

Bidirectional regulation of upstream IGF-I/insulin receptor signaling and downstream FOXO1 in cardiomyocytes

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Abstract

Signaling pathways of IGF-I and insulin receptors play important roles in the regulation of myocardial function. FOXO1 is a member of the forkhead transcriptional factor family, but how insulin and IGF-I receptor signaling regulate FOXO1 in cardiomyocytes is not well understood. This study was carried out to elucidate how IGF-I and insulin receptor signaling modulate FOXO1 in cardiomyocytes. In cardiomyocytes, activation of IGF-I receptor and insulin receptor lead to rapid phosphorylation of FOXO1. Inhibition of phosphatidylinositol 3-kinase/Akt pathway suppressed the effect of insulin and IGF-I on FOXO1 phosphorylation. Prolonged incubation with IGF-I increased ubiquitination of FOXO1 and down-regulated the abundance of FOXO1 proteins, which suggested that IGF-I might modulate FOXO1 degradation. To explore

whether FOXO1 could modulate IGF-I and insulin signaling, a constitutively active FOXO1 was overexpressed in cardiomyocytes. The abundance of insulin receptor and IGF-I receptor was significantly upregulated in the cells overexpressing active FOXO1, accompanied by increased receptor phosphorylation upon insulin/IGF-I stimulation. Interestingly, overexpression of constitutively active FOXO1 also led to activation of MEK and Akt phosphorylation. IGF-I-stimulated MEK and Akt phosphorylation were augmented by overexpression of constitutively active FOXO1. These findings indicate bidirectional regulation of insulin/IGF-I receptor signaling and FOXO1 in cardiomyocytes. FOXO1 may provide feedback control through upregulation of insulin and IGF-I receptor signaling.

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Introduction

Insulin-like growth factor-I (IGF-I) and insulin are part of the complex regulatory mechanisms that help to maintain normal myocardial function (Abel 2004, Saetrum & Wang 2005). Signaling pathways of IGF-I receptor and insulin receptor mediate important biological actions in cardiac muscle, such as carbohydrate metabolism, myocyte size, anti-apoptosis signaling, myocytes differentiation, and myogenesis (Abel 2004, Dorn & Force 2005, Saetrum & Wang 2005). Genetic and pharmacological manipulations of IGF-I and insulin receptor signaling lead to abnormal myocardial structure and function in experimental animals, and may alter myocardial survival during myocardial injuries (Abel 2004, Saetrum & Wang 2005). Although recent research advances have brought new understanding of intracellular signaling, regulation of IGF-I and insulin receptor signaling in cardiac muscle is only partially understood. Understanding the mechanisms of insulin and IGF-I receptor signaling in cardiomyocytes will help elucidate how hormonal signaling modulates myocardial function.

Insulin and IGF-I could prevent skeletal muscle cell atrophy and promote myogenesis through forkhead

transcription factors (FOXO; Hribal *et al.* 2003, Sartorelli & Fulco 2004, Stitt *et al.* 2004). FOXO1 is a member of the forkhead transcriptional factor family that regulates gene expression and thereby modulates cell atrophy and death (Hribal *et al.* 2003). Ample evidence has shown that the effects of IGF-I and insulin on FOXO are mediated through phosphatidylinositol 3-kinase (PI3-K) and protein kinase B/Akt (Hribal *et al.* 2003, Stitt *et al.* 2004). Upon activation of PI3-K and Akt, IGF-I and insulin suppress FOXO1 by phosphorylating and inactivating its transcriptional regulatory actions (Hribal *et al.* 2003). In addition, insulin downregulates FOXO1 activity by augmenting ubiquitination of FOXO1 and enhancing FOXO1 degradation (Matsuzaki *et al.* 2003). These results showed that IGF-I and insulin receptors are upstream regulators of FOXO1, and that FOXO1 is a component of the insulin/IGF-I receptor-signaling pathways. However, whether insulin and IGF-I can modulate FOXO1 in cardiac muscle is not yet known.

In addition to acting as a downstream effector of insulin receptor signaling, members of FOXO transcriptional factor family also participate in feedback control of growth factor signaling. *Drosophila* forkhead-related transcription factor (dFOXO), a homolog of FOXO1 in metazoans, had

been shown to activate insulin receptor transcription in *Drosophila* (Puig *et al.* 2003). FOXO3a, another member in the FOXO family, affected growth factor upstream signaling by increasing the phosphorylation of Akt in neonatal rat cardiomyocytes (Skurk *et al.* 2005). These data suggest the existence of a bidirectional regulatory mechanism between growth factor signaling and FOXO transcription factors. Despite the fact that insulin/IGF-I signaling and FOXO1 signaling contribute to the regulation of myocardial structure and function (Abel 2004, Morris *et al.* 2005, Saetrum & Wang 2005), the cross-talks between IGF-I/insulin receptor signaling and FOXO1 in cardiomyocytes have not been elucidated. Whether FOXO1 can modulate IGF-I receptor is not yet known. In this study, we investigated the relationship between IGF-I/insulin receptor signaling and FOXO1, and observed bidirectional regulation of insulin and IGF-I receptor signaling and FOXO1 in primary cardiomyocytes. IGF-I and insulin regulated FOXO1 in cardiomyocytes as we had anticipated, and FOXO1 provided feedback control of receptor signaling through modulation of IGF-I and insulin receptor ubiquitination.

Materials and Methods

Materials

Anti-FOXO1, anti- α -actinin, anti-actin, and anti-phosphotyrosine (PY99) polyclonal antibodies were purchased from Santa Cruz Biolabs (Santa Cruz, CA, USA). Anti-phospho-FOXO1 and other antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Protein A/G PLUS agarose beads were from Santa Cruz Biolabs and IGF-I was from GroPep (Adelaide, Australia). All other chemicals were purchased from Sigma or Fisher Scientific (Fairlawn, NJ, USA).

Cardiomyocytes culture and transduction of adenoviral constructs

Primary cultures of neonatal cardiomyocytes were prepared from Sprague–Dawley rats according to a protocol as we previously described (Shan *et al.* 2003). The animal experimental procedures were approved by the Institutional Animal Care and Use Committee at University of California, Irvine. The construction of recombinant adenovirus with constitutively active FOXO1 and the control adenovirus Ad-SR had been described in previous publications (Hribal *et al.* 2003, Shan *et al.* 2003). In this constitutively active FOXO1 mutant, all of its three potential Akt phosphorylation sites have been replaced by nonphosphorylatable amino acids (T24A/S253D/S316A), so that the expressed FOXO1 cannot be phosphorylated and excluded out of the nucleus in response to stimulation. The adenoviruses were replicated in 293 cells, purified by Virakit™ from Virapau (Carlsbad, CA, USA), and the viral titers were determined by plaque assay in 293 cells (Wu *et al.* 2000). Cardiomyocytes were plated in 100 mm Petri dishes (approximately 80–85% density) and

maintained with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. When indicated, the cells were infected with equal amounts of Ad-SR or Ad-FOXO1 and incubated at 37 °C, 5% CO₂ for the indicated time period.

Immunoblotting

The cells were rinsed once with PBS and lysed with a lysis buffer (137 mM NaCl, 20 mM Tris–HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mM EDTA (pH 8.0), 3 µg/ml aprotinin, 3 µg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 10 mM NaPP₆, and 2 mM Na₃VO₄). Protein concentrations were determined with the Bradford method as we previously described (Shan *et al.* 2003). Equal amounts of protein from each sample were resolved by SDS–PAGE and then transferred to polyvinylidene difluoride membrane and incubated with a blocking buffer (5% nonfat milk in 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. An alternative blocking buffer containing 2% BSA was used in the experiments involving anti-phosphotyrosine antibodies. The membranes were incubated with primary antibodies overnight at 4 °C, washed thrice (20 mM Tris–HCl (pH 7.5), 137 mM NaCl, and 0.1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000–1:10 000 dilution) for 1 h at room temperature, washed thrice, and then detected with enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL, USA).

Immunoprecipitation

The protein lysates (1000 µg proteins) were preabsorbed with 20 µl protein A/G agarose beads at 4 °C for 30 min on a rocking platform and spun for 5 min at 10 000 g, the supernatants were incubated with specific primary antibody at 4 °C overnight (Su *et al.* 2006). After incubation with 20 µl protein A/G agarose beads for 1.5 h at 4 °C, the immunocomplexes were collected by centrifugation and washed thrice with ice-cold washing buffer (137 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 2 mM EDTA (pH 8.0), 2 mM phenylmethylsulphonyl fluoride, and 2 mM Na₃VO₄). The final products were briefly boiled and resolved with SDS–PAGE and immunoblotted with specific antibodies as indicated.

Statistical analysis

The data were expressed as means ± S.E. based on data derived from multiple independent experiments. The intensity of bands from western blots was scanned with densitometry and digitally analyzed. The statistical significance was tested by Student's *t*-test or ANOVA with *post hoc* analysis when appropriate. A *P* value below 0.05 was considered statistically significant.

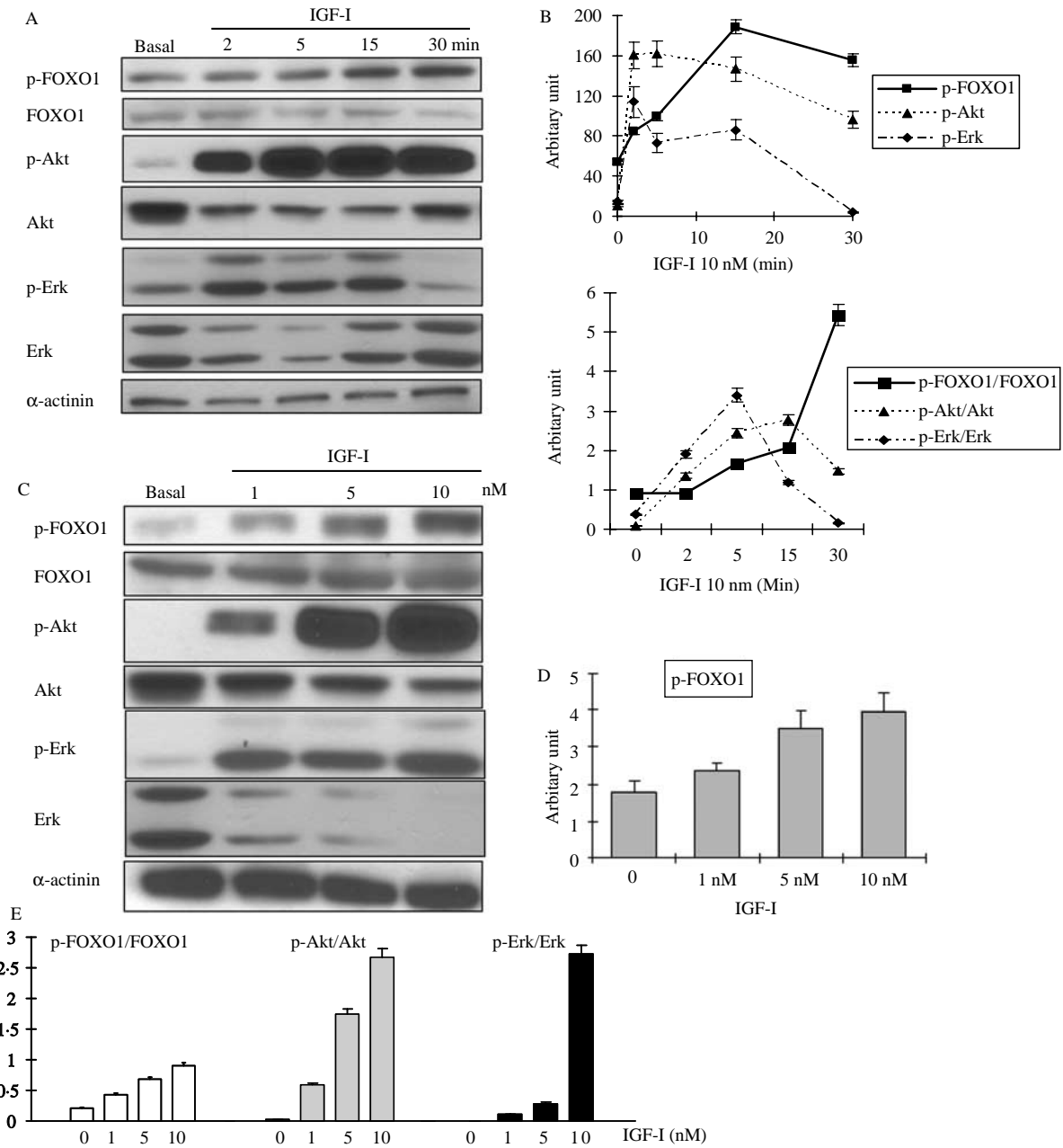


Figure 1 IGF-I stimulated phosphorylation of FOXO1 in cardiomyocytes. Equal amounts of protein lysates were resolved with SDS-PAGE and immunoblotted with specific antibodies. (A) Time-course effect of IGF-I on FOXO1 phosphorylation. The abundance of phospho-FOXO1 was determined by immunoblotting with phospho-specific FOXO1 antibodies. Activation of Akt and Erk was determined by immunoblotting with anti-phospho-Akt or anti-phospho-Erk antibodies. The abundance of FOXO1, Akt, and Erk proteins was determined by immunoblots with specific antibodies. Immunoblotting with α -actinin antibodies served as loading control. (B) Statistical analysis of IGF-I time-course effect. The graphs represent the results from three to six independent experiments analyzed with densitometry. The upper graph represents the abundance of phosphorylated proteins normalized to the abundance of α -actinin. The lower graph represents the ratio of phosphorylated protein to total protein (phosphorylated + unphosphorylated). Data represent means \pm s.e. (C) The dose-response effect of IGF-I on FOXO1 phosphorylation in cardiomyocytes. (D) Statistical analysis of IGF-I dose-response effect. The graph represents the abundance of phosphorylated FOXO1 normalized to the abundance of α -actinin, from four independent experiments analyzed by densitometry. Data represent means \pm s.e. (E) Comparison of IGF-I dose-response effect on FOXO1, Akt, and Erk. This graph represents the ratio of phosphorylated FOXO1 to total FOXO1 protein, phosphorylated Akt to total Akt, and phosphorylated Erk to total Erk.

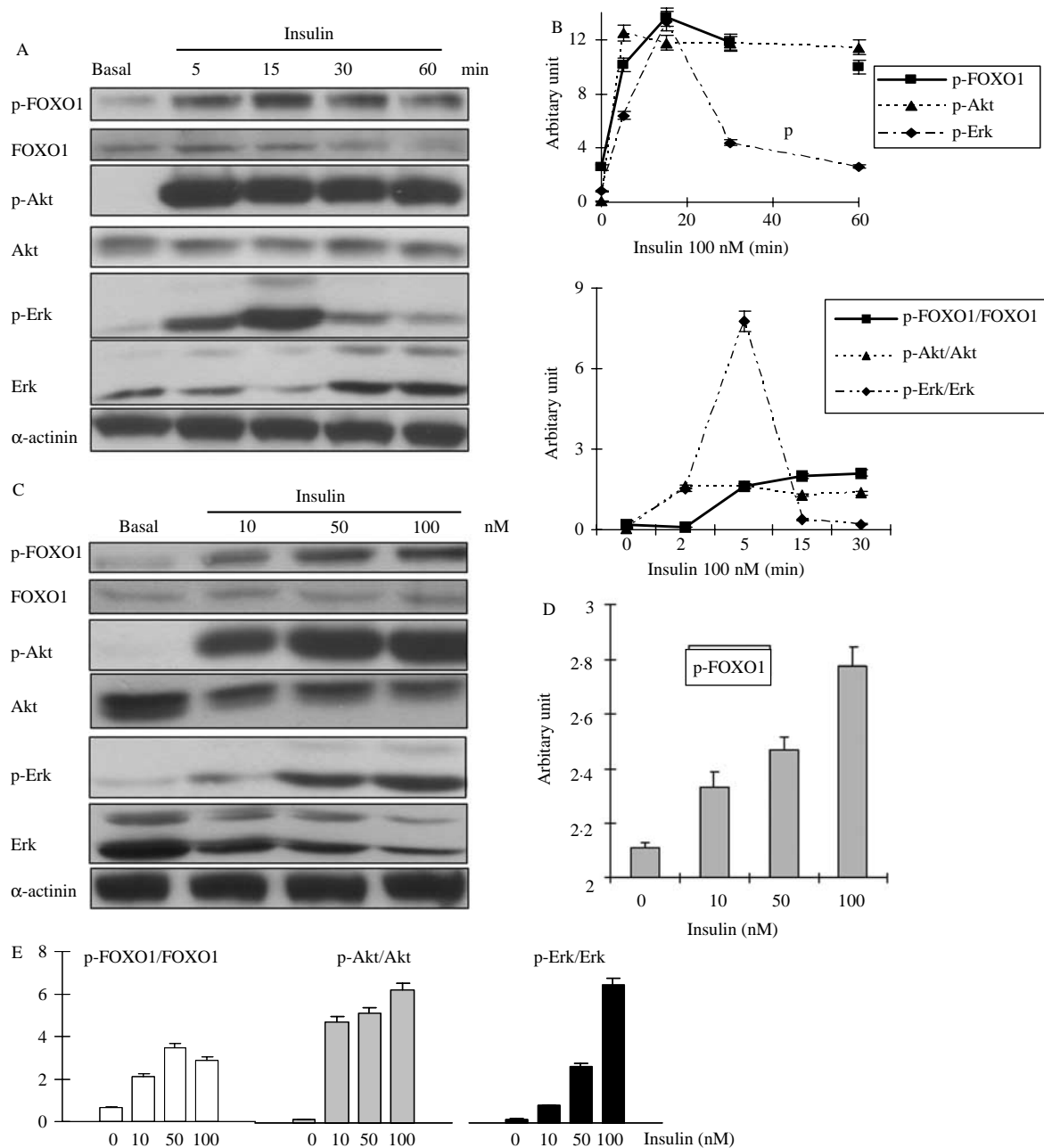


Figure 2 Insulin stimulated phosphorylation of FOXO1 in cardiomyocytes. Equal amounts of protein lysates were resolved with SDS-PAGE and immunoblotted with specific antibodies. (A) Time-course effect of insulin on FOXO1 phosphorylation. The abundance of phospho-FOXO1 was determined by immunoblotting with phospho-specific FOXO1 antibodies. Activation of Akt and Erk was determined by immunoblotting with anti-phospho-Akt or anti-phospho-Erk antibodies. The abundance of FOXO1, Akt, and Erk proteins was determined by immunoblots with specific antibodies. Immunoblotting with α -actinin antibodies served as loading control. (B) Statistical analysis of insulin time-course effect. The graphs represent the results from three to six independent experiments analyzed with densitometry. The upper graph represents the abundance of phosphorylated FOXO1 normalized to the abundance of α -actinin. The lower graph represents the ratio of phosphorylated proteins to total proteins. Data represent means \pm s.e. (C) The dose-response effect of insulin on FOXO1 phosphorylation in cardiomyocytes. (D) Statistical analysis of insulin dose-response effect. The graph represents the abundance of phosphorylated FOXO1, normalized to the abundance of α -actinin, from four independent experiments analyzed by densitometry. Data represent means \pm s.e. (E) Comparison of insulin dose-response effect on FOXO1, Akt, and Erk. This graph represents the ratio of phosphorylated FOXO1 to total FOXO1 protein, phosphorylated Akt to total Akt protein, and phosphorylated Erk to total Erk protein.

Results

IGF-I and insulin phosphorylated FOXO1 via PI3-K/Akt signaling in cardiomyocytes

Growth factor receptor signaling can modulate FOXO1 action through modulation of FOXO1 phosphorylation. To define the effects of IGF-I and insulin on FOXO1 phosphorylation, cardiomyocytes were serum-deprived overnight and stimulated with IGF-I or insulin. Anti-phospho-FOXO1 antibodies were used to determine the abundance of FOXO1 phosphorylation by immunoblotting (Fig. 1). IGF-I stimulation phosphorylated FOXO1 as we had anticipated. Time-course experiment showed that IGF-I-stimulated FOXO1 phosphorylation was preceded by the activation of Akt and Erk. Peak phosphorylation of FOXO1 occurred 15–30 min after IGF-I stimulation. Peak activation of Akt and Erk occurred within 5 min of IGF-I stimulation. Dose-response studies showed that IGF-I phosphorylated FOXO1 at a concentration (5 nM) that specifically interacts with IGF-I receptors in cardiomyocytes (Wang *et al.* 1998).

Insulin stimulation also induced phosphorylation of FOXO1 in cardiomyocytes (Fig. 2). The time-course effects of insulin on FOXO1, Akt, and Erk were similar to that of IGF-I. However, insulin stimulation of FOXO1 phosphorylation peaked earlier than IGF-I stimulation. Dose-response study showed that insulin phosphorylated FOXO1 at a concentration (10 nM) specific for the activation of insulin receptors. These experiments indicate that FOXO1 is a downstream step of insulin receptor and IGF-I receptor signaling in cardiomyocytes.

To determine whether IGF-I/insulin stimulation of FOXO1 phosphorylation is downstream from activation of Akt and Erk, chemical inhibitors of PI3-K and MEK were added to the culture media prior to IGF-I/insulin stimulation. PI3-K inhibitor (LY294002) inhibited IGF-I/insulin effect on FOXO1 phosphorylation (Fig. 3), while MEK inhibitor (PD98059) minimally inhibited phosphorylation of FOXO1 by IGF-I/insulin. These findings demonstrated the indispensable role of PI3-K/Akt-signaling pathway in the regulation of FOXO1 phosphorylation in cardiomyocytes.

IGF-I downregulated the abundance of FOXO1 and increased ubiquitination of FOXO1

To determine whether IGF-I can modulate the abundance of FOXO1 protein, cardiomyocytes were incubated with vehicles or IGF-I (10 nM) after overnight serum deprivation (Fig. 4). The abundance of FOXO1 remained unchanged in the cardiomyocytes treated with vehicles. However, the content of FOXO1 was decreased by 67% after 18 h of IGF-I treatment (Fig. 4B). These findings suggested that IGF-I may modulate FOXO1 protein turnover. Since ubiquitinated FOXO1 leads to its proteosomal degradation (Huang *et al.* 2006), we next studied whether IGF-I can regulate ubiquitination of FOXO1. To this end, cardiomyocytes were treated with vehicle or IGF-I (10 nM) for 20 h, and cell lysates were immunoprecipitated

with anti-ubiquitin monoclonal antibody and immunoblotted with anti-FOXO1 antibody (Fig. 4C). The results showed that IGF-I increased ubiquitination of FOXO1 in cardiomyocytes. Moreover, the effect was inhibited by LY294002, suggesting that the PI3-K/Akt pathway mediated the effect of IGF-I on FOXO1 ubiquitination.

Overexpression of constitutively active FOXO1 upregulated the abundance of IGF-I and insulin receptors and augmented receptor signaling

To explore whether FOXO1 can modulate IGF-I and insulin receptors, cultured neonatal cardiomyocytes were infected with control adenoviral vector or adenoviral vector carrying constitutive FOXO1 (Ad-FOXO1) for 48 h. The abundance of IGF-I and insulin receptors was determined by immunoblotting. The results showed that both insulin and IGF-I receptor proteins were upregulated by approximately 2.5-fold (Fig. 5). To characterize the effects of FOXO1 on IGF-I and insulin receptor signaling, cardiomyocytes were infected with Ad-SR or constitutively active Ad-FOXO1 and then treated with IGF-I (5 nM) or insulin (10 nM; Fig. 6). Cell lysates were immunoprecipitated with specific antibodies against IGF-I or insulin receptor, and then immunoblotted with anti-phosphotyrosine antibodies. IGF-I-stimulated autophosphorylation of IGF-I receptor and insulin-stimulated autophosphorylation of insulin receptor were respectively enhanced in the cardiomyocytes infected with constitutively active FOXO1. In this experiment, augmentation of IGF-I and insulin receptor autophosphorylation can be explained by the increased receptor abundance in the cells infected with Ad-FOXO1 because the stoichiometry of receptor phosphorylation upon insulin/IGF-I stimulation was not altered (Fig. 6C).

To explore signaling pathways downstream from receptor, activation of MEK and Akt was defined by immunoblotting (Fig. 7). Overexpression of constitutive active FOXO1 increased the basal phosphorylation of MEK and Akt, but the protein abundance of MEK and Akt was not altered. IGF-I-stimulated MEK phosphorylation and Akt phosphorylation were accordingly increased in the cardiomyocytes infected with constitutively active FOXO1. Thus, in addition to its effect on receptor, FOXO1 may modulate downstream signaling steps in cardiomyocytes. These findings suggest a feedback regulatory mechanism between insulin/IGF-I receptor signaling and FOXO1.

Discussion

Our data provided novel evidence for bidirectional regulation of IGF-I/insulin receptor signaling and FOXO1 in cardiac muscle cells. We have demonstrated, for the first time, that FOXO1 can modulate IGF-I receptor signaling in addition to its effect on insulin receptor signaling. IGF-I and insulin are known to regulate FOXO1 (Hribal *et al.* 2003, Sartorelli & Fulco 2004, Stitt *et al.* 2004); therefore, we had anticipated

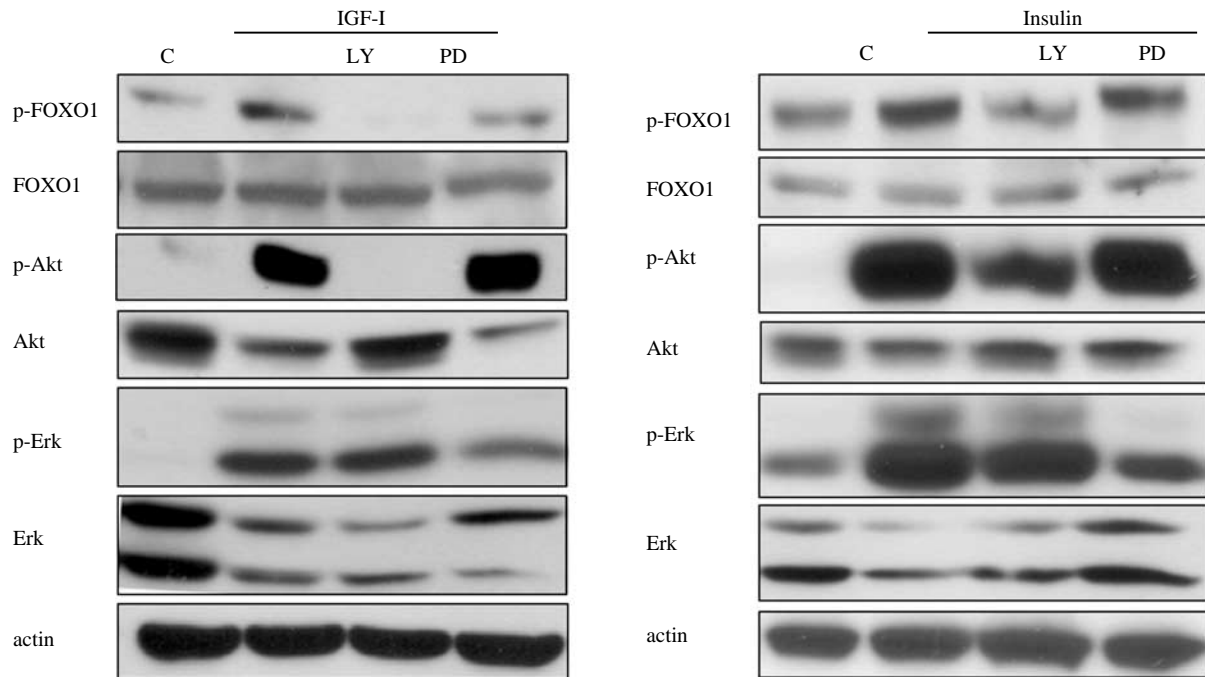


Figure 3 IGF-I and insulin phosphorylated FOXO1 via PI3-K-signaling pathway. Cardiomyocytes were pretreated with MEK inhibitor (PD98059, 15 μ M; PD) or PI3-K inhibitor (LY294002, 15 μ M; LY) for 45 min prior to IGF-I (10 nM) or insulin (100 nM) stimulation. The abundance of phospho-FOXO1 was analyzed with phospho-specific antibodies by immunoblotting. The abundance of actin served as loading control. Phosphorylation of FOXO1 by IGF-I and insulin was suppressed by PI3-K inhibitor LY294002.

that IGF-I and insulin receptor signaling would modulate FOXO1 phosphorylation in cardiomyocytes. The observation that constitutively active FOXO1 upregulated insulin IGF-I receptor and that constitutively active FOXO1 activates MEK and Akt suggest the existence of feedback regulatory mechanisms between FOXO1 and IGF-I/insulin

receptor and between FOXO1 and Akt/Erk. Taken together, this study demonstrated elegantly coordinated regulation between upstream insulin/IGF-I receptor signaling and downstream FOXO1 in cardiac muscle cells.

Cardiomyocytes are a good model to study the interplay of insulin/IGF-I receptor signaling and FOXO1. IGF-I and

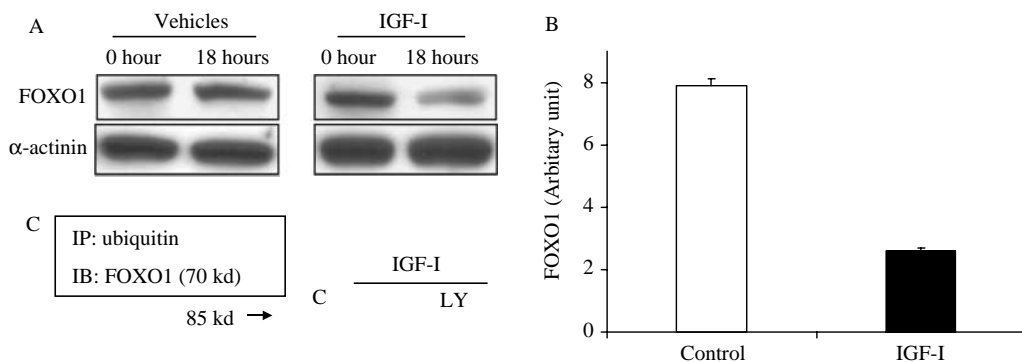


Figure 4 IGF-I downregulated FOXO1 abundance and increased ubiquitination of FOXO1. Serum-deprived cardiomyocytes were incubated with vehicles or IGF-I (10 nM) for 18 h. The abundance of FOXO1 was determined by immunoblotting. (A) The effect of IGF-I on the abundance of FOXO1. Basal level of FOXO1 was unchanged during 18 h of experiments. (B) The results from four independent experiments were summarized in the graph, IGF-I treatment decreased FOXO1 levels by 67% ($P < 0.01$). (C) The effect of IGF-I on FOXO1 ubiquitination was mediated via PI3-K pathway. When indicated, cardiomyocytes were pretreated with PI3-K inhibitor (LY294002) for 45 min, and then incubated with vehicles or IGF-I for 20 h. Cell lysates were immunoprecipitated with anti-ubiquitin antibody (IP: ubiquitin) and then immunoblotted with anti-FOXO1 antibody (IB: FOXO1). PI3-K inhibitor LY294002 suppressed the effect of IGF-I on FOXO1 ubiquitination.

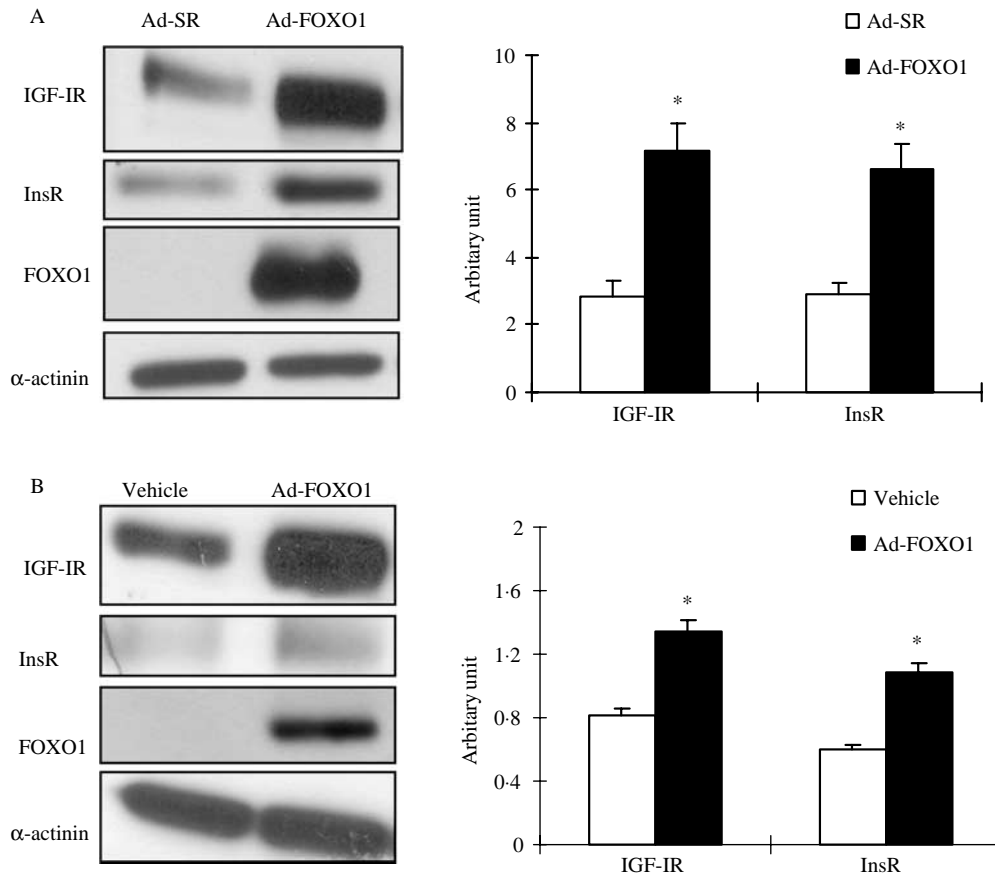


Figure 5 Overexpression of constitutively active FOXO1 upregulated the abundance of IGF-I and insulin receptors. Cardiomyocytes were transduced with control adenoviral vector (Ad-SR) or adenoviral vector carrying active FOXO1 (Ad-FOXO1) for 48 h. The abundance of IGF-I and insulin receptors was determined by immunoblotting with antibodies against IGF-I receptor (IGF-IR) or insulin receptor (InsR) β subunits. The abundance of IGF-I and insulin receptor in the cardiomyocytes transduced with Ad-FOXO1 was compared with (A) the cardiomyocytes transduced with Ad-SR or (B) the cardiomyocytes treated with vehicles. Overexpression of constitutively active FOXO1 upregulated the abundance of IGF-I and insulin receptors by two- to threefold. The graphs represent data summarized from four independent experiments. * $P < 0.05$.

insulin exert pleiotropic actions via overlapping signaling pathways in cardiac muscle cells (Abel 2004, Saetrum & Wang 2005, Taniguchi *et al.* 2006). In our dose-response experiment, moderate concentration of IGF-I and insulin was able to phosphorylate FOXO1 in cardiomyocytes, indicating that these two peptide hormones independently regulated FOXO1 through its specific receptor. FOXO transcription factor family is part of the IGF-I/insulin-signaling network in mammalian cells (Barthel *et al.* 2005, Morris *et al.* 2005). How IGF-I/insulin modulates FOXO transcription factors in cardiomyocytes has rarely been explored in the past. In the present study, we showed that insulin and IGF-I receptor signaling modulated FOXO1 through at least two different mechanisms, by phosphorylating FOXO1 and by downregulating FOXO1 protein. In addition to phosphorylation and ubiquitination, acetylation of FOXO1 in specified sites located in the DNA-binding

domain may inhibit FOXO binding to DNA and thereby prevents FOXO-dependent transcription (Perrot & Rechler 2005). Whether insulin/IGF-I can modulate FOXO1 acetylation and FOXO1-dependent gene transcription in cardiac muscle should be explored in future study.

Previous studies had shown that insulin receptor signaling modulated FOXO by phosphorylating FOXO at Ser/Thr residues and hence prevented FOXO translocation from cytoplasmic compartment into nucleus, and downregulated transcription of target genes (Puig *et al.* 2003). Several groups have shown that FOXO1 is inhibited by insulin through PI3-K pathways (Biggs *et al.* 1999, Brunet *et al.* 1999, Guo *et al.* 1999, Nakae *et al.* 2001, Arden 2004). FOXO1 regulates expression of genes involved in glucose metabolism and apoptosis (Hall *et al.* 2000, Barthel *et al.* 2005). Altering the abundance of FOXO1 could modulate its function. For example, knockdown of FOXO1 expression resulted in more

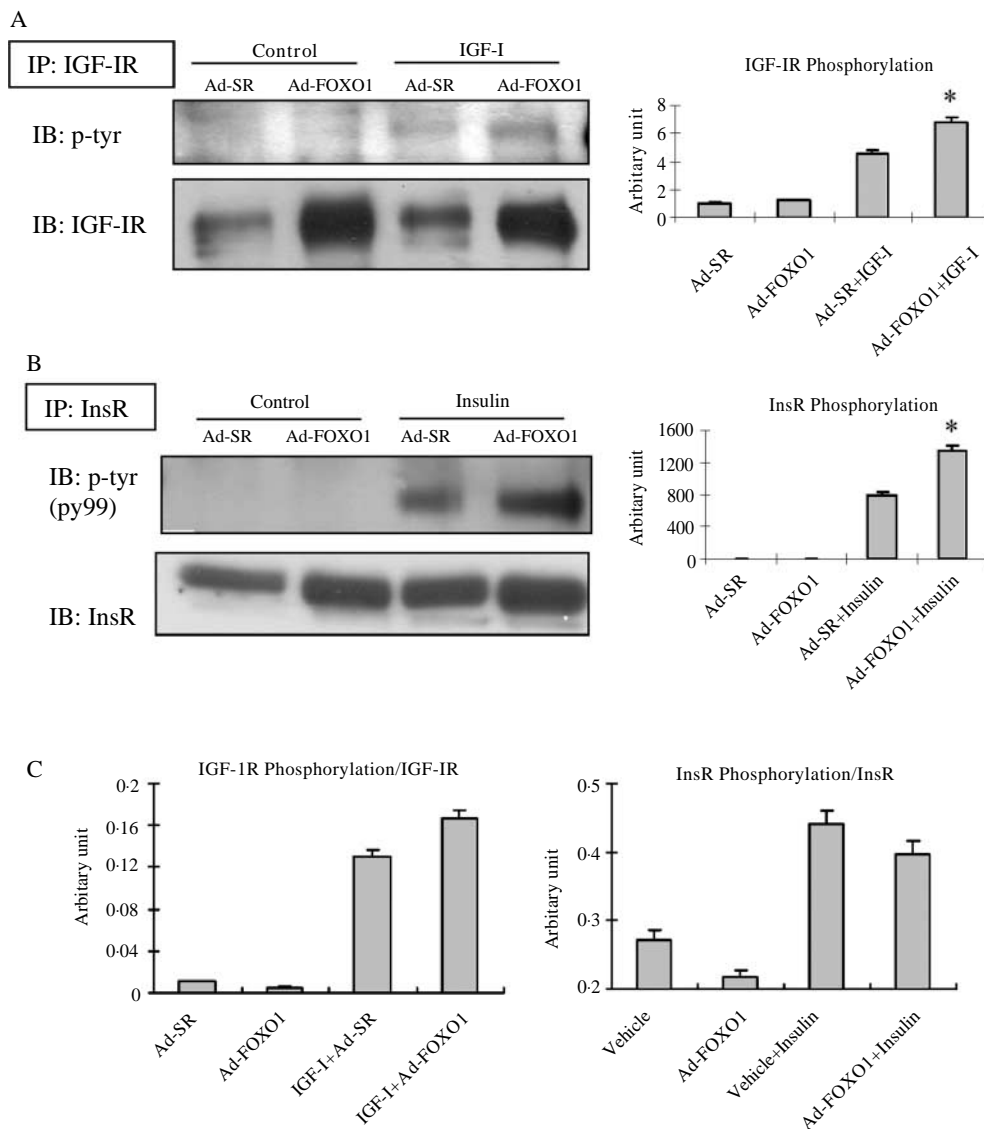


Figure 6 Overexpression of constitutively active FOXO1 augmented phosphorylation of IGF-I and insulin receptors. Cardiomyocytes were transduced with control adenoviral vector (Ad-SR) or adenoviral vector carrying FOXO1 (Ad-FOXO1) for 48 h. To stimulate phosphorylation of IGF-I and insulin receptors, the cells were treated with (A) IGF-I (5 nM) or (B) insulin (10 nM) for 2 min after overnight serum deprivation. At these concentrations, IGF-I and insulin do not cross-react with each other's receptor. Phosphorylation of IGF-I and insulin receptors was investigated by immunoprecipitation (IP) with anti-IGF-I receptor or anti-insulin receptor antibodies and then immunoblotted (IB) with anti-phosphotyrosine antibodies. PVDF membranes were stripped and re-probed with anti-IGF-I receptor or anti-insulin receptor antibodies to determine the abundance of IGF-I and insulin receptor. The bar graph represents data summarized (mean \pm S.E.) from three independent experiments. Overexpression of active FOXO1 augmented phosphorylation of IGF-I receptor by approximately 50%. Overexpression of active FOXO1 augmented phosphorylation of insulin receptor by 71%. (C) Stoichiometry of receptor phosphorylation. This graph showed the stoichiometry of IGF-I and insulin receptor tyrosine phosphorylation in the cardiomyocytes transduced with Ad-SR or Ad-FOXO1. Constitutively active FOXO1 did not alter the stoichiometry of receptor phosphorylation upon IGF-I/insulin stimulation. * $P < 0.05$, Ad-FOXO1 versus Ad-SR-infected cells.

efficient differentiation of myoblasts, and overexpression of FOXO1 increased transcriptional regulatory function (Hall *et al.* 2000, 2003). Insulin phosphorylation of FOXO1 enhanced ubiquitination of FOXO1 and helped target

FOXO1 for degradation (Matsuzaki *et al.* 2003, Aoki *et al.* 2004). In our study, IGF-I has similar effects on FOXO1. IGF-I enhanced FOXO1 ubiquitination and reduced FOXO1 abundance in cardiomyocytes. PI3-K pathway played a pivotal

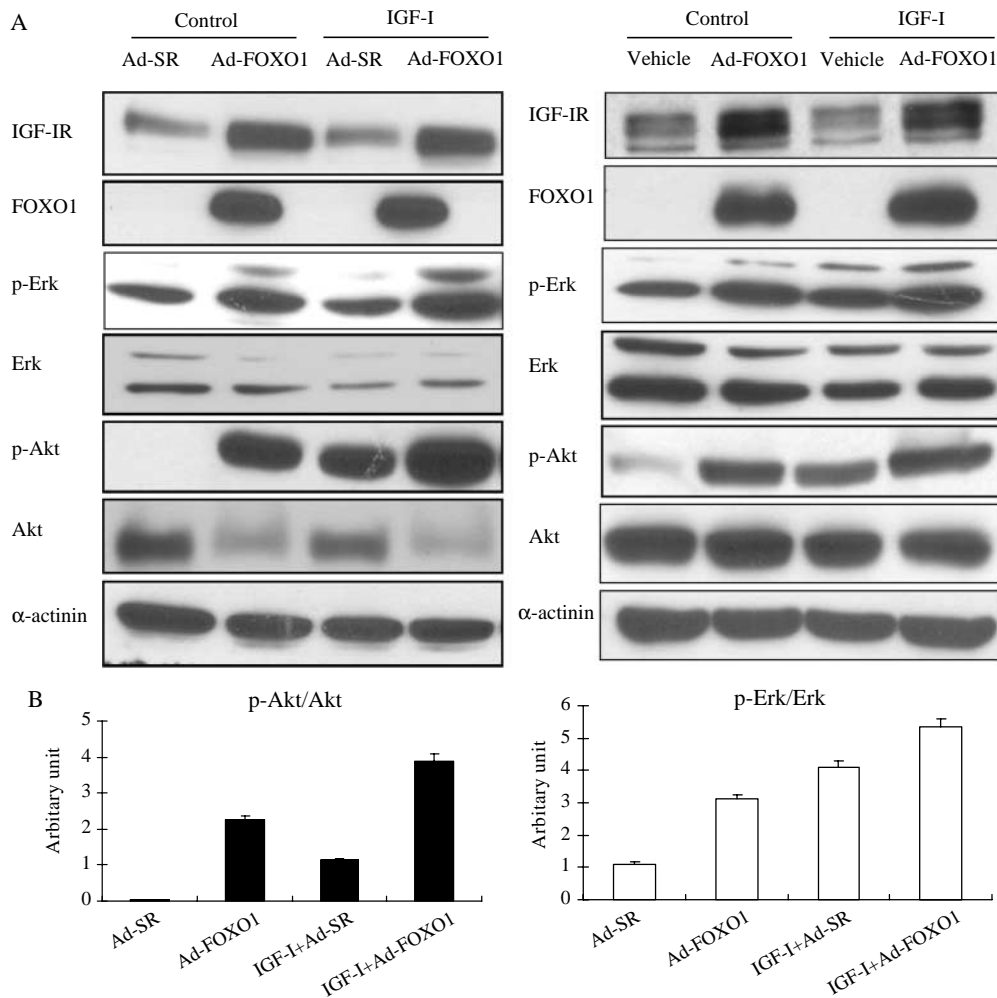


Figure 7 Constitutively active FOXO1 activated PI3-K and MEK signaling. (A) Overexpression of FOXO1 stimulated phosphorylation of MEK and Akt in cardiomyocytes. When stimulated with IGF-I, activation of MEK and Akt was further enhanced. Cardiomyocytes were transduced with control adenoviral vector (Ad-SR) or adenoviral vector carrying FOXO1 (Ad-FOXO1) for 48 h (left panel). The effect of Ad-FOXO1 was also compared with the cells treated with vehicles without adenoviral infection (right panel). Activation of MEK and Akt was determined by immunoblotting with anti-phospho-Akt and anti-phospho-MEK antibodies. Immunoblotting with α -actinin served as loading control. When indicated, the cells were treated with IGF-I (5 nM) after overnight serum deprivation. (B) The effect of IGF-I on Akt and Erk phosphorylation (P). The bar graphs (mean \pm S.E.) represent the results summarized from three independent experiments.

role in this process. PI3-K/Akt pathway mediated IGF-I/insulin receptor signaling and mediated the effect of IGF-I/insulin on phosphorylation and ubiquitination of FOXO1. Moreover, FOXO1 can modulate activation of Akt without altering the abundance of Akt protein. Although both insulin and IGF-I modulate FOXO1 phosphorylation via PI3-kinase pathway, our results suggested that insulin phosphorylation of FOXO1 may be less dependent on PI3-kinase than IGF-I (Fig. 3). Whether differences in post-receptor-signaling pathways can explain the effect of insulin and IGF-I on PI3-kinase-dependent phosphorylation of FOXO1 should be clarified in future study.

Insulin and IGF systems do not diverge in *Drosophila* and share the same prototype insulin-like peptide and its receptors. The existence of a potential feedback control mechanism between FOXO and insulin receptor was first discovered in *Drosophila*. The *Drosophila* equivalent of FOXO, dFOXO, transcriptionally activates upstream targets of the insulin-signaling cascade (Puig *et al.* 2003). Subsequently, it was discovered that dFOXO increased transcription of *Drosophila* equivalent of insulin receptor gene (Puig *et al.* 2003). These investigators proposed that this feedback mechanism allowed the cells to regulate growth and metabolism by responding rapidly to changes in nutrients (Puig *et al.* 2003). When nutrients are abundant,

activation of the dInR pathway will inactivate dFOXO and hence downregulate dInR transcription. Under situations in which nutrients are limiting, the dInR signaling diminishes and frees dFOXO to upregulate dInR. Puig *et al.* (2003) further hypothesized that upregulation of dInR by dFOXO will prepare the flies for growth when nutrients become abundant.

With evolution, the prototype insulin/insulin receptor system developed into specialized insulin and IGF-I systems to coordinate regulation of metabolism and growth in mammals (LeRoith *et al.* 1993, Upton *et al.* 1998). Mammalian regulation of metabolism and growth is considerably more complicated than *Drosophila*, and transcriptional regulatory changes of insulin receptor and IGF-I receptor in mammalian cells do not necessarily synchronize with each other. However, identifying a signaling protein (FOXO1) that serves as a common downstream step of receptor signaling and a common point of feedback regulation of receptor signaling for both insulin receptor and IGF-I receptor entails the evolutionary relationship of these two receptor signaling systems. The interplay between insulin/IGF-I receptor, Akt and Erk signaling, and FOXO1 may provide a new paradigm to understand how insulin and IGF-I modulate cardiac muscle structure and function.

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