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Original article

Insulin suppresses ischemic preconditioning-mediated cardioprotection through Akt-dependent mechanisms☆

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ABSTRACT

It is believed that the diabetic myocardium is refractory to cardioprotection by ischemic preconditioning (IPC) mainly because of impaired insulin signaling to phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB or Akt). However, human as well as animal studies have clearly showed that the hearts of type 2 diabetic humans and animals may exhibit increased signaling through PI3K–Akt but yet are resistant to cardioprotection by IPC or ischemic post-conditioning. Therefore, this study was designed to determine whether activation of insulin signaling prior to IPC is detrimental for cardioprotection and to assess the role of insulin receptors (IRs) and Akt in mediating this effect. Wild-type (WT) hearts, hearts lacking IRs or hearts expressing an active form of Akt (myrAkt1) were perfused ex vivo using a Langendorff preparation and were subjected to IPC (3 cycles of 5 min ischemia followed by 5 min reflow before 30 min no flow ischemia and then by 45 min reperfusion) in the presence or absence of 1 nmol/L insulin. Interestingly, whereas insulin was protective against I/R (30 min no flow ischemia and 45 min reperfusion), it completely abolished cardioprotection by IPC in WT hearts but not in mice lacking insulin receptors (IRs) in cardiomyocytes (CIRKO) or in all cardiac cells (TIRKO). The suppression of IPC-mediated cardioprotection was mediated through downstream signaling to Akt and Gsk3β. In addition, transgenic induction of Akt in the heart was sufficient to abrogate IPC even when insulin was absent, further confirming the involvement of Akt in insulin's suppression of cardioprotection by IPC. These data provide evidence that excessive insulin signaling to Akt is detrimental for cardioprotection by IPC and could explain the failure of the diabetic myocardium to precondition.

1. Introduction

The global incidence of diabetes mellitus is increasing with the estimated number reaching 366 million worldwide by 2030. Cardiovascular disease is the major cause of mortality among patients with diabetes, accounting for nearly 60–80% of the deaths [1,2]. Epidemiological studies and clinical trials have clearly shown that both type 1 and type 2 diabetic individuals are more prone to developing ischemic heart disease, including acute myocardial infarction and post-infarct complications. Moreover, mortality from acute myocardial infarction is almost doubled in diabetic patients compared with nondiabetic individuals [3,4]. Despite the burden of ischemic heart disease among diabetic individuals, effective treatment is currently unavailable.

Ischemic preconditioning (IPC) is considered one of the most protective mechanisms known to reduce ischemic damage in humans and animals [5–7]. IPC consists of subjecting the heart to brief cycles of ischemia and reperfusion (I/R) prior to a more prolonged ischemic period [8]. Recently, post-conditioning, which consists of applying very brief cycles of I/R in the early phase of reperfusion, has also been shown to protect the heart, and this protocol can easily be applied in the clinical setting. Similar to IPC, insulin and insulin-like growth factor 1 (IGF-1) protect the heart from I/R injury as evidenced by their ability to reduce infarct size when given prior to ischemia or at the reperfusion [9–11]. However, despite its cardioprotective property in animal studies, several clinical observations showed that insulin had either no effect or worsened cardiovascular events in type 2 diabetic patients [12–14]. For example, the UK Prospective Diabetes Study (UKPDS) showed that the use of intensive glucose lowering therapy with insulin over 10 years reduced the frequency of micro-vascular endpoints but had minimal effects on diabetes-related mortality or myocardial infarction [15]. Furthermore, in a separate study by Mellbin et al. [16], post-hoc proportional hazard regression analysis showed that although cardiovascular mortality was not affected, the risk of nonfatal myocardial infarction and stroke was 73% higher in patients who were on insulin therapy,

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and these differences persisted in a separate analysis of patients for whom insulin was newly started and in those randomly assigned to insulin in the study. In addition, many investigations have now showed that the failure to precondition the diabetic heart is not always associated with reduced insulin signaling. Indeed, insulin-mediated signal transduction to phosphoinositide 3-kinase (PI3K) and Akt is elevated in the hearts of patients with type 2 diabetes and in obese diabetic *ob*/*ob* mice [17,18]. Despite this activated insulin signaling pathway, cardioprotection by ischemic post-conditioning was not achieved in *ob*/*ob* mice, suggesting that chronic activation of insulin signaling is rather detrimental for cardioprotection. Indeed, excessive insulin signaling exacerbated systolic dysfunction in mice subjected to pressure overload, an effect that was prevented either by lowering hyperinsulinemia or by reducing insulin signaling [19]. Therefore, the main objective of this study is to determine whether activation of insulin signaling prior to IPC is detrimental for cardioprotection and to investigate the mechanisms involved.

2. Methods

2.1. Animals

The 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 the University of Utah. Male Ttr-Insr $^{-}/^{-}$ (TIRKO) mice (on a C57B6j background) and cardiomyocyte-specific insulin receptor knockout (CIRKO) mice (mixed background), previously described [20,21], and their respective wild-type (WT) control mice were used between 10 and 14 weeks of age. Mice harboring α-MHC-tTA and Tet-myrAkt1 genes (Tet-off Akt transgenic: abbreviated as Akt TG), previously described [22], were fed 1 g/kg body weight doxycycline chow (DOX) for 8 weeks before the chow was removed for 2 weeks to allow cardiac-specific induction of the myrAkt1 transgene. Wild-type, Tet-myrAkt1 TG and α-MHC-tTA TG mice were used as controls and were all on a mixed background.

2.2. Heart perfusion

Mice were anesthetized by intra-peritoneal injection of 15 mg of chloral hydrate, and the heart was rapidly excised and arrested in ice-cold buffer. The aorta was then cannulated and retrogradely perfused at constant pressure of 60 mm Hg with 37 °C Krebs buffer containing (in mmol/L) NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, and $CaCl_2$ 2, glucose 11, and gassed with 95% O_2 and 5% CO_2. Left ventricular pressure was monitored from a water-filled balloon placed through the left atrial appendage and connected to a Millar transducer (Millar Instruments, Houston, TX). The balloon was inflated to achieve an end-diastolic pressure of 7–10 mm Hg.

2.3. Perfusion protocols

Hearts from TIRKO, CIRKO, Akt TG and their corresponding WT mice were allowed to stabilize for 20 min before basal hemodynamic parameters and coronary flow were recorded (baseline). Hearts were then subjected to an ischemic preconditioning (IPC) protocol (Fig. 1) consisting of three cycles of 5 min ischemia followed by 5 min reperfusion before 30 min no flow ischemia followed by 45 min reperfusion during which hemodynamic parameters and coronary flow were again recorded. Perfusions were performed in the presence or absence of 1 nmol/L insulin (human insulin 100 U/mL stock, Novolin, Novo Nordisk Inc. Princeton, NJ), that was added to the perfusate and kept for the entire perfusion protocol. In subsequent experiments, WT hearts were stabilized for 20 min before 30 min of no flow ischemia was applied and then followed by 45 min reperfusion (I/R group). Finally, some hearts were used for glycogen assay and western-blotting was perfused with or without insulin for 50 min or subjected to three cycles of IPC (5 min each) and then snap-frozen for analysis.

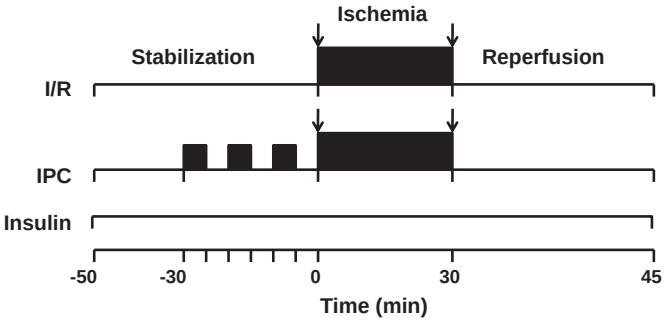

Fig. 1. Perfusion protocol. Male mice were perfused ex vivo using Langendorff perfusion system. Hearts were allowed to stabilize for 20 min before contractile parameters were measured (baseline) and then subjected to either an ischemia/reperfusion protocol consisting of 30 min no flow ischemia followed by 45 min reperfusion or preconditioned by three cycles of 5 min no flow ischemia followed by 5 min reperfusion each, before a 30 min no flow ischemia followed by 45 min reperfusion. Insulin (1 nmol/L) was absent or present during the entire protocol. Stab: stabilization; I/R: ischemia/reperfusion; IPC: ischemic preconditioning.

2.4. Infarct size measurement

Triphenyltetrazolium chloride (TTC) staining was used to assess myocardial tissue viability and to determine myocardial infarct size. Hearts were subjected to IPC as shown in Fig. 1 without the insertion of the balloon. At the end of the 45 min reperfusion, hearts were perfused with 1% TTC in PBS, pH 7.4, at 37 °C for 5 min and then collected and sectioned. The tissue slices were then incubated in 1% TTC in PBS, pH 7.4, at 37 °C for 20 min. Tissues were fixed in 10% PBS-buffered formalin overnight at 4 °C. Both sides of each TTC-stained tissue slice were photographed and digital photographs were acquired. The infracted area (unstained) was measured using the Image-Pro Plus software package, version 5.0 (Media Cybernetics; Silver Springs, USA) and was expressed as % of risk zone.

2.5. Immuno-blotting analysis

Total proteins were extracted from hearts that were frozen either at the end of the 45 min reperfusion period (full IPC protocol) or at the end of the three cycles of IPC (prior to the 30 min ischemia). Hearts were initially pulverized under liquid nitrogen and then homogenized with a Polytron in sample buffer containing (in mmol/L) HEPES 30, pH 7.4, sodium pyrophosphate 50, sodium fluoride 100, EDTA 1, and sodium orthovanadate 10 supplemented with 1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured using a Micro BCA reagent (Pierce, Rockford, IL). Protein extracts were resolved by SDS-PAGE and electrotransferred onto an Immobilon PVDF membrane (Millipore Corp., Bedford, MA). Membranes were probed with the following primary antibodies: mouse anti-Akt1, rabbit anti-p-Akt (Ser 473) and rabbit anti-p-Akt (Thr 308) (1/1000; 1/750; 1/1000 respectively, Cell Signaling Technology Inc. Danvers, MA), mouse anti-Gsk3α/β and rabbit anti-Phospho-Gsk3β (Ser 9) (1/1000 and 1/2000 respectively, Cell Signaling Technology Inc., Danvers, MA), rabbit-anti LC3 (1/1000, Sigma-Aldrich, St. Louis, MO), rabbit anti-p38MAPK, rabbit anti-Phosphop38MAPK, rabbit anti-MEK and rabbit anti-PhosphoMEK (1/1000, Cell Signaling Technology Inc., Danvers MA) and mouse anti-actin (1/1000, Sigma-Aldrich, St. Louis, MO). Alexa Fluor anti-Rabbit 680 (Invitrogen, Carlsbad, CA) and anti-mouse 800 (VWR International, West Chester, PA) were used as secondary antibodies and fluorescence was quantified using the LI-COR Odyssey imager (LI-COR, Lincoln, NE).