Full Paper

Glimepiride Treatment Upon Reperfusion Limits Infarct Size via the Phosphatidylinositol 3-Kinase/Akt Pathway in Rabbit Hearts

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Abstract. The phenomenon termed postconditioning, that is, brief episodes of ischemia/reperfusion at the onset of reperfusion reduce infarct size, is thought to involve the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Treatment with a drug activating PI3K at the onset of reperfusion may confer a similar cardioprotection. The sulfonylurea glimepiride has been shown to activate PI3K in human endothelial cells. We therefore tested in rabbit hearts whether glimepiride can produce postconditioning-mimetic actions. Langendorff-perfused rabbit hearts were subjected to 30 min of global ischemia and 120 min of reperfusion, and infarct size was determined by triphenyltetrazolium staining. Phosphorylation of Akt was analyzed by Western blotting. Glimepiride (10 μ M) treatment for the first 10 min of reperfusion significantly reduced infarct size from $67.2 \pm 1.3\%$ in controls to $35.8 \pm 4.5\%$ ($P < 0.01$). This infarct size-limiting effect of glimepiride was abolished by a selective inhibitor of PI3K (5 μ M LY294002, $65.4 \pm 3.4\%$). Phosphorylation of the PI3K substrate Akt was significantly increased in glimepiride-treated hearts when compared to controls ($P < 0.05$). Glimepiride-induced Akt phosphorylation was inhibited by LY294002. In conclusion, our study demonstrates that glimepiride treatment upon reperfusion reduces infarct size in rabbit hearts via a PI3K/Akt-mediated pathway. The postconditioning-mimetic action of glimepiride may be beneficial for the treatment of diabetic patients with ischemic heart disease.

Keywords: cardioprotection, diabetes mellitus, glimepiride, postconditioning, phosphatidylinositol 3-kinase

Introduction

Patients with diabetes mellitus have an increased risk of coronary artery disease and a worse mortality after acute myocardial infarction (1 – 3). The hyperglycemia is associated with an increased risk for microvascular and macrovascular complications, and the UKPDS (United Kingdom Prospective Diabetes Study) unequivocally demonstrated the benefits of intensified glycemic control on microvascular complications in patients with type 2 diabetes (4). Although the sulfonylureas have been widely used for treatment of type 2 diabetes mellitus, the safety of sulfonylureas with respect to cardiovascular mortality has been under discussion. The sulfonylureas

may interfere with the endogenous cardioprotective form of preconditioning, in which brief episodes of ischemia paradoxically protect the heart against subsequent lethal ischemia, and worsen the prognosis in diabetic patients with acute myocardial infarction (5). However, recent studies have indicated that impairment of preconditioning is not a class effect of sulfonylureas, but specific to glibenclamide (6, 7). Indeed, glimepiride does not affect mitochondrial ATP-sensitive K^+ channel opening, a key element of cardioprotection, and lacks the potential to interfere with preconditioning (7, 8).

More recently, the newly developed concept of postconditioning as a way of reducing reperfusion injury has been receiving much attention. The term postconditioning refers to the phenomenon in which brief repetitive episodes of ischemia/reperfusion at the immediate onset of reperfusion result in a reduction in infarct size (9). Importantly, evidence for the existence of

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postconditioning has also been obtained in humans (10). Postconditioning-induced protection seems to be mediated via the reperfusion injury salvage kinases (RISK) pathway (11). Phosphatidylinositol 3-kinase (PI3K) is a component of the RISK pathway and pharmacological activation of this kinase at reperfusion can mimic postconditioning (11). Interestingly, it has been reported that glimepiride activates PI3K in human coronary artery endothelial cells (12). Accordingly, an attractive hypothesis is that glimepiride activates PI3K in cardiac cells and treatment with glimepiride upon reperfusion can mimic the protection conferred by postconditioning. Here we tested this hypothesis using an isolated rabbit heart model of ischemia/reperfusion.

Materials and Methods

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of Chiba University.

Langendorff heart perfusion

Rabbit hearts were isolated and prepared as previously described in detail (13). Briefly, isolated rabbit hearts were dissected out and mounted on a Langendorff apparatus for perfusion with a modified Krebs-Henseleit solution composed of 119 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.5 mM CaCl_2 , 24.9 mM NaHCO_3 , and 10 mM glucose, which was gassed with 95% O_2 / 5% CO_2 (pH 7.4, 36°C). Krebs-Henseleit solution was delivered at a constant rate of 25–30 ml/min that established an initial mean coronary artery perfusion pressure of >40 mmHg. A water-filled balloon was inserted into the left ventricle for

continuous monitoring of ventricular performance. Initial left ventricular end-diastolic pressure was set between 4 and 8 mmHg. All determinations of ventricular performance, that is, heart rate (HR), left ventricular developed pressure (LVDP: difference between left ventricular end-systolic pressure and end-diastolic pressure), and velocity of contraction (+dP/dt), were obtained by using a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia).

Experimental protocols

Langendorff-perfused hearts were stabilized and randomly assigned to the study groups (Fig. 1). CONT (n = 8): preparations were subjected to 30 min of normothermic global ischemia followed by 120 min of reperfusion. Global ischemia was achieved by complete interruption of coronary perfusion. GM (n = 7): preparations were treated with glimepiride (GM, 10 μM) for the first 10 min of reperfusion. GM + LY (n = 7): preparations were treated with the PI3K inhibitor LY294002 (LY, 5 μM) during glimepiride. LY (n = 4): preparations were treated with LY294002 alone. In a part of the experiments, glibenclamide (10 μM), instead of glimepiride, was administered for the first 10 min of reperfusion (n = 4).

Assessment of infarct size

After 120 min of reperfusion, the heart was removed from the Langendorff apparatus, cut into 6–8 transverse slices from the apex to the base. The slices were incubated for 5 min at 37°C in a 1% solution of triphenyltetrazolium chloride to visualize infarcts. All slices were weighed and photographed after staining. The areas of infarct and the ventricles were measured by computed planimetry, using image-analysis software (Aquacosmos; Hamamatsu Photonics, Hamamatsu).

Fig. 1. Experimental protocols. All hearts were subjected to 30 min of global ischemia (filled boxes) and 120 min of reperfusion. CONT indicates control; GM, administration of glimepiride (10 μM) for first 10 min of reperfusion; GM + LY, co-administration of glimepiride and LY294002 (5 μM) for first 10 min of reperfusion; LY, administration of LY294002 alone for the first 10 min of reperfusion.

Infarct weight was determined with the following equation: % infarct area $\times$ weight of each slice, as described previously (13), and expressed as a percentage of the total tissue weight.

Western immunoblotting

In the second set of experiments, four similar experimental groups (CONT, GM, GM + LY, and LY) of rabbits ($n = 4$ in each group) were subjected to the same experimental procedures and terminated after 10 min of reperfusion for Western blot analysis. For analysis of Akt phosphorylation, left ventricular tissue samples were collected 10 min after reperfusion, immediately frozen in liquid nitrogen, and stored at -80°C until further processing. Approximately 300 mg of snap-frozen ventricular tissue was used for protein extraction. Tissue was homogenized on ice with a Kinematica Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in 1 ml lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% Nonidet P40, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and complete proteinase inhibitor cocktail (one tablet per 10 ml; Roche Diagnostics Corporation, Indianapolis, IN, USA). The lysates were centrifuged at $15,000 \times g$ for 30 min at 4°C to remove cellular debris and isolate total protein. Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IN, USA) using bovine serum albumin as a standard. Equivalent amounts ($50 \mu\text{g}$) of protein samples were mixed with loading buffer and heated for 10 min at 95°C . Samples were separated by 10% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane (GE Healthcare UK, Ltd., Buckinghamshire, England). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), membranes were incubated for 2 h at room temperature in TBST containing 1% nonfat dry milk and 1:1000 dilution of monoclonal antibody against Ser 473 of phospho-Akt. The membranes were then washed three times with TBST containing 1% nonfat dry milk for 10 min before 60-min incubation with a 1:10,000 dilution of horseradish peroxidase-labeled anti-rabbit immunoglobulin G (GE Healthcare UK, Ltd.) in TBST containing 1% nonfat dry milk. Peroxidase activity was visualized by means of an enhanced chemiluminescence substrate system (GE Healthcare UK, Ltd.), followed by exposure to hyperfilms (GE Healthcare UK, Ltd.). To determine total Akt abundance, the membranes were stripped with restore stripping buffer (Pierce Biotechnology, Inc.) and reprobed with polyclonal rabbit Akt antibody. Thereafter, the membranes were

exposed to the identical interventions described above. β -Actin (1:1000 dilution) was detected on immunoblots as a loading control for protein quantity. Optical density for each western blot band was quantified with the Image J 1.36b software (National Institute of Health, Bethesda, MD, USA).

Reagents

Glimepiride was a kind gift from Sanofi-Aventis K.K. (Tokyo). LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glimepiride and LY294002 were dissolved in DMSO before they were added to the experimental solution, and the final concentration of solvent was $\leq 0.1\%$. The rabbit monoclonal antibody for phospho-Akt (ser 473) and rabbit polyclonal anti-total-Akt and β -actin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Statistical analyses

Data are each presented as the mean $\pm$ S.E.M., and the number of experiments is shown as n . Statistical comparisons were made by one-way ANOVA combined with the Fisher post hoc test, as appropriate. A value of $P < 0.05$ was regarded as significant.

Results

Reperfusion with glimepiride improved cardiac dysfunction after ischemia/reperfusion

To evaluate the effect of glimepiride on the post-ischemic cardiac function, the hearts subjected to global ischemia were reperfused with Krebs-Henseleit solution in the presence or absence of glimepiride. There were no significant differences in ventricular performance (heart rate, LVDP, and $+dP/dt$) among the groups before the start of ischemia (Table 1 and Fig. 2). However, administration of glimepiride significantly improved the recovery of LVDP and $+dP/dt$ at the first 10 and 120 min of reperfusion ($P < 0.05$ vs control group). Co-administration of the PI3K inhibitor LY294002 blocked the glimepiride-induced improvement in postischemic LVDP and $+dP/dt$ at 10 and 120 min of reperfusion. Functional parameters at reperfusion in the group treated with LY294002 alone were not significantly different from those of the control group (Table 1).

Reperfusion with glimepiride decreased myocardial infarct size after ischemia/reperfusion

We next examined the ability of glimepiride to reduce infarct size, and the myocardial infarct size is summarized in Fig. 3. Infarct size following global ischemia/reperfusion was $67.2 \pm 1.3\%$ in control hearts. Glimepiride treatment immediately after reperfusion significantly

Table 1. Hemodynamic parameters

Study groups	n	Before ischemia			End time of perfusion with agents (at the first 10 min of reperfusion)			Reperfusion		
		Heart rate (beats/min)	LVDP (mmHg)	+dP/dt (mmHg/s)	Heart rate (beats/min)	LVDP (mmHg)	+dP/dt (mmHg/s)	Heart rate (beats/min)	LVDP (mmHg)	+dP/dt (mmHg/s)
Control	8	178 ± 12	80 ± 3	1678 ± 94	157 ± 7	26 ± 2	752 ± 72	175 ± 11	37 ± 4	931 ± 102
GM	7	176 ± 10	85 ± 3	1832 ± 107	155 ± 11	38 ± 3*	967 ± 73*	161 ± 12	65 ± 2*	1447 ± 46*
GM + LY	7	171 ± 6	86 ± 2	1758 ± 68	149 ± 7	29 ± 2	694 ± 56 [#]	166 ± 28	34 ± 6 [#]	914 ± 174 [#]
LY	4	179 ± 16	85 ± 5	1689 ± 58	163 ± 34	35 ± 3	833 ± 97	185 ± 20	47 ± 15	1068 ± 216

GM indicates glimepiride; LY, LY294002; LVDP, left ventricular developed pressure; and +dP/dt, rate of contraction. Values are reported as the mean ± S.E.M. **P*<0.05 vs control. [#]*P*<0.05 vs GM.

Fig. 2. Changes in left ventricular pressure during ischemia and reperfusion. Representative time course of left ventricular pressure changes in control (CONT), glimepiride-treated (GM), glimepiride plus LY294002-treated (GM + LY), and LY294002-treated (LY) hearts are shown.

reduced infarct size to $35.8 \pm 4.5\%$ (*P*<0.01). Co-administration of LY294002 significantly blocked the glimepiride-mediated reduction in infarct size ($65.4 \pm 3.4\%$, *P*<0.01). Infarct size in the group treated with LY294002 alone for the first 10 min of reperfusion was

Fig. 3. Summarized effects of glimepiride on infarct size. CONT indicates control; GM, glimepiride; and LY, LY294002. Each open circle represents the infarct size in an individual heart, and closed circles with error bars are group means ± S.E.M. **P*<0.01 vs CONT, GM + LY, and LY.

not significantly different from that of the control group ($61.2 \pm 4.3\%$). In contrast, treatment with glibenclamide ($10 \mu\text{M}$), another representative sulfonylurea drug, failed to significantly reduce infarct size ($59.7 \pm 8.7\%$, *n* = 4). These results indicate that treatment with glimepiride but not glibenclamide at the onset of reperfusion affords cardioprotection.

Reperfusion with glimepiride increased the expression of phosphorylated Akt

To further investigate whether glimepiride exerts its cardioprotective effects by activating the PI3K/Akt pathway, lysates of Langendorff-perfused rabbit hearts were analyzed by the Western blot technique for detection of Akt. Figure 4 shows the expression of total Akt and its activated, phosphorylated form (phospho-Akt) after ischemia and reperfusion. Total Akt expression was comparable in all experimental groups. Glimepiride treatment upon reperfusion significantly increased phosphorylation of Akt at Ser⁴⁷³ (phospho-Akt). Co-

Fig. 4. Effects of glimepiride on Akt phosphorylation. **A:** Representative western blot analysis of phosphorylation of Akt at Ser⁴⁷³ (phospho-Akt, top lanes) and total Akt (middle lanes). In each lane, the protein concentration was 50 μ g. β -Actin (lower lanes) was used to demonstrate equal protein loading. **B:** Densitometry results are expressed as average density ratio of p-Akt/total Akt for each group. Data are reported as the mean $\pm$ S.E.M. (n = 4 per group). CONT indicates control; GM, glimepiride; and LY, LY294002. * $P < 0.05$ vs CONT, GM + LY, and LY.

administration of LY294002 inhibited the phosphorylation of Akt in glimepiride-treated hearts to a degree comparable to that in the control.

Discussion

In the present study we have demonstrated that glimepiride treatment upon reperfusion reduces infarct size in rabbit hearts subjected to ischemia/reperfusion and increases the activity of PI3K as well as phosphorylation of Akt. PI3K/Akt is one of the pro-survival kinases, which have been termed the reperfusion injury salvage kinases (RISK), and have recently received much attention in connection with the mechanisms of postconditioning (11). Our results are fully consistent with the hypothesis that pharmacological postconditioning, using a drug that activates the PI3K/Akt pathway at

reperfusion, effectively produces cardioprotection against ischemia/reperfusion injury. While we have shown that glimepiride produces postconditioning-mimetic cardioprotection, glibenclamide has been shown to abolish infarct size-limiting effects afforded by postconditioning in rabbit hearts (14). In addition, glibenclamide administered upon reperfusion failed to reduce infarct size in the present study. Therefore, postconditioning mimetic action is not a class effect of sulfonylurea drugs. A previous study has shown that glimepiride induces Akt phosphorylation in human coronary artery endothelial cells (12). Here we also provide evidence that glimepiride induces phosphorylation of Akt in myocardium. However, the activation mechanisms and downstream effectors of PI3K/Akt remain elusive. It has been suggested that the activation of the RISK pathway confers its cardioprotection through the inhibition of the mitochondrial permeability transition pore (15). Further studies are needed to determine whether postconditioning with glimepiride protects the hearts by inhibiting permeability transition pore.

Previous studies showed that the concentrations of GM needed to increase the level of phosphorylated Akt in endothelial cells were 0.1–10 μ M (12, 16). In our previous study, we have shown that GM at a concentration of 10 μ M blocked sarcolemmal ATP-sensitive K^+ channels but not mitochondrial ATP-sensitive K^+ channels in rabbit ventricular cells (7). In addition, Mocanu et al. demonstrated that in Langendorff-perfused rat hearts, GM at a concentration of 10 μ M did not abolish the infarct-limiting effects of ischemic preconditioning (8). Therefore, we selected the concentration of 10 μ M for GM in this study, although the concentration was somewhat higher than the therapeutic concentrations (17).

The UGDP (University Group Diabetes Program) study concluded that patients treated with tolbutamide sustained an increased cardiovascular mortality (18). Since then, despite a flaw in methodology (19), there is ongoing controversy as to whether the sulfonylureas increase cardiovascular mortality in patients with diabetes mellitus. However, recent experimental and clinical data have shown that impairment of preconditioning, which was presumed to be the most important mechanism for the excess cardiovascular mortality, is not a class effect of sulfonylureas. Glimepiride, unlike glibenclamide, does not affect mitochondrial ATP-sensitive K^+ channel opening and lacks the potential to interfere with the endogenous cardioprotective form of preconditioning (6, 7, 20). The present study further revealed that glimepiride can mimic the postconditioning-like effect. Thus, it should be emphasized that the sulfonylureas differ in their action to endogenous

cardioprotective forms of preconditioning and postconditioning. The characteristics of glimepiride, that is, no interference with preconditioning and pharmacological induction of postconditioning, may be beneficial for the treatment of diabetic patients with ischemic heart disease. Some nice evidence in favor of this idea was provided by a retrospective observational cohort study of type 2 diabetic patients, in which glimepiride was associated with a significantly lower yearly mortality when used in combination with metformin in comparison with glibenclamide (21). Moreover, a population-based case-control study showed a significantly increased risk of myocardial infarction in subjects using glibenclamide, but not glimepiride (22).

Diabetic patients with ischemic heart disease have a substantially worse mortality and morbidity after percutaneous coronary intervention compared with non-diabetics (23). Postconditioning is more clinically applicable to patients undergoing percutaneous coronary intervention and provides a novel strategy for attenuating myocardial reperfusion injury. The postconditioning mimetic action of glimepiride can protect myocardium following percutaneous coronary intervention and may lead to improved clinical outcomes in diabetic patients.

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