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Abstract—Upregulation of the sympathetic nervous system plays a key role in the pathogenesis of insulin resistance. Although the heart is a target organ of insulin, few studies have examined the mechanisms by which β-adrenergic stimulation affects insulin sensitivity in cardiac muscle. In this study, we explored the molecular mechanisms involved in the regulation of the cross-talk between β adrenergic and insulin receptors in neonatal rat cardiomyocytes and in transgenic mice with cardiac overexpression of a constitutively active mutant of Akt (E40K Tg). The results of this study show that β-adrenergic receptor stimulation has a biphasic effect on insulin-stimulated glucose uptake. Short-term stimulation induces an additive effect on insulin-induced glucose uptake, and this effect is mediated by phosphorylation of Akt in threonine 308 through PKA/Ca^{2+}-dependent and PI3K-independent pathway, whereas insulin-evoked threonine phosphorylation of Akt is exclusively PI3K-dependent. On the other hand, long-term stimulation of β-adrenergic receptors inhibits both insulin-stimulated glucose uptake and insulin-induced autophosphorylation of the insulin receptor, and at the same time promotes threonine phosphorylation of the insulin receptor. This is mediated by serine 473 phosphorylation of Akt through PKA/Ca^{2+} and PI3K-dependent pathways. Under basal conditions, E40K Tg mice show increased levels of threonine phosphorylation of the β subunit of the insulin receptor and blunted tyrosine autophosphorylation of the β-subunit of the insulin receptor after insulin stimulation. These results indicate that, in cardiomyocytes, β-adrenergic receptor stimulation impairs insulin signaling transduction machinery through an Akt-dependent pathway, suggesting that Akt is critically involved in the regulation of insulin sensitivity. **(*Circ Res*. 2005;96:180-188.)**

Key Words: glucose uptake ■ isoproterenol ■ insulin resistance ■ protein kinase A ■ L-type Ca^{2+} channel

Insulin resistance plays an important role in the pathogenesis of diabetes,[1,2] obesity,[3] and hypertension,[4,5] and is also a common feature of heart failure.[6,7] Dysregulation of the sympathetic nervous system has been reported in obesity,[8] contributes to the etiology of hypertension,[9,10] leads to an adverse prognosis in heart failure,[11] and is involved in the pathogenesis of insulin resistance.[7,9]

The term "insulin resistance" refers to the action of insulin on glucose homeostasis, and it has been demonstrated that skeletal muscle[12] and adipose tissue[13] are the organs that mainly participate in the development of insulin resistance. Cardiac muscle is also a target of insulin,[14] and impairment of insulin-stimulated cardiac glucose uptake has been described in animal models of diabetes,[15] obesity,[16] and hypertension.[17,18] However, few studies have investigated the cross-talk between β-adrenergic and insulin signaling in the heart.

Binding of insulin to its receptor activates the tyrosine kinase activity of the β-subunit of the receptor,[19] leading to autophosphorylation, as well as tyrosine phosphorylation of several insulin receptor (IR) substrates. These, in turn, interact with phosphatidylinositol 3-kinase (PI3K). Activation of PI3K stimulates the downstream effector Akt, a serine/threonine kinase, which induces glucose uptake through the translocation of the glucose transporter GLUT4 to the plasma membrane.[20] Abnormalities of insulin signaling account for insulin resistance. Several mechanisms have been described as being responsible for the inhibition of insulin-stimulated tyrosine phosphorylation of IR, including proteasome-mediated degradation,[21] phosphatase-mediated dephosphorylation,[22] and kinase-mediated serine/threonine phosphorylation.[23]

The cross-talk between β-adrenergic and insulin signaling has been investigated by Klein et al[24] that have demonstrated in cultured adipocytes that insulin-induced glucose uptake and tyrosine phosphorylation of the IR were inhibited by β_3-adrenergic receptor (AR) stimulation.

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Myocardial βARs consist of β_1 and β_2 subtypes,[25] although recent evidence suggests that a small population of the β_3 subtype also exists in the heart.[26] Ligand binding to different βAR subtypes activates different signaling mechanisms.[27] Therefore, depending on the distribution of βAR subtypes, different cell types have specific molecular mechanisms involved in the regulation of the cross-talk between the insulin and β-adrenergic systems. Because Akt is a serine/threonine kinase, which can be activated by βAR,[28] it is reasonable to hypothesize that, after βAR stimulation, Akt phosphorylates the β-subunit of IR.

In this study, we explored in cardiomyocytes and in transgenic mice the mechanisms involved in the regulation of the cross-talk between the β-adrenergic and insulin systems. We examined (1) the effects of short- and long-term stimulation of βAR on insulin-induced glucose uptake, (2) whether Akt participates in the cross-talk between insulin and βAR, and (3) whether cardiac overexpression of a constitutively active mutant of Akt impairs insulin signaling in vivo.

Materials and Methods

Primary Cultures of Neonatal Rat Ventricular Cardiomyocytes

Primary cultures of neonatal cardiomyocytes were prepared as we have previously described.[29]

Glucose Uptake Assays

Cardiomyocytes were grown in 12-well plates. 2-Deoxyglucose (2DG) uptake was determined by the method of Moyers et al[30] with few modifications (for details, see the online data supplement available at http://circres.ahajournals.org).

Immunoblotting

Cardiomyocytes were grown in 6-well plates. At the end of the stimulation period, the medium was removed, the cells washed twice with ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco PBS, and lysed with 100 μL of ice-cold lysis buffer A (see online data supplement). Phosphorylation of Akt was detected with anti–phospho-Akt (Ser473) (Cell Signaling Technology) and with anti–phospho-Akt (Thr308) (Cell Signaling Technology), Akt was determined with anti-Akt antibody (Cell Signaling Technology). Horseradish peroxidase-conjugated (Cell Signaling Technology) antibody was used as secondary antibody. The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Pharmacia Biotec).

Immunoprecipitation and Detection of Phospho-Akt Substrate

Cardiomyocytes were grown in 60-mm dishes. At the end of the stimulation period, the medium was removed, the cells washed twice with ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco PBS and lysed with 1 mL of ice-cold lysis buffer B (see online data supplement). IR was immunoprecipitated with anti-IR β-subunit (Santa Cruz Biotechnology, Inc) and with protein A/G-Sepharose slurry (Santa Cruz Biotechnology, Inc). Immunoprecipitates were subjected to SDS-PAGE, transferred to a polyvinylidene diflouride membrane, and immunoblotted with anti-IR β-subunit (Santa Cruz Biotechnology, Inc), anti-phosphotyrosine (Cell Signaling Technology), and anti-phosphothreonine (Cell Signaling Technology) antibodies, and with anti–phospho-Akt substrate antibody (Cell Signaling Technology).

Akt Kinase Activity Assay

Kinase activity of Akt was measured by the immune complex kinase assay as we have previously described.[28]

Adenovirus Transduction

Cardiomyocytes were infected with adenoviruses harboring dominant-negative (Ad5 CMV Akt [K179M]) and constitutively active (Akt, E40K) Akt. Adenovirus harboring lacZ (Ad5 · CMV-β-galactosidase) was used as a control. The method of adenovirus infection has been previously described.[31]

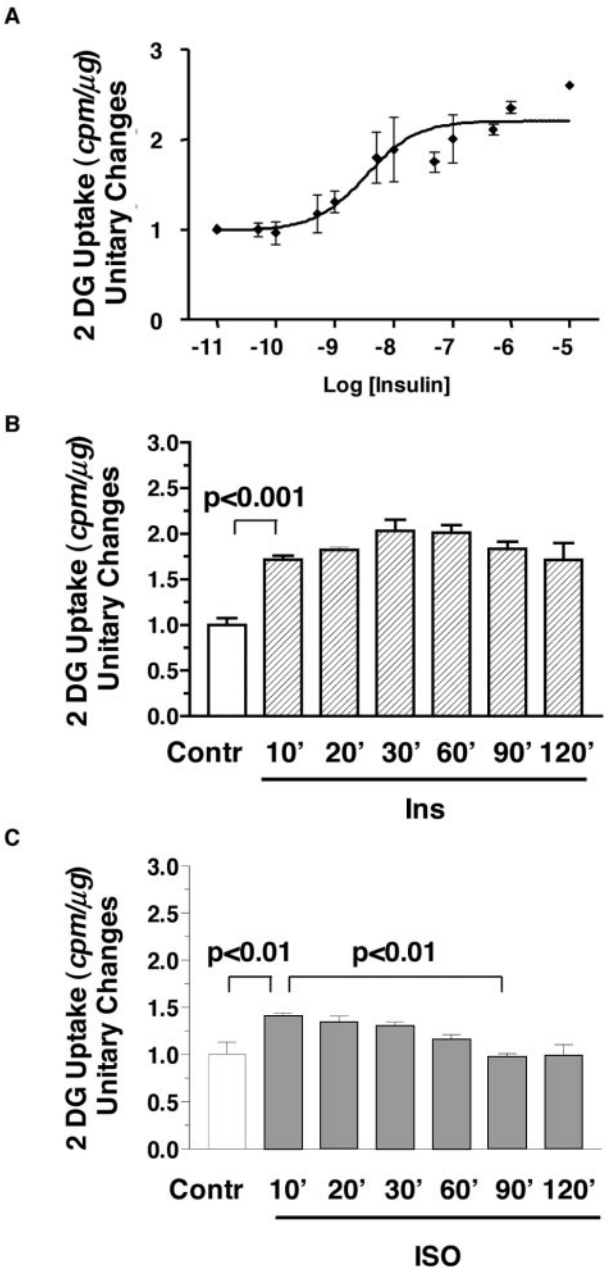

Figure 1. Insulin and βAR stimulation dose- and time-dependently increase 2-deoxyglucose uptake. A, Cardiomyocytes were stimulated for 30 minutes with graded doses of insulin ranging from 10 pmol/L to 10 μmol/L, and the rate of 2-deoxyglucose (2DG) uptake was determined. Graphics show the mean±SEM of 3 independent experiments. B, Cardiomyocytes were stimulated with insulin (Ins, 100 nmol/L) for the times indicated, and the rate of 2DG uptake was determined. C, Cardiomyocytes were stimulated with isoproterenol (ISO, 10 μmol/L), and the rate of 2DG uptake was determined. Values of 2DG uptake (cpm) were adjusted by the protein content (μg). B and C, Mean±SEM of 5 independent experiments.

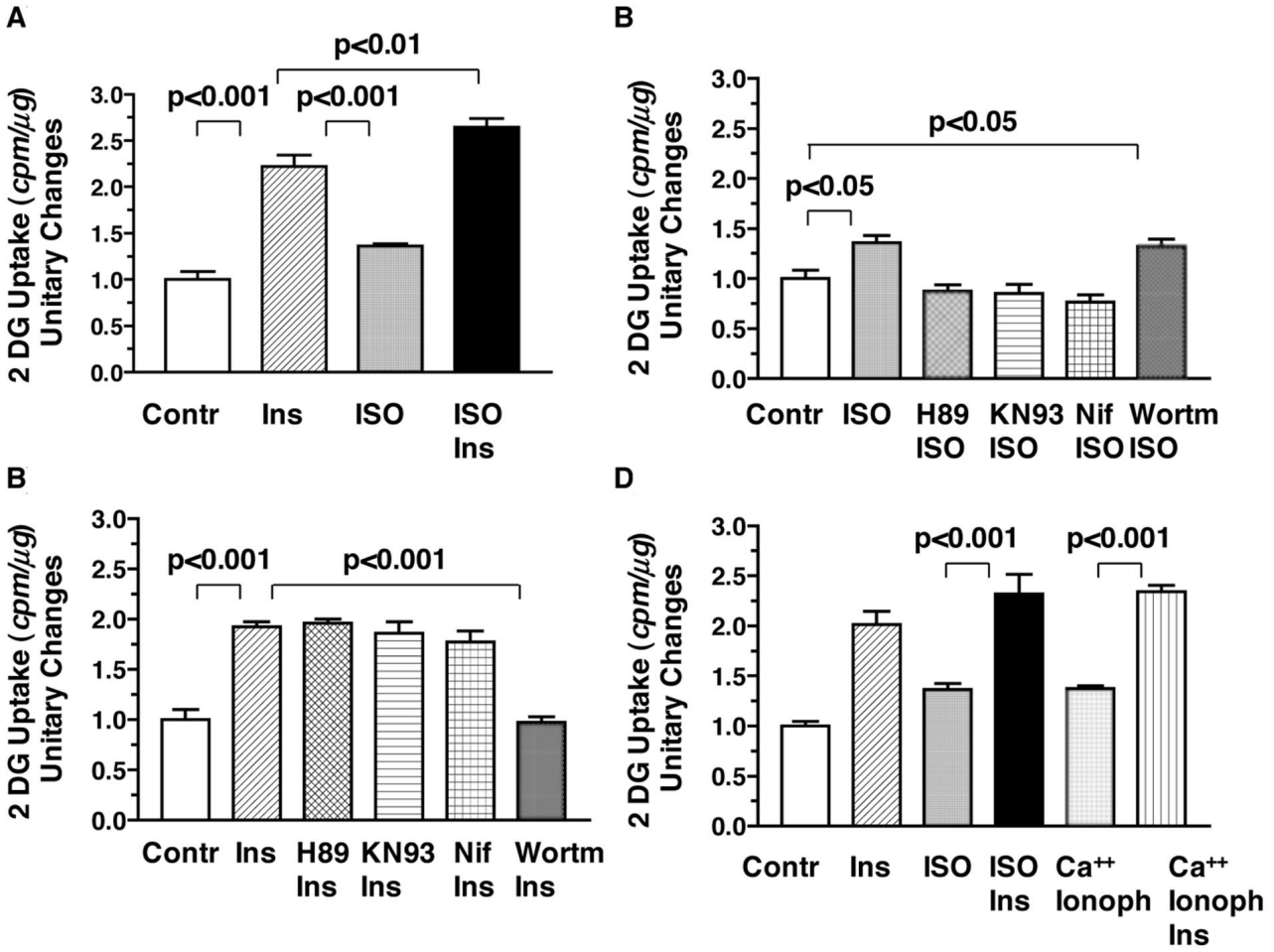

Figure 2. Insulin and βAR stimulation induce 2-deoxyglucose uptake by two different mechanisms. A, Cardiomyocytes were stimulated for 30 minutes with Ins (100 nmol/L), for 10 minutes with ISO (10 μmol/L), and with ISO+Ins (10 and 30 minutes, respectively), and the rate of 2DG uptake was determined. B, Cardiomyocytes were stimulated with ISO for 10 minutes in the absence or presence of pretreatment with H89 (10 μmol/L, 60 minutes), KN93 (0.2 μmol/L, 30 minutes), nifedipine (0.1 μmol/L, 60 minutes), and wortmanin (10 nmol/L, 30 minutes), and the rate of 2DG uptake was determined. C, Cardiomyocytes were stimulated with Ins for 30 minutes in the absence or presence of pretreatment with H89, KN93, nifedipine, and wortmanin, and the rate of 2DG uptake was determined. D, Cardiomyocytes were stimulated for 30 minutes with Ins, for 10 minutes with ISO, with ISO+Ins, and with Ca^{2+} ionophore A23187 (1 μmol/L, 6 hours) with or without Ins, and the rate of 2DG uptake was determined. Values of 2DG uptake (cpm) were adjusted by the protein content of the dish (μg). Graphs show the mean±SEM of 5 independent experiments.

In Vivo Studies

Three-month-old transgenic (Tg) mice with cardiac specific overexpression of constitutively active mutant (E40K) of Akt^{32} and wild-type controls were studied (for details, see online data supplement)

Statistics

Data are given as mean±SEM. Statistical analyses were performed using analysis of variance. The posttest comparison was performed by the method of Tukey. Significance was accepted at $P<0.05$ levels.

Results

Characteristics of Insulin- and ISO-Induced 2DG Uptake in Neonatal Cardiomyocytes

We first assessed the dose-response relationship and the time course of insulin-induced 2DG uptake. Thirty minutes of insulin stimulation dose-dependently increased 2DG uptake. A significant increase in 2DG uptake was observed at 1 nmol/L, and it reached a plateau at 100 nmol/L, with an EC_{50} of 3.25 nmol/L (Figure 1A). Insulin and isoproterenol (ISO), a βAR agonist, both induced 2DG uptake, peaking after 30 (Figure 1B) and 10 (Figure 1C) minutes, respectively.

Short-Term β-Adrenergic Stimulation Increases Insulin-Induced 2DG Uptake Through a PKA/Ca^{2+}-Dependent Pathway

Next, we evaluated the effects of short-term βAR stimulation on insulin-induced 2DG uptake. Ten minutes of stimulation with ISO enhanced 2DG uptake by 36±2% ($P<0.05$ versus control). However, the increases in 2DG uptake were not as large as that obtained with insulin (100 nmol/L), which induced an increase of 122±12%. Pretreatment of cardiomyocytes with ISO for 10 minutes had an additive effect on insulin-induced 2DG uptake (164±9% versus control), suggesting that insulin and ISO use two different mechanisms to stimulate 2DG uptake (Figure 2A). Therefore, we examined the mechanisms that account for ISO and insulin-induced 2DG uptake. βARs act through the cAMP/PKA/L-type Ca^{2+}

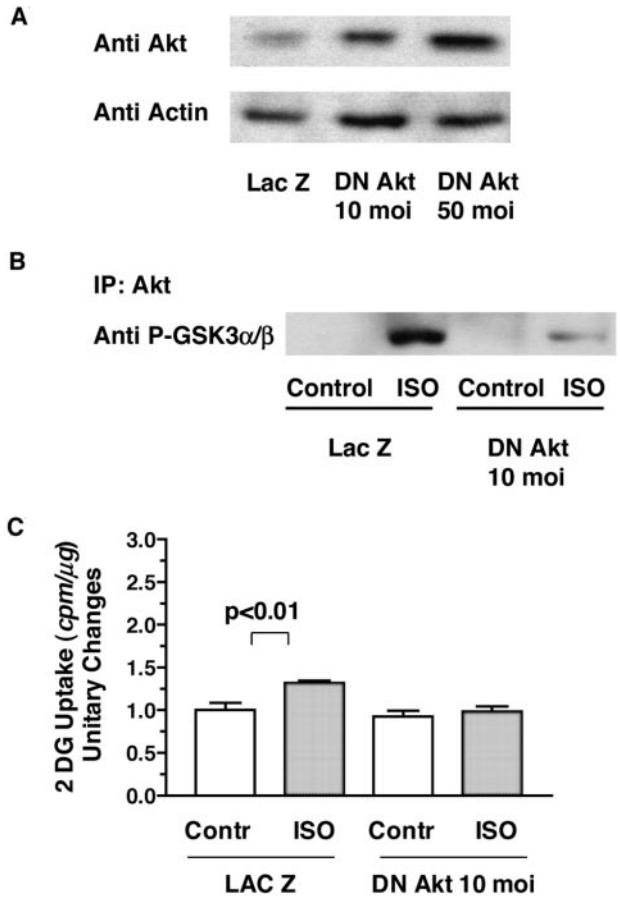

Figure 3. βAR stimulation induces 2-deoxyglucose uptake through an Akt-dependent pathway. A, Cardiomyocytes were infected with the indicated doses of recombinant adenovirus harboring dominant-negative Akt (DN-Akt) or control adenovirus harboring Lac Z, and cultured under serum-free conditions for 48 hours. Top, Immunoblot with anti-Akt antibody shows the expression level of transgenes in infected myocytes. Bottom, Immunoblot with anti-actin antibody shows the expression level of actin in infected myocytes. B, Cardiomyocytes were infected with LacZ and DN-Akt, cultured for 48 hours, and then stimulated with ISO (10 μmol/L) for 10 minutes. Kinase activity of Akt was determined. Similar results were obtained in four other experiments. C, Cardiomyocytes were infected with LacZ and DN-Akt, cultured under serum-free conditions for 48 hours, and then stimulated with ISO for 10 minutes (10 μmol/L). Rate of 2DG uptake was determined. Values of 2DG uptake (cpm) were adjusted by the protein content of the dish (μg). Graph shows the mean±SEM of 5 independent experiments.

channel–dependent pathway,[33] whereas IR uses mainly the IRS1–2/PI3K–dependent pathway. Inhibition of PKA, and kinase Ca^{2+}-calmodulin–dependent, obtained with H89 and KN93, respectively, as well as the Ca^{2+} antagonist, nifedipine, blocked short-term ISO-induced 2DG uptake (Figure 2B), whereas PI3K inhibition by wortmanin did not. In contrast, insulin-induced 2DG uptake was found to be PI3K dependent (Figure 2C). Because the effects of βAR simulation on 2DG uptake seemed to be mediated by a Ca^{2+}-dependent pathway, we investigated the role of Ca^{2+} in the regulation of 2DG uptake. Treatment of cardiomyocytes with Ca^{2+} ionophore A23187 increased 2DG uptake by 37±3% ($P<0.05$ versus control), and enhanced the effect of insulin to the same extent as that of ISO (Figure 2D).

These data suggest that insulin and βAR stimulation induce 2DG uptake by PI3K-dependent and PKA/Ca^{2+}-dependent pathways, respectively.

βAR Stimulation Induces 2DG Uptake Through an Akt-Dependent Pathway

Stimulation of βAR activates Akt,[28] which is the key molecule involved in the regulation of glucose uptake.[20] Therefore, we asked if Akt is involved in ISO-induced glucose uptake. Cardiomyocytes were infected with adenovirus harboring lacZ or dominant-negative Akt (DN Akt) (Figure 3A). Immune complex kinase assays showed that 10 minutes of ISO stimulation increased kinase activity of Akt, whereas overexpression of DN Akt inhibited this response (Figure 3B). Furthermore, overexpression of DN Akt inhibited ISO-induced 2DG uptake (Figure 3C), indicating that Akt is required for ISO-induced 2DG uptake.

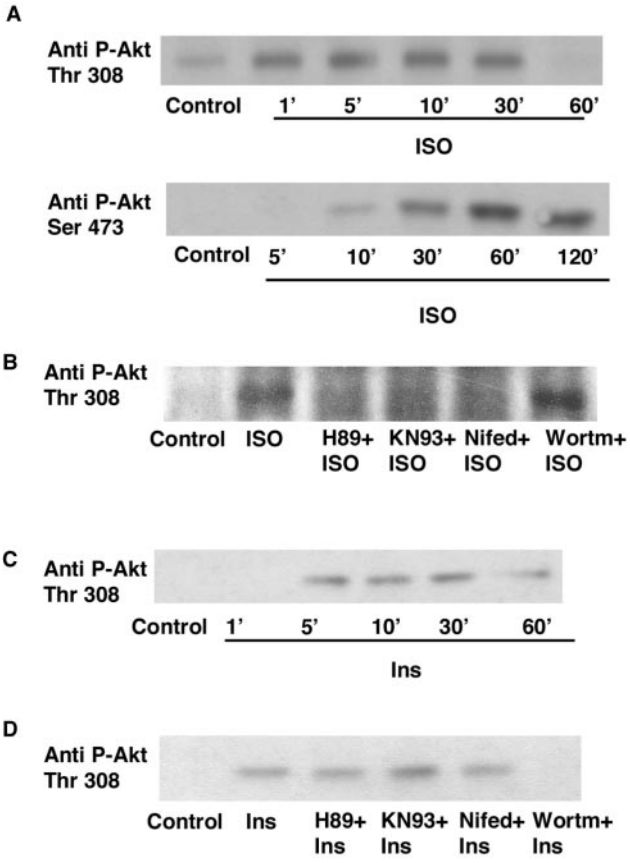

Figure 4. Short-term βAR stimulation and insulin phosphorylate Akt in Thr308 through different pathways. A, Cardiomyocytes were stimulated with ISO (10 μmol/L). Phosphorylation of Akt in threonine and in serine was detected by immunoblotting analyses with anti–phospho-Thr308 Akt (Top), and anti–phospho-Ser473 Akt (bottom) antibodies. B, Cardiomyocytes were preincubated with or without H89, KN93, nifedipine, and wortmanin, and then stimulated with ISO for 10 minutes. Phosphorylation of Akt in threonine was assessed by immunoblotting analysis. C, Cardiomyocytes were stimulated with Ins (100 nmol/L) for the indicated durations. Phosphorylation of Akt in threonine was assessed by immunoblotting analysis. D, Cardiomyocytes were preincubated with or without H89, KN93, nifedipine, and wortmanin and then stimulated with Ins for 30 minutes. Phosphorylation of Akt in threonine was assessed by immunoblotting analysis. All experiments were performed in quadruplicate.

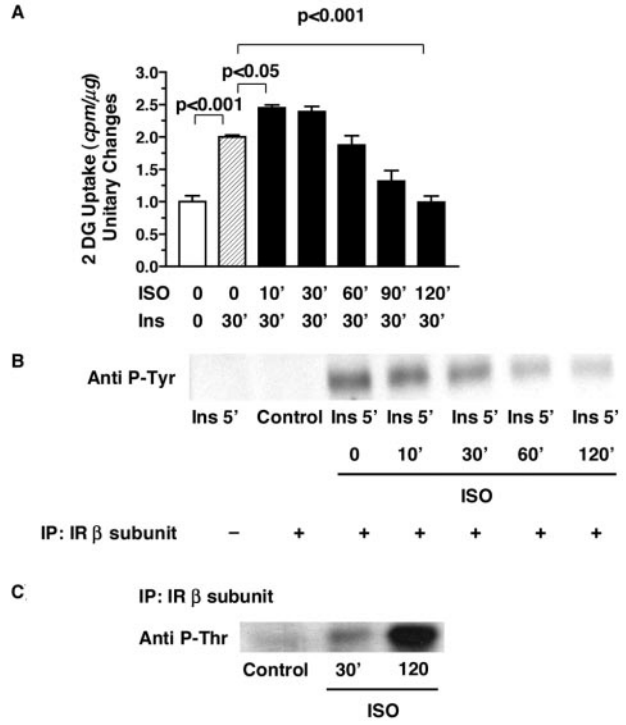

Figure 5. Long-term βAR stimulation inhibits insulin-induced 2-deoxyglucose uptake by interfering with tyrosine phosphorylation of the insulin receptor. A, Cardiomyocytes were preincubated with ISO for the times indicated, and stimulated with Ins for 30 minutes, and the rate of 2DG uptake was determined. Values of 2DG uptake (cpm) were adjusted by the protein content of the dish (μg). Graph shows the mean±SEM of 5 independent experiments. B, Cardiomyocytes were preincubated with ISO (10 μmol/L) for the times indicated and were stimulated with Ins (100 nmol/L) for 5 minutes. Protein lysates were subjected to immunoprecipitation with antibodies against insulin receptor (IR) β-subunit, followed by immunoblotting using anti-phosphotyrosine antibody. C, Cardiomyocytes were stimulated with ISO at a concentration of 10 μmol/L for the times indicated. Protein lysates were subjected to immunoprecipitation with antibodies against IR β-subunit, followed by immunoblotting using anti-phosphothreonine antibody. All experiments were performed in quadruplicate.

Short-Term βAR Stimulation and Insulin Phosphorylate Akt in Thr308 Through Different Pathways

ISO stimulation of cardiomyocytes induces Akt phosphorylation both in threonine (Thr) 308 and in serine (Ser) 473 with different time-course. In particular, ISO-induced Thr phosphorylation was detectable after 1 minute, peaked after 10 minutes, and then progressively decreased, whereas Ser phosphorylation started after 10 minutes, peaked after 60 minutes, and then decreased (Figure 4A). Considering the time course of both ISO-induced 2DG uptake and Akt Thr and Ser phosphorylation, it is likely that ISO-stimulated 2DG uptake was mediated by Thr phosphorylation of Akt. Therefore, we explored the pathway involved in ISO-induced phosphorylation of Akt in Thr308. Treatment of cardiomyocytes with H89, KN93, and nifedipine inhibited, whereas wortmanin did not affect, ISO-induced phosphorylation of Akt in Thr308 (Figure 4B). In contrast, insulin-induced phosphorylation of Akt in Thr308 was detectable after 5 minutes of stimulation (Figure 4C) and was inhibited in the presence of wortmanin (Figure 4D).

It is possible that short-term βAR stimulation and insulin use two different pathways to phosphorylate Akt in Thr308 and consequently have an additive effect on 2DG uptake.

Long-Term βAR Stimulation Inhibits Insulin-Induced 2DG Uptake by Interfering With Tyrosine Phosphorylation of IR

We next determined whether or not long-term ISO stimulation interferes with insulin-induced 2DG uptake. For this

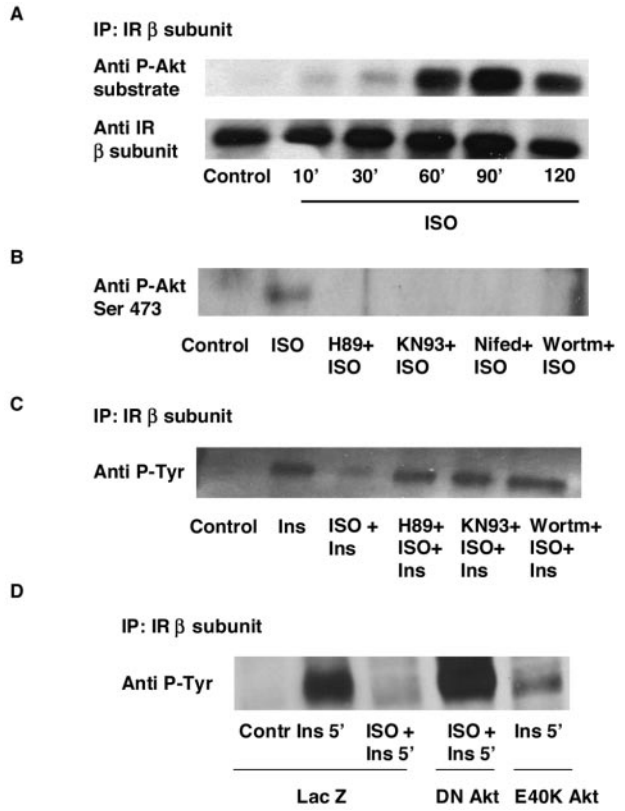

Figure 6. Akt mediates βAR stimulation–induced threonine phosphorylation of the β-subunit of the insulin receptor. A, Cardiomyocytes were stimulated with ISO (10 μmol/L) for the times indicated. Protein lysates were subjected to immunoprecipitation with antibodies against insulin receptor (IR) β-subunit, followed by immunoblotting using phospho-Akt substrate antibody (top). Relative quantities of the IR determined by reblot in this immunoprecipitation experiment are also shown (bottom). B, Cardiomyocytes were stimulated with ISO (10 μmol/L, 60 minutes) in the absence or presence of H89, KN93, nifedipine and wortmanin. Phosphorylation of Akt in serine was detected by immunoblotting analyses. C, Cardiomyocytes were preincubated with or without H89, KN93, nifedipine, and wortmanin then were stimulated with ISO at a concentration of 10 μmol/L for 120 minutes followed by 5 minutes of Ins (100 nmol/L) stimulation. Protein lysates were subjected to immunoprecipitation with antibodies against IR β-subunit, followed by immunoblotting using anti-phosphotyrosine antibody. D, Cardiomyocytes were infected with adenoviruses harboring LacZ, DN-Akt, or E40K Akt cultured under serum-free conditions for 48 hours, and then stimulated with ISO for 120 minutes (10 μmol/L) followed by 5 minutes of Ins stimulation, or with Ins alone. Protein lysates were subjected to immunoprecipitation with antibodies against IR β-subunit, followed by immunoblotting using anti-phosphotyrosine antibody. All experiments were performed in quadruplicate.

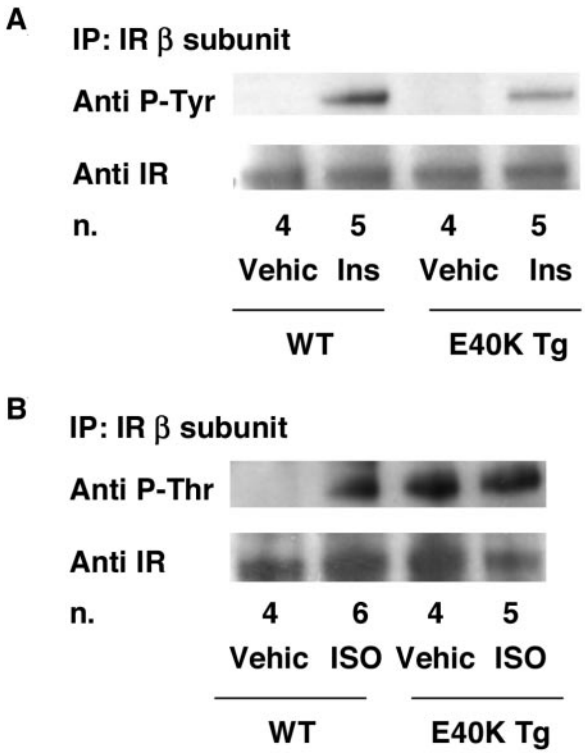

Figure 7. Cardiac overexpression of a constitutively active mutant of Akt interferes with insulin-induced tyrosine phosphorylation of the insulin receptor. A, E40K Tg mice and wild-type controls (WT) were injected with saline (Vehic) or Ins (10 UI/Kg of body weight). After 5 minutes, the hearts were removed, and insulin-induced insulin receptor tyrosine autophosphorylation was determined by immunoprecipitation against the β-subunit of the IR followed by immunoblotting using an anti–phosphotyrosine antibody. B, E40K Tg and WT mice were infused with saline or ISO (0.05 μg/Kg of body weight) for 30 minutes. Hearts were then removed, and insulin receptor threonine phosphorylation was determined by immunoprecipitation against the β-subunit of the IR followed by immunoblotting using an anti–phosphothreonine antibody. In both cases, the expression levels of IR were also determined using an anti-insulin receptor β-subunit antibody.

purpose, cardiomyocytes were incubated with ISO at different time points ranging from 10 to 120 minutes, then were stimulated with insulin for 30 minutes. Insulin stimulation enhanced 2DG uptake detected at 10 and 30 minutes of ISO pretreatment compared with insulin alone. Thereafter, insulin-induced 2DG uptake started to decrease and was completely inhibited after 120 minutes of ISO exposure (Figure 5A), indicating that long-term βAR stimulation is able to induce insulin resistance.

Next, we investigated whether or not long-term ISO stimulation interferes with insulin-induced tyrosine autophosphorylation of the IR. Cardiomyocytes were treated with ISO at different time points, and then stimulated with insulin for 5 minutes. As expected, insulin stimulated tyrosine autophosphorylation of the β-subunit of its receptor. Interestingly, insulin-induced tyrosine phosphorylation of the β-subunit was time-dependently inhibited by ISO pretreatment and was almost totally abolished after 120 minutes of βAR stimulation (Figure 5B).

Because insulin-stimulated tyrosine autophosphorylation of its receptor can be inhibited by either phosphatase-mediated dephosphorylation or kinase-mediated serine/threonine phosphorylation, we asked which mechanism accounts for the inhibition of tyrosine phosphorylation of IR induced by long-term ISO stimulation. Sixty minutes of pretreatment with 50 μmol/L orthovanadate, an inhibitor of tyrosine phosphatases, did not affect the inhibitory effects of long-term ISO stimulation on insulin-induced tyrosine autophosphorylation of the IR (data no shown). By contrast, 120 minutes of ISO stimulation induced threonine phosphorylation of the β-subunit of the IR (Figure 5C). These results suggest that long-term ISO stimulation induces threonine phosphorylation of the β-subunit of IR, which in turn inhibits insulin-induced tyrosine autophosphorylation of the receptor.

Akt Mediates β-Adrenergic Receptor Stimulation-Induced Threonine Phosphorylation of the β-Subunit of IR

Next, we asked if ISO-induced threonine phosphorylation of IR is mediated by Akt. First, we explored whether or not IR is a substrate for Akt. Cardiomyocytes were stimulated with ISO at different time points, then the lysates were immunoprecipitated with an antibody against the β-subunit of IR and blotted with an antibody that binds peptides/proteins that contain phospho-Thr/Ser preceded by Arg/Lys at positions -5 and -3, which are the consensus motifs recognized by Akt. ISO stimulation time-dependently determined the recognition of the β-subunit of IR by anti Akt substrate antibody starting from 60 minutes (Figure 6A). This suggests that ISO stimulation activates Akt, which in turn, recognizes IR as a substrate. Interestingly, the time-course of Akt phosphorylation in Ser induced by ISO (Figure 4A) suggests that the phosphorylation in Ser mediates the interaction of Akt with IR. Therefore, we determined whether or not inhibition of Ser phosphorylation of Akt restores insulin-induced tyrosine phosphorylation of IR after long-term ISO stimulation. ISO-induced Ser phosphorylation of Akt was inhibited by H89, KN93, nifedipine, and wortmanin, indicating that it is a PKA/Ca^{2+}- and PI3K-dependent phenomenon (Figure 6B). Coincidentally, inhibition of both PKA/Ca^{2+}-dependent and PI3K-dependent pathways (Figure 6C) restored insulin-induced tyrosine phosphorylation of IR after long-term βAR stimulation. To explore whether Akt plays a mechanistic role in the regulation of tyrosine phosphorylation of IR, DN Akt, and E40K-Akt mutants were overexpressed in cardiac myocytes. DN Akt restored insulin-induced tyrosine phosphorylation of IR after long-term βAR stimulation, and E40K-Akt blunted insulin-induced tyrosine phosphorylation of IR (Figure 6D).

These data suggest that after long-term βAR stimulation, Ser phosphorylation of IR is mediated by Akt through PKA/Ca^{2+}- and PI3K-dependent pathways.

Cardiac Overexpression of Akt Impairs Insulin Signaling In Vivo

To verify whether activation of Akt impairs insulin signaling in vivo, we examined the hearts of Tg mice with cardiac overexpression of a constitutively active mutant of Akt (E40K) and wild-type controls for insulin-induced phosphorylation of IR. In WT mice, as expected, insulin induced

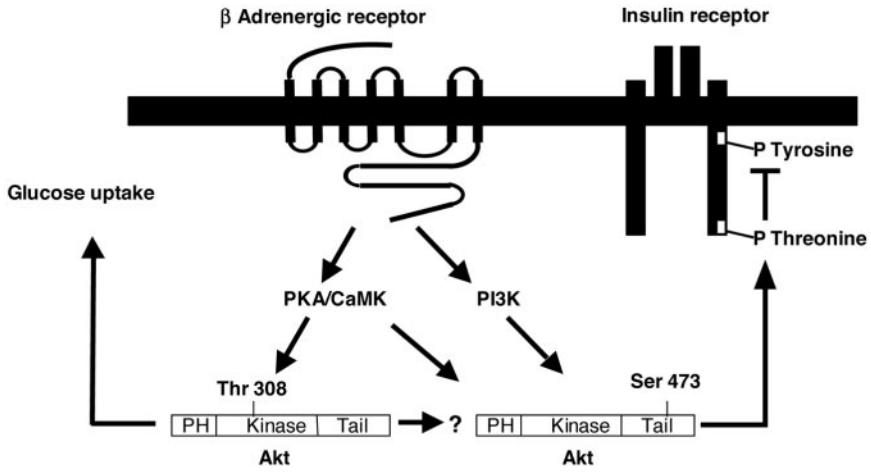

Figure 8. Current hypothesis as to how stimulation of βAR mediates the cross-talk with the IR in cardiac myocytes. Short-term stimulation of β-adrenergic receptors through protein kinase A (PKA) and kinase Ca^{2+}-calmodulin–dependent (CaMK) phosphorylates (threonine 308) and activates Akt, which in turn, promotes glucose uptake. Long-term stimulation of β-adrenergic receptors through a PKA, CaMK, and phosphatidylinositol 3-kinase (PI3K)–dependent pathway phosphorylates (serine 473) and activates Akt, which in turn, phosphorylates the β subunit of the insulin receptor in threonine. Threonine phosphorylation of the insulin receptor inhibits insulin-induced tyrosine autophosphorylation of β-subunit of insulin receptor. This mechanism accounts for the impairment of early steps of insulin signaling and, thus, for β-adrenergic receptor–induced insulin resistance.

tyrosine phosphorylation of its receptor's β-subunit. This phenomenon was blunted in E40K Tg mice (Figure 7A).

Next, we asked whether βAR stimulation, or overexpression of E40K, induces Thr phosphorylation of IR. In WT mice, ISO stimulation induced Thr phosphorylation of IR, and in E40K Tg, Thr phosphorylation was already evident under basal conditions (Figure 7B).

These data suggest that, in vivo, Akt, through Thr phosphorylation of IR, is able to blunt insulin-induced tyrosine phosphorylation of IR.

Discussion

The findings of this study are that, in neonatal cardiomyocytes, (1) short-term stimulation of βARs and insulin increase glucose uptake through two different Akt-dependent mechanisms, (2) long-term stimulation of βARs negatively regulates insulin-induced glucose uptake through inhibition of tyrosine autophosphorylation of IR, and (3) serine phosphorylation of Akt leads to Thr phosphorylation of the IR β subunit, thereby mediating the βAR-induced impairment of insulin signaling, and (4) transgenic mice with cardiac overexpression of E40K Akt have impaired insulin-induced tyrosine phosphorylation of IR.

Our results show that βAR stimulation in cardiomyocytes has a biphasic effect on insulin-stimulated glucose uptake, with an initial additive, followed by an inhibitory action. The additive action on insulin-induced glucose uptake is attributable to the ability of βAR stimulation to increase glucose uptake using molecular mechanisms distinct from those used by insulin. In fact, our data show that insulin-induced glucose uptake is a PI3K-dependent phenomenon, whereas ISO-induced glucose uptake is a PKA/CaMK/Ca^{2+}-dependent and PI3K-independent phenomenon. Our observation is consistent with those previously reported in perfused rat heart,[34] in which a Ca^{2+}-dependent mechanism was required for both α- and βAR-evoked glucose uptake. In addition, we show that Akt kinase activity is required for ISO-induced glucose uptake in cardiomyocytes. In fact, overexpression of dominant-negative mutant Akt inhibited Akt kinase activity, and this was associated with an inhibition of ISO-induced glucose uptake. Akt is involved in insulin-stimulated glucose uptake,[20] and we have previously shown that ISO stimulation activates Akt[28] through a CaMK- and PI3K-dependent mechanism. In this study, we report that threonine 308 phosphorylation of Akt peaks at 10 minutes after ISO stimulation, and this is a PKA/CaMK/Ca^{2+}-dependent phenomenon. On the other hand, it has been reported[35] that phosphorylation in threonine and in serine both stimulate the kinase activity of Akt, and different molecular pathways can account for phosphorylation in threonine or serine. Our finding that ISO-induced threonine 308 phosphorylation of Akt is a PI3K-independent event is consistent with the observation of Alessi et al[36] who reported that threonine 308 phosphorylation of Akt is mediated by PDK1, which is insensitive to wortmanin. On the other hand, insulin-evoked glucose uptake as well as threonine 308 phosphorylation of Akt was selectively mediated by a PI3K-dependent pathway. This is in agreement with the observation that insulin-induced glucose uptake can be a calcium-independent phenomenon.[37] Thus, the different pathways used by βAR and insulin in the activation of Akt can account for the additive effect of short-term ISO stimulation on insulin-induced glucose uptake.

Our finding that long-term βAR stimulation reduces insulin-induced glucose uptake and tyrosine autophosphorylation of IR is partially consistent with that reported by Klein et al[24] who showed a reduction of insulin-induced glucose uptake and tyrosine autophosphorylation of IR in cultured adipocytes stimulated with β_3AR agonist. However, it was reported that this inhibitory effect reached a peak after only 5 minutes, and not after 120 minutes, as we noted in cardiomyocytes, and it is noteworthy that β_3AR stimulation failed to activate Akt in adipocytes. This reinforces the concept that different βAR subtypes drive different cell signaling mechanisms, and thus physiology of different cell types depends on expression levels and/or functional coupling with downstream signaling molecules of the βAR subtypes.

In cultured cardiomyocytes, we found that the reduction of insulin-induced autophosphorylation of IR in response to long-term βAR stimulation was associated with threonine phosphorylation of the β-subunit of IR. The presence of several Akt consensus sites in IR and the evidence that, after

long-term βAR stimulation, immunoprecipitates of the β-subunit of IR are recognized by an anti–phospho-Akt substrate antibody, support the notion that IR is a substrate of Akt. Our data show that long-term ISO stimulation phosphorylates Akt in serine 473, and this is a PKA/CaMK/Ca^{2+}- and PI3K-dependent phenomenon. Although our data do not clarify whether or not threonine 308 phosphorylation is required for the subsequent serine 473 phosphorylation of Akt, they allow to speculate that Akt could be the key molecule involved in the βAR-induced regulation of glucose uptake, mediating both positive and negative feedback on the phosphorylation site (Figure 8).

The possibility that the PI3K-Akt pathway is involved in the pathogenesis of insulin resistance has been reported by Egawa et al,[21] who demonstrated that, in adipocytes, overexpression of a constitutively active mutant of PI3K impairs IRS-1 function most likely by serine/threonine phosphorylation. However, Akt is not the only mechanism that accounts for insulin receptor desensitization, because insulin-induced desensitization of its receptor depends also on a tyrosine phosphatases-dependent mechanism (see online data supplement).

In this study, we recognize two potential limitations. In particular, glucose uptake was measured in absence of glucose in the culture media, and the experiments were performed in neonatal rat cardiomyocytes that are not yet fully mature with regards to the control of glucose metabolism and thus have lower expression levels of GLUT4 compared with adult cardiomyocytes.[38] However, we found that the signal responsible for βAR-induced insulin resistance is mediated by β_1AR subtype (see online data supplement), and cardiomyocytes, in adult rodents as well as in primates, express mainly β_1AR subtype. Therefore, our observation, is not trivial in terms of biological significance; on the contrary, it is possible that our experimental setting underestimates the phenomenon of βAR-induced insulin resistance. In addition, we must consider that, consistent with the molecular machinery that account for βAR stimulation–induced desensitization of IR detected in vitro, adult transgenic mice with cardiac overexpression of constitutively active Akt also showed that Akt is critically involved in the regulation of the first steps of insulin signaling. Therefore, it is possible to speculate that all pathological conditions characterized by sustained stimulation of cardiac βAR induce a state of insulin resistance in the heart through the activation of Akt.

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ON LINE DATA SUPPLEMENT

Methods

Primary Cultures of Neonatal Rat Ventricular Cardiomyocytes

The procedures involving animals were performed with the approval of the animal board of review of our Institution.

Glucose Uptake Assays

Cardiomyocytes were cultured overnight in DMEM containing 5 mM glucose, and then treated as indicated. At the end of the stimulation period, the medium was changed to 1 mL of DMEM without glucose containing 0.2 μCi/mL 2-Deoxy-D-[1-^{3}H]glucose (Amersham Pharmacia Biotec). After 10 min the medium was aspirated, the cells were washed twice in ice-cold Ca^{2+}/Mg^{2+}-free Dulbecco's PBS, and lysed in 500 μL of 0.1 N NaOH. The cell–associated radioactivity was determined by mixing 400 μL of cell lysate with 5 mL of scintillation fluid followed by counting in a β-counter for 10 min. The remaining 100 μL of cell lysate was used for determination of protein content by the Lowry method. Nonspecific 2DG uptake was determined in parallel in the presence of 10 μM of cytochalasin B and subtracted from both basal and stimulated glucose uptake measurements. The results are presented as unitary changes of *cpm/μg* protein.

Immunoblotting

Buffer A: 50 mM HEPES (pH 7.6), 1mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM vanadate, 10 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM dithiothreitol, 1 mM AEBSF.

Immunoprecipitation and Detection of Phospho-Akt Substrate

Buffer B: 50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2

mM vanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4.

In vivo studies

After an overnight fast, the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and a polyethylene catheter (PE-10) was inserted into the right jugular vein for the injection of saline or insulin or isoproterenol. At the end of the stimulation period, the heart was quickly removed and frozen. Frozen tissues were homogenized by a polytron for 1 min in 6 x vol of homogenization buffer (1% Triton X−100, 0.2 mmol phenylmethylsulfonil fluoride, 50 mmol Hepes pH 7.4, 10% glycerol, 150 mmol NaCl, 180 μg/ml aprotinin, 0.1 mmol Na_3VO_4, 100 mmol sodium fluoride). Insoluble protins were removed by centrifugation at 15000 r.p.m. for 45 min. The solubilized proteins were immunoprecipitated with an anti-IR β-subunit (Santa Cruz Biotechnology, Inc), subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane and immunoblotted with polyclonal anti-IR β-subunit (Santa Cruz Biotechnology, Inc), monoclonal anti-phospho-tyrosine (Cell Signaling Technology™), and monoclonal anti-phospho-threonine (Cell Signaling Technology™) antibodies. Horseradish peroxidase-conjugated (Cell Signaling Technology™) antibody was used as secondary antibody. The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Pharmacia Biotec).

Results

Glucose concentration in the culture media affects 2-Deoxy-D-[1-^{3}H]glucose assay sensitivity.

To test whether the concentration of glucose in the culture media affects the measurement of 2-Deoxy-D-[1-^{3}H]glucose (2DG) uptake, cardiac myocytes were stimulated with insulin and 2DG uptake was measured in the absence and i presence of different concentrations of D-Glucose. In absence of D-glucose, insulin increased 2DG

uptake by 96±13%, a response which was not statistically different from that obtained with 1 and 5.5 mM of D-Glucose in the media (85±14% and 62±23%, respectively), while a high concentration of D-Glucose interfered with 2DG uptake detection (Fig. 1).

These results seem to indicate that the absence of D-glucose does not affect the insulin-induced 2DG uptake when compared with low concentrations of D-glucose in the culture media.

Insulin inhibits tyrosine phosphorylation of the insulin receptor through phosphotyrosine phosphatases- and Akt dependent pathways.

We examined the contribution of tyrosine phospatases and Akt in insulin-induced desensitization of the insulin receptor (IR) in cardiac myocytes. First we determined the time-course of insulin-induced tyrosine phosphorylation of IR. Cardiac myocytes were stimulated with insulin (100 nM) at different time points. Tyrosine phoshorylation of IR was detectable after 1 min, peaked after 5 min and then progressively disappeared (Fig. 2, A). Next , we determined the time course of insulin-induced Akt (Ser 473) phosphorylation. Insulin-evoked Akt (Ser 473) phosphorylation was evident after 1 min, peaked after 10 min, and then progressively decreased, but was still detectable after 60 min (Fig. 2 B). To examine the contribution of phosphatases and Akt to the IR insulin-induced desensitization, cardiac myocytes were infected with adenoviruses harboring Lac Z or a dominant negative mutant of Akt (DN Akt), and, after 48 hours stimulated with insulin with or without pre-treatment with sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases. Orthovanadate, DN Akt, and their combination restored insulin-induced tyrosine phosporylation of IR (Fig. 2, C).

These results suggest that insulin-induced desensitization of IR involves both phosphotyrosine phosphatases- and Akt-dependent pathways.

Long-term β adrenergic receptor stimulation dose dependently inhibits insulin-induced 2DG uptake

To verify whether long-term β adrenergic receptor (AR) stimulation interferes with insulin-induced 2DG uptake in a dose dependent fashion, cardiac myocytes were stimulated with graded doses of ISO for 120 min and then with insulin for 30 min. As expected, insulin increased 2 DG uptake by 106±5%, long-term ISO stimulation started to decrease insulin-induced 2DG uptake at a dose of 0.5 μM, and the statistical significance was achieved at a dose of 1μM (Fig. 3).

These results indicate that long-term β AR stimulation dose-dependently inhibits insulin-induced 2DG uptake.

β1 AR mediates threonine phosphorylation of IR

Since neonatal cardiomycytes express both β1 and β2 AR, we examined which β AR subtype accounts for ISO-induced Thr phosphorylation of IR. β1 and β2 AR blockade was induced by betaxolol and ICI 118,551, respectively. ISO-induced IR Thr phosphorylation was inhibited by both propranolol, a β1/β2 antagonist, and betaxolol, while pretreatment with ICI 118,551 did not affect this response (Fig. 4, A). On the other hand, pretreatment with betaxolol and not with ICI 118,551 restored insulin-induced tyrosine phosphorylation of IR following long term ISO stimulation (Fig. 4, B).

These results suggest that β1 ARs selectively mediate Thr phosphorylation of IR following long term ISO stimulation.

Figure Legends

Figure 1

Glucose concentration affects 2-Deoxy-D-[1-^{3}H]glucose uptake. Cardiomyocytes were stimulated for 30 min with insulin (Ins, 100 nM), and the rate of 2-Deoxy-D-[1-^{3}H]glucose (2DG) uptake was determined in the absence and presence of different concentrations of D-Glucose in the culture media. The values of 2DG uptake (*cpm*) in each experiment were adjusted by the protein content of the dish (μg). The graphics show the means±SEM of 4 independent experiments performed in triplicate.

Figure 2

Insulin-induced desensitization of the insulin receptor is mediated by tyrosine phosphatases- and Akt-dependent pathways. A) Cardiomyocytes were stimulated with insulin (Ins, 100 nM) for the indicated durations. Tyrosine phosphorylation of the insulin receptor was determined by immunoblotting analyses using anti-phospho-tyrosine antibody (Anti P-Tyr). B) Cardiomyocytes were stimulated with insulin (Ins, 100 nM) for the indicated durations. Phosphorylation of Akt in serine was detected by immunoblotting analyses with anti-phospho-Ser-473 Akt antibody (Anti P-Akt). C) Cardiac myocytes were infected with adenoviruses harboring Lac Z or a dominant negative mutant of Akt (DN Akt). Forty-eight hours after infection cells were stimulated with insulin (Ins, 100 nM) for the indicated durations in the presence or absence of pre-treatment with orthovanadate (Orthovan, 60', 50 μM). Protein lysates were subjected to immunoprecipitation with antibodies against insulin receptor (IR) β-subunit, followed by immunoblotting using anti-phospho-tyrosine antibody (Anti P-Tyr). The results shown are representative of four experiments.

Figure 3

Long-term Isoproterenol stimulation dose dependently inhibits insulin-induced 2-Deoxy-D-[1-^{3}H]glucose uptake. Cardiomyocytes were preincubated (120 min) with graded

doses of isoproterenol (ISO) ranging from 0.1 to 50 μM and stimulated with insulin (Ins, 100 nM, 30'), and 2-Deoxy-D-[1-^{3}H]glucose (2DG) uptake was determined. The values of 2DG uptake (*cpm*) in each experiment were adjusted by the protein content of the dish (μg). The graphics show the means±SEM of 6 independent experiments performed in triplicate

Figure 4

β1 AR induces threonine phosphorylation of the insulin receptor. A) Cardiomyocytes were preincubated with propranolol (Prop, 10 μM, 30 min), betaxolol (BTX, 10 μM, 30 min), and with ICI 118,551 (ICI, 1 μM, 30 min), and then stimulated with isoproterenol (ISO, 10 μM) for 120 min. Protein lysates were subjected to immunoprecipitation with antibodies against insulin receptor (IR) β-subunit, followed by immunoblotting using anti-phospho-threonine antibody. B) Cardiomyocytes were stimulated with insulin (Ins 100 nM) for 5 min, and with ISO for 120 min followed by 5 min of Ins stimulation in the presence or absence of pre-treatment with BTX or ICI. Protein lysates were subjected to immunoprecipitation with antibodies against IR β-subunit, followed by immunoblotting using anti-phospho-tyrosine antibody. The results shown are representative of four experiments.

Figure 1

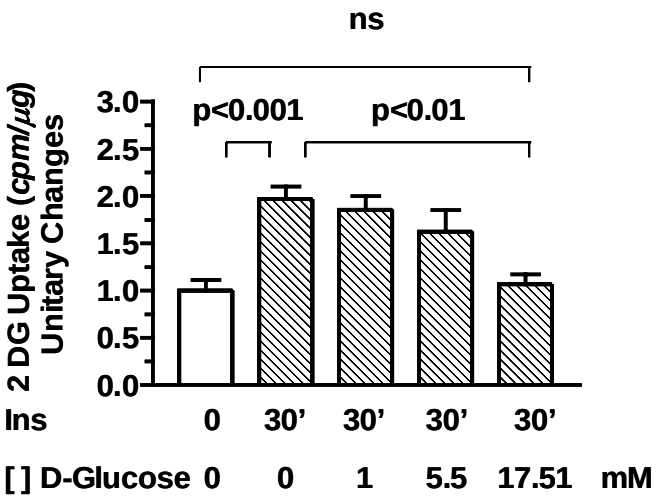
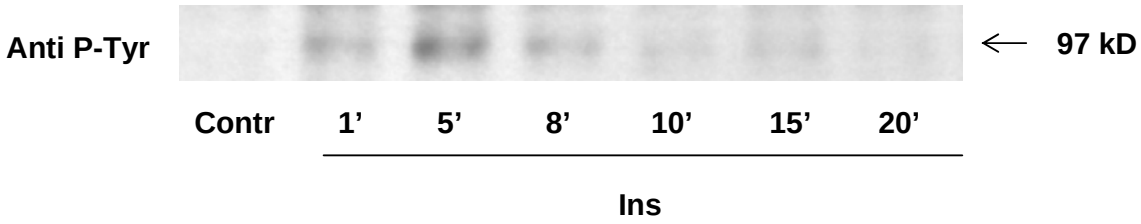


Figure 2

A)



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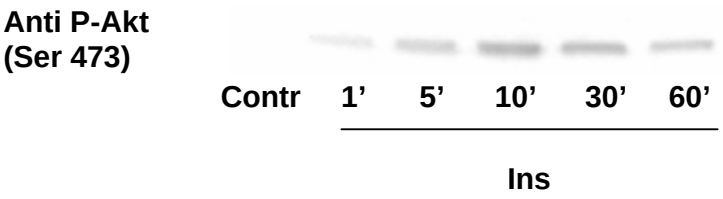

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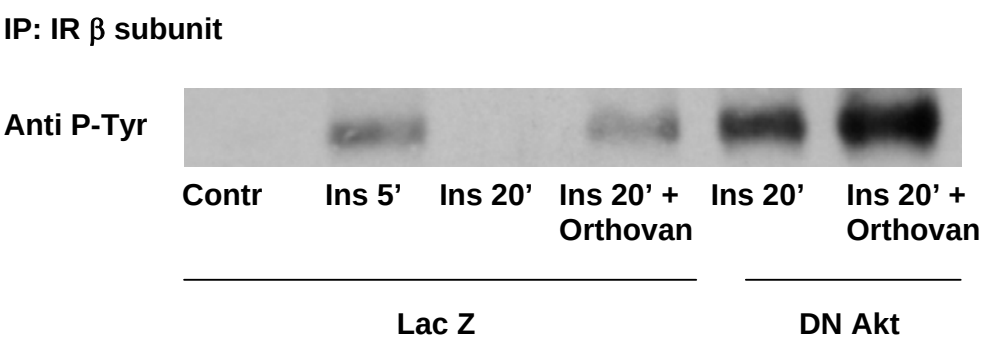


Figure 3

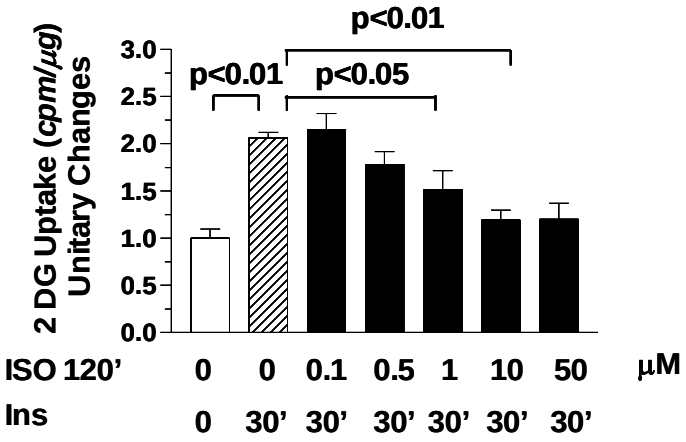
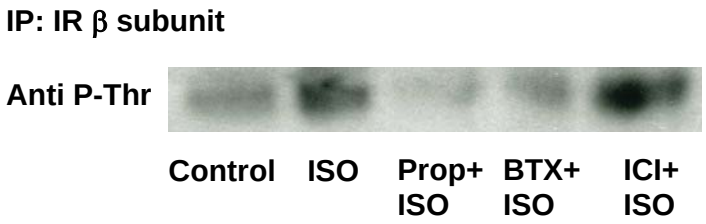


Figure 4

A)



B)