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Continually high insulin levels impair Akt phosphorylation and glucose transport in human myoblasts

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Abstract

Chronic hyperinsulinemia is both a marker and a cause for insulin resistance. This study analyzes the effect of long-term exposure to high insulin levels on the insulin-signaling pathway and glucose transport in cultured human myoblasts. Human myoblasts were grown in the presence of low (107 pmol/L, SkMC-L) or high (1430 pmol/L, SkMC-H) insulin concentrations for 3 weeks. Glucose transport, insulin receptor (IR), and IR substrate 1 (IRS1) phosphorylation, phosphatidylinositol 3′ -kinase (PI3K) activity, as well as Akt-Ser473 phosphorylation have been investigated at the end of the incubation period and after a further short-term insulin stimulation. At the end of the incubation period, IR, IRS1, p85/PI3K, Akt, and GLUT4 protein expression levels were similar in both culture conditions. Basal glucose transport was similar in SkMC-L and SkMC-H, but after short-term insulin stimulation significantly increased ($P < .01$) only in SkMC-L. IR binding was down-regulated in SkMC-H ($P < .01$), but IR and IRS1 tyrosine phosphorylation and PI3K activity were significantly higher ($P < .01$) in SkMC-H than SkMC-L. Despite increased PI3K activation, Akt-Ser473 phosphorylation was similar in SkMC-L and SkMC-H. After a short-term insulin stimulation (10 nmol/L insulin for 10 minutes), IR and IRS1 tyrosine phosphorylation, PI3K activation, and Akt-Ser473 phosphorylation significantly increased ($P < .01$ and $P < .05$ for Akt) in SkMC-L but not in SkMC-H. Serine phosphorylation of IRS1 was similar in SkMC-L and SkMC-H. Moreover, in the SkMC-H, insulin stimulation was associated with the inhibition of IRS1 tyrosine dephosphorylation ($P < .05$). In summary, continuous exposure of cultured myoblasts to high insulin levels induces a persistent up-regulation of IR, IRS1, and PI3K activity associated with the demodulation of insulin signaling. Moreover, the impairment of the insulin-signaling steps between PI3K and Akt is concomitant with the desensitization of glucose transport. These alterations may contribute to the derangement insulin-signaling pathway states of hyperinsulinemia such as obesity and type 2 diabetes.

1. Introduction

Chronic hyperinsulinemia accompanies most states of insulin resistance as an expression of compensatory insulin secretion. As such, fasting plasma insulin concentration has been taken as a marker for insulin resistance [1]. In longitudinal studies, hyperinsulinemia and insulin resistance have been shown to precede and predict the appearance of hyperglycemia in nondiabetic subjects [1]. Moreover, in vivo and in vitro studies have suggested that long-term exposure of insulin-dependent tissues to elevated plasma insulin concentration can exacerbate insulin resistance [2-4]. In a previous in vivo study, we showed that 96-hour euglycemic physiological hyperinsulinemia (+72 pmol/L) in healthy individuals caused a 20% to 40% reduction in insulin-mediated glucose use [5]. Similarly, insulin-mediated glucose use decreased by 39% in normal rats after 7 days of euglycemic hyperinsulinemia [6]. Skeletal muscle tissue accounts for most of the insulin-mediated glucose use and is the major site of insulin resistance [7-9]. Skeletal muscle biopsies from type 2 diabetic patients indicate that at high insulin concentration there is an impaired insulin-mediated glucose transport, insulin receptor substrate 1 (IRS1) tyrosine phosphorylation, phosphatidylinositol 3′-kinase (PI3K) activation, and Akt activity [3,9-13]. In addition, myotubes from obese patients with impaired glucose

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tolerance have an impaired insulin-induced glucose uptake associated with defective IRS2-associated PI3K activation [14]. However, from in vivo and ex vivo studies, it is difficult to link alterations in insulin signaling and action to hyperinsulinemia because of the effects of concomitant hyperglycemia [15], elevated FFA [16] and, possibly, cytokines [17]. In vitro studies have explored the consequence of short incubation (24 hours) of L6 myoblasts with high insulin levels showing a PI3K-mediated reduction in IRS1/2 protein expression and desensitization of insulin-signaling pathway [18]. Moreover, 24-hour pretreatment of L6 myotubes with high insulin and glucose caused a decrease of insulin-stimulated GLUT4 translocation together with a reduced activation of insulin-signaling cascade [19]. This study was performed in human myoblasts to determine how strictly controlled experimental conditions, characterized by moderately elevated insulin concentrations (1430 pmol/L) and normal glucose levels maintained for 3 weeks, affect insulin signal transduction network and glucose transport.

The data obtained could be a useful tool to understand the effect of an in vivo hyperinsulinemic/normoglycemic metabolic milieu such as that of obesity.

2. Materials and methods

2.1. Cell culture

Myoblasts of human skeletal muscle (SkMC 2561) and all chemicals for cell culture were obtained from Cambrex (Walkersville, MD). These cells were preferred over terminally differentiated myotubes because of their ease in handling and maintenance. Moreover, this cell line expresses myogenic markers such as myoD, Myf 5, myogenin, and sarcomeric myosin (data not shown), but no fusion and differentiation into myotubes took place under any of the culture conditions used in this study. SkMC cells were maintained in skeletal basal medium (SkBM, Cambrex) supplemented with 1% fetal bovine serum, 10 μg/mL human epidermal growth factor, 50 mg/mL bovine serum albumin, 50 mg/mL fetuin, 0.38 mg/mL dexamethasone, 50 mg/mL gentamicin, and 50 μg/mL amphotericin B. SkMC cells were grown to semiconfluence and detached from plates with 0.25% trypsin-EDTA. Cell culture was carried out at 37 °C in a humidified incubator in an atmosphere of 5% CO_2. Cells were incubated in the presence of low (107 ± 20 pmol/L, SkMC-L) or high (1430 ± 120 pmol/L, SkMC-H) insulin while maintaining normal glucose (5.5 mmol/L) concentrations for 3 weeks. Cell medium was replaced everyday, and cells were splitted every 5 days through the 3-week incubation period.

At the end of each incubation period, cells were washed with phosphate-buffered saline before short-term insulin stimulation (10 nmol/L for 10 minutes). Insulin stimulation was also repeated after 3-minute incubation with HEPES buffer (pH 4.5) at 4 °C and extensive washing with HEPES buffer (pH 7.8). In addition, for exploration of a possible cause of basal sustained IRS1 phosphorylation, cells were acid washed after maximal phosphorylation to remove insulin bound to surface receptors and resuspended in insulin-free HEPES buffer (pH 7.8) at 37 °C for different times and time course of residual phosphorylation was assessed.

2.2. Measurement of 2-deoxy-D-[^{3}H]glucose uptake

2-Deoxy-D-[^{3}H]glucose (2-DG; Amersham Biosciences, Buckinghamshire, UK) uptake was determined using the procedure described by Baque et al [20] by the addition of 0.5 μCi 2-DG per well and 1.5 mmol/L unlabeled 2-DG, followed by 3 washes in ice-cold phosphate-buffered saline after 6 minutes of exposure. Cells were subsequently lysed with 0.5% sodium dodecyl sulfate (SDS) for 30 minutes at 4 °C and aliquots measured for radioactivity in 10 mL of EcoLite (ICN, Costa Mesa, CA) on a β-counter (Wallac 1211 RACKBETA, SW, Wallac, Turku, Finland).

2.3. Insulin receptor binding and immunodetection of phosphotyrosine-containing protein

All chemicals for cellular studies were obtained from Sigma-Aldrich (St Louis, MO). For insulin receptor (IR) binding measurement, cells were acid washed and then incubated with 66 pmol/L A14-[^{125}I]insulin in binding buffer

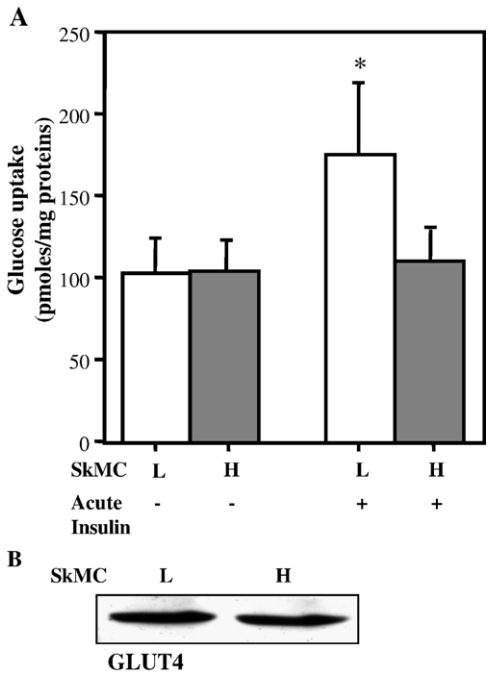

Fig. 1. A, 2-Deoxyglucose uptake in myoblasts grown at low (SkMC-L) or high (SkMC-H) insulin concentrations and normal glucose levels (5.5 ± 0.7 mmol/L) and after a further insulin stimulation with 10 nmol/L insulin for 10 minutes as reported in Materials and methods. B, GLUT4 protein expression in SkMC-L and SkMC-H. Cell lysates were analyzed by immunoblotting with specific antibodies. Results were normalized for β-actin. *$P < .05$ vs SkMC-L.

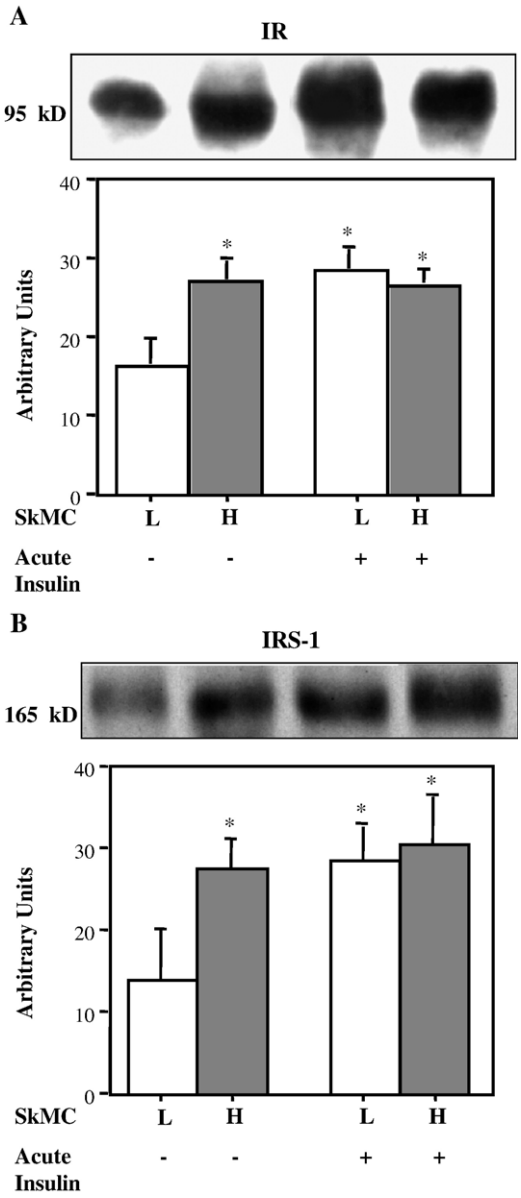

Fig. 2. Tyrosine phosphorylation of IR (A) and IRS1 (B) in cells grown at low (SkMC-L) or high (SkMC-H) insulin concentrations and after a further insulin stimulation as reported in Materials and methods. IR and IRS1 were isolated from cell lysates by immunoprecipitation with anti-IR or IRS1 antibody and analyzed by immunoblotting with antiphosphotyrosine antibody. Blots were quantified by densitometry and normalized for milligrams of protein. *$P < .01$ vs SkMC-L.

(50 mmol/L HEPES with 1% bovine serum albumin, pH 7.8) at 4 °C for 16 hours, as previously reported [21]. Nonspecific binding was determined in the presence of 16.6 μmol/L unlabeled insulin [21]. For the study of protein expression, cells were washed extensively in ice-cold buffer (137 mmol/L NaCl, 120 mmol/L Tris, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, and 100 μmol/L Na_3VO_4, pH 7.6) and then incubated with lysis solution (10% glycerol, 1% Nonidet P-40, 120 mmol/L NaCl, 100 mmol/L NaPP, 20 mmol/L Tris, 10 mmol/L Na_3VO_4, 100 mmol/L NaF, 10 mmol/L EDTA, 2 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5 μg/mL pepstain, and 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.4). Cells were scraped, vortexed, incubated for 60 minutes a 4 °C on a rotating device, and then centrifuged at 20 000*g* for 90 minutes at 4 °C. One aliquot of the supernatant was used for protein concentration measurement (DC Protein Assay, BioRad Laboratories, Hercules, CA). Aliquots of lysates were immunoprecipitated with anti-IR, anti-IRS1 (Upstate Biotechnology, Lake Placid, NY), and anti-GLUT4 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 20 hours at 4 °C on a rotating device. Protein A–Sepharose (Sigma-Aldrich) was then added for additional 2-hour incubation. Sepharose-bound immunocomplexes were washed extensively in a washing buffer, boiled for 5 minutes in Laemmli sample buffer under reducing conditions, and supernatants were resolved in SDS–polyacrylamide gel electrophoresis (7% Tris acrylamide). Anti–phospho-Akt (Ser473) and anti-Akt (Cell Signaling, Beverly, MA) were detected by direct immunoblotting. Protein electrotransfer to nitrocellulose (Schleicher & Schuell, Dassel, Germany) was performed in a buffer containing 192 mmol/L glycine, 25 mmol/L Tris, 3.4 mmol/L SDS, and 20% methanol for 4 hours at 480 mA. Nitrocellulose filters were blocked in Tris-buffered saline

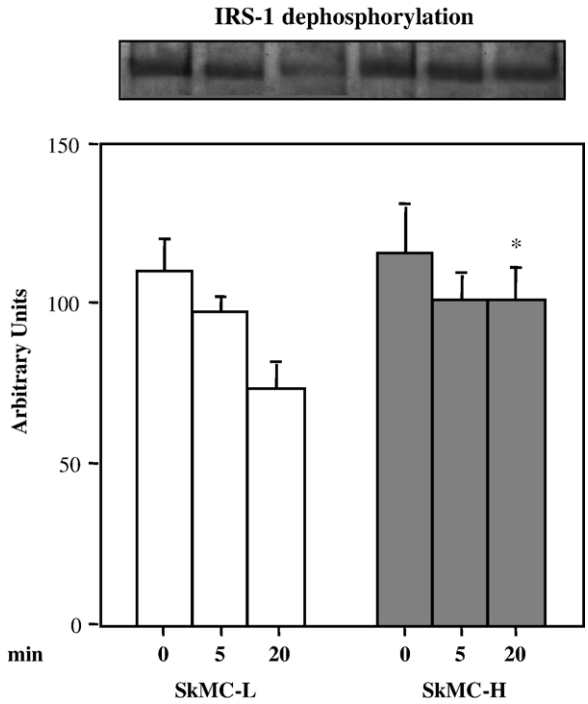

Fig. 3. Time course of loss of phosphotyrosine content of IRS1. Maximal phosphorylation was obtained by incubating cells with 10 nmol/L insulin at 37°C for 10 minutes. After acid washing, cells were reincubated in an insulin-free medium. At the indicated times, cells were solubilized, IRS1 immunoprecipitated, and phosphotyrosine IRS1 content was assessed by immunoblotting with antiphosphotyrosine antibody. Blots were quantified by densitometry and normalized for milligrams of protein. *$P < .05$ vs SkMC-L.

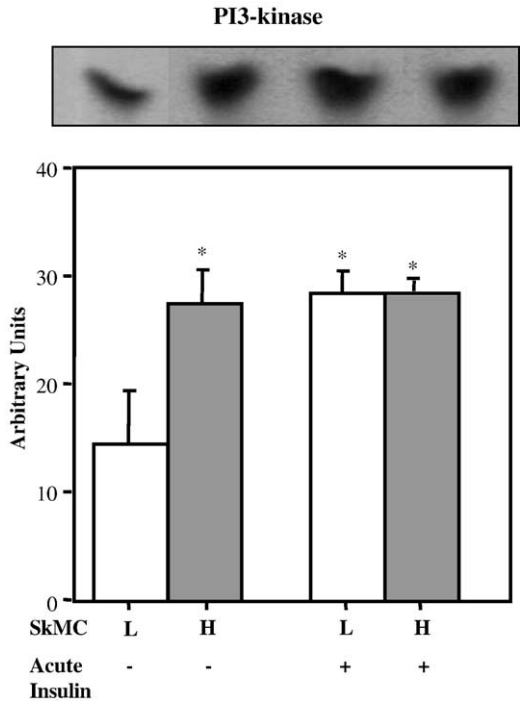

Fig. 4. PI3K activity in cells grown at low (SkMC-L) or high (SkMC-H) insulin concentrations and after a further insulin stimulation as reported in Materials and methods. PI3K was isolated from cell lysates by immunoprecipitation with anti-IRS1 antibody and incubated with phosphatidylinositol and γ-^{32}P-ATP for 15 minutes. Reaction was analyzed by autoradiography after thin-layer chromatography, quantified by densitometry, and normalized for milligrams of protein. *P < .05 vs SkMC-L.

(TBS), 0.1% Tween-20, and 5% milk for 1 hour at room temperature with rocking. Filters were incubated with the indicated antibodies or with anti-phosphotyrosine (Transduction Laboratories, Lexington, KY) or anti-phosphoserine (Zymed Lab, San Francisco, CA) antibodies diluted in blocking solution (1 μg/mL) for 20 hours at 4 °C, washed extensively, and then incubated with appropriate secondary antibody diluted in blocking solution (1:2000) for 90 minutes at room temperature. Bound antibodies were detected using procedures carried out according to the manufacturer's instructions (ECL, Amersham Biosciences). Bands of interest were quantified with a densitometer (GS 690, BioRad Laboratories) using MultiAnalyst/PC-PC Software for Bio-Rad's Image Analysis Systems, Version 1.02 (BioRad Laboratories), and all results were normalized for milligrams of protein [22].

2.4. PI3K activity

Phosphorylation of phosphatidylinositol was measured using a modification of the method of Backer et al [23]. Confluent cells grown in 100-mm dishes were washed and solubilized in 1 mL lysis buffer. The lysate was centrifuged at 12 000g for 5 minutes, and the supernatant was immunoprecipitated with anti-IRS1 antibody and protein A–sepharose. The immunoprecipitate was repeatedly washed and resuspended in 50 μL of 10 mmol/L Tris (pH 7.5), containing 100 mmol/L NaCl and 1 mmol/L EDTA. Ten microliters of 100 mmol/L $MgCl_2$ and 20 μg of phosphatidylinositol (Sigma-Aldrich) were added to each tube. The phosphorylation reaction was started by addition of 10 μL of 440 μmol/L ATP containing 30 μCi of (γ-^{32}P) ATP (Amersham Biosciences). After 15 minutes the reaction was stopped by adding of 20 μL of 8 mol/L HCl. The organic phase was extracted with 160 μL of $CHCl_3$/methanol (1:1 vol/vol) and applied to a silica gel thin-layer chromatography plate (Sigma-Aldrich), which was developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2 vol/vol/vol/vol), dried, and visualized by autoradiography. Spots comigrating with authentic phosphatidylinositol 3-phosphate (Sigma-Aldrich) were quantified with a densitometer as reported above.

2.5. Statistical analysis

Data represent means ± SD of 3 independent experiments, each done in duplicate. Results obtained in cells cultured in the presence of low insulin levels were considered as basal values. Data comparison was performed by paired and unpaired Student t test, as appropriate, with level of statistical significance set at P < .05.

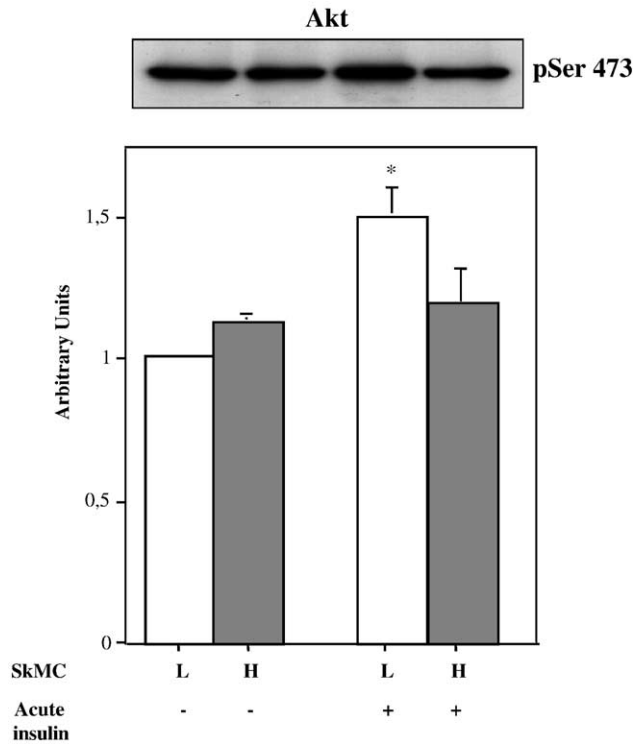

Fig. 5. Akt-Ser473 phosphorylation in cells grown at low (SkMC-L) or high (SkMC-H) insulin concentrations and after a further insulin stimulation as reported in Materials and methods. Phosphoserine 473 Akt was detected by immunoblotting with a specific antiphosphoserine 473 antibody. Blots were quantified by densitometry and normalized for milligrams of protein. *P < .05 vs SkMC-L.

3. Results

3.1. Glucose transport

At the end of the incubation period, glucose transport measured by the uptake of 2-DG was not different in SkMC-H as compared with SkMC-L. Nevertheless, short-term insulin stimulation induced a 73% increase in glucose transport in SkMC-L ($P < .01$ vs basal), whereas no change occurred in SkMC-H (Fig. 1A). GLUT4 protein expression was similar in both culture conditions (Fig. 1B).

3.2. IR and IRS1

Pretreatment with increased insulin concentration for 3 weeks induced in SkMC-H a 30% decrease of A14-[^{125}I]insulin binding compared with SkMC-L (2.5% ± 0.2% vs 3.73% ± 0.25%, $P < .01$), thus confirming the inverse relationship between the ambient insulin concentration and the receptor density on the cell surface. At the same time SkMC-H showed a 72% increase of IR tyrosine phosphorylation (Fig. 2A) and a 100% increase of IRS1 tyrosine phosphorylation (Fig. 2B) as compared with cells grown at low insulin (SkMC-L). Short-term insulin stimulation caused a prompt and significant increase in the tyrosine phosphorylation of IR and IRS1 (77% and 106%, respectively, $P < .01$ vs basal) in control cells, but no further increase above baseline occurred in SkMC-H (Fig. 2A and B). When IRS1 serine phosphorylation was determined, no increase occurred at the end of both culture conditions as well as after short-term insulin stimulation (data not shown). After prolonged hormone exposure, both cell types were acid washed before repeated short-term insulin stimulation to exclude an effect of IR occupancy. The response of IRS1 to short-term insulin treatment was not changed either in SkMC-L nor in SkMC-H, suggesting the development of an intrinsic defect in the insulin signaling. Finally, we evaluated the possible role of tyrosine dephosphorylation in determining the basal high levels of IRS1 tyrosine phosphorylation. After a short-term insulin stimulation, the time course of IRS1 dephosphorylation was significantly decreased in SkMC-H as compared with that observed in SkMC-L (−33%, $P < .05$) (Fig. 3).

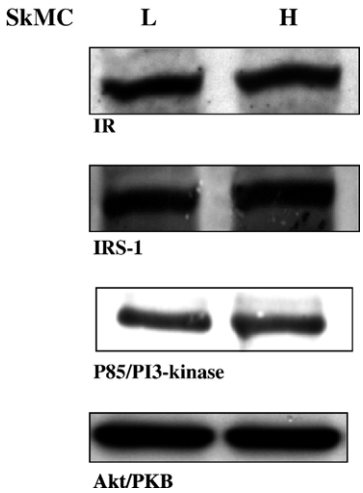

Fig. 6. IR, IRS1, p85/PI3K, and Akt protein expression in cells grown at low (SkMC-L) or high (SkMC-H) insulin concentrations as reported in Materials and methods. Cell lysates were analyzed by immunoblotting with specific antibodies. p85/PI3K was isolated from cell by immunoprecipitation with anti-IRS1 antibody and analyzed by immunoblotting, with anti-p85/PI3K antibodies.

3.3. PI3K activation and Akt phosphorylation

The pattern of PI3K activation mirrored that of IRS1 phosphorylation in the 2 experimental conditions. As shown in Fig. 4, in SkMC-H, PI3K activity was 90% greater than that in control myoblasts ($P < .01$). Similar to IR and IRS1 phosphorylation, PI3K activity also increased in SkMC-L in response to short-term insulin stimulation (97%, $P < .01$), whereas no significant changes occurred in SkMC-H. The activity of Akt was evaluated by the phosphorylation of Ser473, because, together with phosphorylation of Thr308, it represents the final step of Akt activation [24]. Opposite to the activation pattern of PI3K, no difference in Akt-Ser473 phosphorylation between SkMC-L and SkMC-H occurred at the end of both culture conditions (Fig. 5). Nonetheless, short-term insulin stimulation caused a significant increase of Akt phosphorylation in SkMC-L ($P < .05$), whereas no significant change was observed in SkMC-H. These results suggest that high insulin levels impair the insulin-signaling steps between PI3K and Akt (Fig. 5).

3.4. Protein expression

The expression of IR, IRS1, and Akt immunoprecipitated and blotted with the corresponding antibody was not affected by the different culture conditions of myoblasts (Fig. 6). The possibility that incubation with high insulin levels could affect p85 binding to IRS1 was also tested. After cell solubilization, activated IRS1 was immunoprecipitated and probed by immunoblotting with anti–p85 antibody. As shown in Fig. 5, an efficient binding between p85 and IRS1 occurred in both control cells and SkMC-H. Thus, aberrant protein expression cannot account for the defects of the insulin-signaling pathway in SkMC-H in basal conditions and after short-term insulin stimulation.

4. Discussion

We have investigated the effect of long-term exposure of human skeletal myoblasts to moderately elevated insulin concentration on several steps of insulin signaling as well as on glucose transport. Myoblasts grown for 3 weeks in the presence of ~1400 pmol/L insulin, have, as compared with cells incubated in the presence of physiological insulin concentration (~100 pmol/L), an increased basal tyrosine phosphorylation of the IR, and IRS1 along with increased PI3K activity. More importantly, our results show that in the presence of continually increased insulin levels there is an

uncoupling between PI3K activity and Akt-Ser473 phosphorylation possibly leading to impaired GLUT4 translocation to the plasma membrane [25]. This conclusion is supported by the observation that total GLUT4 protein expression is not affected in SkMC-H as compared with SkMC-L myoblasts. The uncoupling of PI3K/Akt was not reported in studies with L6 myoblasts [18] or myotubes [19], which have assessed the effect of 24-hour incubation with high insulin and high insulin/high glucose concentration on basal and insulin-stimulated signaling pathway and glucose transport. On the contrary, Oku et al [26] reported that sustained hyperglycemia impairs the insulin-signaling steps between PI3K and Akt/protein kinase B in skeletal muscle and that reduced Akt/protein kinase B activation underlies hyperglycemia-induced insulin resistance in this tissue. Thus, our results suggest a common effect of high insulin or high glucose levels on the efficiency of the insulin-signaling steps between PI3K and Akt. We can now only speculate as to how high insulin levels may impair Akt activation. Possible mechanisms might include phosphoinositide-dependent protein kinase (PDK1) and PDK2 inhibition [27] or impaired Akt phosphorylation by PDK1 due to a defective translocation of Akt to plasma membrane [27,28]. In addition, we cannot rule out other potential mechanisms.

A second interesting result is the dissociation between increased basal activation of insulin signaling in response to long-term exposure to increased insulin levels and the lack of response to a second short-term insulin stimulation. In fact, SkMC-H myoblasts do not respond to insulin stimulation in terms of IR and IRS1 tyrosine phosphorylation, PI3K activation, and Akt-Ser473 phosphorylation or to glucose transport. Decreased protein expression cannot account for these defects. In fact, expression of IR IRS1, p85/PI3K, and Akt is similar in SkMC-L and SkMC-H myoblasts. Moreover, desensitization of insulin signaling is not the result of persistent occupation of IRs because removal of insulin by acid wash from SkMC-H myoblasts does not revert the impairment in IRS1 phosphorylation. Likewise, increased insulin levels in the incubation medium do not affect IRS1 serine phosphorylation as compared with cell maintained in low insulin levels. Thus, taken together, our data suggest that the increased basal phosphorylation of down-regulated IR of SkMC-H could represent one of the levels of desensitization of the insulin-signaling pathway after a short-term insulin stimulus. In addition, the decreased IRS1 tyrosine dephosphorylation found in SkMC-H myoblasts could further contribute for both the high basal IRS1 phosphorylation and the desensitization of IRS1 phosphorylation to short-term insulin stimulation. Our results are only partially in keeping with data obtained in muscle and adipose cells. Interestingly, NIH3T3 cells expressing a constitutive activation of the IR kinase displayed increased basal tyrosine phosphorylation of both β subunits of IR and IRS1 with little or no further stimulation by insulin. In these cells, IRS1 protein content was reduced by 55% [29]. In L6 myoblasts, long insulin treatment (10^2-10^3 nmol/L) desensitized PI3K/Akt pathway to a second short-term insulin stimulation. However, desensitization was correlated to a reduction in IRS1 protein levels possibly because of increased serine phosphorylation and subsequent commitment of the protein to proteosomal degradation [18]. Moreover, L6 myotubes preincubated for 24 hours with high glucose (25 mmol/L) and insulin (100 mmol/L) showed a reduced short-term insulin-mediated glucose uptake and insulin signaling concomitant with a 50% reduction in total cellular IRS1 protein [19]. Similarly incubation of 3T3-L1 adipocytes with 100 nmol/L insulin leads to impaired insulin-mediated glucose transport associated with 70% reduction of expression and 50% decrease of phosphorylation of IRS1 [30]. Our study differs from those reported above with regard to human cell culture model. Thus, our experimental conditions could explain, almost in part, the different result found in this study. Moreover, we cannot rule out that our results are related to the cell type used. Although data obtained in this cell line do have not to be directly extrapolated to mature myocytes, myoblasts express many of the myogenic markers such as myoD, Myf 5, myogenin, and sarcomeric myosin (data not shown). Moreover, myoblasts have been previously used to study insulin signaling and insulin action [18,31]. In conclusion, this study underlines that moderately elevated insulin levels, maintained for a long period, impair insulin signaling and glucose transport. Whether this effect has a role in skeletal muscle insulin resistance is a matter for further appropriately designed studies.

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