Biochemical and Biophysical Research Communications 467 (2015) 451–457

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Insulin/NFκB protects against ischemia-induced necrotic cardiomyocyte death

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ARTICLE INFO

Article history:
Received 23 September 2015
Accepted 30 September 2015
Available online 9 October 2015

Keywords:
Insulin
Cell signaling
Ischemia
Heart
Cell death

ABSTRACT

In the heart, insulin controls key functions such as metabolism, muscle contraction and cell death. However, all studies have been focused on insulin action during reperfusion. Here we explore the cardioprotective action of this hormone during ischemia. Rat hearts were perfused *ex vivo* with an ischemia/reperfusion Langendorff model in absence or presence of insulin. Additionally, cultured rat cardiomyocytes were exposed to simulated ischemia in the absence or presence of insulin. Cytoprotective effects were measured by myocardial infarct size, trypan blue exclusion, released LDH and DNA fragmentation by flow cytometry. We found that insulin protected against cardiac ischemia *ex vivo* and *in vitro*. Moreover, insulin protected cardiomyocytes from simulated ischemia by reducing necrotic cell death. Protective effects of insulin were dependent of Akt and NFκB. These novel results show that insulin reduces ischemia-induced cardiomyocyte necrosis through an Akt/NF-κB dependent mechanism. These novel findings clarify the role of insulin during ischemia and further support its use in early GIK perfusion to treat myocardial infarction.

1. Introduction

Ischemic heart disease (IHD) is characterized by atherosclerotic narrowing of the arteries of the heart, resulting in a reduced blood supply to the heart. IHD is the major cause of death worldwide. However, myocardial ischemia also occurs during heart transplantation. After ischemia, reperfusion takes place, restoring the blood flow and nutrients. Both ischemia and reperfusion can induce cardiac death by necrosis and/or apoptosis [1]. The glucose-insulin-potassium (GIK) infusion [2,3] was initially used during the reperfusion in the infarcted heart [4]. GIK was also used as a cardioprotective solution to provide metabolic support based on that insulin could control energy metabolism, muscle contraction and cell death [5–8]. However, clinical trials using GIK infusion during reperfusion showed negative results [9]. Apstein proposes that these negative results could be explained, in part, because GIK did not start before reperfusion [6]. Therefore we hypothesized that insulin has cardioprotective effect when is applied during the ischemic period. Cardiomyocyte death is mainly inhibited by activation of PI3K/Akt pathway. Akt phosphorylates and inactivates several proteins including BAD, caspase 9, FoxO, etc [8]. However, insulin also controls transcriptional factors including NFκB. This one has also shown to be protective against ischemia [10,11]. Recently we showed that insulin stimulates cardiomyocyte mitochondrial fusion and metabolism by activating Akt/NFκB signaling pathway [9]. Therefore, the aim of this work was to investigate the protective role of insulin during ischemia and understand the contribution of NFκB on insulin-dependent cardioprotection.

2. Materials and methods

2.1. Cultured cardiomyocytes

Primary cultured cardiomyocytes (2–3 days old) Sprague-Dawley rats were prepared and used as described in Ref. [12]. Rats were obtained from the Animal Breeding Facility, Facultad Ciencias Quimicas y Farmaceuticas, Universidad de Chile, Chile. All studies conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8th

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http://dx.doi.org/10.1016/j.bbrc.2015.09.171
0006-291X/

Edition, 2011) and were approved by our Institutional Ethics Review Committee.

2.2. Langendorff system

Langendorff studies were performed according to Ibacache et al. [13]. Briefly, the hearts were mounted in a Langendorff system, perfused retrograde via the ascending aorta at 12 mL/min with physiological Krebs Henseleit buffer. After stabilization, hearts were subjected to 45 min low flow ischemia (0.25 mL/min) followed by 60 min reperfusion. Insulin (Actrapid) was added during ischemia or ischemia/reperfusion (Fig. 1A). Then, the hearts were sliced from into 2 mm slices. They were counterstained with 2,3,5-triphenyltetrazolium chloride (Sigma). For each slice, measuring the size of the infarct area was performed by planimetry as described in Ref. [13]. The infarct size was expressed as % of the ventricular volume [13].

2.3. Simulated ischemia/reperfusion

Ischemia was performed as described in Ref. [14]. Briefly, cells were incubated in ischemia-mimicking solutions containing (in mM) HEPES (5), 2-deoxy-D-glucose (10), NaCl (139), KCl (12), $MgCl_2$ (0.5), $CaCl_2$ (1.3), and lactic acid (20), pH 6.2, under 100% nitrogen (O_2 <1%) at 37 °C for 8 h. Subsequently for reperfusion, this medium was replaced with DMEM/M199 medium containing 10% FBS and cells were cultured under normoxia.

2.4. Western blot

After treatments, cells were lysed and equal amounts of protein were separated by SDS–PAGE (5–20% gels) and electrotransferred to PVDF membranes. The membranes were incubated with primary antibodies (anti-pS473 Akt, anti-pT308 Akt, pS137 BAD, anti-pS536 p65, anti-p65, anti-GAPDH, anti-Akt total, anti-β actin or anti-IκB, Cell Signaling) and re-blotted with HRP-linked secondary antibody. The bands were detected using ECL and quantified by image densitometry using ImageJ software (NIH). Protein content was normalized by GAPDH or β-actin level.

2.5. Immunofluorescence studies

Cardiomyocytes were fixed with paraformaldehyde 4% PBS, permeabilized with 0.01% triton, 3% bovine serum albumin was used to block the samples. They were incubated with rabbit anti-p65 NFκB overnight at 4°C, and cells were washed and incubated with secondary fluorescent antibody.

2.6. Cell death assays

Cell death was studied by: a) Exclusion Trypan blue: cells were counted by double-blind; b) Apoptotic subG1 population [14]; and c) released LDH (Cytotoxicity assay kit, Promega).

2.7. Statistical analysis

Results were expressed as mean ± SEM (n: 4–7 independent cell preparations). The comparisons between groups versus control were performed in GraphPad Prism 5 software using t-Student or ANOVA for two or more than two groups, respectively. $p \leq 0.05$ was set as significance level.

3. Results

3.1. Effect of insulin on infarct size ex vivo

To understand the protective role of insulin against ischemia/reperfusion, hearts were exposed to ischemia/reperfusion in absence or presence of 10 or 100 nM insulin only during ischemia or during ischemia/reperfusion. Infarct size was measured as

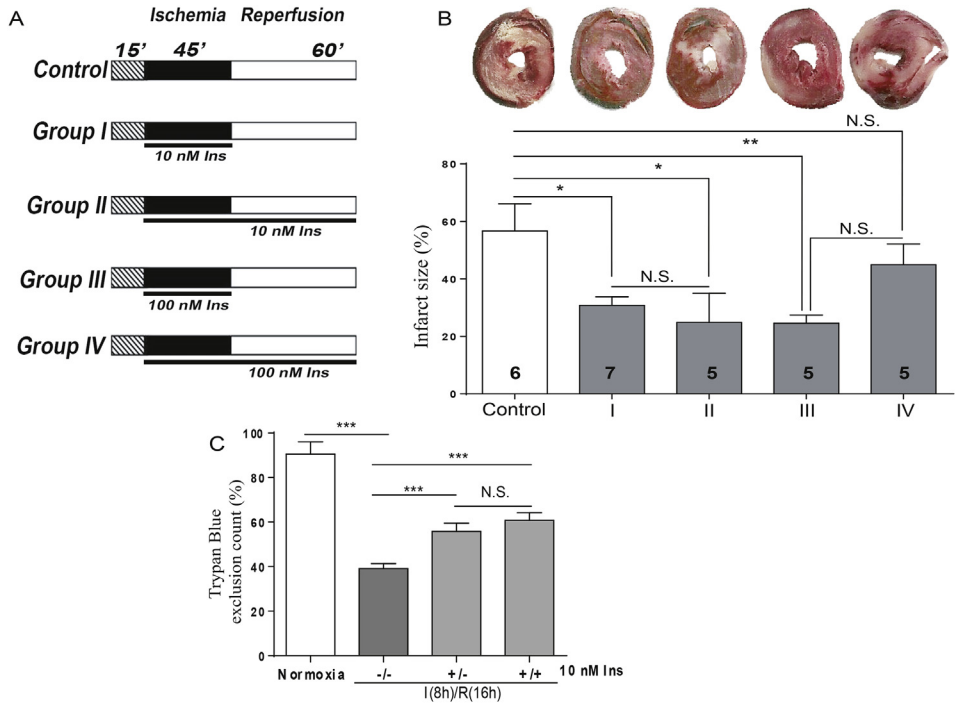

Fig. 1. Effect of insulin against ischemia/reperfusion (I/R) *ex vivo* and *in vitro*. **Panel A**, Experimental protocol for Langendorff. **Panel B**, % infarct size of *ex vivo* experiments. **Panel C**, cultured cardiomyocytes were submitted to simulated I/R in absence (−/−) or presence of 10 nM insulin (Ins) during ischemia (+/−) or for complete I/R protocol (+/+). Cell viability was measured by trypan blue exclusion. Values are mean ± SEM. N: 4–7 independent experiments. Data were analyzed by one-way ANOVA and Tukey's multiple comparison test. *p < 0.05, **p < 0.01 and ***p < 0.001. N.S. no significant changes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

described in Methods. Fig. 1A shows that insulin reduced infarct size. However, no differences between insulin treatment during ischemia or ischemia/reperfusion were found (Fig. 1B). This result was novel because most of studies have shown protective effects of insulin during reperfusion [15–18]. These results were confirmed *in vitro* where cultured cardiomyocytes were exposed to ischemia/reperfusion in the absence or presence of 10 nM insulin during ischemia or ischemia/reperfusion. Similar to Langendorff, insulin protects the cells with no differences between its presence during ischemia or ischemia/reperfusion (Fig. 1C). These results suggest that insulin protected from ischemia/reperfusion, primarily by acting during ischemia.

3.2. Effect of insulin on cell death during simulated ischemia

We studied whether insulin protects against ischemia alone. In order to test this, ischemia was performed by 2, 4 and 8 h. We observed a time-dependent decrease in cell viability of 20, 35 and 50%, respectively (Fig. 2A). We chose 8 h to evaluate cell death. Then, we studied the effects of different insulin concentrations (1, 10, and 100 nM) for 8 h of simulated ischemia. The results showed that 10 nM insulin was the optimal concentration to prevent and/or inhibit the cell viability decrease in simulated ischemia (Fig. 2B). Next, we analyzed the type of cell death that insulin prevents on cardiomyocytes exposed to ischemia. Fig. 2C shows that ischemia increased necrotic cell death detected by released LDH. Also apoptotic subG1 cell population increased (Fig. 2D). However insulin reduces both released LDH and subG1 cells induced by ischemia (Fig. 2C and D). Insulin did not decrease active caspase-3 and cleaved PARP in ischemia (Fig. 2E). Therefore, these results suggest that insulin protects and/or prevents ischemia damage by inhibiting necrosis cell death.

3.3. Effect of insulin on Akt activation during simulated ischemia

Akt activation was determined because is a key mediator of insulin signaling controlling cell death. Levels of phospho-Akt on serine 473 and threonine 308 were increased with insulin treatment in ischemia (Fig. 3A). These effects were inhibited in a dose-dependent manner by the Akt inhibitor (0–5 uM) Akt VIII (Fig. S1A). Downstream, insulin increased BAD phosphorylation on Serine 137 (Fig. 3B). These results, suggest that insulin activated Akt and triggered BAD phosphorylation during simulated ischemia.

3.4. Effect of insulin on NFκB during simulated ischemia

Insulin treatment in simulated ischemia reduced the NFκB repressor IκBα levels (Fig. 4A). Moreover, insulin augmented p65 NFκB nuclear translocation in cardiomyocytes (Fig. 4B) and increased p65 NFκB phosphorylation on Serine 536 during ischemia (Fig. S1B). This effect was abolished by BAY 11-7082 (Fig. S1B**).**

3.5. Effect of Akt and NFκB inhibition on protective role of insulin during ischemia

In order to evaluate the role of Akt and NFκB on insulin-induced cardioprotection during ischemia. Akt and NFκB were inhibited by AKTi VIII and BAY 11-7082, respectively. Both Akt and NFκB inhibition abolished insulin-induced cardioprotection during simulated ischemia (Fig. 4C and D). These results suggest that Akt and NFκB participate on insulin-induced cardioprotection and/or prevention of ischemic damage.

4. Discussion

The main finding of this work involved the clarification of the role of insulin on cardioprotection during ischemia. We found that insulin protects cardiomyocytes by reducing necrotic cell death. Protective action of insulin involves NFκB through an Akt-dependent mechanism.

In the normal heart, insulin promotes glucose uptake and its utilization via glycolysis, regulates long-chain fatty acid uptake and protein synthesis [8]. However, during ischemia/reperfusion, insulin promotes cardioprotection [15–18]. Dogs subjected to myocardial ischemia/reperfusion and treated with insulin 10 min before reperfusion showed significant cardioprotective effects as evidenced by improved cardiac function, improved coronary blood flow, reduced infarct size, and myocardial apoptosis [17]. In rabbits, insulin given just before reperfusion also reduced infarct size [15]. In isolated rabbit hearts, infusion of insulin prior to ischemia significantly reduced myocardial infarction through a PI3K-dependent mechanism [19]. In rats, administration of insulin after reperfusion reduced post-ischemic myocardial apoptotic death [18]. Moreover in rats, insulin infusion 10 min before the ischemia and continuing for 2 h, significantly reduced infarct size, decreased apoptosis and improved cardiac function after ischemia [16]. These data unveiled the use of GIK infusion as a cardioprotective treatment during reperfusion [5–7]. However, clinical trials using GIK infusion during reperfusion as a cardioprotective agent showed negative results [9]. Here, we showed that cardioprotective actions of insulin are more important during ischemia than reperfusion. This result could explain most of the controversial results obtained with GIK infusion. In this sense, Apstein proposes that negative results obtained in the CREATE-ECLA trial regarding the effect of GIK infusion on mortality in acute myocardial infarction is due, in part, because GIK infusion was not started early and well before reperfusion [6]. Our results suggest that the main effect of insulin was associated with the reduction of necrosis during ischemia rather than the reduction of apoptosis during reperfusion. We propose that insulin administration early during reperfusion could reduce some of the necrotic process initiated during ischemia, inducing cardioprotection. After reperfusion was initiated effect of insulin in apoptosis should be negligible, hence explaining the null results obtained in CREAT-ECLA trial.

Only few works have explored the cardioprotective effect of insulin administered just before or during ischemia [16,19]. However, none of them has characterized the effect on cardiomyocyte death during this stage. Xing et al. and first preclinical studies using GIK infusion in the early 70's showed that treatment with insulin during ischemia decreased plasma creatine kinase and LDH activities [16,20]. Although Xing et al. described a reduction of apoptosis after reperfusion [16], histological analysis [20] and plasma enzymatic activities [16,20] suggest also an anti-necrotic action of insulin.

Several studies involved the insulin-mediated activation of PI-3K/Akt pathway to explain the protective effect of GIK [2,21,22]. Akt phosphorylates and inactivates several pro-apoptotic proteins including BAD [23]. We found that basal and insulin-dependent Akt and BAD phosphorylation were increased during ischemia. BAD is a pro-apoptotic member of the Bcl-2 family which exerts its proapoptotic action by binding Bcl-2 and Bcl-XL [23]. Akt-dependent BAD phosphorylation at serine 136 creates a consensus site for interaction with the 14-3-3 protein. BAD then binds to 14-3-3, releasing Bcl-2 or Bcl-XL, with the consequent promotion of cell survival [23,24]. Bcl-2 has been associated with prevention of mitochondrial permeability transition, an event resulting in either apoptosis or necrosis [25,26]. In fact, Bcl-2 and Bcl-XL can inhibit the mitochondrial permeability transition itself [27]. The possible

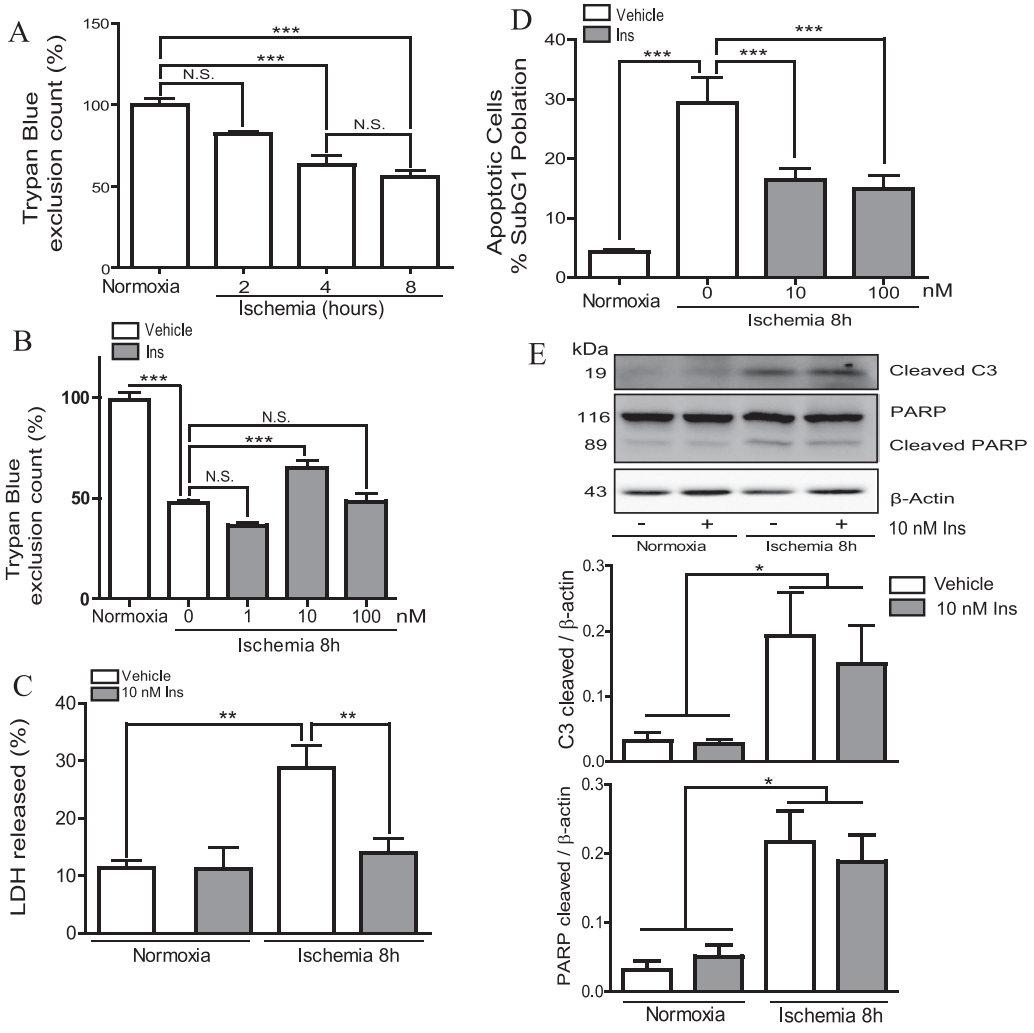

Fig. 2. Insulin protects against ischemia-induced cardiomyocyte death. **Panel A**, Time-course of the effect of simulated ischemia on cell viability. 8 h was chosen as the best time for ischemia. ***p < 0.001 vs normoxia. **Panel B**, in the absence or presence of insulin (0, 1, 10 and 100 nM) and then cell viability was measured as described in Methods. **Panel C**, in the absence or presence of 10 nM insulin. Then released LDH was assayed as described in Methods. **Panel D**, in the absence or presence of 10 or 100 nM insulin. Then the apoptotic subG1 population was measured as described in Methods. **Panel E**, protein levels of active caspase 3 and PARP were measured by Western blot. Values are mean ± SEM. N:4–6 independent experiments. These data were analyzed by one or two-way ANOVA and Tukey's multiple comparison test. *p < 0.05, **p < 0.01 and ***p < 0.001. N.S. no significant changes.

mechanism involved an inhibition of VDAC and ANT activities by Bcl-2 [28,29]. Therefore, BAD regulation of Bcl-2 and Bcl-XL could block the mitochondrial permeability transition, and could therefore block mitochondrial permeability transition-dependent necrosis in addition to their well-established ability to inhibit apoptosis [26].

In addition to BAD phosphorylation, our results showed that insulin-dependent Akt activation also stimulates NFκB, visualized by a decreased NFκB repressor IκBα protein levels associated with a p65 translocation to the nucleus. Dan et al. described that the Akt-dependent mTOR and IKK interaction stimulates IKK activity which phosphorylates IκBα leading to NFκB activation [30]. Moreover, we have previously shown that insulin activates NFκB in cardiomyocytes through an Akt-dependent pathway [31]. Here, we showed that insulin activated NFκB and prevented cardiomyocyte death induced by ischemia. Inhibition of both Akt and NFκB by using Akti and BAY during ischemia, respectively, abolished insulin-dependent reduction of cell death.

Besides insulin, NFκB is activated by pro-inflammatory cytokines and endogenous ligands for toll-like receptors that are generated during ischemia [32]. NFκB activation in turn induces pro-inflammatory proteins [32]. Because inflammation can trigger heart injury, NFκB blocking using pharmacological inhibitors or decoy oligonucleotides reduces myocardial infarction in animal models [32–35]. In addition, NFκB knockout mice have less heart failure and lower mortality compared with wild-type mice after myocardial infarction [36]. Our results showed that interventions blocking NFκB should be analyzed with caution because NFκB also blocks necrosis during myocardial ischemia. Misra et al. revealed that NFκB activation was required to decrease ischemia-induced cell death in a murine model of acute myocardial infarction via induction of c-IAP1 and Bcl-2 [10].

NFκB is a well-known anti-apoptotic transcription factor [37]. However, NFκB has also less known anti-necrotic action [37]. NFκB exerts its protective effects by upregulating expression of several genes [38]. Among these are Bcl-2-family members, A1/Bfl-1 and Bcl-XL, the caspase-8 regulator FLIPL, the caspase inactivators: cellular inhibitor of apoptosis proteins (c-IAP)1 and c-IAP2 and X chromosome-linked IAP (XIAP), TRAF1 and TRAF2 and serpin SPI2a [38]. Several of these proteins, including Bcl-2 family members and

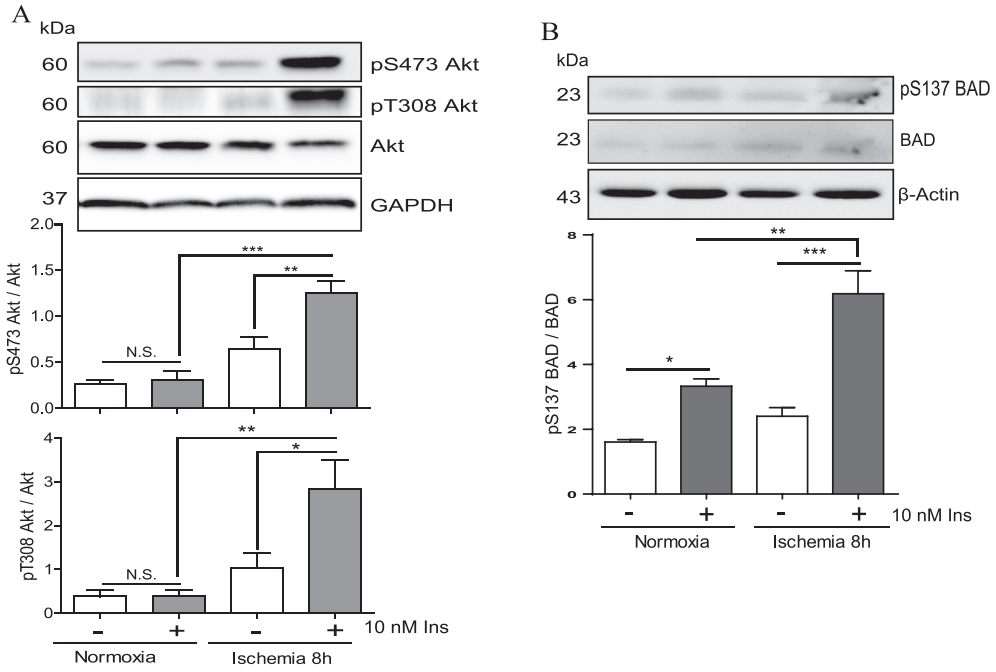

Fig. 3. Insulin activates Akt in ischemic cardiomyocytes. Cardiomyocytes were submitted to simulated ischemia for 8 h in absence or presence of 10 nM insulin (Ins). **Panel A**, protein levels of phospho-S473 Akt, phospho-T308 Akt and total Akt were measured by Western blot. **Panel B**, protein levels of phospho-S137 BAD were determined by Western blot. Values are mean ± SEM. N:3–5 independent experiments. Data were analyzed by two-way ANOVA and Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. N.S. no significant changes.

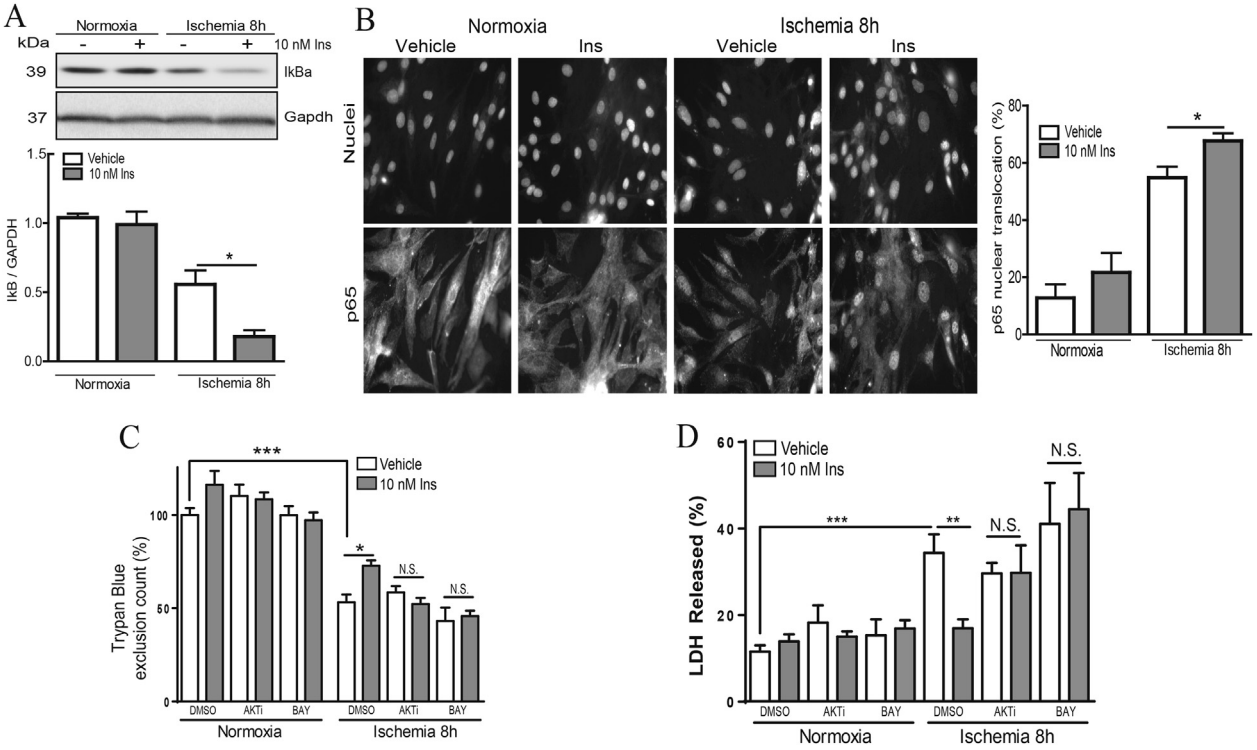

Fig. 4. Insulin protects against ischemia-induced cell death through Akt and NFκB. Cardiomyocytes were submitted to ischemia for 8 h in the absence or presence of 10 nM insulin (Ins). **Panel A**, IκBα levels were measured by Western blot. **Panel B**, p65 nuclear translocation was measured by immunofluorescence. **Panel C-D**, Akti VIII (Akti, 0.1 uM) and BAY11-7082 (BAY, 0.05 uM) inhibitors were pre-incubated 30 min before ischemia, then cell viability and LDH released were measured as described before. Values are mean ± SEM. N: 3–5 independent experiments. Data were analyzed by two-way ANOVA and Tukey's multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. N.S. no significant changes.

serpin SPI2a, have been directly associated with the prevention of necrosis [26,39]. Therefore, insulin dependent NFκB activation could be considered as a mechanism to decrease necrosis during ischemia.

Because insulin reduced cardiomyocyte necrosis during ischemia, insulin could be used as a cardioprotective agent in organ preservation solutions during transplantation. Several organ preservation solutions have been used in heart transplantation, including University of Wisconsin, histidine-tryptophane-ketoglutarate, St. Thomas' Hospital, crystalloid cardioplegia and blood [40–42]. Insulin has been added to some of these solutions to maintain a steady state of metabolism of the donor heart *ex vivo* [43,44]. None of these works evaluated insulin as a cardioprotective agent. However, protective effect of insulin seems to be tissue specific, since in liver transplant, insulin in University of Wisconsin solution exacerbates the ischemic injury and decreases the graft survival rate [45]. Therefore, future research should clarify the contribution of insulin as cardioprotective agent in organ preservation solutions.

In conclusion, this study shows that insulin decreased necrosis in cardiomyocytes during ischemia through an Akt/NFκB pathway. These novel findings thus clarify the role of insulin during ischemia, joining the large body of literature supporting the use of insulin in GIK perfusion to treat myocardial infarction.

Acknowledgments

This work was supported by CONICYT: 21110381 (AD), 21120406 (to CH), FONDECYT 1110346 (LG), FONDAP 15130011 (LG, MC).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.171.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.09.171.

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