Arrhythmia/Electrophysiology

K_ATP Channel Activation Induces Ischemic Preconditioning of the Endothelium in Humans In Vivo

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Background—Endothelial dysfunction contributes to ischemia-reperfusion injury (IRI) and is reduced by ischemic preconditioning (IPC). IPC may involve activation of ATP-sensitive potassium channels (K_ATP). We determined whether modulation of K_ATP channels occurs in endothelial IPC in humans.

Methods and Results—IRI of the forearm was induced by inflating a blood pressure cuff to 200 mm Hg for 20 minutes in healthy volunteers. K_ATP activation was modulated by intra-arterial glibenclamide (blocker) and diazoxide (opener). Endothelial function (response to intra-arterial acetylcholine) was assessed with forearm plethysmography before and after (1) 15-minute reperfusion, (2) IRI preceded by IPC (3 five-minute periods of ischemia), (3) IRI preceded by IPC with glibenclamide, (4) IPC followed by glibenclamide before IRI, (5) IRI preceded by diazoxide, and (6) IRI preceded by coinfusion of glibenclamide with diazoxide. IRI caused endothelial dysfunction (P=0.002), which IPC prevented (P=0.68). Glibenclamide abolished IPC when given contemporaneously with (P=0.003) or during IRI (P=0.0005). Diazoxide prevented endothelial dysfunction after IRI (P=0.68) but not when confused with glibenclamide.

Conclusion—Glibenclamide abolishes and diazoxide mimics endothelial IPC in humans. The time course of the effect of glibenclamide suggests involvement of K_ATP channels as effectors of endothelial IPC in vivo. These data may have implications for understanding the therapeutic role of agents that modulate K_ATP channel function. (Circulation. 2004; 110:2077-2082.)

Key Words: ischemia ■ reperfusion ■ potassium channels ■ ischemic preconditioning

Ischemia-reperfusion injury (IRI) complicates myocardial infarction and stroke and contributes to the associated tissue injury and mortality; reducing IRI may improve the outcome of reperfusion therapy for these conditions.1 One successful approach in the experimental setting is ischemic preconditioning (IPC), whereby prior sublethal ischemia induces a state of protection against subsequent prolonged IRI. IPC reduces the size of experimental myocardial infarction in a variety of animal models.2–7

The mechanism of IPC is complex, involving a variety of “triggers” believed to be released in tissues during ischemia, including adenosine, bradykinin, endogenous opiates, and reactive oxygen species.10 These triggers activate multiple signaling pathways that appear to converge on the mitochondria, with evidence for activation of mitochondrial ATP-sensitive potassium channels (K_ATP) as a key step in the effector mechanism of IPC. Although mitochondrial K_ATP channels have yet to be identified at the molecular level, K_ATP openers (including diazoxide) mimic IPC,11–13 and K_ATP blockers (including glibenclamide) inhibit IPC in animal models.4,11,14 How opening of mitochondrial K_ATP channels causes IPC is unclear, but depolarizing of mitochondria with reduction in intramitochondrial calcium,15 reduced mitochondrial swelling,16 and activation of the mitochondrial permeability transition pore,17 has been suggested. The situation is further complicated by recent animal data that suggest that opening of K_ATP channels is involved in the triggering of preconditioning via release of reactive oxygen species from the mitochondria.13

In humans, IPC has been demonstrated to protect human cardiomyocytes from ischemic damage in vitro.18–20 Although K_ATP channel blockers reverse the protective effect of IPC in isolated cardiomyocytes, it remains unclear to what extent K_ATP channels contribute to IPC in humans in vivo. In patients undergoing coronary angioplasty, episodes of cardiac ischemia (caused by angioplasty balloon inflation) induce resistance of the myocardium to subsequent episodes of ischemia. This protection is prevented by administration of glibenclamide, consistent with a preconditioning effect that is dependent on K_ATP channels.21 We have shown recently that forearm ischemia causes endothelial dysfunction that is prevented by IPC.22 This model is suited to exploring the

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mechanism of preconditioning in the absence of any confounding effects caused by altered collateral blood supply or direct effects of $K_{ATP}$ channel blockers on ECG indices of ischemia. The aims of the present study were to test the hypotheses that the $K_{ATP}$ channel blocker glibenclamide blocks IPC and that the $K_{ATP}$ activator diazoxide mimics IPC in the human endothelium in vivo. In addition, we sought to investigate the timing of the involvement of $K_{ATP}$ channels in IPC to determine whether they were involved as triggers or effectors of IPC.

**Methods**

After local ethics committee approval and written informed consent were obtained, 42 healthy nonsmoking adult volunteers (4 females) aged between 20 and 40 years were recruited. Seventeen subjects underwent repeat studies (participating in up to 5 protocols), but these were separated in time by at least 34 days.

**Techniques**

**Assessment of Resistance-Vessel Endothelial Function**

All studies were performed in a quiet, temperature-controlled (25°C to 26°C) laboratory. The volunteers were asked to abstain from caffeine for 12 hours before the study. Brachial artery cannulation with a 27-gauge needle (Coopers Needle Works) was performed in the nondominant arm under aseptic conditions with 2 mL of 2% lidocaine (subcutaneous) for anesthesia. Drugs were administered in saline (0.9% [wt/vol] sodium chloride) and infused at a rate of 0.5 mL/min. Bilateral forearm blood flow was measured with calibrated mercury-in-silastic strain gauges as described previously. During recording periods, the hands were excluded from the circulation by inflation of wrist cuffs to 200 mm Hg. Forearm blood flow in response to infusion of endothelium-dependent dilator acetylcholine (ACh; 25, 50, and 100 nmol/min, each dose for 3 minutes; Clinalfa) was measured 10 minutes after cannulation to establish baseline endothelial function. In some studies, the response to the endothelium-independent dilator nitroglycerine (NTG; 4, 8, and 16 nmol/min, each dose for 3 minutes) was determined.

**Ischemia-Reperfusion Injury**

The nondominant forearm was rendered ischemic by inflating a 12-cm blood pressure cuff to a pressure of 200 mm Hg around the upper arm for 20 minutes as described previously. Endothelial function was assessed at baseline and 15 minutes after reperfusion.

**Ischemic Preconditioning**

The nondominant forearm was rendered ischemic by inflating a 12-cm blood pressure cuff to a pressure of 200 mm Hg around the upper arm for 5 minutes, after which the cuff was deflated and the arm reperfused for 5 minutes. This cycle was then repeated twice so that the arm was exposed to 3 episodes of brief ischemia in total.

**Experimental Protocols**

**Protocol 1: Effects of IRI on Endothelial Function**

Baseline endothelial function was established, after which the nondominant arm underwent IRI, and the response to ACh was reassessed after reperfusion for 15 minutes (n = 13; Figure 1A).

**Protocol 2: Effects of IPC on Endothelial Dysfunction Caused by IRI**

Baseline endothelial function was established, after which the nondominant arm underwent the preconditioning protocol, followed by IRI as above. The response to ACh was reassessed after 15 minutes of reperfusion (n = 10; Figure 1B). In 5 subjects, the response to NTG was determined at baseline and after IPC followed by IRI.
Protocol 3: Effect of K_{ATP} Blockade on Endothelial IPC
After baseline endothelial function was established, glibenclamide (10 μg/min; a gift from Aventis Pharma Deutschland, Frankfurt, Germany) was infused for 5 minutes and continued during the 30 minutes of the IPC protocol. After this, the arm underwent IRI, and the response to ACh was reassessed after reperfusion for 15 minutes (n=9; Figure 1C). When administered during IPC, any effect of glibenclamide may be explained by an action on the initiation (triggering) of IPC. To determine whether glibenclamide had an effect independent of the triggering of IPC, the same dose was administered for 35 minutes during IRI, after which the response to ACh was reassessed (n=6; Figure 1D). In 6 subjects, the effect of glibenclamide alone (10 μg/min for 35 minutes) on endothelial function was assessed by determining the response to ACh before and 35 minutes after cessation of the infusion.

Protocol 4: Effect of K_{ATP} Stimulation on Endothelial IRI
After baseline endothelial function was established, diazoxide (800 μg/min; Goldshield Pharmaceuticals Ltd) was infused in 8 subjects for 20 minutes into the nondominant arm. After a further 10 minutes (to allow baseline blood flow to be restored), the arm underwent the IRI protocol, after which the response to ACh was assessed (Figure 1E). In 5 subjects, the effect of diazoxide alone (800 μg/min for 20 minutes) on endothelial function was assessed by determining the response to ACh before and 45 minutes after cessation of the infusion.

Protocol 5: Effect of Coinfusion of Glibenclamide and Diazoxide on Endothelial IRI
After baseline endothelial function was established, glibenclamide (10 μg/min) was infused for 35 minutes, during which diazoxide (800 μg/min) was coinfused for 20 minutes. The arm underwent the IRI protocol, after which the response to ACh was reassessed (Figure 1F).

Calculations and Statistics
Resting forearm blood flow in both arms in the last minute of each recording period was used for analysis. The mean ratio of flow in the infused/noninfused was calculated in the immediately preceding baseline period before each drug infusion, and changes in blood flow were expressed as a percentage change relative to this baseline flow ratio.

All data were analyzed with GraphPad Prism version 3.00 and expressed as mean±SEM. To assess the affects of each protocol, dose-response curves were compared by paired 2-way ANOVA with Bonferroni posttests as appropriate. In all cases, a value of P<0.05 was considered statistically significant.

Results
All subjects tolerated the procedures without complications. Neither diazoxide nor glibenclamide altered heart rate, blood pressure, or blood glucose.

Effect of IRI and IPC on Dilator Function
ACh caused dose-dependent increases in forearm blood flow (Figure 2A). Fifteen minutes after IRI (when baseline blood flow had returned to normal), the response to ACh was significantly blunted (P=0.002; Figure 2A). IPC before IRI prevented endothelial dysfunction (P=0.40; Figure 2B). IPC followed by IRI had no effect on the response to NTG in 5 subjects (P=0.34; data not shown).

Effect of K_{ATP} Blockade on Endothelial IPC
Glibenclamide alone did not alter the response to ACh (P=0.52). However, glibenclamide blocked the protective effect of IPC on endothelial function when infused during the triggering phase of IPC (P=0.003; Figure 3A) and during IRI (P=0.0005; Figure 3B).

Effect of K_{ATP} Stimulation on Endothelial IRI
Diazoxide caused a 52±6.9% (mean±SEM) increase in blood flow, but this returned to normal by 45 minutes after the infusion was stopped (n=7). Prior infusion of diazoxide had no direct effect on the response to ACh at 45 minutes (P=0.50). Diazoxide infusion prevented endothelial dysfunction caused by IRI (Figure 4A). However, when diazoxide was administered with glibenclamide (given at a dose that blocked IPC), the protective effect of diazoxide on IRI-induced endothelial dysfunction was abolished (n=7; Figure 4B).

Discussion
This study has confirmed our previous observations that IRI of the human forearm causes endothelial dysfunction and that this can be prevented by IPC. Moreover, these findings implicate K_{ATP} channels in the mechanism of IPC, because IPC was blocked by glibenclamide and mimicked by diazoxide.

Endothelial dysfunction caused by IRI has been well described in experimental animals and in humans. Such endothelial damage may play a pathogenic role in tissue injury during IRI by predisposing to neutrophil infiltration of tissues or microvascular obstruction, both of which have been implicated in the pathogenesis of tissue damage. Consequently, the protective effect of IPC to limit IRI may be, in part, a consequence of its salutary effect on the endothelium, promoting more effective reperfusion after relief of vascular obstruction. Mechanistically, IPC of the vascular endothelium in animal models resembles that described in other cell types, including the myocardium, and may be directly rele-
vant to other tissue types in humans. In the present study, IRI substantially reduced the response of the forearm resistance vascular bed to ACh. ACh may have a preconditioning effect, and by using a different agonist (e.g., substance P), we might have demonstrated a greater degree of endothelial dysfunction caused by IRI. Nonetheless, the present findings confirm the results of our previous study, in which we also demonstrated that the response of the smooth muscle to NTG was unaffected by IRI, which indicates that the site of injury is the endothelium. IPC completely preserved the response to ACh but, as expected, had no effect on the response to NTG. These observations exclude an effect of IPC to augment vascular smooth muscle function, which might also increase the dilator response to ACh, and indicate that IPC acts to preserve endothelium-dependent dilatation.

A large body of published data implicates activation of K<sub>ATP</sub> channels in the mechanism of IPC in a variety of tissues and animal models. There is evidence for involvement of K<sub>ATP</sub> channels on the sarcolemma and the mitochondria, based on the actions of known pharmacological modulators of channel activity. How the activation of K<sub>ATP</sub> channels causes IPC has yet to be determined. Mitochondrial channels may be activated to trigger IPC via a mechanism that is dependent on production of reactive oxygen species to activate second-messenger pathways. In contrast, other data suggest that K<sub>ATP</sub> channels are involved after IPC is triggered and distal to second-messenger pathways. How this increases resistance to ischemic damage is unclear, but it has been suggested that K<sub>ATP</sub> activation closes the mitochondrial permeability transition pore, limiting the rise in intramitochondrial calcium and reducing mitochondrial swelling. K<sub>ATP</sub> channels are also present on vascular smooth muscle cells and endothelium, and similar mechanisms of protection may be involved in blood vessels.

In human tissue, mitochondrial K<sub>ATP</sub> channels are involved in preconditioning in vitro, but it is unclear at which point in the preconditioning mechanism they are activated. In humans in vivo, the action of K<sub>ATP</sub> modulators has been investigated in patients undergoing angioplasty, in which the preconditioning stimulus is a brief period of ischemia caused by angioplasty balloon inflation. In such studies, ECG changes during a second balloon inflation are taken as a measure of IRI. The preconditioning inflation reduces the magnitude of these ECG changes, with evidence that K<sub>ATP</sub> channel blockers limit the protection induced by the preconditioning inflation. However, whether this represents a direct effect of K<sub>ATP</sub> channel blockade on the ECG rather than on ischemia is unclear.

The present study sought to clarify further the role of K<sub>ATP</sub> channels in the mechanism of IRI using a human in vivo model that would allow direct assessment of K<sub>ATP</sub> channels in the mechanism of IPC. Glibenclamide was used to block both mitochondrial and sarcolemmal K<sub>ATP</sub> channels. The dose used was based on that which had previously been shown to block the effects of K<sub>ATP</sub> channel openers. In the present study, intra-arterial infusion of glibenclamide did not affect...
basal blood flow or the response to ACh, consistent with the results of other investigators. However, when infused during the preconditioning protocol, glibenclamide abolished the protective effect of IPC on IRI, with recurrence of endothelial dysfunction. These observations suggest that IPC activates K\textsubscript{ATP} channels to limit IRI. To confirm a role for K\textsubscript{ATP} channels in the mechanism of IPC, we used diazoxide (a selective activator of the mitochondrial K\textsubscript{ATP} channel) to induce pharmacological preconditioning in the forearm as a prelude to IRI. At the dose infused, diazoxide caused an increase in forearm blood flow, which returned to baseline by 45 minutes. There was no direct effect of diazoxide on the response to ACh. However, diazoxide administered 15 minutes before IRI preserved the response to ACh after IRI. Moreover, when glibenclamide was infused at a dose that blocked IPC, the protective effect of diazoxide was also blocked. Taken together with the results using glibenclamide, these data clearly implicate K\textsubscript{ATP} channel activation in the mechanism of endothelial preconditioning in humans and, to a lesser extent, in swine myocardium. Circ Res. 1990;66:1133–1142.


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