

Insulin Suppresses Atrophy- and Autophagy-related Genes in Heart Tissue and Cardiomyocytes Through AKT/FOXO Signaling

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Key words

- diabetes
- atrogenes
- lysosomal/autophagic genes

Abstract

Insulin is an important regulator of the ubiquitin-proteasome system (UPS) and of lysosomal proteolysis in cardiac muscle. However, the role of insulin in the regulation of the muscle atrophy-related Ub-ligases atrogin-1 and MuRF1 as well as in autophagy, a major adaptive response to nutritional stress, in the heart has not been characterized. We report here that acute insulin deficiency in the cardiac muscle of rats induced by streptozotocin increased the expression of atrogin-1 and MuRF1 as well as LC3 and Gabarapl1, 2 autophagy-related genes. These effects were associated with decreased phosphorylation levels of Akt and its downstream target Foxo3a; this phenomenon is a well-known effect that permits the maintenance of Foxo in the nucleus

to activate protein degradation by proteasomal and autophagic processes. The administration of insulin increased Akt and Foxo3a phosphorylation and suppressed the diabetes-induced expression of Ub-ligases and autophagy-related genes. In cultured neonatal rat cardiomyocytes, nutritional stress induced by serum/glucose deprivation strongly increased the expression of Ub-ligases and autophagy-related genes; this effect was inhibited by insulin. Furthermore, the addition of insulin in vitro prevented the decrease in Akt/Foxo signaling induced by nutritional stress. These findings demonstrate that insulin suppresses atrophy- and autophagy-related genes in heart tissue and cardiomyocytes, most likely through the phosphorylation of Akt and the inactivation of Foxo3a.

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Introduction

Diabetes is associated with a marked increase in the risk of cardiac disease [1], which is generally associated with a loss of heart mass [2]. In fact, insulin plays an important role in regulating cardiac size, but the underlying mechanisms of this anabolic response remain unknown.

Cardiac proteins are in a dynamic state of continual synthesis and degradation that is highly selective and critical for normal cellular function. The ubiquitin-proteasome system (UPS) is responsible for the degradation of most intracellular proteins and plays a key role in the regulation of many biological processes. In a variety of atrophy models, the processes of ubiquitination and degradation are regulated by 2 muscle-specific Ub-ligases, atrogin-1/Muscle Atrophy F-box (MAF_x) and Muscle RING Finger 1 (MuRF1) [3, 4]. These 2 genes, which are known as atrophy-related genes or atrogenes, are muscle atrophy markers and could be considered master genes

for muscle wasting [5]. Although several other genes among the atrogenes are of potential interest, including genes encoding lysosomal proteases (cathepsin B and L), their roles in heart muscle wasting have not been determined. The expression of atrogenes in skeletal and cardiac muscles is inhibited by the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway through the phosphorylation and nuclear exclusion of Forkhead box class O (Foxo) transcription factors [6–8]. Although the importance of the UPS in regulating protein degradation in cardiac muscles in diabetes is well documented [9, 10], it is still unclear how atrophy-related genes are directly regulated by insulin signaling. Autophagy occurs in most cells to remove protein aggregates and abnormal organelles that would otherwise lead to toxicity and dysfunctional muscles. Autophagy has recently been implicated in a number of genetic myopathies, and lysosomal degradation has been suggested to contribute to the increased protein breakdown in

skeletal muscle wasting [11,12]. Autophagy is also induced in the heart in response to starvation [13,14] and ischemia [15,16]. However, little is known about the regulation of autophagy in the heart under insulin deficiency conditions [17].

The aim of the present study was to investigate the impact of insulin deficiency on the expression levels of atrophy-related genes (atrogin-1, MuRF1) and lysosomal/autophagy-related genes (LC3, Gabarap1 and cathepsin L) in heart tissue from streptozotocin-treated rats and in cardiomyocytes under nutritional stress. In addition, we examined alterations in Akt/Foxo signaling in heart tissue and in cardiomyocytes by analyzing the protein content and phosphorylation levels of the Akt, Foxo1, and Foxo3a transcriptional factors.

Materials and Methods



Animals and treatment

Male Wistar rats were housed in a room with a 12/12-h light/dark cycle and were given free access to water and a normal lab chow diet. All experiments and protocols were performed in accordance with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation and approved by the School of Medicine of Ribeirão Preto of the University of São Paulo-Ethical Commission of Ethics in Animal Research (No. 157/2008). Streptozotocin (STZ, 135 mg/kg body weight) was used to induce a diabetic state. This dose has been chosen because a single injection of STZ at doses lower than 135 mg/kg was unable to induce hyperglycemia in juvenile rats. STZ was dissolved in 0.01 M citrate buffer (pH 4.5) and injected under anesthesia into the jugular vein. Control rats received citrate buffer. Previous studies from our lab have shown that no changes in mortality were observed until 8 days after STZ injection [18]. STZ-injected and control rats of similar body weight (70–80 g) were killed by cervical dislocation for heart excision 3 days after injection. A group of STZ-injected rats was subcutaneously injected with NPH insulin (4 U) twice daily for 3 days, and animals were killed 12 h after the last injection. To measure plasma glucose levels, blood samples were taken from the tip of the tail before euthanasia. Plasma was stored at -70°C until assayed, and the glucose concentration was determined by a glucose oxidase colorimetric method [19].

Primary cultures of neonatal rat cardiac myocytes

The hearts of neonatal (1 day old) Wistar rats were excised, and the ventricles were minced and transferred to a sterile buffer. The tissue was then subjected to 6–7 subsequent enzymatic digestions with collagenase, each performed at 37°C for 12 min. The solution obtained from each digest was then transferred to a tube containing 1 ml of newborn calf serum (NCS) and centrifuged. Each cell pellet was resuspended in NCS, and dissociated cells were pooled. To separate myocytes from non-myocytes, the cell suspension was layered onto discontinuous Percoll density gradients consisting of 2 phases. After washing to remove all traces of Percoll, the myocytes were cultured in DMEM containing 5% fetal calf serum, penicillin and streptomycin (P/S, 1%), and 10% horse serum for 48 h [20]. The next day, a group of myocytes was cultured in glucose- and serum-free media for 2 h, and during the last hour, insulin ($10\,000\ \mu\text{U}\cdot\text{ml}^{-1}$), triciribine (an Akt inhibitor; $10\ \mu\text{M}$) and/or wortmanin (a PI3K inhibitor; $0.5\ \mu\text{M}$) was either added to or omitted from the incubation medium. Triciribine and wortmanin were added 30 min before insulin.

Quantitative PCR

Real time RT-qPCR was used to assess the expression levels of the transcripts of the ubiquitin ligases (atrogin-1 and MuRF1) and lysosomal/autophagy-related genes (LC3, Gabarap1 and cathepsin L). The hearts from control, diabetic and diabetic+insulin rats were rapidly removed and immediately frozen in liquid nitrogen. Total RNA was isolated from tissue and cardiomyocytes with TRIZOL reagent (Invitrogen) and was used to generate cDNA. The RNA quality was quantified by absorption spectrophotometry at 260 and 280 nm. cDNA was generated from the total mRNA from each heart in a first-strand synthesis reaction with Superscript II Reverse transcriptase (Invitrogen) at 42°C for 2 h, according to the manufacturer's instructions. Relative quantitative real time RT-PCR was subsequently performed with an Applied Biosystems 7500 system and SYBER Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primers were designed with Primer Express software (Applied Biosystems). Primers for atrogin-1 (forward 5'-CTT TCA ACA GAC TGG ACT TCT CGA-3', and reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'); MuRF1 (forward 5'-TCG ACA TCT ACA AGC AGG AA-3', and reverse 5'-CTG TCC TTG GAA GAT GCT TT-3'); LC3 (forward 5'-TTT GTA AGG GCG GTT CTG AC-3', and reverse 5'-CAG GTA GCA GGA AGC AGA GG-3'); Gabarap1 (forward 5'-CCC AGT TGT GGC AGT AGA CA-3', and reverse 5'-GAC TGA TCC TGA GGC TCC TG-3'); cathepsin L (forward 5'-GTG GAC TGT TCT CAC GCT CAAG-3', and reverse 5'-TCC GTC CTT CGC TTC ATA GG-3'); and cyclophilin (forward 5'-AAG GAC TTC ATG ATC CAG GG-3', and reverse 5'-TGA CAT CCT TCA GTG GCT TG-3'). The relative quantitation of mRNA levels was plotted as the fold increase compared to the control group values. Transcripts of interest were normalized to cyclophilin levels, and the level of the target transcripts was calculated using the standard curve method [21].

Western blotting

Hearts (control, diabetic and diabetic+insulin) and cardiomyocytes (control, fasted, and fasted+insulin) were harvested and homogenized at 4°C in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 5 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at $12\,000g$ at 4°C for 30 min. The supernatant was retained, and the protein content was determined by the Lowry method using bovine serum albumin (BSA) as a standard [22]. An equal volume of sample buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, pH 6.8) was added to the supernatant, and the mixture was boiled and subjected to SDS-PAGE analysis on 10–18% acrylamide gels [23]. The gels were electroblotted onto nitrocellulose membranes [24] and blotted with anti-cathepsin B (1:75, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cathepsin L (1:75, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Gabarap (1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-LC3/II (1:1 000, MBL International Corporation, Japan), anti- β -actin (1:3 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Akt (1:2 000, Cell Signaling, Danvers, MA, USA), anti-phospho-[Ser⁴⁷³]-Akt (1:1 000, Cell Signaling, Danvers, MA, USA), anti-phospho-[Thr³⁰⁸]-Akt (1:1 000, Cell Signaling, Danvers, MA, USA), anti-Foxo1 (1:1 000, Cell Signaling, Danvers, MA, USA), anti-Foxo3a (1:1 000, Cell Signaling, Danvers, MA, USA), anti-phospho-[Ser²⁵³]-Foxo3a (1:1 000, Cell Signaling, Danvers, MA, USA), and anti-phospho-[Thr²⁴]-Foxo1/anti-phospho-[Thr³²]-

Foxo3a (1:1 000, Cell Signaling, Danvers, MA, USA). The primary antibody was detected with a peroxidase-conjugated secondary antibody (1:5 000, Cell Signaling, Danvers, MA, USA); ECL reagents were used for visualization. Band intensities were quantified with ImageJ (version 1.4 National Institute of Health, USA). The densitometry of the bands was analyzed 3 times, and the average value is reported.

Drugs

All drugs and reagents were purchased from Sigma Chemicals (St. Louis, MO, USA), Calbiochem EMD Biosciences (Inc. La Jolla, CA, EUA), GE Healthcare Life Sciences (Piscataway, NJ, USA), Bio-Rad (Hercules, CA, USA), MBL (Japan), Cell Signaling Technology (Danvers, MA, USA), or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

The means of the heart samples from different groups of animals were analyzed with Student's nonpaired *t*-test. The paired *t*-test was used to compare the means of the cardiomyocyte samples

incubated in the presence or absence of insulin. A *p*-value of ≤ 0.05 was taken as the criterion of significance.

Results

Plasma glucose, body, and heart weight

The plasma glucose concentration 3 days after STZ treatment was drastically increased compared to the values observed in the saline-injected controls. The body weight of the diabetic rats was 21% lower than that of the control animals (Table 1). Compared with the control group, the wet heart weight was significantly decreased by 30% in the diabetic group, expressed as either the absolute weight or relative to the total body weight. Insulin administration was sufficient to normalize blood glucose levels and to prevent the loss of body and heart weight induced by diabetes (Table 1).

Changes in the expression levels of Ub-ligases and lysosomal/autophagy pathway components in the heart tissue of diabetic rats

The mRNA levels of the ubiquitin ligases atrogin-1 and MuRF1 were drastically increased (4- and 3.5-fold, respectively) in the heart tissue of acutely diabetic rats, and these effects were restored to normal levels after insulin administration (Fig. 1a). The gene expression of both LC3 and Gabarap1 was increased (5.5- and 7-fold, respectively) by diabetes (Fig. 1a). An increase (50%) in LC3II protein content in the heart tissue of diabetic rats relative to control rats was also observed. The insulin deficiency did not promote changes in the cardiac protein content of Gabarap (Fig. 1b). Insulin administration blocked the diabe-

Table 1 Plasma glucose levels, body weight, and heart weight in control and diabetic rats treated with or without insulin (4U).

	Control (n=7)	Diabetic (n=7)	Diabetic+Insulin (n=8)
Blood glucose (mg/dl)	118±5	465±11*	90±2.0#
Body weight (g)	89±2.7	71±1.5*	87±3.5#
Heart weight/Body weight (mg/100g)	388.6±2.6	273±10.6*	379±2.4#

**p*≤0.05 vs. control. #*p*≤0.05 vs. diabetic

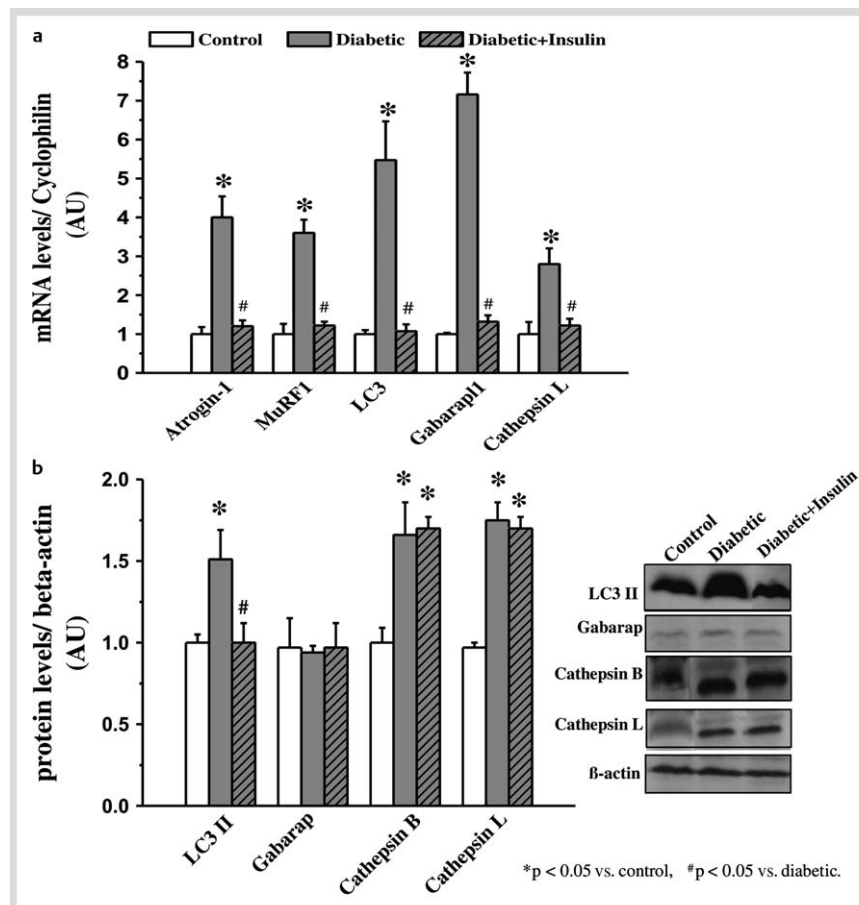


Fig. 1 The mRNA expression levels of Atrogin-1, MuRF1, LC3, Gabarap1 and Cathepsin L (normalized to cyclophilin mRNA) **a** and the protein content **b** of LC3 II, Gabarap, Cathepsin B and L (% of control group, normalized to β -actin) in the heart tissue of control, diabetic and diabetic + insulin rats.

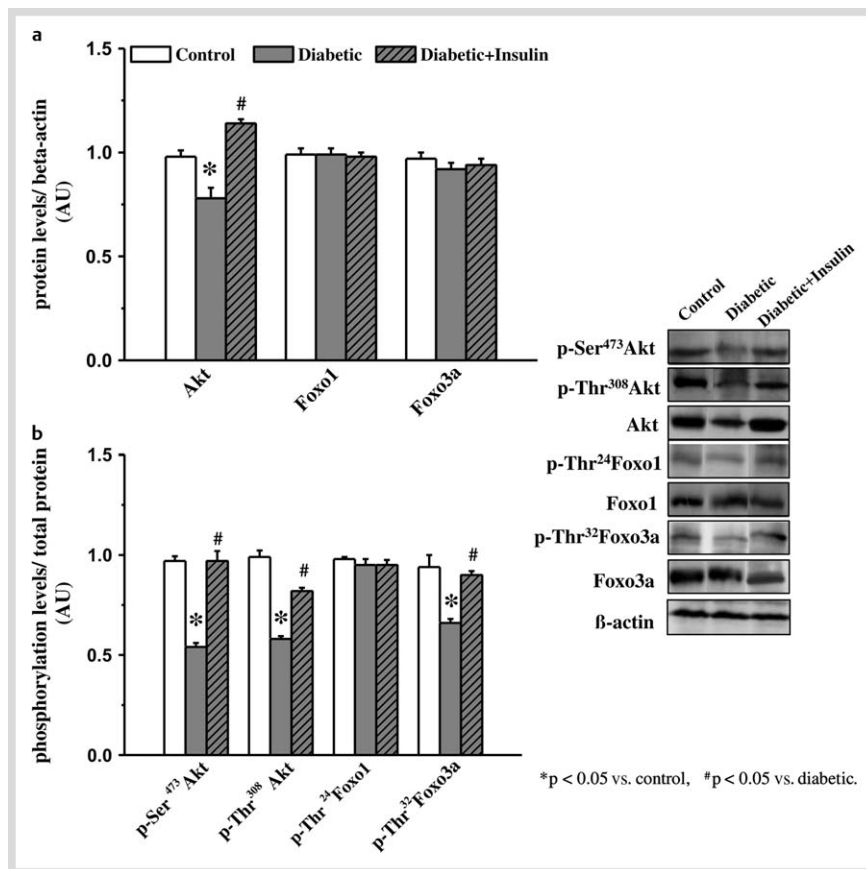


Fig. 2 The protein content of Akt, Foxo 1 and Foxo 3a (% of control group, normalized to β -actin) **a** and the phosphorylation levels of Akt (Ser⁴⁷³), Foxo1 (Thr²⁴) and Foxo3a (Thr³²) (normalized to Akt, Foxo 1 and Foxo 3a, respectively) **b** in the heart tissue of control, diabetic and diabetic + insulin rats.

tes-induced increase in LC3 and Gabarap1 mRNA and in the LC3II protein content. The atrogin-1, MuRF1, and LC3I proteins were not detected by immunoblotting in the heart tissue of any group. The changes in the lysosomal components of the heart tissue of diabetic rats were investigated by measuring the expression levels of 2 proteases, cathepsin B and L. Diabetes induced increases in the gene expression of cathepsin L (2.5-fold) and the protein contents of cathepsin B and L (62 and 75%, respectively) relative to those of the control group (Fig. 1b). Insulin treatment restored the diabetes-induced increase in cathepsin L mRNA but did not affect the heart tissue protein content of either cathepsin B or L. Altogether, these data indicate that insulin exerts an inhibitory control of the processes of ubiquitination and lysosomal/autophagy activity in heart tissue.

Changes in Akt/Foxo signaling in the heart tissue of diabetic rats

Acute insulin deficiency significantly decreased the protein content of Akt (22%) as well as the levels of phosphorylation of Akt at serine⁴⁷³ and threonine³⁰⁸ (43% and 41%, respectively) (Fig. 2a, b). The phosphorylation levels of Foxo3a were also reduced (28%) in the heart tissue of diabetic rats (Fig. 2b), but no changes in the Foxo3a protein content were observed (Fig. 2a). There were no apparent alterations in the protein content or in the phosphorylation levels of Foxo1 in the heart tissue of diabetic rats. The administration of insulin restored the protein content of Akt and the phosphorylation levels of Akt and Foxo3a (Fig. 2a, b).

Changes in the expression of Ub-ligases and lysosomal/autophagy pathway components as well as Akt/Foxo signaling in fasted cardiomyocytes

In cultured neonatal rat cardiomyocytes, the nutritional stress induced by glucose/serum deprivation strongly increased the gene expression of the ubiquitin ligases (atrogin-1 and MuRF1) and lysosomal/autophagy pathway genes (LC3, Gabarap1, and cathepsin L) (Fig. 3), an effect that was associated with low levels of phosphorylation of both Akt^{Ser} (50%) and Foxo3a^{Ser} (30%) (Fig. 4b). All of these effects were prevented when the cardiomyocytes were incubated in the presence of insulin (Fig. 3, 4). Furthermore, the addition of the Akt and PI3K inhibitors (tricitriline and wortmanin, respectively) blocked the insulin effects on the mRNA of Ub-ligases and autophagy genes and the phosphorylation of Akt and Foxo (Fig. 3, 4b). The incubation of cardiomyocytes with inhibitors in the absence of insulin did not change genes expression and Akt/Foxo phosphorylation (data not shown). These data convincingly demonstrate that insulin inhibits the fasting-induced expression of Ub-ligases and lysosomal/autophagy genes, most likely through the activation of Akt/Foxo signaling in cardiomyocytes.

Discussion

The anabolic effects of insulin on the heart involve multiple mechanisms, including an increase in protein synthesis [25,26] and the repression of catabolic pathways and factors, such as myostatin [27] and glucocorticoid receptor signaling [28]. The present data provide new insights in this field by revealing that insulin acts *in vivo* to rapidly and widely repress the expres-

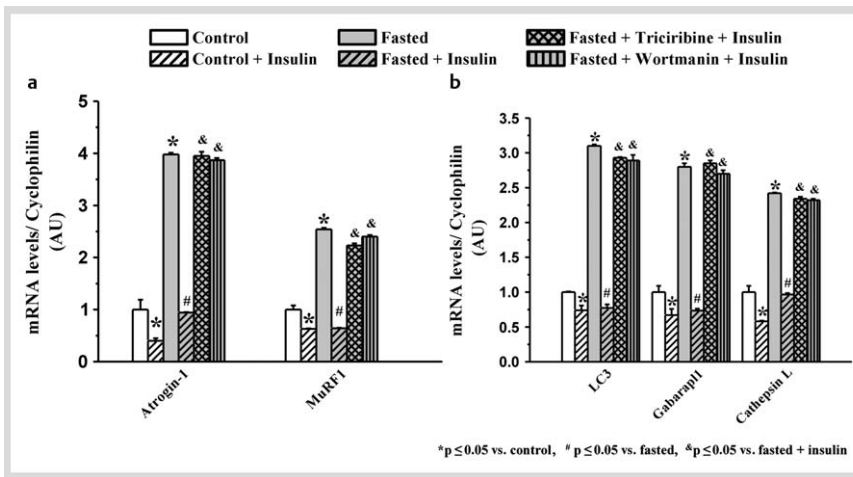


Fig. 3 The effect of insulin on the mRNA levels of Atrogin-1, MuRF1, LC3, Gabarapl1, and Cathepsin L (normalized to cyclophilin mRNA) in cardiomyocytes with or without nutrient deprivation for 2 h and/or incubated with Akt (10 μ M triciribine) and PI3K (0.5 μ M wortmanin) inhibitors for 30 min before insulin.

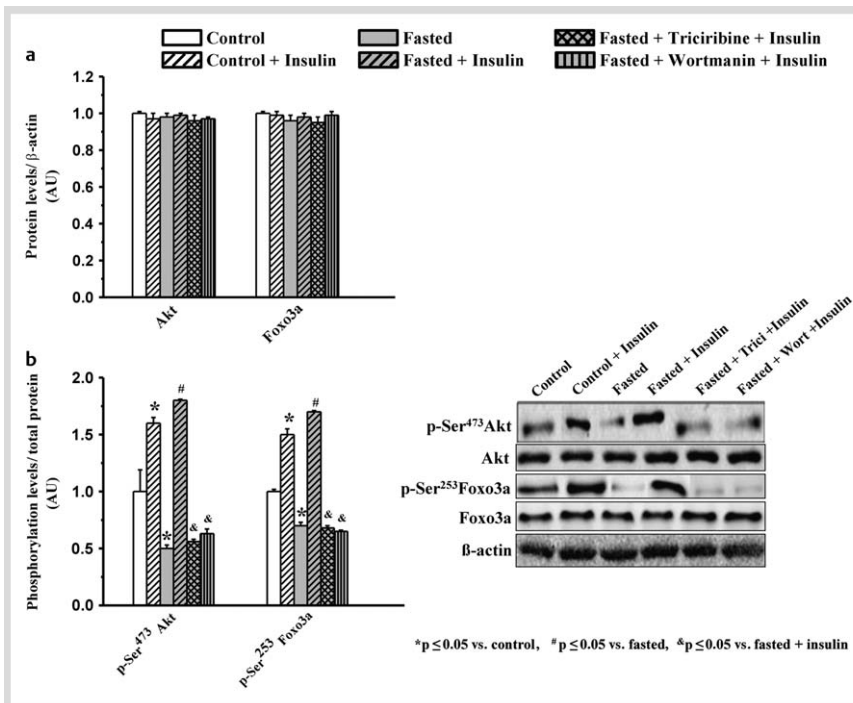


Fig. 4 The effect of insulin on the protein content **a** of Akt and Foxo3a (% of control group, normalized to β -actin) and phosphorylation levels **b** of Akt (Ser⁴⁷³) and Foxo3a (Ser²⁵³) (normalized to Akt and Foxo3a, respectively) in cardiomyocytes with or without nutrient deprivation for 2 h and/or incubated with Akt (10 μ M triciribine) and PI3K (0.5 μ M wortmanin) inhibitors for 30 min before insulin.

sion of atrogin-1 and MuRF1, thereby affecting UPS activity, which is responsible for most proteolysis in the cell [29]. Acute insulin deficiency was sufficient to promote a decrease in the heart weight in rats. Given that the expression levels of atrophy-related genes were upregulated in the heart tissue of diabetic rats, we suggest that this atrophic effect was due, at least in part, to an increase in the activity of the UPS. Consistent with our conclusions, the UPS has been reported to be the major proteolytic pathway for protein degradation in the cardiac muscle of diabetic mice. The heart tissue of diabetic rats exhibits an increase in the mRNA of ubiquitin [10] and subunits C2 and C8 of the proteasome [9]. We also found that insulin treatment prevented the transcriptional upregulation of the atrophy-related Ub-ligases in rat neonatal cardiac myocyte cultures *in vitro*. To our knowledge, this study is the first to directly demonstrate an *in vitro* inhibitory effect of insulin on Ub-ligases expression in myocytes, a finding that strongly supports similar conclusions from both *in vivo* and *in vitro* experiments in skeletal muscle [30,31]. The autophagic pathway is a vital process that presumably participates in the removal of dysfunctional cytosolic components

and serves as a catabolic energy source during starvation [32]. However, autophagy may be enhanced under a variety of atrophic conditions, as evidenced by the expression of a number of proteins involved in lysosomal degradation, including LC3 and Gabarapl1, in multiple types of skeletal muscle atrophy. Indeed, the expression level of LC3II is highly correlated with the number of autophagosomes [33]. In the present study, LC3 mRNA and, consequently, LC3II protein levels were enhanced in the heart tissue of diabetic animals and in fasted cardiomyocytes, suggesting that the autophagic flux is increased. Similar results were observed for Gabarapl1 mRNA. Fasting has previously been shown to increase the protein content of LC3II and Gabarap in HeLa cells and cardiomyocytes [34,35]. The enhancement of lysosomal/autophagy genes and proteins that was observed in the present study was completely prevented by the addition of insulin *in vivo* and *in vitro*, a finding that suggests that this hormone plays an important role in the control of the heart protein balance through the lysosomal/autophagic process. In agreement with this notion, insulin prevented the diabetes-induced increase in cathepsin L mRNA expression. Intriguingly, the protein content of cathepsin B

and L was upregulated by insulin deficiency but was unresponsive to insulin treatment.

In skeletal muscle, Akt, once activated by insulin or IGF-1, phosphorylates and inhibits Foxo3a, a transcriptional factor involved in the induction of atrophy-related Ub-ligases [7,8] and lysosomal/autophagy genes [36], and prevents the activation of the UPS and autophagy and the loss of muscle weight. Skurk et al. [6] were the first to demonstrate the existence of a similar Akt/Foxo signaling axis in cardiac myocytes both in vitro and in vivo that is regulated by multiple physiological stimuli of myocyte growth, including the presence of insulin. Sengupta et al. [35] reported that either glucose deprivation or the overexpression of Foxo3 leads to decreased cell size and the induction of LC3 mRNA and GFP-LC3 puncta, which would represent the autophagic vacuoles, in cultured rat neonatal cardiomyocytes. In agreement with these studies, we observed that the phosphorylation levels of Akt and Foxo3a are decreased in the heart tissue of diabetic animals and in cardiomyocytes under nutritional stress and that these effects are prevented by insulin. In addition, the downregulation of Ub-ligases and lysosomal/autophagy genes induced by insulin in fasted cardiomyocytes was abolished by PI3K/Akt inhibitors. AMP-activated protein kinase (AMPK) is a sensor of energy status that maintains cellular energy homeostasis and may activate autophagy in cardiomyocytes [37]. However, we have observed that AMPK phosphorylation (activation) is not altered by fasting condition and/or insulin in cardiomyocytes (data not shown). Altogether, these results provide evidence for an important role of the Akt/Foxo3a-dependent pathway in mediating the antiproteolytic effect of insulin on cardiac myocytes.

In spite of accelerated protein degradation described herein and by Hu et al. [9] in heart from diabetic rodents, we did not rule out the possibility that heart dysfunction or cell death could contribute to heart atrophy in our model. Borges et al. [38] have demonstrated that STZ-diabetic rats exhibited a heart dysfunction associated with a reduced heart rate, mean arterial pressure, inotropism and lusitropism in a basal condition, effects that were prevented by insulin treatment. In addition, apoptosis and caspase-3 activation has been related to Akt inhibition in heart from diabetic rats [39].

In summary, the present study demonstrates that insulin has a marked direct inhibitory effect on Ub-ligases and lysosomal/autophagy-related genes in both heart tissue and in cardiomyocytes through Akt/Foxo3a signaling, raising the possibility that enhanced proteasomal degradation and autophagy contribute in concert to the loss of cardiac mass under insulin deficiency.

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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