Glimepiride, a Novel Sulfonylurea, Does Not Abolish Myocardial Protection Afforded by Either Ischemic Preconditioning or Diazoxide

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Background—The sulfonylurea glibenclamide (Glib) abolishes the cardioprotective effect of ischemic preconditioning (IP), presumably by inhibiting mitochondrial K<sub>ATP</sub> channel opening in myocytes. Glimepiride (Glim) is a new sulfonylurea reported to affect nonpancreatic K<sub>ATP</sub> channels less than does Glib. We examined the effects of Glim on IP and on the protection afforded by diazoxide (Diaz), an opener of mitochondrial K<sub>ATP</sub> channels.

Methods and Results—Rat hearts were Langendorff-perfused, subjected to 35 minutes of regional ischemia and 120 minutes of reperfusion, and assigned to 1 of the following treatment groups: (1) control; (2) IP of 2 × 5 minutes each of global ischemia before lethal ischemia; or pretreatment with (3) 30 μmol/L Diaz, (4) 10 μmol/L Glim, (5) 10 μmol/L Glib, (6) IP + Glim, (7) IP + Glib, (8) Diaz + Glim, or (9) Diaz + Glib. IP limited infarct size (18.5 ± 1% vs 43.7 ± 3% in control, P < 0.01) as did Diaz (22.2 ± 4.7%, P < 0.01). The protective actions of IP or Diaz were not abolished by Glim (18.5 ± 3% in IP + Glim, 22.3 ± 3% in Diaz + Glim; P > 0.01 vs control). However, Glib abolished the infarct-limiting effects of IP and Diaz. Patch-clamp studies in isolated rat ventricular myocytes confirmed that both Glim and Glib (each at 1 μmol/L) blocked sarcolemmal K<sub>ATP</sub> currents. However, in isolated cardiac mitochondria, Glim (10 μmol/L) failed to block the effects of K<sub>ATP</sub> opening by GTP, in contrast to the blockade caused by Glib.

Conclusions—Although it blocks sarcolemmal currents in rat cardiac myocytes, Glim does not block the beneficial effects of mitochondrial K<sub>ATP</sub> channel opening in the isolated rat heart. These data may have significant implications for the treatment of type 2 diabetes in patients with ongoing ischemic heart disease. (Circulation. 2001;103:3111-3116.)

Key Words: potassium • myocardial infarction • diabetes mellitus • ion channels
Heart perfusion was started with a saline solution of 0.12% Evans blue. After 1 to 4 hours at −20°C, hearts were sliced into 1-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 10 to 15 minutes. The tissue slices were then fixed in 10% formalin. At the end of this procedure, in the risk zone the viable tissue was stained red and the infarcted tissue appeared pale. The slices were drawn onto acetate sheets. With the use of a computerized planimetry package (Summa Sketch II, Summagraphics), the percentage of infarcted tissue within the volume of myocardium at risk was calculated.

### Infarct Size Measurement

At the end of the reperfusion period the snare was tightened to reocclude the coronary artery, and a saline solution of 0.12% Evans blue was infused slowly by way of the aorta. This procedure delineated the nonischemic zone of the myocardium as a dark blue area. After 1 to 4 hours at −20°C, hearts were sliced into 1-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 10 to 15 minutes. The tissue slices were then fixed in 10% formalin. At the end of this procedure, in the risk zone the viable tissue was stained red and the infarcted tissue appeared pale. The slices were drawn onto acetate sheets. With the use of a computerized planimetry package (Summa Sketch II, Summagraphics), the percentage of infarcted tissue within the volume of myocardium at risk was calculated.

### Methods

#### Animals

Male Sprague-Dawley rats (350 ± 30 g body weight) were used. All animals were obtained from the same source (Charles River UK Limited, Margate, UK), fed a standard diet, housed under the same conditions, and received humane care in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

#### Isolated Heart Perfusion

Rats were anesthetized with sodium pentobarbital (55 mg/kg intraperitoneally) and given heparin sodium (300 IU). Hearts were excised, arrested in ice-cold buffer, and mounted on a constant-pressure (80 mm Hg) Langendorff perfusion system. They were perfused retrogradely with a modified bicarbonate-buffer containing the following chemicals (in mmol/L): NaCl 135.0, KCl 6.0, CaCl 2 2.0, MgCl 2 1.0, NaHCO 3 25.0, KCl 4.8, MgSO 4 1.2, KH 2 PO 4 1.2, CaCl 2 1.7, and glucose 12.0. All solutions were filtered through a Whatman 2.0-micronfilter and gassed with 95%O 2 /5% CO 2 (pH 7.35 to 7.50 at 37°C). Temperature was continuously monitored by a thermoprobe inserted into the right ventricle. A latex, fluid-filled, isovolumic balloon was introduced into the left ventricle through the left atrial appendage and inflated to give a preload of 8 to 10 mm Hg. Left ventricular developed pressure, heart rate, and coronary flow were registered at regular intervals. A surgical needle was passed under the left main coronary artery, and the ends of the thread were passed through a small plastic tube to form a snare. Regional ischemia was induced by tightening the snare, and reperfusion was started by releasing the ends of the thread.

#### Treatment Protocols

The experimental protocols are presented in Figure 1. Glibenclamide, glibempire, and diazoxide were dissolved in dimethyl sulfoxide and added to the Krebs-Henseleit buffer such that the final dimethyl sulfoxide concentration did not exceed 0.02%. The hearts were randomly assigned to 1 of 9 treatment groups: (1) Control hearts (n = 9) were perfused with 0.02% dimethyl sulfoxide for 20 minutes during stabilization before 35 minutes of regional ischemia and 120 minutes of reperfusion. (2) IP hearts (n = 7) were treated with 2 periods of 5 minutes each of global ischemia with a 10-minute intervening reperfusion before 35 minutes of regional ischemia and 120 minutes reperfusion. (3) Hearts (n = 9) were perfused with 30 μmol/L diazoxide for 20 minutes immediately before regional ischemia. (4) Hearts (n = 6) were perfused with 10 μmol/L glibempire for 20 minutes immediately before regional ischemia. (5) Hearts (n = 6) were perfused with 10 μmol/L glibempire for 20 minutes immediately before regional ischemia. (6) Hearts (n = 8) underwent the IP protocol in the presence of 10 μmol/L glibempire. The drug was added to the perfusate 20 minutes before starting the preconditioning protocol and was present throughout this protocol. The buffer was switched to normal Krebs-Henseleit buffer after the onset of regional ischemia. (7) Hearts (n = 7) underwent the IP protocol in the presence of 10 μmol/L glibempire as in group 6. (8) Hearts (n = 9) were coperfused with 30 μmol/L diazoxide and 10 μmol/L glibempire for 20 minutes immediately before regional ischemia. (9) Hearts (n = 8) were coperfused with 30 μmol/L diazoxide and 10 μmol/L glibempire for 20 minutes immediately before regional ischemia.

#### Infarct Size Measurement

At the end of the reperfusion period the snare was tightened to reocclude the coronary artery, and a saline solution of 0.12% Evans blue was infused slowly by way of the aorta. This procedure delineated the nonischemic zone of the myocardium as a dark blue area. After 1 to 4 hours at −20°C, hearts were sliced into 1-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 10 to 15 minutes. The tissue slices were then fixed in 10% formalin. At the end of this procedure, in the risk zone the viable tissue was stained red and the infarcted tissue appeared pale. The slices were drawn onto acetate sheets. With the use of a computerized planimetry package (Summa Sketch II, Summagraphics), the percentage of infarcted tissue within the volume of myocardium at risk was calculated.

#### Patch-Clamp Studies

Ventricular myocytes were isolated from adult rat hearts by enzymatic dissociation as previously described. Cells were stored at 10°C to 12°C and bathed in a solution containing the following constituents (in mmol/L): NaCl 135.0, KCl 9.0, CaCl 2 2.0, MgCl 2 1.0, NaH 2 PO 4 0.33, sodium pyruvate 5, and HEPES 10.0, pH 7.4. The intracellular (pipette) solution contained the following constituents (in mmol/L): KCl 140.0, MgCl 2 1.0, EGTA 5.0, ATP 2.0, ADP 0.1, GTP 0.1, and HEPES 10.0, pH 7.2. Currents were recorded by using conventional patch-clamp techniques with an Axopatch 200B amplifier, analyzed with pCLAMP 8 software, and expressed relative to cell size as picoamps per picofarad. Experiments were performed at 30°C.

#### Studies in Isolated Cardiac Mitochondria

Mitochondria were isolated from rat hearts by using a previously described technique. After extraction the mitochondria were kept on ice, and an aliquot was suspended in KCl buffer containing (in mmol/L) KCl 45.0, potassium acetate 25.4, TES 5.0, EGTA 0.1 (pH 7.4), MgCl 2 1.0, and 10 μmol/L cytochrome c. Substrates for respiration were 2.5 mmol/L ascorbate and 0.25 mmol/L N’,N’,N’,N’-tetramethyl-P-phenylene diamine. Aliquots of mitochondria were incubated with the mitochondrial membrane potential–sensitive dye tetramethylrhodamine methyl ester (TMRM, 200 nmol/L) at room temperature for 5 minutes before drug intervention. Where indicated, the physiological K ATP channel opener GTP (50 μmol/L) was added to the TMRM-stained mitochondria 2 minutes before measurements of fluorescence in the absence or presence of 5-hydroxydecanoate (a mitochondrial K ATP channel inhibitor, 100 μmol/L), glibenclamide (10 μmol/L), and glibempire (10 μmol/L). The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (1 μmol/L) was used as a positive control. Cytofluorometric analysis was done on a Coulter Epics flow cytometer equipped with a 488-nm argon laser. The TMRM signal was analyzed in the FL2 channel equipped with a band-pass filter at 580 ± 30 nm; the photomultiplier value of the detector was 631 V. Data were acquired on a logarithmic scale. Arithmetic mean values of the median fluorescence intensities were determined for each sample for graphic representation. Experiments were performed on mitochondria isolated from 6 individual rats, each experiment representing 15 000 mitochondria.

#### Statistical Analysis

All values are expressed as mean ± SEM. Data were analyzed by 1-way ANOVA and Fisher’s protected least significant difference test.
Figure 2. Infarction developed in risk zone in hearts preconditioned (IP) in presence of 10 μmol/L glibenclamide (glib) or glimepiride (glim). *P<0.001.

Figure 3. Infarction developed in risk zone in hearts pretreated with both 30 μmol/L diazoxide (diaz) and 10 μmol/L glibenclamide (glib) or glimepiride (glim) before regional ischemia. *P<0.001.

**Results**

**Exclusions**
We used a total of 76 rat hearts for the Langendorff perfusion study. Of these, 6 were excluded owing to poor function during stabilization.

**Hemodynamic Data**
Baseline data relating to cardiac function and coronary flow rates before regional ischemia where similar in all experimental groups. During regional ischemia, coronary flow and left ventricular developed pressure decreased to a similar extent in all groups. An increase in coronary flow during the first minutes of reperfusion was indicative of successful reflow, but coronary flow subsequently declined in all groups during the following 120-minute reperfusion period. During reperfusion, left ventricular developed pressure recovered gradually, though never reaching stabilization values.

**Infarct Size Data**
The risk zone volume was similar in all experimental groups, at ~0.5 cm³. Infarct size is represented as the percentage of tetrazolium-negative tissue in the ischemic risk zone. As expected, IP significantly reduced the amount of infarcted tissue in the risk zone compared with control hearts (18.6±1.5% vs 43.7±3.0%, P<0.01; Figures 2 and 3). Glimepiride or glibenclamide alone did not influence infarct size (glibenclamide 44.7±5%, glimepiride 41.4±4.7%). However, when administered before and during the IP protocol, glibenclamide abolished the protective effect of preconditioning (36.3±4% in glibenclamide+IP vs 18.6±1.5% in IP, *P<0.05*), whereas glimepiride did not (18.5±2.7% in glimepiride+IP vs 18.6±1.5% in IP; Figure 2).

With regard to potential effects on the mitochondria, we used the K<sub>ATP</sub> channel opener diazoxide to investigate the differences between glibenclamide and glimepiride. Diazoxide alone given before ischemia also conferred protection against ischemia/reperfusion injury (infarct/risk zone, 22.2±4.7%; *P<0.05* vs control). This beneficial effect was lost in the presence of glibenclamide (22.2±4.7% in diazoxide vs 38.8±5% in diazoxide+glibenclamide; *P<0.05*) but not in the presence of glimepiride (22.4±2.9% in diazoxide+glimepiride vs 22.2±4.7% in diazoxide; *P>0.05*; Figure 3).

**Patch-Clamp Studies**
To test whether glimepiride and diazoxide affect currents through sarcolemmal K<sub>ATP</sub> channels of rat ventricular myocytes, we used patch-clamp techniques to record whole-cell membrane currents at a holding potential of 0 mV in 6 mmol/L K⁺ solution. Under these conditions, the K<sub>ATP</sub> channel opener pinacidil activated a substantial outward K<sub>ATP</sub> current, which was blocked by both 1 μmol/L glibenclamide (Figure 4A) and 1 μmol/L glibenclamide (Figure 4B). The effectiveness of glimepiride in blocking sarcolemmal K<sub>ATP</sub> channels was confirmed in 16 additional cells. In experiments in which we tested different concentrations, half blockage occurred with ~10 mmol/L glibenclamide. In similar experiments, we looked for current activation by diazoxide (at 30 and 300 μmol/L). Figure 4B shows that no activation of current was detectable in response to diazoxide at 300 μmol/L, but the subsequent application of pinacidil (200 μmol/L) to the same cell activated substantial K<sub>ATP</sub> current. The results from several cells (Figure 4C) show that diazoxide caused no activation of sarcolemmal K<sub>ATP</sub> current at either 30 or 300 μmol/L. These results suggest that glibenclamide and glimepiride are potent blockers of sarcolemmal K<sub>ATP</sub> channels in rat ventricular myocytes and that diazoxide does not activate these channels under our experimental conditions.

**Mitochondrial Membrane Potential**
Ascorbate was used in all experiments as a mitochondrial respiratory substrate. Application of ascorbate to the mitochondria caused an instantaneous increase in intensity of TMRM fluorescence, concomitant with mitochondrial membrane polarization. The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (1 μmol/L), used as a positive control to collapse membrane potential in the mitochondria, resulted in a large reduction in intensity of TMRM fluorescence (Figure 5A). Treatment of mitochondria with the physiological mitochondrial K<sub>ATP</sub> channel opener GTP (50 μmol/L) significantly (*P<0.0001*) decreased the TMRM fluorescence from 153±3.9 arbitrary fluorescence units in untreated mitochondria to 135±2.9 (Figure 5A). GTP significantly decreased the mitochondrial membrane potential by 14±0.9% of the control value (Figure 5B). 5-Hydroxydecanoate, glimepiride, or glibenclamide alone had no effect on membrane potential (Figure 5B). Both glibenclamide and 5-hydroxydecanoate prevented the changes in membrane potential induced by GTP (150±4.7 and 150±3.8
arbitrary units, respectively, compared with control (153 ± 6), whereas glimepiride did not block these changes (132 ± 6 arbitrary units; Figures 5A and 5B).

Discussion

Diabetes is a common and widespread disease. In the diabetic population, 90% of patients have type 2 diabetes. In these patients there is an increased risk of cardiovascular complications followed by higher morbidity and mortality than in a nondiabetic population with coronary artery disease. The most common treatment approach in type 2 diabetes is administration of oral sulfonylureas, such as glibenclamide, which block K<sub>ATP</sub> channels, thereby stimulating insulin release by pancreatic β-cells. Unfortunately, K<sub>ATP</sub> channel blockade is not specific to the pancreas and can affect other tissues as well. It is well established that glibenclamide can also block sarcolemmal K<sub>ATP</sub> channels in a number of other cell types, including vascular smooth muscle cells, cardiac myocytes, and vascular endothelium, as well as the K<sub>ATP</sub> channels situated on the inner membrane of mitochondria.

Figure 4. Effects of sulfonylureas and diazoxide on sarcolemmal K<sub>ATP</sub> current. A, Membrane current recorded from isolated rat ventricular myocyte by using whole-cell patch-clamp technique. Cell was held at 0 mV throughout, and extracellular solution contained 6 mmol/L K<sup>+</sup>. Application of K<sub>ATP</sub> channel opener pinacidil activated outward K<sub>ATP</sub> current, which was completely blocked by 1 μmol/L glimepiride. B, Recording from different cell under same conditions as in A. Diazoxide (300 μmol/L) did not activate K<sub>ATP</sub> current, but subsequent application of pinacidil (200 μmol/L) activated substantial current that was inhibited by 1 μmol/L glibenclamide. C, Mean ± SEM sarcolemmal K<sub>ATP</sub> (glibenclamide-sensitive) current at 0 mV in absence of K<sub>ATP</sub> channel openers (6K), in presence of diazoxide (30 and 300 μmol/L) or pinacidil (200 μmol/L). n=5 cells in each case. *P<0.0001 vs 6K, t test.

Figure 5. Effects of sulfonylureas and GTP on mitochondrial membrane potential. A, Representative flow cytometric profile of isolated cardiac mitochondria stained with TMRM showing mitochondrial membrane potential-associated fluorescence. Effect on control of (a) 50 μmol/L GTP, (b) GTP in presence of 10 μmol/L glimepiride, (c) GTP in presence of 100 μmol/L 5-hydroxydecanoate, (d) GTP in presence of 10 μmol/L glibenclamide, and (e) 1 μmol/L carbonyl cyanide m-chlorophenylhydrazone CCCP. B, Mean ± SEM percent change from control of median TMRM fluorescence, n=6. ***P<0.0001.
There is also substantial evidence to suggest that in diabetic patients with acute myocardial infarction, these oral agents should be avoided. Initial concern for issue this was raised in the early 1970s when the University Group Diabetes Program assessed the efficacy of oral hypoglycemic treatment compared with insulin and diet alone in the prevention of cardiovascular complications. They demonstrated a significantly higher cardiovascular mortality in patients on sulfonylureas compared with diet alone. Nonetheless, these agents have continued to be extensively used because, one suspects, of the lack of a plausible mechanism for the University Group Diabetes Program study results. The United Kingdom Prospective Diabetes Study, a large-scale clinical study of >5000 patients, attempted to answer the question of whether improved glycemic control reduced the risk of cardiovascular death in patients who were taking insulin and sulfonylureas. In that study no detrimental effect of sulfonylureas was noted, and the United Kingdom Prospective Diabetes Study is often cited as proof that sulfonylureas such as glibenclamide do not pose a risk to patients with type 2 diabetes. Unfortunately, what the study failed to ascertain was the effect that these agents had on these type 2 diabetic patients in the setting of acute coronary syndromes, ie, in patients directly at risk of myocardial infarction (presenting with chest pain or unstable angina).

In this context, one of the most potent mechanisms of protection against myocardial ischemia/reperfusion injury is ischemic preconditioning. This endogenous protective response has been demonstrated in all species, including humans, and has been described as the beneficial adaptive response of the myocardium to repeated episodes of sublethal ischemia. A substantial body of evidence implicates mitochondrial KATP channel opening as playing a central role in ischemia. A substantial body of evidence implicates mitochondrial KATP channels, known to play a crucial role in preconditioning protection. To examine this hypothesis, the second aim of our study was to ascertain whether glimepiride abolished the protective role of diazoxide, a known opener of mitochondrial K<sub>ATP</sub> channels at specific doses. It has been shown that diazoxide, when administered before ischemia, protects the infarcting myocardium; this beneficial effect being lost in the presence of glibenclamide. Our results confirm these studies with respect to glibenclamide but also demonstrate that glimepiride does not appear to abolish this protective effect; ie, the protection conferred by diazoxide is not lost even when the mitochondrial K<sub>ATP</sub> opener is given in the presence of this sulfonylurea. The most plausible explanation would be that glimepiride does not affect mitochondrial K<sub>ATP</sub> opening, whereas glibenclamide blocks this channel. We do note, however, that 10 μmol/L glibenclamide may not be specific, and we cannot exclude the possibility that at this concentration, glibenclamide abolishes other mechanisms involved in preconditioning.

Diazoxide has been shown to cause a decrease in mitochondrial membrane potential, although the exact process by which it does so remains controversial. Although diazoxide has been proposed to directly open mitochondrial K<sub>ATP</sub> channels, it may in addition have a nonspecific effect on electron transport of the respiratory chain. To concentrate on the mitochondrial K<sub>ATP</sub> channel specifically, the physiological mitochondrial K<sub>ATP</sub> channel opener GTP was therefore used to investigate the action of the two sulfonylureas. GTP produced a decrease in mitochondrial membrane potential, which was blocked by glibenclamide, as well as by a suitable agent known to block mitochondrial K<sub>ATP</sub> channels, viz, 5-hydroxydecanoate. Under the same conditions, glimepiride failed to inhibit the effects of GTP on mitochondrial membrane potential. These data indicate that glimepiride has no effect on mitochondrial K<sub>ATP</sub> channel opening by GTP.

We believe that more studies, basic as well as clinical, are needed to fully elucidate and characterize the role of this sulfonylurea. At present, we believe that our study undertaken in the isolated rat heart demonstrates that glimepiride appears to be significantly less harmful to the ischemic heart than is the more conventionally used sulfonylurea glibenclamide. Further work in other species and in vivo are warranted. However, the present data may have important implications for the treatment of type 2 diabetes patients at risk of myocardial infarction, and appropriate clinical studies would need to be designed to ascertain the true nature of the role and place of such sulfonylureas in ischemic heart disease patients.
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