as described in Gao et al. Guinea pigs, weighing 300 to 500 g, were sacrificed with sodium pentobarbital solution (1 mL of 390 mg/ml by peritoneal injection. The heart was isolated and placed in Ca²⁺ free Tyrode solution and the aorta was cannulated. The heart was perfused with 50 mL of Ca²⁺ free Tyrode solution followed by 100 mL of Tyrode solution with 30 μmol/L CaCl₂ and 0.4 mg/mL collagenase kept at 37°C. The heart was then placed in Ca²⁺ free Tyrode solution at room temperature for 2 hours. Afterward, a piece of the ventricle is dissected out and teased into smaller pieces in Kraft-Bühe (KB) solution containing (in mM, KCl 83; K₂HPO₄ 30; MgSO₄ 5; Na-Pyruvic Acid 5; β-OH-Butyric Acid 5; Creatine 5; Taurine 20; Glucose 10; EGTA 0.5; KOH 2; Na₂-ATP 5; pH 7.2). The dissociated cells are then kept in KB solution at room temperature for at least 1 hour before the experiment. All solutions are bubbled with 100% O₂. The isolated cells were stored in KB solution.

An Axopatch 1A amplifier (Axon Instruments, Inc) and the patch clamp technique were employed to observe cell membrane current. Patch-pipette resistances were 2 to 3 mol/L before sealing. The pipette solution contained (in mM): K-Aspartic Acid 125; KCl 15; KOH 10; MgCl₂ 1; HEPES 10; EGTA 11; Mg-ATP 1; pH 7.2. The external Tyrode solution contained (in mM): NaCl 137.7; NaOH 2.3; KCl 4; MgCl₂ 1; HEPES 5; CaCl₂ 1; CdCl₂ 1; Glucose 10; pH 7.4. Under these conditions, the L-type Ca²⁺-current, the Na+/Ca²⁺ exchange current, and the Na⁺/K⁺ pump current are blocked.

Cells were held at −70 mV and exposed to two 3-minute applications of 2 mmol/L NaCN (n=4) interspersed with 3 minutes of normal Tyrode solution. Cells from the same hearts were also held at −70 mV for 12 minutes and served as control cells (n=4). Another group of cells were also held at −70 mV and exposed to 2 three-minute applications of 100 μmol/L diazoxide (n=4) interspersed with three minutes of normal Tyrode solution. Again, control cells (n=4) isolated from the same hearts were held at −70 mV membrane potential for 12 minutes. After the preconditioning treatment, the cells were held at −20 mV to measure the membrane current change induced by a longer period of metabolic inhibition (MI). After the membrane current reached equilibrium, MI was achieved by application of 2 mmol/L NaCN and zero glucose (to the external solution), to mimic ischemia. All experiments were carried out at room temperature (24±0.5°C). Both NaCN and diazoxide were dissolved in Tyrode solution and prepared at target concentrations. All patch clamp data were digitized by the data acquisition program pClamp (Axon Instruments, Inc) for later analysis.

Statistical Analysis

All data are presented as mean±SEM. Comparisons between groups were made with ANOVA (Systat v5.02. Systat, Inc, Evanston, IL). As indicated, multiple comparisons were made with the post hoc test. Statistical significance was considered at P<0.05.
Results

Preconditioning in Isolated Whole Organ Studies

Coronary Flow
The application of either NaCN or diazoxide slightly increased coronary flow, but these increases did not reach statistically significant. The coronary flow for all groups decreased during the regional ischemia. Coronary flow in the NaCN group was significantly higher than that in control (17.6 ± 1.7 versus 29.3 ± 4.1 mL/min, control versus NaCN, P<0.05) during regional ischemia. There was no significant difference between groups during the reperfusion phase.

Left Ventricle Pressures
The brief infusion of NaCN significantly increased end diastolic pressure compared with control (7.4 ± 1.3 versus 27.6 ± 4.4 mm Hg, control versus NaCN, P<0.05). The brief infusion of diazoxide did not induce a change in end diastolic pressure, nor was a change observed with 5-HD pretreatment. There were no significant differences between groups during the regional ischemia and reperfusion phase. The peak developed pressure in all groups decreased during the regional ischemia and recovered slightly during the reperfusion phase. There were no significant differences between groups during the reperfusion phase.

Infarct Size
Infarct sizes for control, IPC, NaCN, and NaCN +5-HD are expressed as a percentage of the region at risk (Figure 1, Panel A). Control hearts had an infarct size of 40 ± 3%. IPC significantly reduced infarct size to 21 ± 2% (P<0.05 versus control). Preconditioning via metabolic inhibition with NaCN also significantly reduced infarct size (18 ± 3%; P<0.05 versus control). However, mitoK_{ATP} channel blocked by 5-HD prior to preconditioning with NaCN reversed infarct size comparable to those in hearts not preconditioned (36 ± 3%; P<0.05 versus NaCN).

Infarct sizes for control, IPC, diazoxide, diazoxide +5HD and IPC +5-HD are shown in Figure 1, Panel B. Preconditioning with the mitoK_{ATP} channel opener, diazoxide significantly reduced infarct size to 26 ± 3% (P<0.05 versus control). These preconditioning effects were reduced by pretreatment with the mitoK_{ATP} channel inhibitor, 5-HD (33 ± 2%; P<0.05 versus diazoxide). 5-HD pretreatment also abolished the infarct size limiting effect of IPC (37 ± 2%; P<0.05 versus IPC).
Sarcolemmal Membrane Current Induced by NaCN Pretreated Myocyte

**The Time Course of Appearance of Outward Current**

Representative data are shown in Figure 2 (Panels A and B) for control and NaCN pretreated myocytes isolated from the same guinea pig heart. The membrane voltage, which is set by the user, is at the top of panel A and B, and the corresponding measured current is plotted below. In the control cell (Figure 2, Panel A), a rapid increase in the outward current induced by exposure to NaCN appears after 12.5 minutes. In the cell exposed to 2 episodes of NaCN during the pretreatment phase a decrease in the time to activation of the outward current induced by NaCN exposure is observed (Figure 2, Panel B). The average time (Figure 2, Panel C) to induction of an outward current was significantly shorter in the NaCN treated cells (5.1 ± 1.0 minutes; *P < 0.05* versus control) compared with the control cells (11.6 ± 1.8 minutes.). Figure 3 panel A and B are sample recordings demonstrating the marked reduction in time to outward current from 6.5 minutes to 2 minutes, in response to pretreatment of diazoxide. Brief exposure of diazoxide followed by metabolic inhibition induced by application of NaCN also markedly reduced the average time (Figure 3, Panel C) to activation of the outward current (5.5 ± 1.4 minutes versus 2.0 ± 0.8 minutes; control versus diazoxide pretreatment; *P < 0.05*). Representative data are shown in Figure 3 (Panels A and B) for a control and a diazoxide pretreated myocyte isolated from the same heart.

**Discussion**

Several conclusions can be drawn from these studies. First, preconditioning with NaCN reduced infarct size following prolonged ischemia. The effect of preconditioning with NaCN were largely eliminated if mitoK_ATP channel was blocked by 5-HD in the whole organ model. Second, short periods of inhibition of mitochondrial oxidative phosphorylation using NaCN induced a rapid appearance of the sarcolemmal membrane outward current in isolated myocyte exposed to long term NaCN. Third, diazoxide, a mitochondrial K_ATP channel opener, caused infarct size reduction, which was largely eliminated by 5-HD pretreatment in the whole organ model. Also, the sarcolemmal outward current, which was initiated by continuous application of NaCN, appeared earlier after diazoxide exposure, suggesting a sarcolemmal current is altered by opening the mitochondrial K_ATP channel. Finally, we have demonstrated a correlation between a reduction in infarct size in the whole organ and a decreased time to the appearance of a sarcolemmal outward current. Taken together, our results suggest the activation of the mitochondrial K_ATP channels...
may lead to preconditioning and a compensatory sarcolemmal outward current.

The Opening of Mitochondrial KATP Channels is Myoprotective

Sodium cyanide uncouples oxidative phosphorylation leading to a decrease in cellular ATP levels, which may result in opening mitochondrial K\textsubscript{ATP} channels. Therefore, it is not unreasonable to presume that both sodium cyanide and the mitochondrial K\textsubscript{ATP} channel opener diazoxide may exert their preconditioning effects through similar mechanisms. Herein, both agents were shown to reduce infarct size, the standard measurement for determining the effectiveness of preconditioning, and these reductions were reversed by 5-hydroxy decanoic acid, which is a mitoK\textsubscript{ATP} channel blocker.

The depressed high-energy phosphate levels has been suggested to be a determinant of myocardial ischemic injury and that their improvement could result in the protection of the ischemic heart.\textsuperscript{14} Also, the preconditioned heart can preserve high-energy phosphate levels.\textsuperscript{14} However, in NaCN preconditioning, a possible explanation of our results is that the depletion of high energy phosphate caused by ischemia was altered by NaCN pretreatment. It is conceivable that the more rapid depletion of high-energy phosphate caused by NaCN preconditioning in face of the subsequent ischemic insult leads to more rapid activation of mitoK\textsubscript{ATP} channels, which may be myoprotective.

Early Appearance of Sarcolemmal Outward Current Initiated by NaCN Preconditioning as Well as Diazoxide Preconditioning May Be Myoprotective

Other studies have demonstrated the application of sodium cyanide induces an outward current, which occurs with a variable time delay of roughly 2 to 20 minutes.\textsuperscript{15,16} Preconditioning is thought to decrease the time to induction of the outward current, as well as be a self-protective response of the myocyte to ischemia.\textsuperscript{15} In the present study, brief exposure to 2 mmol/L sodium cyanide before the subsequent simulated ischemia significantly decreased the time necessary to induce the outward current. This result suggests that a brief period of metabolic inhibition, to simulate ischemic preconditioning, is protective in our isolated cell model.

To elucidate the role of mitochondrial ATP-dependent potassium channels and their potential interaction with sar-
Coleman membrane current, diazoxide, a specific mitochondrial agonist, was applied to the isolated cells. In these studies, diazoxide was applied as a pretreatment prior to NaCN application. Notably, diazoxide preconditioning resulted in an earlier appearance of sarcolemmal outward current as seen in cyanide preconditioning. These data suggest a coupling of the response in the sarcolemma to the mitochondrial stimulus.

Although our results are consistent with others in demonstrating a reduced time to the rapid activation of an outward sarcolemmal current, it is important to note that we did not measure mitochondrial current. Thus, we are not able to directly assess the role of mitochondrial KATP channel openers. However, it should be noted that in previous studies with the same protocol, the observed outward current has been assumed to be \( i_{\text{KATP}} \), a finding with which we disagree (see next section).

**Identification of Protective Sarcolemmal Outward Current**

Previously, we have demonstrated in guinea pig ventricle myocytes, that the protective inward rectifier may not be \( i_{\text{KATP}} \). This conclusion is based on our finding that the amplitude and timing of appearance of the outward current is unaffected by glybenclamide, a blocker of the sarcolemmal KATP channel. These results are in agreement with those from other laboratories who note that this current declines on its own, even when glybenclamide is not present. The most likely possible candidates for this inwardly rectifying current is \( i_{\text{K1}} \), which is the major background potassium current involved in maintaining the resting potential and generating final repolarization of the action potential. Supporting the role for \( i_{\text{K1}} \) as the outward current, is the recent observation that preconditioning is eliminated when \( i_{\text{K1}} \) is knocked down with dominant negative Kir 2.1 gene. Purpose of this increased outward current is still unknown, although its presence helps the cell maintain a more hyperpolarized membrane potential, and thus enhance excitability.

One of the major roles of these inwardly rectifying potassium channels is to maintain the resting membrane potential of the cell. During ischemia, rapid activation of these channels can help maintain a membrane potential closer to the electrochemical potassium membrane potential (\( E_k \)). Therefore, the cell can preserve excitability and limit the calcium and hydrogen influx earlier, which result in increasing cell survival. As potassium is the ion that contributes most to the resting membrane potential, by keeping the membrane potential closer to \( E_k \) (which is more negative than the resting membrane potential), the cell will maintain its excitability. We believe that control of these sarcolemmal ion currents is very important to increase the cell survival during the ischemia.

**Study Limitations**

A possible limitation of this study is that aerobic metabolism was blocked by NaCN, but anaerobic metabolism was not blocked. The latter might contribute a small amount of ATP generation during ischemia, but did not obviously affect our results.

A second concern is that the cell isolation may be affected by the isolation procedure. We suggest that this was not the case as cell membrane current determinations clearly discriminated between control and study cells.

**Clinical Implication**

Recently, “hyperpolarized” cardioplegia, usually with an ATP sensitive potassium (\( K_{\text{ATP}} \)) channel opener as an adjunct or sole arresting agent, has been shown to be superior to conventional cardioplegia. These agents should tend to keep \( E_m \) more hyperpolarized during prolonged ischemia. Snabaiti et al used a sodium channel blocker to hyperpolarize \( E_m \) and also documented significant improvements over conventional hyperkalemic cardioplegia. These data suggest that hyperpolarization during ischemia can maintain cell excitability and is therefore myoprotective. In preconditioning, quick activation of this protective current during ischemia may be one mechanism of myoprotection. If this hypothesis is correct, then combination of the protective effects of ischemic preconditioning with conventional cardioplegia may have a benefit to surgical patients.

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