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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 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 Gsk3. 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.

Keywords

insulin; cardioprotection; insulin signaling; ischemia; reperfusion

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Disclosure statement

None of the authors has any conflict of interest to disclose.

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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 non diabetic 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) protects 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 microvascular 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 hazards 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, 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 postconditioning 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 10–14 weeks of age. Mice harboring β -MHC-tTA and Tet-*myrAkt1* genes (Tet-off Akt transgenic; abbreviated as Akt TG), previously described [22], were fed 1g/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 -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 mmHg with 37°C Krebs buffer containing (in mmol/L) NaCl 118, KCl 4.7, NaHCO₃ 25, MgSO₄ 1.2, KH₂PO₄ 1.2, and CaCl₂ 2, glucose 11, gassed with 95% O₂ and 5% CO₂. 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 mmHg.

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 100U/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 were perfused with or without insulin for 50 min or subjected to three cycles of IPC (5 min each) and then snap-frozen for analysis.

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 infarcted 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, sodium orthovanadate 10 supplemented with 1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was measured using Micro BCA reagent (Pierce, Rockford, IL). Protein extracts were resolved by SDS-PAGE and electro-transferred 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-Gsk3 / and rabbit anti-Phospho-Gsk3 (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). Alexafluor 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).

2.6. Glycogen assay

For glycogen measurements, three different sets of hearts from both TIRKO and WT mice were used (1) hearts that were perfused for 50 min with or without 1 mmol/L insulin; (2) hearts that were subjected to three cycles of IPC but no prolonged ischemia or reperfusion and (3) hearts that were subjected to full I/R or IPC protocols in the absence or presence of 1 nmol/L insulin as outlined in Fig. 1. At the end of each perfusion protocol, hearts were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Total glycogen was measured by acid extraction followed by enzymatic hydrolysis with amylo- $-1,4-$ $-1,6-$ glucosidase and measurement of glucose as previously described [23]. Total glycogen was expressed in μmol glucose/g wet tissue weight.

2.7. Metabolomic analysis

Extraction of metabolites—only hearts that were perfused with full I/R or full IPC in the absence or presence of 1 nmol/L insulin were used. At the end of the 45 min reperfusion, hearts were snap-frozen in liquid nitrogen and stored at -80°C for further analysis. Frozen heart samples (~ 50 mg) were homogenized in cold methanol (-20°C) containing D4-succinate as an internal standard at a final concentration of 80% MeOH (aq) and 20% tissue homogenate. Samples were vortexed and incubated for one hour at -20°C to precipitate protein. Following incubation, cell debris was pelleted by centrifugation (14,000 g for 5 min at 4°C) and the supernatant reserved. A second extraction of the pellet was performed by the addition of -20°C MeHO (aq) to a final concentration of 50% MeOH (aq) and 50% tissue homogenate. Each sample was mixed by vortex, incubated for one hour at -20°C , and centrifuged (14,000 g for 5 min at 4°C) to remove cell debris. The two extracts were combined and dried *en vacuo*.

Gas chromatography-mass spectrometry (GC-MS) analysis—GC-MS analysis was performed with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples were suspended in 40 μL of 40 mg/mL O-methoxylamine hydrochloride (MOX) in pyridine and incubated for one hour at 30°C . 25 μL of this solution was added to autosampler vials, then 20 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was added using the autosampler and incubated for 60 minutes at 37°C with shaking. 1 μL of the sample was injected to the gas chromatograph inlet in the split mode at a 10:1 split ratio with the inlet temperature held at 250°C . The gas chromatograph had an initial temperature of 95°C for one minute followed by a $40^{\circ}\text{C}/\text{min}$ ramp to 110°C and a hold time of 2 minutes. This was followed by a second $5^{\circ}\text{C}/\text{min}$ ramp to 250°C , a third ramp to 350°C , then a final hold time of 3 minutes. A 30 m Phenomenex-ZB5MSi column with a 5 m long guard column was employed for chromatographic separation. Data was collected using MassLynx 4.1 software (Waters). Known metabolites were identified and their peak area was recorded using QuanLynx. Data was transferred to an Excel file where each sample was normalized to the internal standard D4-succinate.

2.8. Statistical analysis

All values were expressed as means \pm standard error of the mean (sem). Comparisons between two groups were evaluated by Student's *t*-test. To compare results from more than two groups, a two-way ANOVA followed by Bonferroni test was performed using GraphPad Prism software, version 5 (GraphPad Software, Inc. La Jolla, CA). A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Insulin suppressed cardioprotection by IPC in wild-type mice but not in TIRKO mice

To determine the role of insulin receptors in cardioprotection, we subjected TIRKO and wild-type (WT) hearts to I/R or IPC in the absence or presence of 1 nmol/L insulin, present for the entire duration of I/R or IPC protocol (Fig. 1). Percent (%) recovery of left ventricular developed pressure (LVDP) and rate pressure product (RPP) was determined. Consistent with previously published results showing that the presence of insulin at the reperfusion protected rat hearts from I/R injury [10], we demonstrated that the presence of insulin prior to ischemia and during reperfusion protected WT hearts from ischemia. Thus, % recovery of LVDP and RPP was significantly ($p < 0.05$) elevated in WT hearts subjected to I/R with insulin compared to WT hearts subjected to I/R without insulin (Fig. 2A, B). In contrast, the protective effect of insulin against I/R was lost in TIRKO hearts, consistent with absent insulin signaling. Of note, the absence of insulin receptors in TIRKO hearts did not exacerbate ischemic injury relative to WT hearts perfused without insulin. Three cycles of IPC in the absence of insulin protected WT and TIRKO hearts from ischemia as evidenced by the increase in % recovery of LVDP and RPP compared to the I/R without insulin (Fig. 2A, B). Interestingly, when insulin was present for the entire IPC protocol, cardioprotection was lost in WT hearts as indicated by poor recovery of contractile function. In contrast, IPC protected TIRKO hearts from ischemia despite the presence of insulin, indicating that abolition of IPC by insulin requires the presence of insulin receptors in the heart.

To determine the effect of insulin on infarct size, a separate group of WT and TIRKO hearts were subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin as outlined in Fig. 1, without insertion of the balloon. Consistent with the functional data, infarct size, expressed as % of risk zone, was significantly ($p < 0.05$) bigger in WT hearts subjected to I/R without insulin or to IPC with insulin (Fig. 2C). In contrast, while TIRKO hearts subjected to I/R were susceptible to infarction, IPC was extremely efficient in reducing infarct size in these animals independently of insulin (Fig. 2C).

3.2. Insulin's suppression of IPC-mediated cardioprotection requires cardiomyocyte insulin receptors

Having demonstrated that insulin abolished cardioprotection by IPC and that this effect was abolished in hearts lacking the insulin receptors (IRs) in the entire heart, we next sought to determine whether this effect was mediated via cardiomyocyte IRs. To achieve this goal, we subjected hearts that lack IRs specifically in cardiomyocytes (isolated from CIRKO mice) to I/R or IPC in the absence or presence of 1 nmol/L insulin and measured functional recovery and infarct size. Similar to TIRKO mice, CIRKO mice exhibited reduced heart size (Table 1), confirming the previously known role of insulin signaling in cardiac growth regulation [21]. Independently of the background strain, insulin protected wild-type hearts from I/R as evidenced by enhanced functional recovery of LVDP and RPP and reduced infarct size (Fig. 3A, B). However, the beneficial effect of insulin on functional recovery and infarct size reduction after I/R was completely abolished in CIRKO mice. Similar to results obtained in TIRKO mice, insulin suppressed IPC in WT mice (different background strain) as evidenced

by worse functional recovery and bigger infarct size after ischemia. In contrast, IPC protected the heart from ischemic injury in CIRKO mice despite the presence of insulin during the perfusion protocol (Fig. 3A, B). Thus insulin's suppression of IPC is mediated via cardiomyocyte IRs because CIRKO hearts were refractory to insulin's abolition of IPC as suggested by their enhanced functional recovery when insulin was present. It is important to note that cardiac function at baseline as assessed by echocardiography is indistinguishable between TIRKO, CIRKO and their respective WT controls (data not shown). In addition, the responses of TIRKO and CIRKO mice to IPC in the absence of insulin were similar to their controls, excluding the possibility of additional protective mechanisms related to the absence of IRs in the heart.

3.3. Insulin's abolition of IPC occurs despite elevated Akt phosphorylation

Cardioprotection by insulin was previously shown to involve downstream signaling molecules such as phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB or Akt) and glycogen synthase 3 (Gsk3) [10, 24–25]. To explore whether insulin's effect on IPC required the activation of downstream signaling to Akt and Gsk3, we first measured Akt(Ser473) and Gsk3 (Ser9) phosphorylation at the end of the 45 min perfusion in TIRKO, CIRKO and their corresponding WT hearts subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin. Second, we determined whether insulin-mediated activation of downstream signaling occurred prior to ischemia by measuring both Akt(Ser473) and Gsk3 (Ser9) phosphorylation at the end of the three cycles of IPC. As expected, Akt(Ser473) and Gsk3 (Ser9) phosphorylation at the end of the reperfusion was significantly ($p < 0.005$) enhanced in WT hearts subjected to I/R in the presence of insulin compared to WT hearts subjected to I/R without insulin (Fig. 4). The effect of insulin on the phosphorylation of these two downstream effectors of insulin signaling was abolished in TIRKO hearts (Fig. 4). Similarly, while insulin was able to enhance Akt phosphorylation on Ser473 in WT hearts, it failed to do so in CIRKO hearts (Fig. 1S). Interestingly, when compared to I/R, IPC attenuated Akt and Gsk3 phosphorylation independently of insulin. However, WT hearts subjected to IPC with insulin still exhibit higher phospho Akt/total Akt and phospho Gsk3/total Gsk3 ratios compared to WT hearts subjected to IPC without insulin (Figs. 4, 1S).

When applied prior to 30 min ischemia, insulin phosphorylated Akt and Gsk3 in WT hearts but not in TIRKO and CIRKO hearts respectively (Fig. 2S). However, although significant ($p < 0.05$), this effect of insulin on downstream effectors was less pronounced compared to WT hearts examined at the end of the reperfusion. Taken together, these results suggest that insulin's activation of downstream signaling is involved in its suppression of cardioprotection by IPC.

3.4. Insulin suppression of IPC-mediated cardioprotection is Akt dependent

To further investigate the involvement of Akt in mediating insulin's effect on IPC, we used animals with 2 weeks induction of an activated form of Akt in the heart. As shown in Figs. 5A–D, withdrawal of the DOX chow for two weeks resulted in small but significant increase in Akt signaling and one of its downstream targets S6 as evidenced by elevated phosphorylated Akt (Ser473 or Thr308)/total Akt and phosphorylated S6 (Ser253/236)/total S6 ratios. It is worth noting that total Akt was increased by 15% ($p=0.06$) in Akt TG hearts compared to WT hearts, which was associated with a modest but significant 6% increase in heart weight (Fig. 5E) and heart weight/body weight ratio (Fig. 5F), consistent with the previously known role of Akt in promoting cardiac growth [26]. The presence of insulin resulted in an equivalent increase in phospho Akt/total Akt (~5 fold) and phospho S6/total S6 (~2 fold) ratios respectively in WT and Akt TG (Figs. 5A). When Akt TG mice and their littermate controls were subjected to IPC in the absence of insulin, Akt TG hearts exhibited

significantly ($p < 0.05$) lower recovery of LVDP compared to their control hearts perfused under the same conditions. Furthermore and similar to control hearts perfused with insulin, % recovery of LVDP and RPP was reduced in Akt TG hearts (Fig. 5G and H). Taken together, these data suggest that similar to the effect of insulin, short-term activation of Akt1 in the heart abolished cardioprotection by IPC.

3.5. Insulin's suppression of IPC does not involve MAP kinase pathway

In addition to Akt activation, we investigated the effect of insulin on MAP kinase pathway. As shown in Fig. 6A–C, MAP kinase pathway, as measured by the phosphorylation of p38MAPK or its downstream target MEK, was not modified by insulin in WT hearts. By contrast, we observed a small but significant ($p < 0.005$) increase in the ratio of phosphorylated p38MAPK/ total p38MAPK in Akt TG hearts subjected to IPC in the presence of insulin (Fig. 6B). However, this elevation did not translate into the activation of its downstream target MEK (Fig. 6C).

3.6. Insulin's suppression of IPC is associated with less glycogen depletion and more lactate production at the reperfusion

To evaluate the involvement of metabolic regulation by insulin in I/R and IPC, we first measured pre-ischemic cardiac glycogen content in TIRKO hearts and their WT controls before ischemia. As shown in Fig. 7A, insulin increased glycogen content in WT hearts but not in TIRKO hearts. When both TIRKO and WT hearts were subjected to the IPC stimulus, glycogen levels dropped significantly (Fig. 7B). However, insulin was able to significantly enhance glycogen content by 37% in WT hearts. Interestingly, after IPC stimulus, glycogen levels were high in TIRKO hearts independently of insulin (Fig. 7B). Next, we measured post-ischemic glycogen content and lactate levels in TIRKO and WT hearts at the end of the 45 min reperfusion. As shown in Fig. 7C and consistent with higher Gsk3 phosphorylation (Fig. 4C), WT hearts subjected to I/R in the presence of insulin had 58% ($p < 0.05$) more glycogen compared to WT hearts subjected to I/R without insulin. Similarly, the combination of insulin with IPC in WT hearts increased glycogen content by 3.8 fold ($p=0.08$) compared to IPC without insulin (Fig. 7C). In the other hand, lactic acid trended higher in WT/I/R (+ insulin) compared to WT/I/R without insulin (Fig. 7D). By contrast, there was a modest increase in lactic acid in WTIPC (+insulin) compared to WTIPC (-insulin) that did not reach statistical significance (Fig. 7D). It is worth noting that lactic acid levels were overall reduced within the IPC groups compared to the I/R groups independently of the genotype.

4. Discussion

This study was designed to determine the effect of insulin on ischemic preconditioning (IPC)-induced cardioprotection in mice. The major results of this work are: (1) insulin suppressed functional recovery and enhanced infarct size in wild-type mice subjected to IPC; (2) insulin's abolition of IPC required the presence of cardiomyocyte insulin receptors (IRs); (3) the effect of insulin on cardioprotection by IPC is mediated through downstream signals to Akt but is independent of MAPK signaling. Taken together, these findings demonstrate for the first time that persistent activation of Akt signaling by insulin is detrimental to cardioprotection by IPC, which may explain in part the failure to precondition the diabetic heart.

4.1. Insulin suppressed IPC-mediated cardioprotection

IPC is a potent cardioprotective phenomenon that has been shown to limit infarct size in dogs [8] and post-conditioning with repetitive I/R cycles at the onset of reperfusion is as effective as IPC in protecting the heart from a prolonged ischemic insult [27], which could

be of great relevance in the clinical setting. Similar to IPC and post-conditioning, insulin was shown to protect the heart from ischemic injury when administered at the onset of reperfusion [10]. However, the effect of insulin on IPC has never been explored before and could be of great importance for the clinical utility of IPC or post-conditioning in patients with type 2 diabetes, who most often exhibit hyperinsulinemia. In addition, although it is still debated, several studies found that the diabetic heart is refractory to cardioprotection by IPC or required a greater IPC stimulus to achieve this protection [24, 28–34] and that this resistance to IPC was mainly related to impaired PI3K-Akt signaling [24, 35]. However, recent studies have clearly challenged this idea by showing that instead of reduced insulin signaling, PI3K-Akt signaling is rather enhanced in the hearts of type 2 diabetic humans and animals [17–18]. Thus, we hypothesized that insulin's activation of PI3K-Akt signaling prior to IPC could be detrimental to cardioprotection. Using a combination of pharmacologic and genetic approaches, we demonstrated that insulin administered prior to IPC abolished its cardioprotection in Langendorff-perfused mouse heart. This was evidenced by worse functional recovery and increased infarct size in WT hearts subjected to IPC in the presence of 1 nmol/L insulin. Although this is the first study to directly assess the acute effect of insulin on IPC, Drenger et al. [36] showed that correction of hyperglycemia in streptozotocin-induced diabetic rats using insulin not only increased infarct size but also abolished ischemic and sevoflurane post-conditioning. Similarly, post-conditioning failed to protect the hearts of obese and diabetic *ob/ob* mice despite enhanced basal insulin signaling to Akt [17]. By contrast, our data are not supported by a prior study on isolated human right atrial trabeculae obtained from patients taking insulin and in which IPC was shown to be effective in recovering developed force [37]. This could be due in part to differences in the responses of right atrial tissue versus whole heart to the IPC stimulus or to the experimental protocol of IPC (hypoxic, glucose-free buffer with pacing versus Langendorff-perfused heart with 11 mmol/L glucose and no pacing).

4.2. Insulin's abolition of IPC required the presence of cardiomyocyte IRs

We next sought to examine the involvement of the insulin receptors (IRs) in mediating insulin's suppression of IPC and asked whether IRs of specific cell types within the heart were responsible for this effect. We used mice with either a complete ablation of IRs in the heart (TIRKO) or with cardiomyocyte-specific deletion of IRs (CIRKO) and showed that the negative effect of insulin on IPC required the presence of IRs in cardiomyocytes as CIRKO mice exhibited the same cardioprotection by IPC in the presence of insulin as TIRKO mice (Figs. 2 and 3). Importantly, our data demonstrated that the efficacy of IPC to reduce infarct size was actually potentiated when IRs are absent in the hearts as evidenced by reduced infarct size in TIRKO and CIRKO hearts subjected to IPC in the absence of insulin (Figs. 2C and 3C). These results on functional recovery and infarct size limitation in CIRKO mice are unique to the IPC stimulus and contrast with previous findings showing that functional recovery after myocardial infarction (induced by coronary artery ligation) in CIRKO mice was impaired [38]. The failure to protect the CIRKO heart from myocardial infarction was attributed to limited substrate utilization and mitochondrial dysfunction. However, in the context of IPC, the lack of cardiomyocyte IRs might be beneficial.

4.3. Insulin's suppression of IPC requires downstream activation of Akt and Gsk3 β

After establishing the involvement of IRs in mediating insulin's abolition of cardioprotection by IPC, we next examined downstream signaling to Akt and Gsk3 in TIRKO and CIRKO hearts subjected to I/R or IPC in the absence or presence of insulin. Whether examined at the end of reperfusion or at the end of the three IPC cycles, acute insulin treatment induced Akt phosphorylation on Ser473 and Gsk3 phosphorylation on Ser9 in hearts of TIRKO and CIRKO controls (Figs. 4, 1S and 2S). However, the fold induction of Akt phosphorylation was much higher when examined at the end of the

reperfusion, suggesting that insulin's abolition of IPC-mediated cardioprotection may have occurred during the reperfusion period. Indeed, insulin mediated cardioprotection again I/R in the absence of IPC is believed to be mainly mediated during early reperfusion [10, 39]. Downstream of Akt, phosphorylation and inactivation of Gsk3 β has been implicated as one of the main mechanisms by which IPC confers protection [40–41]. However, the use of animal models that lack the phosphorylation site on Gsk3 β that is required for its inactivation did not confirm a role for this protein in cardioprotection [42]. Our results demonstrated that despite increased Gsk3 β phosphorylation and inhibition by insulin, the heart could no longer be protected by this protocol, suggesting the existence of Gsk3 β - independent mechanisms mediating the insulin's effect on IPC.

4.4. Moderate induction of Akt in the heart abolished cardioprotection by IPC

To directly assess the role of Akt activation in cardioprotection by IPC, we used hearts with inducible activation of Akt (myrAkt1) [22]. We hypothesized that if insulin-abolished cardioprotection was associated with downstream activation of Akt signaling, then Akt TG hearts, with moderate expression of myrAkt1, should be refractory to cardioprotection by IPC even in the absence of insulin. As detailed in the methods section, we induced the myrAkt transgene for two weeks, which resulted in a modest but significant increase in Akt expression and one of its downstream targets S6 (Figs. 5B–D) and enhanced cardiac growth. The induction of Akt and its downstream target S6 were not influenced by the IPC stimulus as equivalent changes in their expression were also observed in these animals at baseline when the hearts were not perfused [43]. As predicted, Akt1 induction in the heart was sufficient to abrogate IPC-mediated cardioprotection as evidenced by the poor functional recovery of the Akt TG hearts (Figs. 5G and H) in the absence of insulin. The mechanisms by which activation of Akt abolished IPC-mediated cardioprotection have not been fully investigated in the present study but could involve the inhibition of glucose uptake via feedback impairment of insulin receptor substrate (IRS)1/2. Although, we did not assess IRS 1/2 expression or glucose uptake in WT and Akt TG hearts subjected to IPC, a recent study showed that moderate induction of myrAkt1 in the heart (equivalent to ours) did not impair insulin-mediated IRS 1/2 but reduced glucose uptake [43]. It should be noted however that our data on Akt TG are not in agreement with a recent study by Kunuthur et al. [44] showing that mice deficient in Akt1 were resistant to infarct limitation by IPC. However, it should be noted that in this study, Akt phosphorylation on Thr308 was enhanced when normalized to total Akt, which could explain their resistance to IPC. This increase in Akt phosphorylation on Thr308 is probably caused by Akt2 activation.

4.5. Metabolic changes associated with insulin's abrogation of IPC

To explore the involvement of metabolic regulation in insulin's suppression of IPC, we first assessed glycogen content in TIRKO and WT hearts at different time points of perfusion. Next, we performed a metabolomic analysis of key metabolites using GC-MS. When glycogen was measured before ischemia and in the absence of the IPC stimulus, insulin increased glycogen synthesis in WT but not in TIRKO hearts (Fig. 7A). This could be due to enhanced Gsk3 β phosphorylation on serine 9, leading to less inactivation of glycogen synthase as previously suggested [45]. Interestingly, TIRKO hearts exhibited less glycogen content compared to WT hearts independently of insulin, which could be due to higher glycogen breakdown. Our results contrast prior report showing that glycogen depletion prior to ischemia is beneficial for functional recovery after no flow ischemia [46], which is mainly due to less lactate accumulation during ischemia and thus less cellular acidosis. In the present study, we did not measure lactate levels at the onset of ischemia but at the end of the 45 min reperfusion and found that insulin did not significantly enhanced lactate levels in WT hearts despite higher glycogen content. This could be related to stimulation of glucose oxidation by insulin and reduced anaerobic glycolysis. One other possibility could be that

the beneficial effect of insulin on functional recovery and infarct size is independent of insulin's effect on glycogen or lactate. Indeed, Jonassen et al. [10] demonstrated that the protective effect of insulin on I/R is independent of glucose presence at the reperfusion because the use of pyruvate in the reperfusion instead of glucose did not negate insulin-mediated cardioprotection. Therefore, the authors concluded that cardioprotection by insulin is mediated via Akt and p70S6 kinase but does not involve insulin-stimulated glucose uptake. Compared to control perfusion, three cycles of IPC significantly reduced glycogen content in the heart independently of the genotype (Fig. 7B). This observation is supported by prior studies highlighting glycogen depletion as one of the mechanisms by which IPC protects the heart [47–48]. Despite glycogen depletion by the IPC stimulus, insulin induced a modest but significant ($p < 0.05$) increase in glycogen content in WT hearts, but whether this increase is causal for poor functional recovery remains to be determined. When glycogen and lactate were examined at the reperfusion, we also observed a higher glycogen content in WT hearts perfused in the presence of insulin. Interestingly, although glycogen accumulation was similar between WT I/R (+insulin) and WTIPC (+insulin), functional recovery was completely different, suggesting that glycogen content does not correlate with functional recovery. Furthermore, we analyzed several other metabolites in the heart at the end of the 45 min reperfusion and showed that lactate was higher in the I/R groups compared to the IPC groups and that tricarboxylic (TCA) cycle intermediates such as pyruvate, malate and fumarate accumulated more in the I/R groups compared to IPC groups. (Fig. 3S). However, none of these metabolites showed a significant variation in the IPC group with the exception of succinate, which was significantly ($p < 0.05$) increased in WTIPC (+insulin) compared to WTIPC (–insulin). Succinate can be used as by complex II of the electron transport chain, which can lead to higher production of reactive oxygen species (ROS) [49], possibly exacerbating cellular damage in this group. The exact mechanisms by which these insulin-mediated metabolic changes affect functional recovery after IPC required further investigations. Finally, other mechanisms could explain the detrimental effect of insulin on IPC including inhibition of autophagy, alteration of nitric oxide or calcium signaling, modulation of the mitochondrial KATP channel and perturbation of Na⁺/K⁺ ATPase activity.

4.6. Study limitation

There are limitations in the present study. We used an *ex-vivo* perfusion system to assess cardioprotection by IPC, although this is an established method to conduct such studies, it does not recapitulate the *in-vivo* situation such as changes in the systemic milieu that could affect cardioprotection. This is particularly true for TIRKO mice which are known to be hyperinsulinemic between 20–25 weeks of age [50]. In addition, only glucose (11 mmol/L) was used as substrate without fatty acids, which are known to be the main substrate for the post-ischemic heart [51]. Furthermore, only one dose of insulin was used in this study (1 nmol/L), which is somewhat supraphysiologic, but was aimed to mimic the hyperinsulinemic state observed in diabetes. In addition, signaling molecules downstream of Akt that mediate insulin's effect on IPC have not yet been identified and could be the subject of future investigations. Finally, lactate levels were only assessed at the end of the 45 min reperfusion and not during ischemia.

5. Conclusions

The present study suggests that insulin may inhibit cardioprotection by IPC through downstream activation of PI3K-Akt signaling, which could in part explain the resistance of the diabetic heart to cardioprotection by IPC in the context of increased insulin signaling. Additional work is needed to determine the mechanisms by which insulin inhibits IPC-mediated cardioprotection and to examine whether reducing insulin signaling in the hearts of type 2 diabetic animals could sensitize their hearts to IPC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Insulin abrogates ischemic preconditioning (IPC)-mediated cardioprotection
- Insulin's suppression of IPC is mediated via cardiomyocyte insulin receptors
- Insulin's abolition of IPC is Akt-dependent

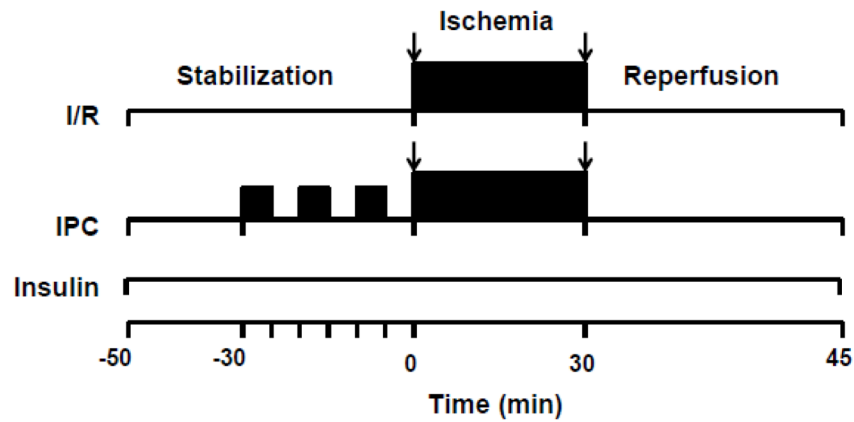


Fig. 1.

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.

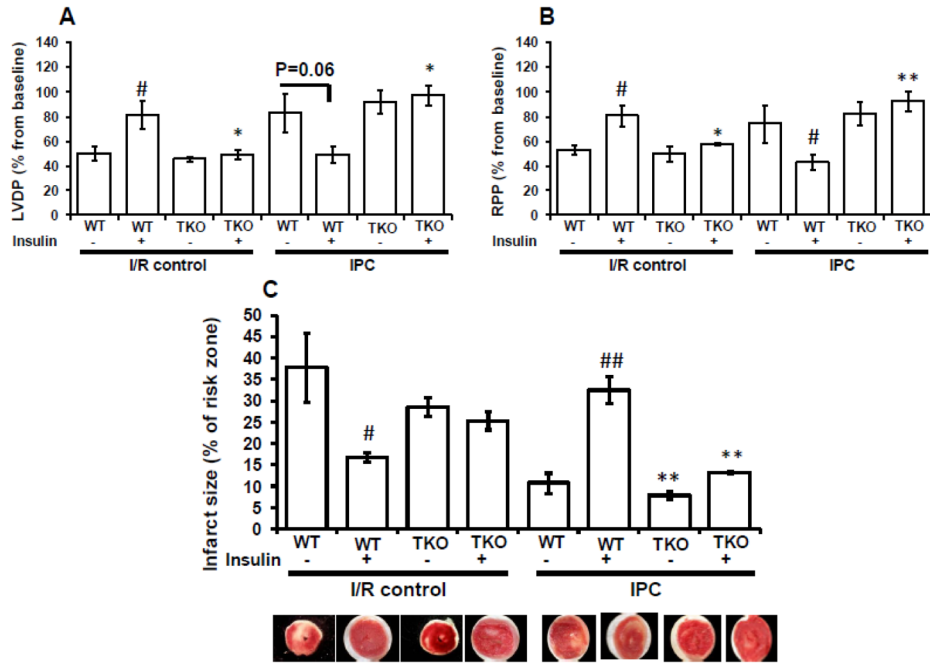


Fig. 2. Insulin protected against I/R and abolished cardioprotection by IPC in WT but not in TIRKO hearts. Hearts were isolated from mice lacking insulin receptors in the entire heart (TIRKO) and their corresponding WT controls were subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin. Contractile parameters were recorded at the end of the stabilization period (baseline) and 10 min before the end of the reperfusion and are expressed as % from baseline values. **(A)** % recovery of left ventricular developed pressure (LVDP); **(B)** % recovery of rate pressure product (RPP) and **(C)** infarct size expressed as % of risk zone in TIRKO and their corresponding WT controls. Data are mean \pm SEM (n = 3 mice per group for I/R and n = 7–9 mice per group for IPC). * p<0.05; ** p<0.005 versus WT perfused under the same condition; # p<0.05; ## p<0.005 versus (-insulin) within the same genotype.

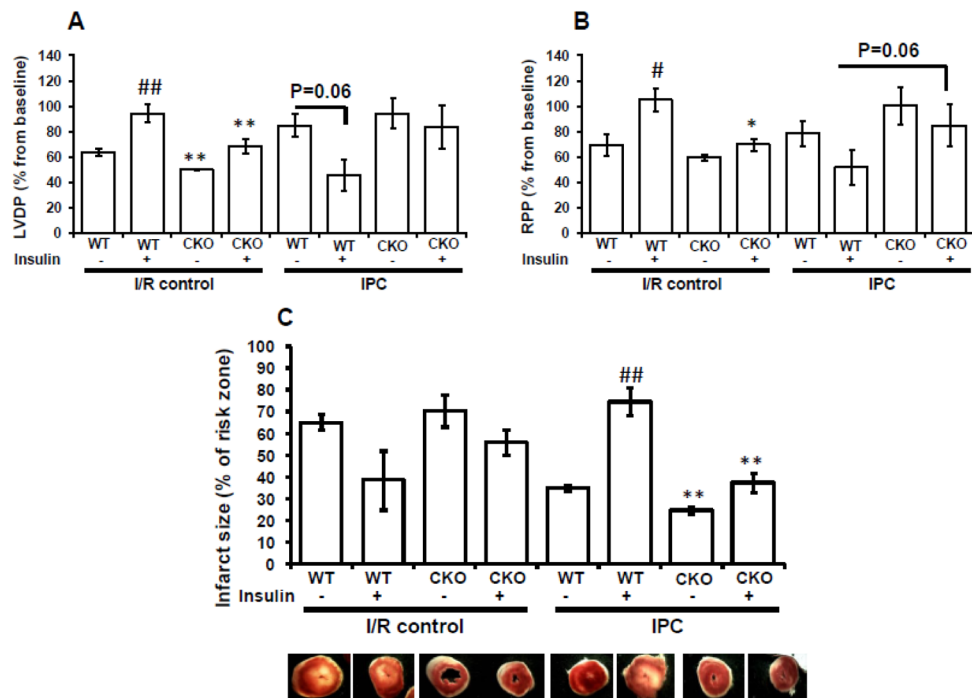
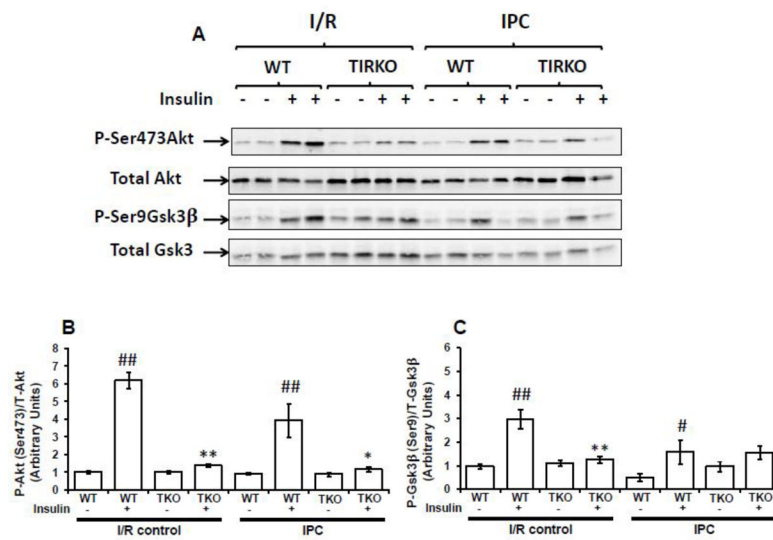


Fig. 3. Insulin's abolition of contractile recovery is mediated through cardiomyocyte insulin receptors. Hearts isolated from mice lacking insulin receptors specifically in cardiomyocytes (CIRKO) and their corresponding WT controls were subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin during the entire perfusion protocol. **(A)**: % recovery of LVDP; **(B)**: % recovery of RPP and **(C)** infarct size in CIRKO mice respectively and their corresponding WT controls subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin. Data are mean \pm SEM ($n = 3-6$ mice per group for I/R and $n = 7-8$ mice per group for IPC). * $p < 0.05$; ** $p < 0.005$ versus WT perfused under the same condition; # $p < 0.05$; ## $p < 0.005$ versus (-insulin) within the same genotype.

**Fig. 4.**

The effect of insulin on Akt and Gsk3 phosphorylation in TIRKO and their corresponding WT controls. **(A)** Representative western-blot of Akt phosphorylation on Ser473 and total Akt expression and Gsk3 phosphorylation on Ser9 and total Gsk3 expression in TIRKO and WT hearts subjected to I/R or to a full IPC protocol in the absence or presence of 1 nmol/L insulin. **(B)** and **(C)** are the corresponding densitometry of phosphoAkt/total Akt ratios and phosphoGsk3/total Gsk3 ratios. Data are mean \pm SEM ($n = 4$ mice per group for I/R and $n = 4$ mice per group for IPC). * $p < 0.05$; ** $p < 0.005$ versus WT perfused under the same condition; # $p < 0.05$; ## $p < 0.005$ versus (-insulin) within the same genotype.

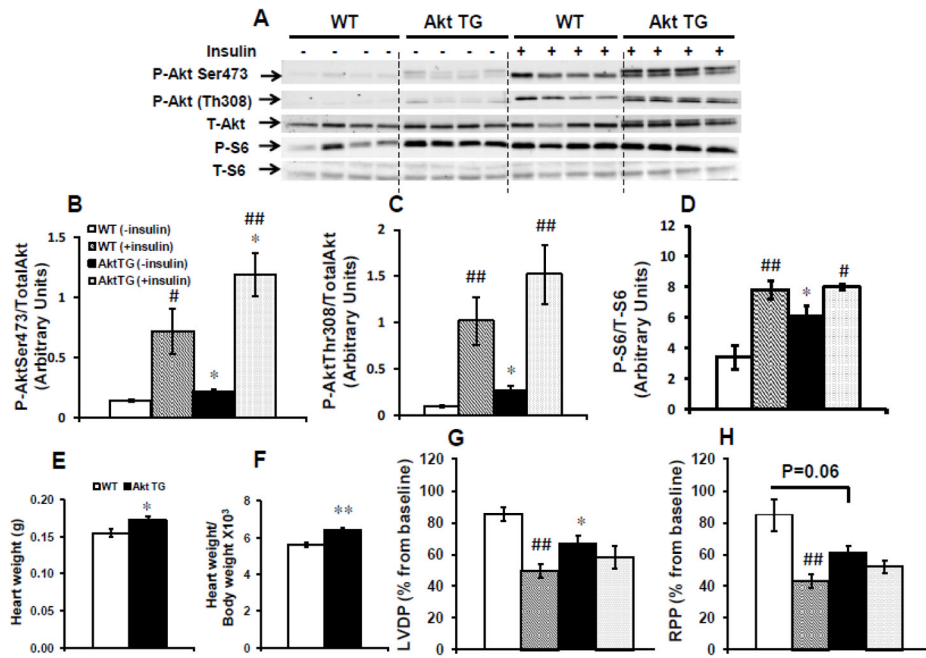


Fig. 5. Akt activation in the heart abolished cardioprotection by IPC. Hearts isolated from Akt TG and WT mice were subjected to the full IPC protocol as shown in Fig. 1A in the presence or absence of 1 nmol/L insulin. **(A)** Representative western-blots of Akt phosphorylation on Ser473 and Thr308, total Akt, S6 phosphorylation on Ser235/236 and total S6 protein; **(B)**, **(C)** and **(D)** the corresponding densitometry of phosphoSer473Akt/total Akt, phosphoThr308Akt/total Akt and phosphoSer235/236 S6/total S6 ratios respectively. **(E)** heart weights; **(F)** heart weight/body weight ratios $\times 10^3$; **(G)** % recovery of left ventricular developed pressure (LVDP); **(H)** % recovery of rate pressure product (RPP) in Akt TG and WT mice. Data are mean \pm SEM (4 mice per group and per genotype for western-blots and $n = 5-8$ mice per group and per genotype for functional recovery). * $p < 0.05$; ** $p < 0.005$ versus WT perfused in the same condition; # $p < 0.05$; ## $p < 0.005$ versus (- insulin) within the same genotype.

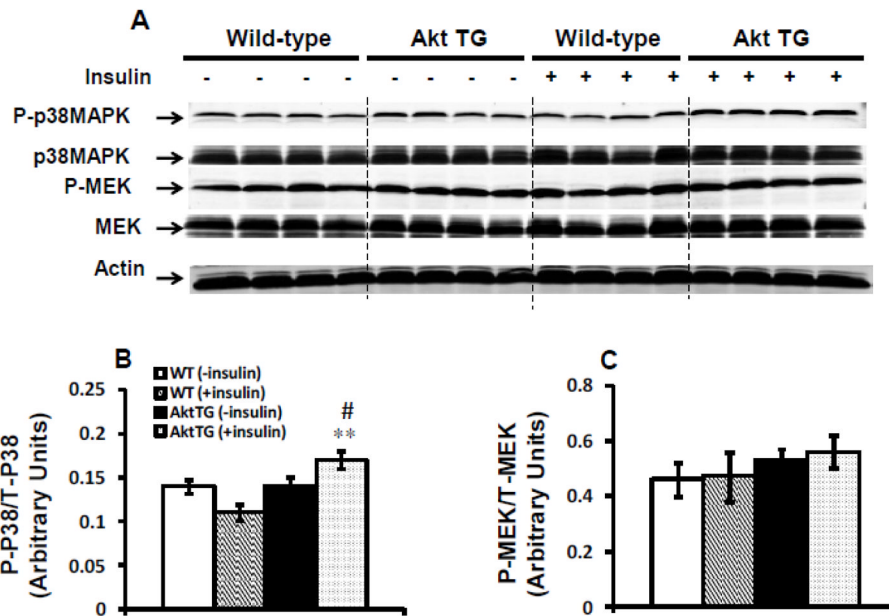


Fig. 6. Insulin does not modulate mitogen-activated protein kinase (MAPK) pathway during IPC. (A) Representative western-blots of phosphop38MAPK, phosphoMEK, total p38MAPK and total MEK expression in Akt TG and WT hearts subjected to the full IPC protocol in the presence or absence of 1 nmol/L insulin. (B) and (C) The corresponding densitometry of phosphop38/total p38 and phosphoMEK/total MEK ratios respectively. Data are mean \pm SEM (n = 4 mice per group and per genotype). **p<0.005 versus WT perfused in the same condition; #p<0.05 versus (- insulin) within the same genotype.

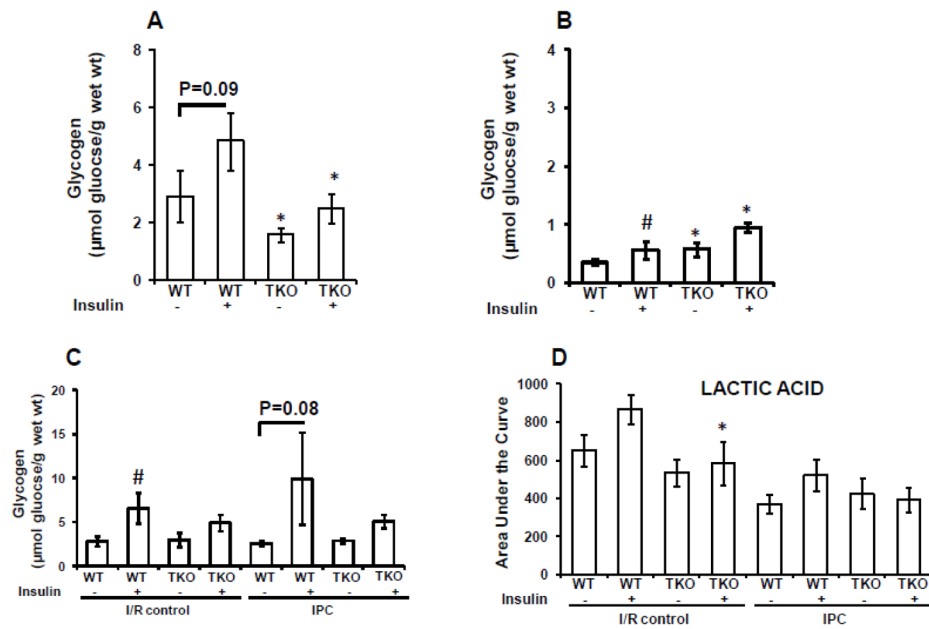


Fig. 7. Glycogen and lactate levels in TIRKO and WT hearts. **(A)** Pre-ischemic glycogen content in TIRKO and WT hearts subjected to 50 min perfusion without IPC in the absence or presence of 1 nmol/L insulin. **(B)** Same as **(A)** but hearts received three cycles (5 min each) of IPC. **(C)** Post-ischemic (at the end of the 45 min reperfusion) glycogen content in TIRKO and WT hearts subjected to I/R or IPC with or without 1 nmol/L insulin. **(D)** Lactate levels measured by GC-MS at the end the reperfusion in TIRKO and WT hearts subjected to I/R or IPC in the absence or presence of 1 nmol/L insulin. Data are mean \pm SEM ($n = 3-4$ mice per group for **A** and **B** and $n = 4-7$ mice per group for **C** and **D**). * $p < 0.05$ versus WT perfused in the same condition; # $p < 0.05$ versus (- insulin) within the same genotype.

Table 1

Morphometric parameters of TIRKO and CIRKO mice and their corresponding wild-type controls.

	WT	TIRKO	WT	CIRKO
N	14	17	9	8
BW (g)	26.9 ± 0.5	27.2 ± 1	31.7 ± 1.1	34.7 ± 2.3
HW (mg)	173.3 ± 4.6	149.6 ± 5 ^{**}	171.3 ± 7.4	146.1 ± 7.5
HW/BW (mg/g)	6.5 ± 0.2	5.5 ± 0.1 ^{**}	5.4 ± 0.1	4.2 ± 0.12 ^{**}

BW: body weight; HW: heart weight; HW/BW: heart weight/body weight ratio. Data are mean ± STE.

^{**}
p<0.005 versus WT of the same strain.