Original Article

Preconditioning the Diabetic Heart

The Importance of Akt Phosphorylation

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Conflicting evidence exists whether diabetic myocardium can be protected by ischemic preconditioning (IPC). The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is important in IPC. However, components of this cascade have been found to be defective in diabetes. We hypothesize that IPC in diabetic hearts depends on intact signaling through the PI3K-Akt pathway to reduce myocardial injury. Isolated perfused Wistar (normal) and Goto-Kakizaki (diabetic) rat hearts were subjected to *1*) 35 min of regional ischemia and 120 min of reperfusion with infarct size determined; *2*) preconditioning (IPC) using 5 min of global ischemia followed by 10 min of reperfusion performed one, two, or three times before prolonged ischemia; or *3*) determination of Akt phosphorylation after stabilization or after one and three cycles of IPC. In Wistar rats, one, two, and three cycles of IPC reduced infarct size 44.7 ± 3.8% ($P < 0.05$), 31.4 ± 4.9% ($P < 0.01$), and 34.3 ± 6.1% ($P < 0.01$), respectively, compared with controls (60.7 ± 4.5%). However, in diabetic rats only three cycles of IPC significantly reduced infarction to 20.8 ± 2.6% from 46.6 ± 5.2% in controls ($P < 0.01$), commensurate with significant Akt phosphorylation after three cycles of IPC. To protect the diabetic myocardium, it appears necessary to increase the IPC stimulus to achieve the threshold for cardioprotection and a critical level of Akt phosphorylation to mediate myocardial protection.
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Diabetes is a major risk factor for ischemic heart disease. Ischemic heart disease is the leading cause of death in the western world (1) and accounts for >50% of deaths in the diabetic population (2). After a myocardial ischemic event, diabetes is associated with increased adverse outcomes in terms of both morbidity and mortality over the short and long term (3–6). In patients with diabetes, the mortality rate after an acute myocardial infarction or coronary bypass surgery is almost double that of nondiabetic subjects (7,8). Therefore, reducing the consequences of coronary artery disease using strategies that target ischemia-reperfusion injury would be particularly beneficial in this population.

Unfortunately, results from the current literature regarding whether the diabetic myocardium can be protected by the phenomenon of ischemic preconditioning (IPC) are conflicting (9). Although some studies have reported that the diabetic heart can be protected using IPC (10–12), the majority have reported no protective effect (13–19).

The importance of the phosphatidylinositol 3-kinase (PI3K)-Akt prosurvival pathway in IPC was first demonstrated by Tong et al. (20) and has been further supported by subsequent studies (21). However, the cellular signaling pathways that specifically mediate the effects of IPC in the diabetic myocardium have not been elucidated. Recent data using type 2 diabetic models have provided clues as to the possible defects in the cell survival cascades in a variety of tissues, as well as the heart, that may be responsible for the conflicting results seen. For example, defects in the insulin receptor β, insulin receptor substrate-1, and GLUT-4 protein (22), protein kinase B (23), basal and insulin-stimulated Akt, extracellular signal-related kinase, and PI3K (24) have been demonstrated in diabetic animal models.

Therefore, the aims of our study were *1*) to determine whether the inbred lean model of type 2 diabetes, namely the Goto-Kakizaki (GK) rat (25,26), can be protected by IPC and *2*) to determine the role of the PI3K-Akt signaling pathway in mediating the protective effects of IPC in this diabetic model.

RESEARCH DESIGN AND METHODS

Male Wistar rats (300–550 g, $n = 47$) were obtained from Charles River UK Limited (Margate, U.K.), and male GK rats (300–550 g, $n = 71$) were obtained from Taconic (Denmark). All animals received humane care in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986 (Her Majesty's Stationery Office, London, U.K.).

Isolated perfused rat heart. Rats were fed a standard chow diet, heparinized with sodium heparin (300 IU), and anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. Hearts were rapidly excised, mounted on a Langendorff system, and perfused with a modified Krebs-Henseleit buffer. All hearts were subjected to 35 min of regional ischemia and 120 min of reperfusion as previously described (27). Infarct size (expressed as a percent-

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IPC, ischemic preconditioning; PDK, phosphatidylinositol-3-phosphate–dependent kinase; PI3K, phosphatidylinositol 3-kinase.

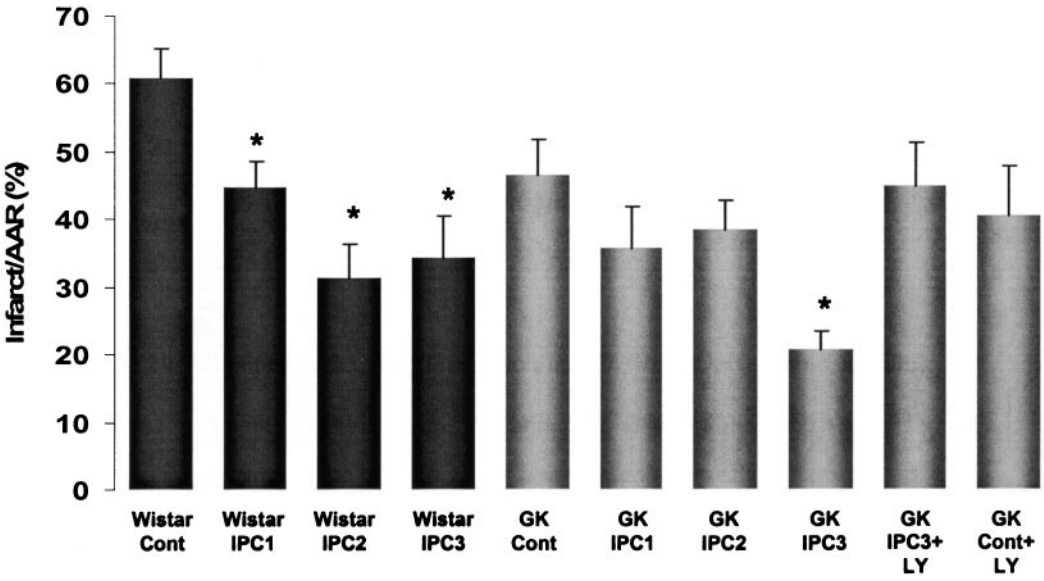

FIG. 1. The reduction in infarct size (expressed as a percentage of the area at risk [AAR]) achieved by one, two, and three cycles of IPC in Wistar hearts. In the diabetic GK rat, one and two cycles of IPC did not reduce infarct size significantly. Only three cycles of IPC was sufficient stimulus to achieve a significant reduction in infarct size in diabetic GK hearts. Pharmacologically inhibiting PI3K during IPC using LY294002 (LY) abolished the protection associated with three cycles of IPC in GK hearts. *$P < 0.05$.

age of the area at risk) was determined by triphenyltetrazolium chloride staining.

Infarct study treatment protocols. Hearts were randomly assigned to one of the following groups: *1*) Wistar vehicle control ($n = 8$) with 0.02% DMSO given alone during stabilization; *2*) Wistar one-cycle IPC ($n = 8$) comprising 5 min of global ischemia followed by 10 min of reperfusion before the index ischemia; *3*) Wistar two-cycle IPC ($n = 8$) before the index ischemia; *4*) Wistar three-cycle IPC ($n = 8$) before the index ischemia; *5*) GK vehicle control ($n = 8$) with 0.02% DMSO given alone during stabilization; *6*) GK one-cycle IPC ($n = 9$) before the index ischemia; *7*) GK two-cycle IPC ($n = 9$) before the index ischemia; *8*) GK three-cycle IPC ($n = 8$) before the index ischemia; *9*) GK three-cycle IPC plus LY294002 ($n = 6$), in which hearts were given the PI3K inhibitor LY294002 (15 μmol/l) (21) starting 5 min before and throughout the preconditioning protocol with a 5-min washout before the lethal ischemia; or *10*) GK control plus LY294002 ($n = 6$), in which hearts were given LY294002 during stabilization.

Western blot analysis. Hearts ($n = 5$ per group) were randomly assigned to the treatment groups 1, 2, 4, 5, 6, 8, 9, and 10 as described above. Myocardial samples were taken at the end of stabilization or 5 min after the last IPC cycle and freeze-clamped in liquid nitrogen before being stored at −80°C. The phosphorylation state of Akt (phospho-Akt, Ser 473) and total level of Akt protein were analyzed by SDS-PAGE immunoelectrophoresis using antibodies obtained from New England BioLabs as described previously (21). Levels of phosphorylated proteins were normalized to their total protein levels, and equal protein loading was confirmed by β-actin probing of membranes (Abcam, Cambridge, U.K). Relative densitometry was determined using the computer software package NIH Image 1.63.

Blood glucose and HbA_{1c} assessment. Samples for nonfasting blood glucose ($n = 92$) and HbA_{1c} (A1C) ($n = 45$) were taken immediately after excision of the heart. Blood glucose measurements (millimoles per liter) were determined using an ABL 700 series blood gas analyzer (Radiometer, Copenhagen, Denmark), and A1C measurements (percent) were determined by an antibody-colorimetric assay using a Cobas Mira Plus analyzer (Roche Diagnostic Systems).

Statistical analysis. All values are expressed as means ± SE. Infarct size and Western blot results were analyzed by one-way ANOVA and Fisher's protected test of least significant difference. Differences were considered statistically significant when $P < 0.05$. Infarct size, glucose, and A1C correlations were calculated by linear regression analysis.

RESULTS

The threshold for preconditioning is elevated in the diabetic myocardium. In normal Wistar rats, one, two, and three cycles of IPC significantly reduced infarct size represented as a percentage of the area at risk (44.7 ± 3.8% [$P < 0.05$], 31.4 ± 4.9% [$P < 0.01$], and 34.3 ± 6.1% [$P < 0.01$], respectively) compared with control hearts (60.7 ± 4.5%). However, in diabetic GK rats, only three cycles of IPC reduced infarct size significantly compared with GK control hearts (20.8 ± 2.6 vs. 46.6 ± 5.2%; $P < 0.01$). Both one and two cycles of IPC failed to reduce infarct size significantly compared with GK control hearts (35.8 ± 6.2 and 38.5 ± 4.5 vs. 46.6 ± 5.2%, respectively; NS). However, the infarct reduction afforded by three cycles of IPC in the GK rat (20.8 ± 2.6%) was completely abolished in the presence of the PI3K inhibitor LY294002 administered 5 min before the IPC protocol until 5 min after (44.9 ± 6.4%; $P < 0.01$), suggesting that PI3K may be important as a trigger in IPC. LY294002 did not influence infarct size in the GK control group (40.6 ± 7.2%) (Fig. 1).

Impaired Akt phosphorylation is responsible for this threshold elevation. In diabetic GK hearts, one cycle of IPC induced significant phosphorylation (in arbitrary units [AU]) of Akt compared with GK control hearts (24.7 ± 5.0 AU vs. 6.2 ± 0.9 AU, respectively; $P < 0.05$), although this was not commensurate with a reduction in infarct size. However, three cycles of IPC induced significant phosphorylation of Akt compared with both GK control and GK one-cycle IPC hearts (42.4 ± 3.2 vs. 6.2 ± 0.9 AU [$P < 0.01$] and 24.7 ± 5.0 AU [$P < 0.05$], respectively), and this was associated with a significant reduction in infarct size. Inhibiting PI3K with LY294002 during the IPC protocol partially but significantly abrogated the phosphorylation of Akt in GK three-cycle IPC to levels similar to those seen with one-cycle IPC (42.4 ± 3.2 vs. 20.8 ± 2.1 AU, respectively; $P < 0.01$). In normal Wistar rats, one and three cycles of IPC led to significant phosphorylation of Akt compared with Wistar control hearts (72.4 ± 7.5 and 74.2 ± 9.1 vs. 37.1 ± 5.7 AU, respectively; $P < 0.01$ for both) (Fig. 2).

Blood glucose and A1C. Diabetic GK rats were characterized by significantly higher levels of blood glucose (16.1 ± 0.6 mmol/l in GK rats vs. 9.0 ± 0.3 mmol/l in Wistar rats; $P < 0.01$) and A1C (4.4 ± 0.1% in GK rats vs. 3.0 ± 0.04% in Wistar rats; $P < 0.01$) when compared with normal Wistar rats. A1C values >3.45% were considered diabetic using our assay. Regression analysis, however, did not demonstrate any correlation between infarct size and glucose ($R^2 = 0.003$) or A1C ($R^2 = 0.163$).

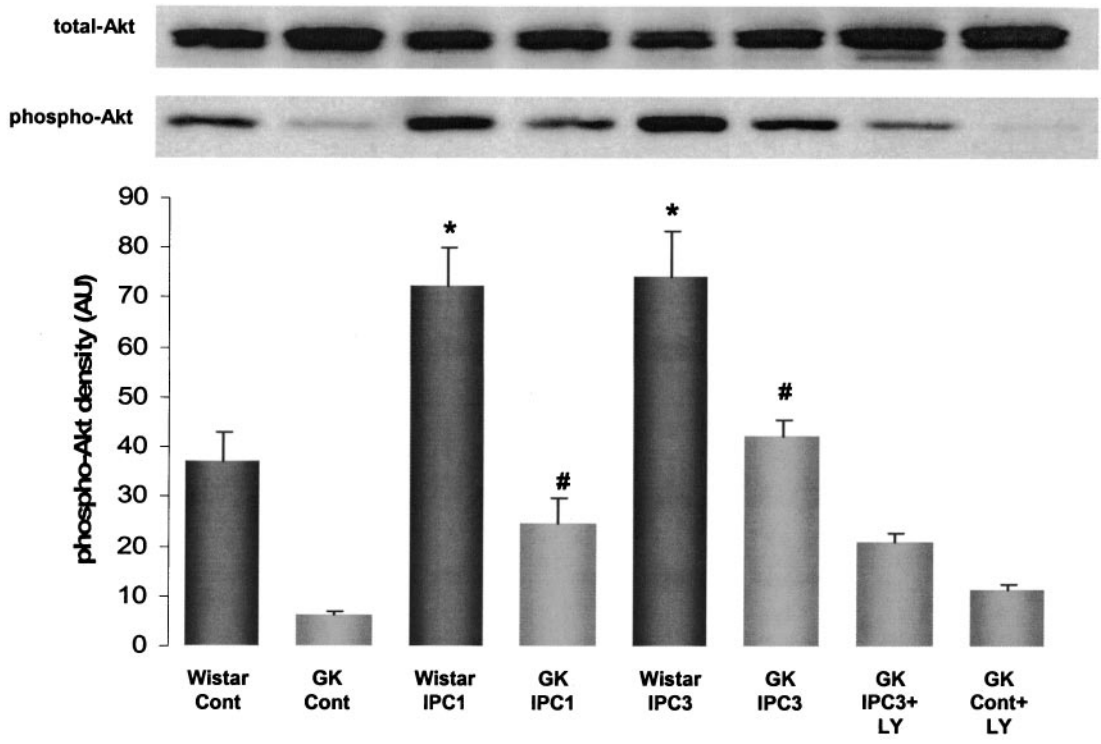

FIG. 2. Representative Western blots demonstrating phosphorylated and total Akt levels (densities in arbitrary units). IPC induced phosphorylation of Akt in both Wistar and diabetic GK hearts, although the increase was more marked in the Wistar hearts. In Wistar hearts, both one and three cycles of IPC resulted in a significant increase in Akt phosphorylation. Although one cycle of IPC resulted in significant phosphorylation of Akt in GK hearts, this was not sufficient to reduce infarct size. Only three cycles of IPC produced significant phosphorylation of Akt that was commensurate with a significant reduction in infarct size in an LY294002 (LY)-sensitive manner (see Fig. 1). *$P < 0.05$, #$P < 0.05$.

DISCUSSION

We report two findings for the first time. First, the type 2 diabetic myocardium can benefit from the cardioprotective effects of ischemic preconditioning, provided that the preconditioning stimulus is increased to reach the threshold necessary to achieve myocardial protection. In our study, this threshold required a preconditioning stimulus of three cycles of 5 min of ischemia and 10 min of reperfusion before the prolonged ischemic insult. Second, it appears that impairment of prosurvival signaling cascades may be responsible for this elevated threshold. We demonstrated that although one cycle of IPC induced a significant phosphorylation of Akt, this did not result in a reduction of infarct size. However, three cycles of IPC in the diabetic heart resulted in a significantly greater phosphorylation of Akt than one cycle, and this was commensurate with a significant reduction in infarct size, suggesting that a certain level of activated Akt is critical to mediate the protective effects of preconditioning. Furthermore, Western blot analysis did not demonstrate any significant differences in the total levels of Akt protein in diabetic GK hearts in all groups, indicating that the impairment in phosphorylation of Akt was not a result of lower levels of total Akt in diabetic hearts but rather in the signaling process leading to Akt activation. Whether this impairment in Akt activation is due to abnormalities in upstream mediators of the Akt pathway (i.e., PI3K or phosphatidylinositol-3-phosphate–dependent kinase [PDK]) remains to be elucidated. However, it should be noted that our data does not prove a direct causal relationship between impaired Akt phosphorylation and the elevated threshold for protection. The use of specific Akt inhibitors would help to clarify the role of Akt in protection in this setting.

Interestingly, our current study supports the findings of Kondo and Kahn (28) who demonstrated that different defects in components of cell survival kinase cascades in diabetic models are not species specific but organ specific within the same species. In both type 1 and 2 diabetic mice, they showed that in the retina, the reduction in the phosphorylation of PDK1 and Akt was due to reduced total levels of PDK1 and Akt when compared with controls after insulin stimulation. However, within the same mice, the total levels of PDK1 and Akt were the same in the liver, and the reduction in PDK1 and Akt phosphorylation was due to impaired activation of these proteins.

Evidence suggests that other components of cellular prosurvival pathways are defective in diabetic tissues as well as the heart. Hyperglycemia has been shown to inhibit the prosurvival effect of vascular endothelial growth factor, leading to retinal cell apoptosis via tyrosine nitration of PI3K that results in Akt inactivation and increased p38 mitogen-activated protein kinase activation (29). Similar results have been demonstrated in rat hearts exposed to hyperglycemic conditions, which led to tyrosine nitration and apoptosis through the action of inducible nitric oxide (NO) synthase and NO release (30). To our knowledge, our data demonstrate for the first time that the impairment in cellular signaling cascades is responsible for the inconsistent results reported in previous diabetic preconditioning experiments.

The lack of correlation between glucose, A1C levels, and infarct size in our study suggests that the severity of diabetes does not predict the extent of myocardial infarc-

tion. Rather, once a minimum level of diabetes is reached, the threshold for preconditioning is elevated.

Until recently, most animal studies have focused on ischemic preconditioning in the diabetic heart using chemically induced type 1 diabetic animal models (10–14). However, the conflicting data obtained from these studies should be interpreted with caution. Many of the animals died as a result of the chemical induction of diabetes, and others displayed characteristics of stress, probably as a consequence of the toxicity of the drugs (alloxan or streptozotocin) used. The nonspecific effects of these drugs on the myocardium are not well known. In addition, these animal models of diabetes simulate type 1 diabetes (31), which in the clinical scenario is the least common form in the human population. In this regard, chemically induced models of diabetes do not represent the most appropriate model in which to study the effects of myocardial protection. Animal models of type 2 diabetes should be used, as it is this form of diabetes that is prevalent worldwide and is associated with increased cardiovascular risk.

The GK rat is a selectively inbred model of type 2 diabetes developed from the Wistar rat. Type 2 diabetes in this rat has many similarities to the human form of the disease (25,26), and it has been used extensively as a type 2 diabetic research model (32). This appears to be a more appropriate diabetic research model because worldwide the most common form of diabetes is type 2 diabetes, and its prevalence is steadily increasing (33). To date, only one study has addressed the issue of preconditioning in a model of type 2 diabetes using an obese and lean animal. In that study, Kristiansen et al. (34) used the GK rat to address the issue of ischemic preconditioning and demonstrated that, in their model, the diabetic heart failed to show any reduction in infarct size when subjected to an IPC protocol of four cycles of 2 min of ischemia followed by 3 min of reperfusion, a preconditioning stimulus that we would argue was insufficient to reach the threshold necessary to activate cardioprotective mechanisms in the type 2 diabetic heart. Furthermore, this study did not provide any mechanistic data to explain the lack of protection afforded by IPC in the model. In contrast to their study, the diabetic hearts in our study were able to be protected by IPC, although the IPC stimulus required was elevated. This would suggest that, provided the IPC stimulus is sufficient, the diabetic heart can be protected from ischemic-reperfusion injury.

Interestingly, our data support the observations made by Kristiansen et al. (34) with respect to the smaller infarct size they observed in their diabetic animal hearts compared with nondiabetic hearts after an episode of ischemia-reperfusion. This appears to be a common finding in studies comparing the myocardial infarct size in diabetic animal models with "normal" hearts (10,34,35) and suggests that, in diabetic hearts, a myocardial adaptation occurs *after* a prolonged ischemic insult that attempts to limit the damage sustained from the ischemic injury. However, the exact mechanisms responsible for this adaptation have yet to be elucidated. In our study, the Wistar control animals also demonstrated larger infarct sizes compared with the diabetic GK animals, raising an important question as to whether a "normal" Wistar heart is the appropriate control with which to compare the type 2 diabetic GK hearts. GK rats have been developed by selective inbreeding of glucose-intolerant Wistar rats since 1975, resulting in a species with many biochemical and metabolic similarities to human type 2 diabetes. Therefore, as in clinical studies, it could be argued that a more appropriate control to compare a treatment in diabetic populations would be a nontreated diabetic rather than a "healthy" species. Nevertheless, we still thought it appropriate to add this control specifically to demonstrate that the concept of preconditioning is observed in nondiabetic hearts. Importantly, this should not detract from the fact that the most important findings from our study, namely the requirement for a sufficient preconditioning stimulus to induce protection, was observed within the diabetic GK group.

In conclusion, we report for the first time that the type 2 diabetic myocardium can be protected by IPC, but that the threshold required to achieve this protection is elevated compared with that in nondiabetic hearts. We find that this elevation in threshold may be required to achieve sufficient phosphorylation of Akt to execute the IPC protective signal. The findings from this study suggest that the human diabetic population may be more resistant to the protective effects of IPC but that, provided the preconditioning stimulus is sufficient, the diabetic myocardium can be protected. One intriguing aspect would be to investigate whether this resistance to IPC-induced protection is reversed in diabetic species treated with either insulin or oral hypoglycemic drugs.

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