

Insulin resistance affects the cytoprotective effect of insulin in cardiomyocytes through an impairment of MAPK phosphatase-1 expression

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Abstract

Objective: Insulin protects cardiomyocytes from apoptosis. Insulin resistance usually refers to a defect in the ability of insulin to stimulate glucose uptake. It is unknown, however, whether or not insulin resistance compromises the cell-protective effect of the hormone. Caspases are a family of cysteine proteases that regulate apoptosis. We explored the effects of insulin resistance on hypoxia-induced caspase-3 activation in cardiomyocytes.

Methods: Experiments were performed in cultured neonatal rat cardiomyocytes. Insulin resistance was induced by treating cardiac myocytes with isoproterenol, a β -adrenergic receptor agonist.

Results: Twelve hours of hypoxia-induced caspase-3 cleavage, which was inhibited by treatment with insulin, while pre-treatment with isoproterenol abolished the insulin effect. Hypoxia-induced cleavage of caspase-3 was mediated by p38 mitogen-activated protein kinase (MAPK). Insulin inhibited hypoxia-induced phosphorylation of p38 through MAPK phosphatase-1 (MKP-1). Insulin-induced MKP-1 expression was mediated by extracellular signal-regulated protein kinases (ERK) 1/2, c-Jun NH2-terminal kinases (JNK) MAPK, and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. Isoproterenol stimulation failed to induce expression of MKP-1; moreover, insulin resistance induced by long-term β -adrenergic stimulation inhibited insulin-evoked expression of MKP-1 by impairing insulin-induced phosphorylation of both ERK1/2 and JNK without affecting Akt kinase activity. Furthermore, concomitant activation of Akt, ERK 1/2, and JNK was required for insulin to exert its protective effect against the hypoxia-induced cleavage of caspase-3.

Conclusions: The results of this study lead to the conclusions that, in cardiac myocytes, antiapoptotic signals induced by insulin are mediated by more than one signaling pathway, and that long-term β -adrenergic receptor stimulation impairing some of these pathways affects the cytoprotective action of insulin.

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1. Introduction

Insulin resistance is characterized by the reduced ability of insulin to stimulate the uptake and disposal of glucose by

muscle [1], and is a common feature of diabetes mellitus [2], hypertension [3], obesity [4], and metabolic syndrome [5]. Insulin resistance is accompanied by an impairment of cardiac function and observed in heart failure and dilated cardiomyopathy [6]. It is noteworthy that some of these conditions are characterized by dysregulation of the sympathetic nervous system [7–9], resulting in enhanced stimulation of adrenergic receptors (ARs). However, in the heart, pathophysiological consequences of the cross-talk

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between the sympathetic nervous system and the insulin sensitivity remain unclear.

Insulin is a pleiotropic hormone and regulates several biological processes. In particular, insulin protects cardiac myocytes from oxidative stress-induced apoptosis through a phosphatidylinositol 3 kinase (PI3K)/Akt-dependent pathway [10]. Since loss of cardiomyocytes by apoptosis plays a pivotal role in the pathogenesis of left ventricular dysfunction [11], elucidating whether insulin resistance impairs the cytoprotective effect of insulin is crucial for better understanding of the mechanism mediating the development of left ventricular dysfunction in patients with insulin resistance [12].

In cardiac myocytes, ligand binding of catecholamines to β ARs induces apoptosis [13], as well as insulin resistance [14]. Interestingly, both β ARs and the insulin receptor share the PI3K/Akt pathway which exerts an antiapoptotic effect, suggesting that additional mechanisms are required for insulin to exert its protective effect. The MAPK superfamily plays an important role in the regulation of apoptosis [15]. Recent evidence indicates that the family of enzymes known as MAPK phosphatases (MKPs), which inactivate MAPKs via dephosphorylation, is involved in the regulation of apoptosis. Therefore, we hypothesized that the differential regulation of MAPK and MKPs by insulin and β AR stimulation may account for the cytoprotective action of insulin.

This study was performed in order to: 1) explore whether the condition of insulin resistance induced by β AR stimulation affects the cytoprotective effect of insulin; 2) better clarify the molecular mechanism, which is involved in the cytoprotective effect of insulin but is eventually compromised by β AR stimulation-induced insulin resistance.

2. Methods

2.1. Primary cultures of neonatal rat ventricular cardiomyocytes

The procedures involving animals were performed with the approval of the animal reviewer board of our Institution. Primary cultures of neonatal cardiomyocytes were prepared as described [16]. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Cells were cultured in the cardiac myocyte culture medium containing Dulbecco's

modified Eagle's medium/Ham's F-12 supplemented with 5% horse serum, 4 μ g/mL transferrin, 0.7 ng/mL sodium selenite, 2 g/L bovine serum albumin (fraction V), 3 mmol/L pyruvic acid, 15 mmol/L HEPES, 100 μ mol/L ascorbic acid, 100 μ g/mL ampicillin, 5 μ g/mL linoleic acid, and 100 μ mol/L 5-bromo-2'-deoxyuridine.

The cells, after overnight starvation, were exposed to hypoxia. Hypoxia was induced in a medium saturated previously for 10 min at 1 atm with 95% N₂ and 5% CO₂, and containing (mM) concentrations of 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 26.2 NaHCO₃, 1 NaH₂PO₄, 1.8 CaCl₂, and 0.01 glycine and 0.001 (% w/v) phenol red using a hypoxia chamber (temperature: 37 °C, atmosphere: 5% CO₂ and 95% N₂) [17]. The pH, PO₂ and PCO₂ of the medium resulted to be 7.36 \pm 0.2, 45.3 \pm 1.2 mm Hg, 35.3 \pm 0.8 mm Hg, and 7.32 \pm 0.9, 32.6 \pm 1.1 and 37.9 \pm 2.1 mm Hg, before and at the end of hypoxia, respectively.

2.2. Immunoblotting

For detection of cleaved caspase-3, cells were lysed with 100 μ L of ice-cold lysis buffer A (see Supplementary data). For phospho-ERK 1/2, phospho-JNK, phospho-p38, and MKP-1 detection, cells were lysed with 100 μ L of ice-cold lysis buffer B (see Supplementary data). Cleavage of caspase-3, phosphorylation of ERK 1/2, JNK, and p38 MAPK, expression of MKP-1 were detected by immunoblot analyses, using specific primary antibodies.

2.3. Akt kinase activity assay

Kinase activity of Akt was measured by the immune complex kinase assay as we have previously described [18].

2.4. Glucose uptake assays

Cardiomyocytes were grown in twelve-well plates (0.5 \times 10⁶ cells/well). 2-Deoxyglucose (2DG) uptake was determined as described [14].

2.5. Adenovirus transduction

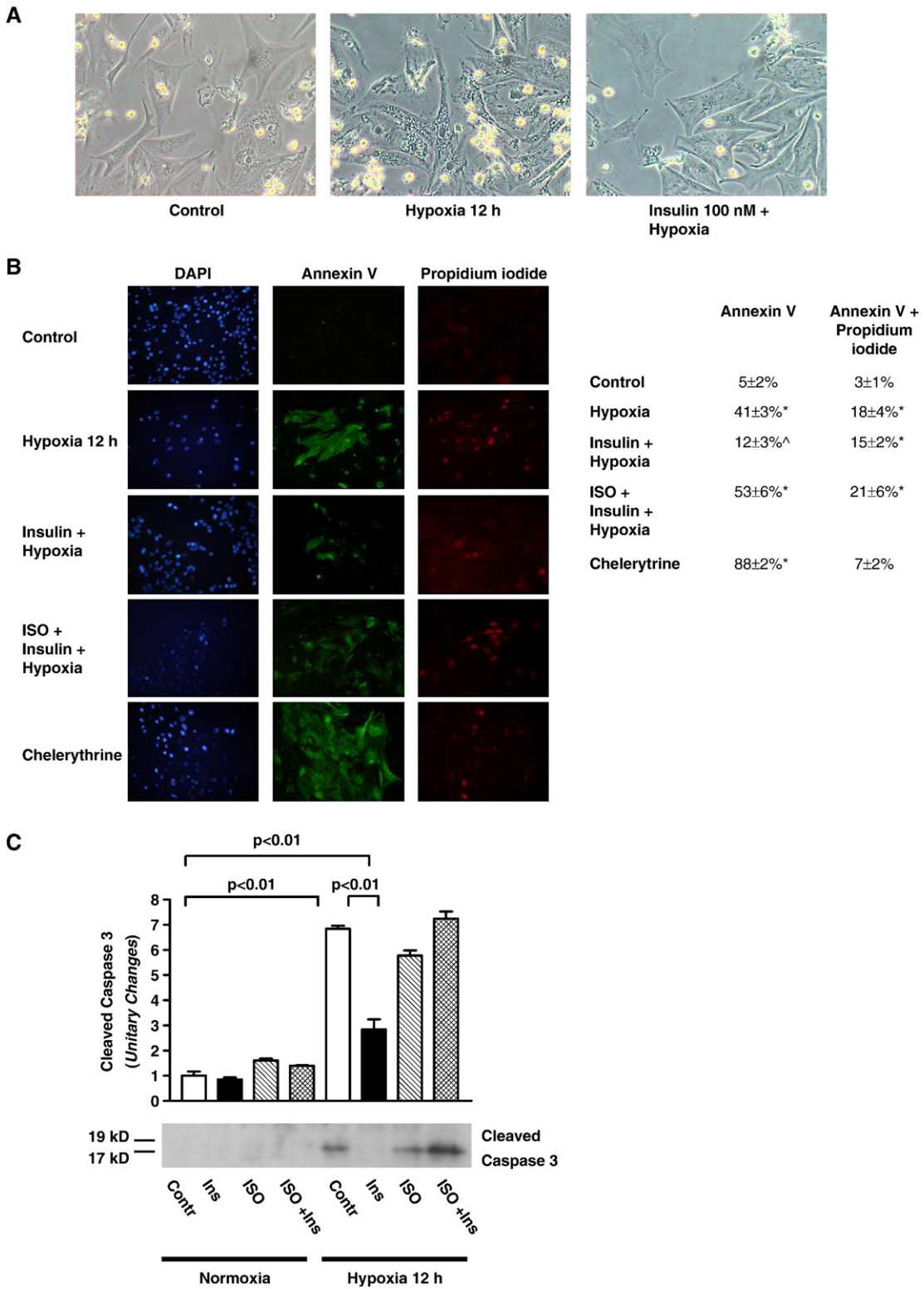
Adenovirus harboring dominant negative Akt (Ad5 CMV Akt (K179M)) was obtained from Professor G. Condorelli

Fig. 1. A) Morphological changes in cardiac myocytes induced by hypoxia, obtained by phase-contrast microscopy. Twelve hours of hypoxia induced the characteristic morphological changes of apoptosis. Many dead cells were also observed (middle panel). Insulin treatment completely prevented the occurrence of these morphological changes (right panel). B) Insulin reduces hypoxia-induced apoptosis. Cardiac myocytes were incubated with serum-free medium overnight and then exposed to hypoxia for 12 h in the presence or absence of insulin (100 nM). Insulin stimulation was conducted in the presence and absence of isoproterenol (ISO, 10 μ M, 2 h before insulin exposure). Cells were stained with annexin-V-fluorescein (green), and propidium iodide (PI) (red), and counterstained by DAPI (blue) (left). The percentage of apoptotic and necrotic cells was calculated by dividing the number annexin-V-positive/PI-negative cells and annexin-V-positive/PI-positive cells with the total number of nuclei detected with DAPI staining, respectively (right). Stimulation (30 min) with chelerythrine (6 μ M) was used as positive control for apoptosis. Values represent the mean \pm SEM of four culture preparations. * p <0.05 vs control, ^ p <0.05 vs hypoxia. C) Insulin reduces hypoxia-induced (12 h) caspase-3 cleavage. Cleavage of caspase-3 was assessed by immunoblot analysis. Treatment with Ins (100 nM) reduced, while ISO (10 μ M) did not affect the amount of hypoxia-induced caspase-3 cleavage. A representative immunoblot is shown. Bar graph represents densitometric analysis, mean \pm SEM, expressed as fold increase in protein cleavage over that in unstimulated cells, of four independent experiments performed in triplicate.

(University of California, San Diego, USA). Adenovirus harboring lacZ (Ad5-CMV-β-galactosidase) was used as a control. The method of adenovirus infection has been described [19].

2.6. Annexin-V staining

Cardiac myocytes growth on coverslips were stained with Annexin-V-fluorescein+propidium iodide (Annexin-V-



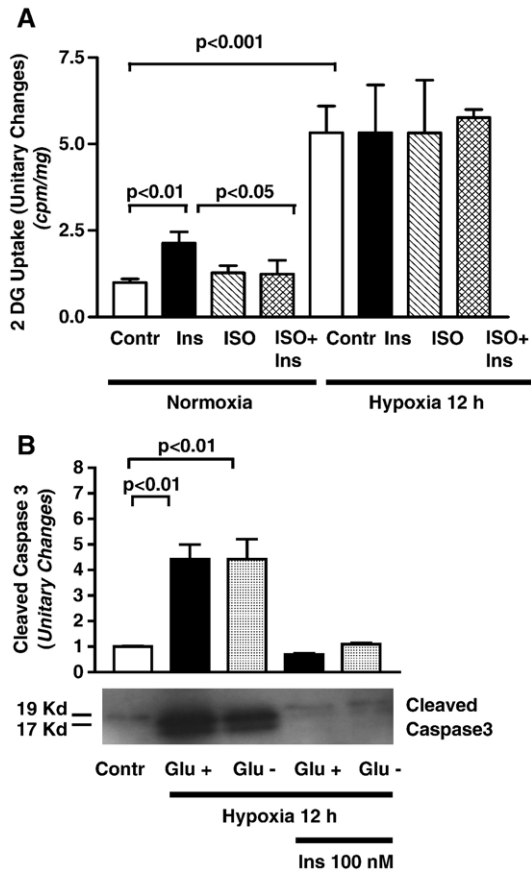


Fig. 2. A) Hypoxia stimulates 2-Deoxyglucose (2 DG) uptake and abolishes the ability of insulin to promote 2 DG uptake. Cardiac myocytes were stimulated with insulin (Ins, 100 nM, 30 min), isoproterenol (ISO, 10 μ M, 2 h) and ISO (2 h)+Ins (30 min) during normoxia and hypoxia (12 h). The values of 2 DG uptake (cpm) in each experiment were adjusted by the protein content of the dish (μ g). The graph shows the mean \pm SEM of four independent experiments performed in triplicate. B) Cardiac myocytes were exposed to hypoxia in media with (Glu +) and without glucose (Glu -), in the presence or absence of insulin (Ins, 100 nM). Cleavage of caspase-3 was assessed by immunoblot analysis. A representative immunoblot is shown. Bar graph represents densitometric analysis, mean \pm SEM expressed as fold increase in protein cleavage over that in unstimulated cells, of three independent experiments.

FLUOS Staining Kit, Roche) according to the instructions of the manufacturer. Cells undergoing early apoptosis were stained with Annexin V, but not with propidium iodide (PI), while necrotic cells or cells in late apoptosis stages were stained with both annexin-V and PI.

2.7. Cardiac myocytes morphological analysis

Cell morphology was examined by phase-contrast microscopy as previously reported [20].

2.8. Statistics

Data are given as mean \pm SEM. Statistical analyses were performed using the analysis of variance. The post-test com-

parison was performed by the method of Tukey. Significance was accepted at $p < 0.05$ levels.

3. Results

3.1. Hypoxia induces morphological changes and cleavage of caspase-3 in cardiac myocytes with insulin resistance

Cardiac myocytes were subjected to hypoxia for 12 h in the presence or absence of insulin. Hypoxia-induced characteristic morphological changes of apoptosis, such as pyknosis, vacuolization, and cell shrinkage. Furthermore, many round, floating cells, and few remaining adherent cells were detected (Fig. 1A). Morphology of myocytes treated with insulin before hypoxia was quite similar to that detected in control conditions. Hypoxia significantly increased the percentage of both annexin-V positive/PI-negative and annexin-V positive/PI-positive cardiac myocytes (Fig. 1B). Insulin treatment before hypoxia significantly reduced the rate annexin-V positive/PI-negative, while unaffected the rate of annexin-V positive/PI-positive cells. To determine whether or not the condition of insulin resistance compromises the cytoprotective of insulin on hypoxia-induced cell death, cardiac myocytes were stimulated 2 h before insulin exposure with isoproterenol (ISO, 10 μ M), a β AR agonist. ISO stimulation abolished the ability of insulin to reduce the percentage of hypoxia-induced annexin-V positive/PI-negative cells.

Hypoxia-induced myocyte death was associated with cleavage of caspase-3. Immunoblot analyses showed that, in control conditions cleaved caspase-3 was undetectable, while hypoxia enhanced the amount of cleaved caspase-3 by 7 fold, and insulin treatment reduced hypoxia-induced caspase-3 cleavage (Fig. 1C).

To determine whether or not the condition of insulin resistance compromises the protective effect of insulin on hypoxia-induced cleavage of caspases, cardiac myocytes were stimulated 2 h before insulin exposure with ISO (10 μ M). During normoxia, cardiac myocytes stimulated with ISO did not show increases in the amount of cleaved caspase-3, while the exposure of cells to ISO and insulin followed by hypoxia dramatically increased the amount of cleaved caspase-3 (Fig. 1C).

These results show that in neonatal cardiac myocytes hypoxia induces morphological and biochemical changes consistent with both apoptosis and necrosis, that insulin prevents the occurrence of apoptosis, and that β AR stimulation-induced insulin resistance inhibits the protective effect of insulin on hypoxia-induced apoptosis.

3.2. Glucose is not required for the protective effect exerted by insulin

Since glucose uptake reduces hypoxia-induced apoptosis in cardiac myocytes [21] we hypothesized that the absence of insulin-evoked reduction of hypoxia-induced caspase-3

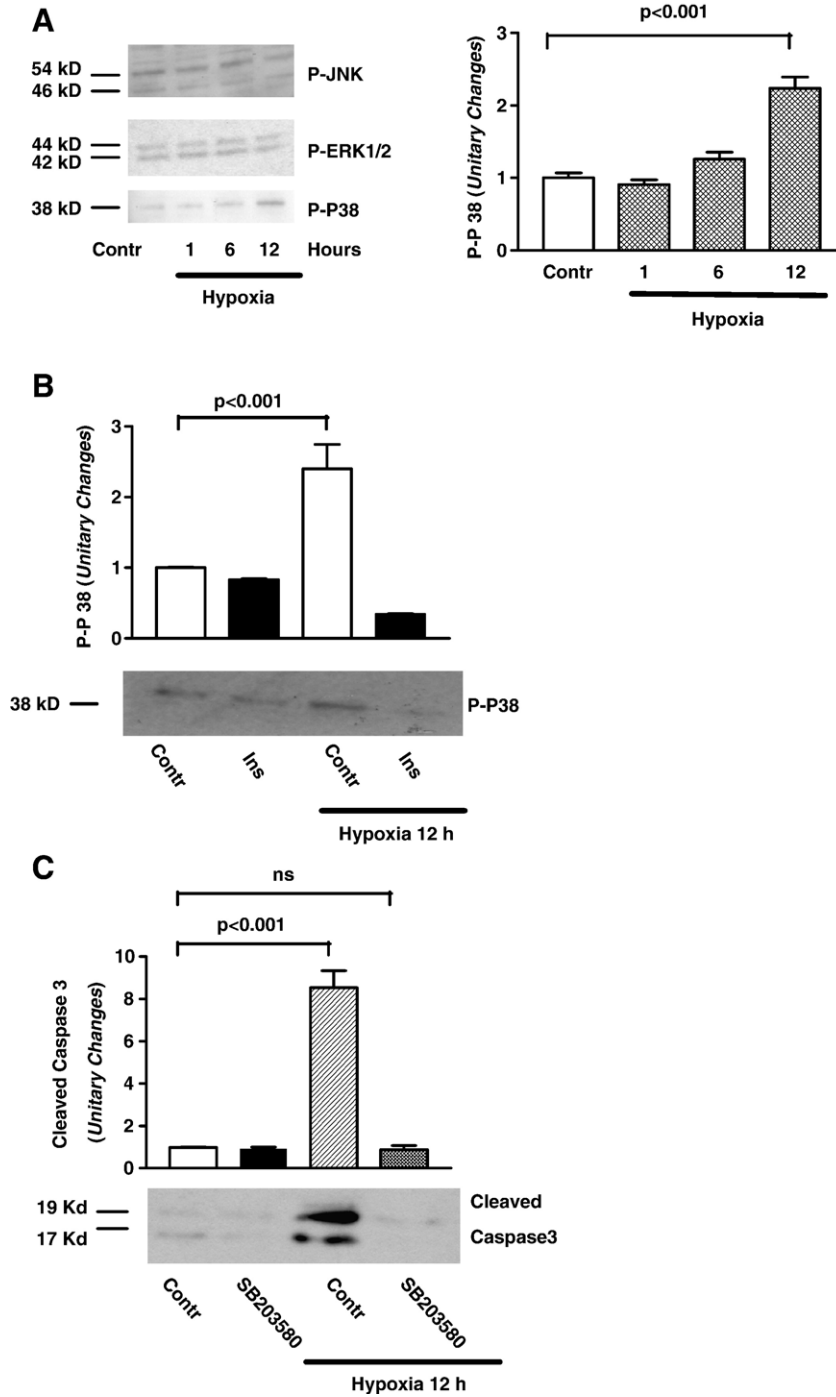


Fig. 3. A) Hypoxia selectively induces the phosphorylation of p38 MAPK. Cardiac myocytes were exposed to hypoxia for the indicated times. Phosphorylation of ERK 1/2, JNK and p38 MAPK was detected by immunoblotting. B) Insulin inhibits hypoxia-induced phosphorylation of p38 MAPK. Cardiac myocytes were exposed to hypoxia in the presence or absence of insulin (Ins, 100 nM). Phosphorylation of p38 MAPK was detected by immunoblotting. C) Cardiac myocytes were exposed to hypoxia in the presence or absence of an inhibitor of p38 MAPK, SB203580 (10 μ M, 60 min before insulin exposure). Cleavage of caspase-3 was assessed by immunoblot analysis. Representative immunoblots of four independent experiments are shown. Bar graph represents densitometric analysis, means \pm SEM expressed as fold increase in phosphorylation or cleavage over that in unstimulated cells.

cleavage during β AR-induced insulin resistance is due to an impairment of insulin-stimulated glucose uptake. Therefore, we measured insulin-stimulated 2-Deoxyglucose (2DG) uptake during normoxia and hypoxia in the presence or

absence of β AR stimulation. During normoxic conditions, as expected, insulin increased 2DG uptake by 80% ($p < 0.01$, vs control), and 2 h of ISO stimulation inhibited this response (Fig. 2A). Twelve hours of hypoxia increased 2DG

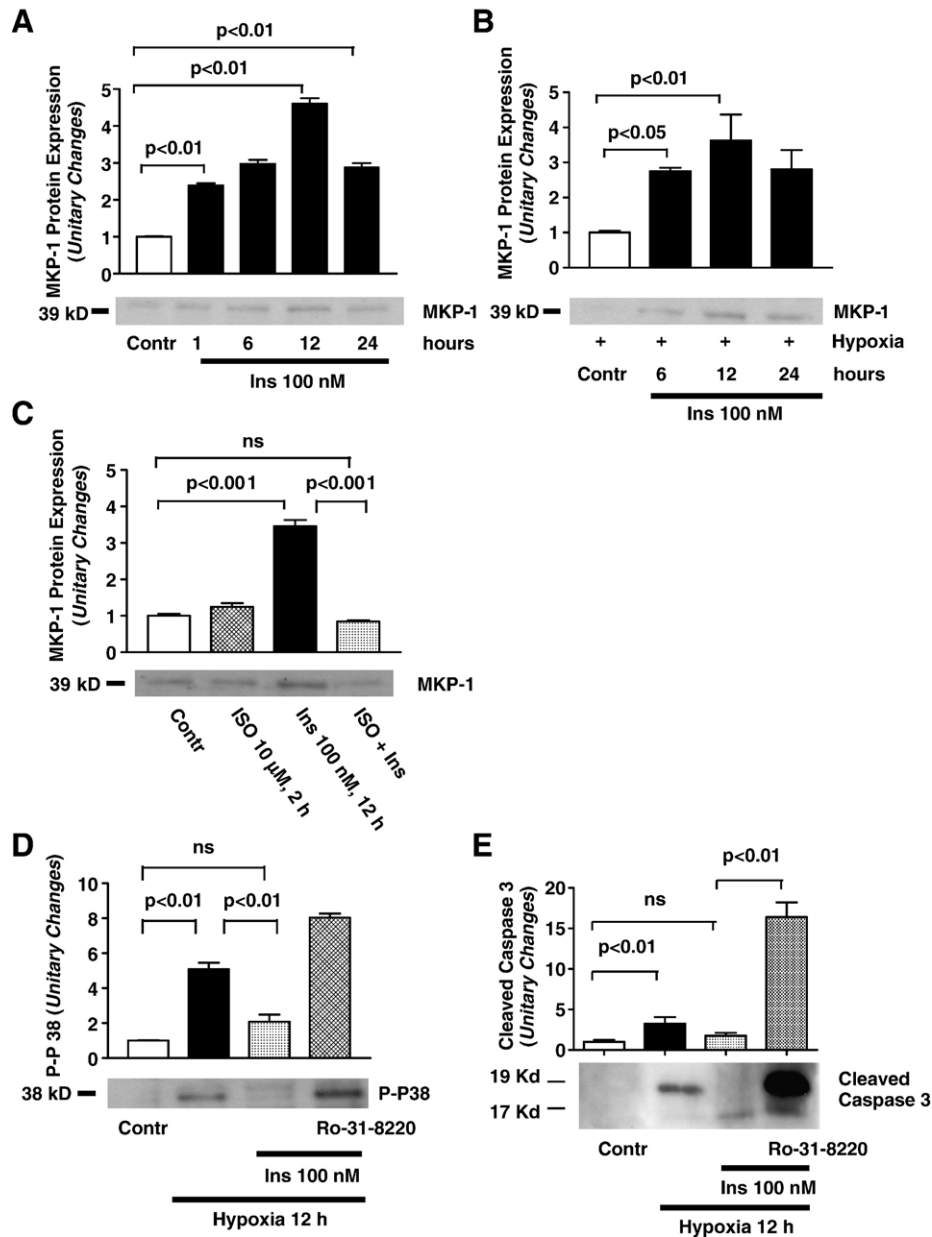


Fig. 4. A) Insulin time-dependently stimulates MKP-1 protein expression. Cells were exposed to insulin (Ins) for the times indicated. MKP-1 protein expression was assessed by immunoblot analysis using anti-MKP-1 antibody. B) Hypoxia does not affect insulin-induced MKP-1 protein expression. Cells were stimulated with Ins and exposed to hypoxia for the indicated times. MKP-1 protein expression was assessed by immunoblot analysis. C) β AR stimulation-induced insulin resistance inhibits insulin-evoked MKP-1 protein expression. Insulin resistance was induced by pre-treating cardiomyocytes with isoproterenol (ISO) 2 h before insulin exposure. MKP-1 protein expression was assessed by immunoblot analysis. D) MKP-1 accounts for insulin-induced p38 MAPK dephosphorylation. Cardiac myocytes were exposed to hypoxia in the absence or presence of insulin, and Ro-31-8220 (5 μ M), an inhibitor of MKP-1. E) MKP-1 is involved in insulin inhibition of hypoxia-induced caspase-3 cleavage. Cardiac myocytes were exposed to hypoxia in the absence or presence of insulin, and Ro-31-8220. Representative immunoblots of four independent experiments are shown. Bar graphs represent densitometric analysis, mean \pm SEM, expressed as fold increase in protein expression or phosphorylation or cleavage over that in unstimulated cells.

uptake (530%, $p < 0.001$ vs control normoxia) and compromised insulin-stimulated 2DG (538%, p : NS vs control hypoxia) (Fig. 2A). Furthermore, ISO stimulation in the presence or absence of insulin did not affect 2DG uptake compared to control conditions. These results show that hypoxia dramatically increases glucose uptake and blunts the effects of hormonal stimulation. Next, we examined whether

or not glucose is required for the protective effect of insulin on hypoxia-induced cleavage of caspases. For this purpose, cardiac myocytes were exposed to hypoxia in the presence or absence of glucose (5.5 mM) in the culture media. Hypoxia induced the cleavage of caspase-3, and insulin treatment prevented this response independently from the presence of glucose in the culture media (Fig. 2B).

3.3. Hypoxia-induced caspase-3 cleavage is mediated by phosphorylation of p38 MAPK

Since MAPK mediates cardiomyocyte apoptosis during ischemia and/or reperfusion [22], we determined which MAPK

mediates hypoxia-induced caspase-3 cleavage, and then whether insulin affects the molecular pathway that accounts for hypoxia-induced caspase-3 cleavage. Hypoxia did not affect the phosphorylation of either ERK or JNK, but, did induce phosphorylation of p38 MAPK (Fig. 3A), which was inhibited by insulin (Fig. 3B). To determine whether activation of p38 MAPK accounts for hypoxia-induced cleavage of caspase-3, cardiomyocytes were pre-treated with SB203580 (10 μ M), an inhibitor of p38 MAPK. Hypoxia-induced cleavage of caspase-3 was abolished by treatment with SB203580 (Fig. 3C), suggesting that p38 MAPK plays a critical role in mediating hypoxia-induced activation of caspase-3.

3.4. Hypoxia-induced phosphorylation of p38 MAPK is inhibited by insulin through a MKP-1 dependent pathway

MKP-1, a member of a family of enzymes known as MAPK phosphatases, plays a critical role in the negative regulation of p38 MAPK and promotes cell survival [23]. Therefore, we examined whether or not MKP-1 accounts for insulin inhibition of hypoxia-induced phosphorylation of p38 MAPK. Insulin time-dependently induced protein expression of MKP-1, which was first evident after 1 h of stimulation and peaked after 12 h (Fig. 4A). Insulin-induced expression of MKP-1 was also detectable during hypoxia (Fig. 4B), while pre-treatment with ISO abolished insulin-induced expression of MKP-1 (Fig. 4C). To verify whether MKP-1 accounts for insulin-induced inhibition of hypoxia-evoked phosphorylation of p38 MAPK, cardiomyocytes were pre-treated with Ro-31-8220 (5 μ M), an inhibitor of MKP-1, and then stimulated with insulin. Ro-31-8220 abolished inhibition of hypoxia-evoked phosphorylation of p38 induced by insulin (Fig. 4D), suggesting that MKP-1 is critically involved in insulin-induced inactivation of p38 MAPK. Then, we examined whether MKP-1 accounts for insulin-induced inhibition of hypoxia-evoked caspase-3 cleavage. Pre-treatment with Ro-31-8220 (5 μ M) abolished inhibition of hypoxia-evoked caspase-3 cleavage induced by

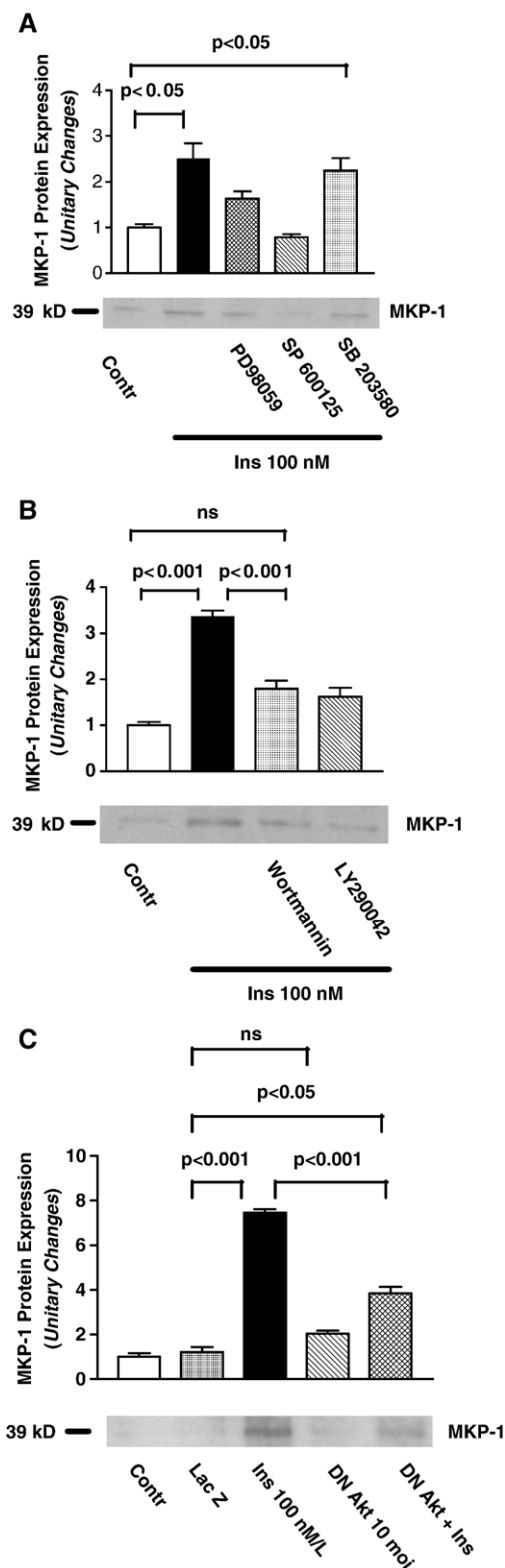


Fig. 5. A) Insulin-induced MKP-1 protein expression is an ERK1/2- and JNK-dependent phenomenon. Cardiac myocytes were stimulated with insulin (Ins, 12 h) in the absence or presence of ERK 1/2 inhibitor, PD98059 (20 μ M, 60 min before Ins exposure), JNK inhibitor, SP600125 (10 μ M, 60 min before Ins exposure) and p38 inhibitor, SB203580 (10 μ M, 60 min before Ins exposure). MKP-1 protein expression was assessed by immunoblot analysis. B) Insulin-induced MKP-1 protein expression also requires a PI3K-dependent pathway. Cardiac myocytes were stimulated with Ins in the absence or presence of 2 different inhibitors of PI3K, wortmannin (10 nM, 30 min before Ins exposure) and LY290042 (10 μ M; 30 min before Ins exposure). MKP-1 protein expression was assessed by immunoblot analysis. C) Akt is required for insulin-induced MKP-1 protein expression. Cardiac myocytes were infected with adenovirus harboring a dominant negative mutant of Akt (DN Akt, 10 MOI) or Lac Z, further cultured for 48 h, and then stimulated with Ins for 12 h. MKP-1 protein expression was assessed by immunoblot analysis. Representative immunoblots of three independent experiments are shown. Bar graphs represent densitometric analysis, mean \pm SEM, expressed as fold increases in protein expression over that in unstimulated cells.

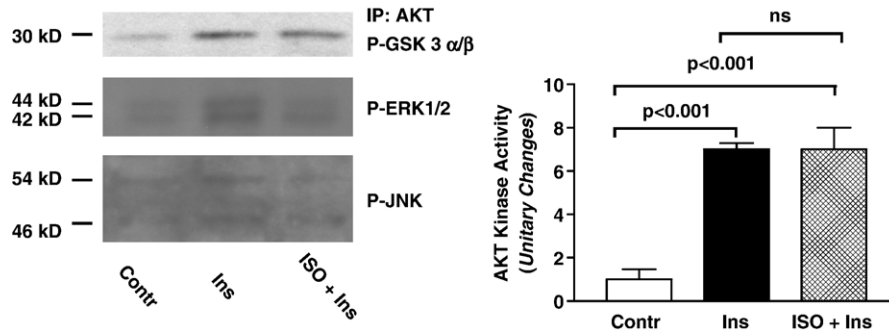


Fig. 6. Long-term β AR stimulation does not inhibit insulin-stimulated Akt kinase activity (top). Cardiac myocytes were pre-treated with or without ISO for 2 h and then stimulated with Ins for 20 min. The kinase activity of Akt was determined by the immune complex kinase assay using paramyosin-GSK3 α (peptide 7-25) fusion protein as a substrate. GSK3 α (peptide 7-25) phosphorylated at Ser-21 was quantified by immunoblot analysis using the anti-phospho GSK3 α/β (Ser-21/Ser-9) antibody. IP indicates immunoprecipitation. Long-term β AR stimulation inhibits insulin-induced ERK 1/2 phosphorylation (middle). Cardiac myocytes were pre-treated with ISO for 2 h and then stimulated with Ins for 10 min. Phosphorylation of ERK 1/2 was assessed by immunoblot analysis using anti-phospho-ERK 1/2 antibody. Long-term β AR stimulation time-dependently inhibits insulin-induced JNK phosphorylation (bottom). Cardiac myocytes were pre-treated with ISO for 2 h before Ins stimulation (30 min). Phosphorylation of JNK was assessed by immunoblot analysis using anti-phospho-JNK antibody. Representative immunoblots of four independent experiments are shown. Bar graph represents densitometric analysis, mean \pm SEM, expressed as fold increase in phosphorylation over that in unstimulated cells.

insulin, suggesting that MKP-1 is critically involved in cytoprotective effect of insulin (Fig. 4E).

3.5. Insulin-induced MKP-1 expression requires both ERK and JNK MAPK, and PI3K/Akt pathways

We examined how insulin regulates MKP-1 protein expression. MKP-1 induction is generally regulated by MAPK family members [24], as well as by PI3K [25]. Therefore, to evaluate the potential contribution of MAPK and PI3K signaling pathways to insulin-mediated MKP-1 induction, myocytes were pre-treated with PD98059, a specific MEK inhibitor, SP600125, an inhibitor of JNK, SB203580, a specific inhibitor of p38 MAPK, wortmannin and LY290042, two different inhibitors of PI3K, followed by insulin exposure. Pre-treatment with PD98059, SP600125, wortmannin or LY290042 completely blocked insulin-induced expression of MKP-1 expression (Fig. 5A and B, respectively). In contrast, SB203580, did not affect insulin-induced MKP-1 expression.

Akt is a serine/threonine kinase that regulates several PI3K-dependent transcriptional activities. We examined the role of Akt in mediating insulin-induced MKP-1 protein expression. Myocytes were transduced with adenoviruses harboring dominant negative (DN) Akt (10 MOI). DN Akt significantly reduced insulin-induced expression of MKP-1, suggesting that Akt is required for MKP-1 expression (Fig. 5C).

Collectively, insulin regulates MKP-1 expression through a complex pathway which includes both ERKs and JNK MAP kinases, and the PI3K/Akt pathway.

3.6. β AR stimulation-induced insulin resistance compromises insulin-evoked MKP-1 expression by inhibiting ERK1/2 and JNK phosphorylation

Since MKP-1 is involved in the cytoprotective action of insulin, we determined whether β AR-induced insulin

resistance compromises insulin-evoked MKP-1 protein expression. First, β AR stimulation failed to induce expression of MKP-1 (data not shown). On the other hand, insulin resistance induced by long-term stimulation of β AR inhibited insulin-evoked expression of MKP-1 (Fig. 4C). Next, we explored the molecular pathway that accounts for impaired expression of MKP-1 during β AR-induced insulin resistance. In cardiac myocytes, insulin-induced MKP-1 expression is regulated by both ERK and JNK MAPK, and by the PI3K-dependent pathway. Therefore, we examined whether β AR-induced insulin resistance affects insulin signaling involved in the expression of MKP-1. Long-term stimulation (120 min) with ISO inhibited insulin-induced phosphorylation of both ERK 1/2 and JNK, but did not affect the insulin-evoked kinase activity of Akt (Fig. 6). These data indicate that β AR-stimulation-induced insulin resistance compromises an arm of the molecular pathway that is involved in the expression of insulin-induced MKP-1.

3.1. Akt and phosphorylation of ERK1/2 and JNK are required for the protective effect of insulin

Since the protective action of insulin against hypoxia-induced caspase-3 cleavage is mediated by MKP-1, whose expression is regulated by PI3K/Akt-, ERK1/2-, and JNK-dependent mechanisms, we asked if these three components involved in insulin-induced MKP-1 expression are also required for the protective action of insulin. Inhibition of Akt activity was obtained by overexpression of a dominant negative mutant of Akt, while ERK 1/2 and JNK were inhibited by PD98059 and SP600125, respectively. Adenovirus-mediated expression of the dominant negative mutant of Akt abolished the effect of insulin on hypoxia-induced caspase-3 cleavage (Fig. 7A). Similarly, pre-treatment of cardiac myocytes with either PD98059 or SP600125 abolished the effect of insulin on hypoxia-induced caspase-3

cleavage (Fig. 7B and C). These results indicate that insulin requires simultaneous activation of Akt, ERK 1/2 and JNK to exert its protective effect against the hypoxia-induced cleavage of caspase-3.

4. Discussion

Insulin resistance is a common feature of several diseases which increase the cardiovascular risk, and, at the same time, worsen the prognosis of ischemic heart disease. Several

pathogenic mechanisms account for the worsening of the cardiovascular prognosis during insulin resistance. However, it is still unknown whether the condition of insulin resistance compromises the cytoprotective action of insulin. In this study we demonstrated that in neonatal cardiac myocytes: 1) insulin prevents hypoxia-evoked caspase-3 cleavage, 2) the cytoprotective action of insulin on hypoxia-induced caspase-3 cleavage requires MKP-1, 3) insulin-induced MKP-1 expression is mediated by ERK 1/2 and JNK MAPK and PI3K/Akt pathways; 4) β AR stimulation-induced insulin resistance compromises the cytoprotective action of insulin by impairing ERK 1/2 and JNK phosphorylation.

Caspases play a key role in the regulation of apoptosis, and caspase-3 is known to be an important molecule in the cellular suicide cascade [26]. Hypoxia is one of the components of ischemia and previous reports have documented apoptosis in cardiac myocytes subjected to hypoxia [27,28]. Although in some cell types mild hypoxia can induce cellular phenotype more resistant to acute metabolic stress [29] and then, mimic the effect of cytoprotective agents, here we found that treatment with insulin prevents cleavage of caspase-3 evoked by a severe hypoxia. This observation is not trivial, since activation of caspase-3 also impairs cardiac contractility by cleaving myofibrillar proteins and decreasing the myofibrillar ATPase activity [30]. Thus, the protective effect of insulin on hypoxia-induced caspase-3 cleavage could be not limited to just the reduction of apoptosis but also include the preservation of cardiac contractility. In contrast, the condition of insulin resistance, by impairing the protective effect of insulin on caspase-3, can worsen the cellular response to hypoxia by enhancing the degree of apoptosis and by inducing contractile dysfunction.

Several mechanisms can account for the antiapoptotic action of insulin. For instance, glucose uptake and glycolysis protect cardiac myocytes from hypoxia-induced apoptosis [21]. Our results indicate that insulin exerts its protective action during hypoxia independently of glucose uptake, since the insulin-mediated reduction of hypoxia-induced caspase-3 cleavage was also obtained in the absence of glucose in culture

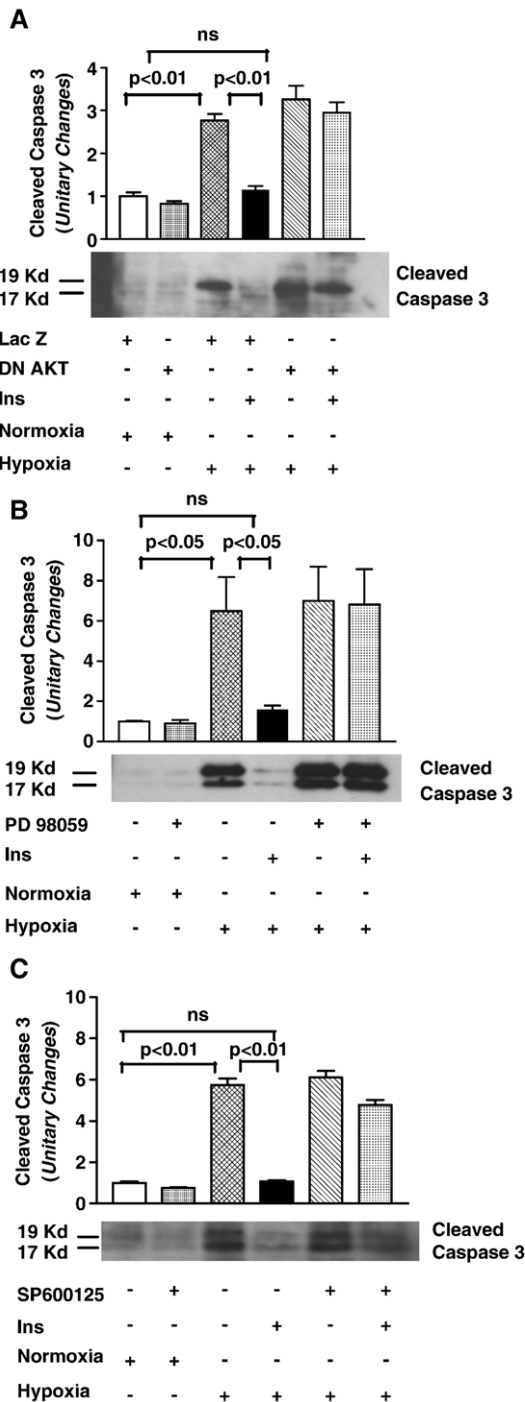


Fig. 7. A) Overexpression of a dominant negative mutant of Akt compromises the effect of insulin on hypoxia-induced cleavage of caspase-3. Twenty-four hours after plating, myocytes were infected with adenovirus harboring a dominant negative mutant of Akt (DN Akt), 10 MOI or Lac Z, and further cultured for 48 h, then were exposed to hypoxia (12 h) in the presence or absence of insulin (Ins, 100 nM). B) ERK 1/2 inhibition compromises the effect of insulin on hypoxia-induced caspase-3 cleavage. Cardiac myocytes were exposed to hypoxia in the presence or absence of insulin. Inhibition of ERK 1/2 was obtained by pre-treating cells with PD98059 (20 μ M). C) JNK inhibition compromises the effect of insulin on hypoxia-induced caspase-3 cleavage. Cardiac myocytes were exposed to hypoxia in the presence or absence of insulin. Inhibition of JNK was obtained by pre-treating cells with SP600125 (10 μ M). Cleavage of caspase-3 was assayed by immunoblot analysis. Representative immunoblots of five independent experiments are shown. Bar graphs represent densitometric analysis, mean \pm SEM expressed as fold increase in protein cleavage over that in unstimulated cells.

media. Our results do not rule out the possibility that glucose uptake and metabolism are protective for ischemic myocardium but indicate that the protective action of insulin is a multifactorial and complex process due to the activation of different antiapoptotic pathways, including insulin-induced glycogen and ATP sparing, cross-activation of insulin-like growth factor 1, *bad* phosphorylation. In this regard, insulin protects neonatal cardiac myocytes from oxidative stress-induced apoptosis through two different pathways, PI3 Kinase/Akt and ERKs, which are independently activated, and these two pathways may exert their protective action in a cooperative fashion, by phosphorylating different serine residues of *Bad* [10]. Our data seems to further extend this hypothesis since parallel pathways are required for the insulin-induced inhibition of hypoxia-evoked caspase-3 cleavage and do not act in cooperative fashion. In fact, we found that hypoxia induces cleavage of caspase-3 through a p38 MAPK-dependent pathway, and insulin stimulation of the expression of MKP-1, which dephosphorylates p38 MAPK, inhibits this phenomenon (Fig. 8). Our data does not rule out the possibility that insulin exerts its protective effect by the synergistic actions of PI3K/Akt and ERK 1/2 on *Bad* phosphorylation, but does describe another molecular mechanism involving expression of MKP-1 that contributes to the protective effect of insulin.

Although MKP-1 is involved in the regulation of several physiological functions, such as gene expression, cell growth, apoptosis, and immune responsiveness [31], the importance of MKP-1 in mediating the regulation of these processes remains

unclear. Here we report that MKP-1 plays a pivotal role in the protective action of insulin. Our data is consistent with that of Wu [23], who showed in primary mouse embryonic fibroblasts that MKP-1 promotes cell survival by attenuating p38 MAPK-mediated activation of caspase-3 and apoptosis. Furthermore, the protective role of MKP-1 has been also reported in vivo. In particular, Kaiser et al. have demonstrated that, in both cultured neonatal cardiomyocytes and the adult heart, p38 MAPK functions as a pro-death signaling effector, and that enhanced expression of MKP-1 protects the heart from p38-dependent injury [32]. Altogether, these data support the concept that MKP-1 plays a key role in insulin-mediated cellular protection.

In cardiac myocytes, insulin-induced MKP-1 protein expression is a complex phenomenon which requires ERK 1/2, JNK MAPK and PI3K/Akt pathways. β AR-induced insulin resistance compromises this molecular machinery. We found that β AR stimulation activates the molecular pathways involved in insulin-induced MKP-1 expression only in part and with different kinetics. Actually, ISO induced a shorter ERK 1/2 phosphorylation compared to that induced by insulin and failed to activate JNK (see Supplementary data). Furthermore, we found that sustained β AR stimulation impaired insulin-induced ERK 1/2 and JNK phosphorylation, while it did not affect insulin-induced Akt activity. Thus, it is possible that, in cardiac myocytes, β AR stimulation induces only partial phosphorylation/activation of protein kinases involved in MKP-1 transcription. On the other hand, long-term

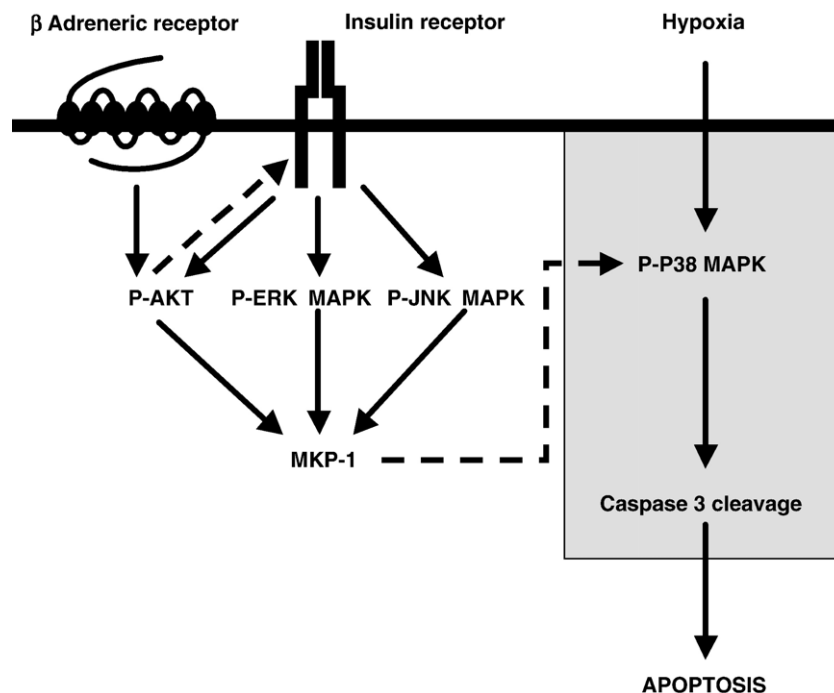


Fig. 8. Current hypothesis as to how stimulation of β AR-induced insulin resistance compromises the protective effect of insulin on hypoxia-induced apoptosis. Hypoxia induces cleavage of caspase-3 through p38 MAPK-dependent pathway. Insulin stimulation phosphorylates/activates Akt, ERK and JNK MAPK, which, in turn, promote the expression of MKP1, a MAPK phosphatase, that inactivates p38 MAPK. Sustained β AR stimulation exerts a detrimental effect on insulin receptor function through Akt, and, thus, removes the inhibitory effect of insulin on hypoxia-induced caspase-3 cleavage/activation (dashed lines indicate negative regulation of pathway).

stimulation of β AR by impairing insulin receptor function [14] inhibits insulin-induced ERK 1/2 and JNK activation. This compromises the protective machinery activated by insulin. These results suggest that phosphorylation/activation of Akt or ERK 1/2 is not capable of preventing hypoxia-induced caspase-3 cleavage. Our data are apparently in contrast with the notion that overexpression of Akt [33,34] or ERK [35] is sufficient to protect cardiac myocytes from apoptosis. However, it should be noted that this concept comes from experimental models characterized by the overexpression of a single antiapoptotic kinase, presumably associated with the integrity of the other antiapoptotic pathways, while our observation is obtained in an experimental model in which one arm of the insulin-activated antiapoptotic pathway is impaired.

In summary, antiapoptotic signals induced by insulin are mediated by more than one signaling pathway, and long-term β adrenergic receptor stimulation impairs these pathways, thereby inhibiting the cytoprotective action of insulin (Fig. 8). This has important pathophysiological implications since one of the main goals of treatment for cardiovascular diseases is to preserve cardiac contractility. This can be achieved in an experimental model by reduction of apoptosis, attained by inhibition of caspase activities [36,37]. It is possible to speculate that all treatments that are able to interfere with the development of insulin resistance, or, alternatively, increase the insulin sensitivity, in cardiovascular diseases may be beneficial for survival of cardiac myocytes and increased contractility.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: [10.1016/j.cardiores.2007.07.012](https://doi.org/10.1016/j.cardiores.2007.07.012).

References

- [1] Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* 2000;106:165–9.
- [2] Olefsky J, Farquhar JW, Reaven G. Relationship between fasting plasma insulin level and resistance to insulin-mediated glucose uptake in normal and diabetic subjects. *Diabetes* 1973;22:507–13.
- [3] Reaven GM. Insulin resistance, hyperinsulinemia, and hypertriglyceridemia in the etiology and clinical course of hypertension. *Am J Med* 1991;90:7S–12S.
- [4] Kolterman OG, Insel J, Saekow M, Olefsky JM. Mechanisms of insulin resistance in human obesity: evidence for receptor and postreceptor defects. *J Clin Invest* 1980;65:1272–84.
- [5] Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). *Jama* 2001;285:2486–97.
- [6] Shah A, Shannon RP. Insulin resistance in dilated cardiomyopathy. *Rev Cardiovasc Med* 2003;4(Suppl 6):S50–7.
- [7] Mancica G, Grassi G, Giannattasio C, Seravalle G. Sympathetic activation in the pathogenesis of hypertension and progression of organ damage. *Hypertension* 1999;34:724–8.
- [8] Scherrer U, Randin D, Tappy L, Vollenweider P, Jequier E, Nicod P. Body fat and sympathetic nerve activity in healthy subjects. *Circulation* 1994;89:2634–40.
- [9] Francis GS, Cohn JN, Johnson G, Rector TS, Goldman S, Simon A. Plasma norepinephrine, plasma renin activity, and congestive heart failure. Relations to survival and the effects of therapy in V-HeFT II. The V-HeFT VA Cooperative Studies Group. *Circulation* 1993;87:VI40–8.
- [10] Aikawa R, Nawano M, Gu Y, Katagiri H, Asano T, Zhu W, et al. Insulin prevents cardiomyocytes from oxidative stress-induced apoptosis through activation of PI3 kinase/Akt. *Circulation* 2000;102:2873–9.
- [11] Condorelli G, Morisco C, Stassi G, Notte A, Farina F, Sgaramea G, et al. Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat. *Circulation* 1999;99:3071–8.
- [12] Morisco C, Lembo G, Trimarco B. Insulin resistance and cardiovascular risk: new insights from molecular and cellular biology. *Trends Cardiovasc Med* 2006;16:183–8.
- [13] Communal C, Singh K, Sawyer DB, Colucci WS. Opposing effects of beta (1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein. *Circulation* 1999;100:2210–2.
- [14] Morisco C, Condorelli G, Trimarco V, Bellis A, Marrone C, Condorelli G, et al. Akt mediates the cross-talk between beta-adrenergic and insulin receptors in neonatal cardiomyocytes. *Circ Res* 2005;96:180–8.
- [15] Sugden PH, Clerk A. “Stress-responsive” mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ Res* 1998;83:345–52.
- [16] Morisco C, Zebrowski DC, Vatner DE, Vatner SF, Sadoshima J. Beta-adrenergic cardiac hypertrophy is mediated primarily by the beta(1)-subtype in the rat heart. *J Mol Cell Cardiol* 2001;33:561–73.
- [17] Scorziello A, Pellegrini C, Forte L, Tortiglione A, Gioielli A, Iossa S, et al. Differential vulnerability of cortical and cerebellar neurons in primary culture to oxygen glucose deprivation followed by reoxygenation. *J Neurosci Res* 2001;63:20–6.
- [18] Morisco C, Zebrowski D, Condorelli G, Tschlis P, Vatner SF, Sadoshima J. The Akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. *J Biol Chem* 2000;275:14466–75.
- [19] Condorelli G, Morisco C, Latronico MV, Claudio PP, Dent P, Tschlis P, et al. TNF-alpha signal transduction in rat neonatal cardiac myocytes: definition of pathways generating from the TNF-alpha receptor. *FASEB J* 2002;16:1732–7.
- [20] Yamamoto S, Seta K, Morisco C, Vatner SF, Sadoshima J. Chelerythrine rapidly induces apoptosis through generation of reactive oxygen species in cardiac myocytes. *J Mol Cell Cardiol* 2001;33:1829–48.
- [21] Malhotra R, Brosius III FC. Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes. *J Biol Chem* 1999;274:12567–75.
- [22] Abe J, Baines CP, Berk BC. Role of mitogen-activated protein kinases in ischemia and reperfusion injury: the good and the bad. *Circ Res* 2000;86:607–9.
- [23] Wu JJ, Bennett AM. Essential role for mitogen-activated protein (MAP) kinase phosphatase-1 in stress-responsive MAP kinase and cell survival signaling. *J Biol Chem* 2005;280:16461–6.
- [24] Cook SJ, Beltman J, Cadwallader KA, McMahon M, McCormick F. Regulation of mitogen-activated protein kinase phosphatase-1 expression by extracellular signal-related kinase-dependent and Ca²⁺-dependent signal pathways in Rat-1 cells. *J Biol Chem* 1997;272: 13309–19.
- [25] Begum N, Ragolia L, Rienzie J, McCarthy M, Duddy N. Regulation of mitogen-activated protein kinase phosphatase-1 induction by insulin in vascular smooth muscle cells. Evaluation of the role of the nitric oxide signaling pathway and potential defects in hypertension. *J Biol Chem* 1998;273:25164–70.

- [26] Condorelli G, Roncarati R, Ross J, Pisani A, Stassi G, Todaro M, et al. Heart-targeted overexpression of caspase3 in mice increases infarct size and depresses cardiac function. *Proc Natl Acad Sci U S A* 2001;98:9977–82.
- [27] Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, et al. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994;75:426–33.
- [28] Long X, Boluyt MO, Hipolito ML, Lundberg MS, Zheng JS, O'Neill L, et al. p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J Clin Invest* 1997;99:2635–43.
- [29] Crawford RM, Jovanović S, Budas GR, Davies AM, Lad H, Wenger RH, et al. Chronic mild hypoxia protects heart-derived H9c2 cells against acute hypoxia/reoxygenation by regulating expression of the SUR2A subunit of the ATP-sensitive K⁺ channel. *J Biol Chem* 2003;278:31444–55.
- [30] Communal C, Sumandea M, de Tombe P, Narula J, Solaro RJ, Hajjar RJ. Functional consequences of caspase activation in cardiac myocytes. *Proc Natl Acad Sci U S A* 2002;99:6252–6.
- [31] Camps M, Nichols A, Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J* 2000;14:6–16.
- [32] Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF, et al. Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *J Biol Chem* 2004;279:15524–30.
- [33] Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. *Circulation* 2001;104:330–5.
- [34] Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000;101:660–7.
- [35] Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Kleivitsky R, et al. The MEK1–ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J* 2000;19:6341–50.
- [36] Yaoita H, Ogawa K, Maehara K, Maruyama Y. Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation* 1998;97:276–81.
- [37] Holly TA, Drincic A, Byun Y, Nakamura S, Harris K, Klocke FJ, et al. Caspase inhibition reduces myocyte cell death induced by myocardial ischemia and reperfusion in vivo. *J Mol Cell Cardiol* 1999;31:1709–15.