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ARTICLE

Isoform-specific defects of insulin stimulation of Akt/protein kinase B (PKB) in skeletal muscle cells from type 2 diabetic patients

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Abstract
Aims/hypothesis The serine/threonine kinase Akt/protein kinase B (PKB) is required for the metabolic actions of insulin. Controversial data have been reported regarding Akt defective activation in the muscle of type 2 diabetic patients. Because three Akt isoforms exist, each having a distinct physiological role, we investigated the contribution of isoform-specific defects to insulin signalling in human muscle.
Methods The phosphorylation pattern and kinase activity of each Akt isoform were compared in primary myotubes from healthy control participants and type 2 diabetic patients. Phosphorylation of Ser^{473} and of Thr^{308} in each isoform was determined after immunoprecipitation in myotubes treated or not with insulin.
Results Muscle cells from diabetic patients displayed defective insulin action and a drastic reduction of insulin-stimulated activity of all Akt isoforms. This was associated with specific defects of their phosphorylation pattern in response to insulin, with impaired Akt2- (and to a lower extent Akt3-) Ser^{473} phosphorylation, and with altered Akt1-Thr^{308} phosphorylation. These defects were not due to faulty phosphoinositide-dependent protein kinase 1 (PDK1) production or activation. Rather, we found higher levels of the Akt2-Ser^{473}-specific protein phosphatase PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1) in muscle from diabetic patients, which may contribute to the alteration of Akt2-Ser^{473} phosphorylation.
Conclusions/interpretation These results suggest that several mechanisms affecting Akt isoforms, including deregulated production of PHLPP1, could underlie the alterations of skeletal muscle insulin signalling in type 2 diabetes. Taking into account the recently described isoform-specific metabolic functions of Akt, our results provide mechanistic insight that may contribute to the defective regulation of glucose and lipid metabolisms in the muscle of diabetic patients.

Keywords Akt isoforms · Insulin signalling · Insulin resistance · Myotubes · PDK1 · PHLPP1/2 · PI 3-kinase · Skeletal muscle

Abbreviations

GSK-3	glycogen synthase kinase-3
PDK1	phosphoinositide-dependent protein kinase 1
PH domain	pleckstrin homology domain
PHLPP	PH domain leucine-rich repeat protein phosphatase

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PI3K phosphoinositide 3-kinase
PKB protein kinase B

Introduction

Type 2 diabetes is characterised by insulin resistance in peripheral tissues, particularly in skeletal muscle, which is responsible for more than 75% of whole-body glucose uptake in response to insulin [1]. The action of insulin is initiated through the binding of the hormone to its receptor, stimulating a cascade of phosphorylation events leading to activation of phosphoinositide 3-kinase (PI3K). In turn, PI3K produces phosphatidylinositol 3,4,5-trisphosphate (PIP_3), a lipid second messenger relaying the metabolic effects of insulin through activation of the serine/threonine protein kinase B (PKB)/Akt [2, 3]. PIP_3 recruits Akt at the plasma membrane, where its kinase activity is elicited by two phosphorylation steps. Akt is first phosphorylated at Ser^{473} by the rictor–mammalian target of rapamycin (mTOR) complex [4], followed by phosphorylation of Thr^{308} by phosphoinositide-dependent protein kinase 1 (PDK1) [5]. Phosphorylation of both residues confers maximal activity to the enzyme and stabilises its active conformation [4, 6].

Akt exists in three isoforms—Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ—coded for by distinct genes and exhibiting more than 80% amino acid sequence identity [7]. The three Akt isoforms share the same structural organisation with an amino-terminal pleckstrin homology domain (PH domain), a kinase catalytic domain encompassing Thr^{308}, and a carboxy-terminal regulatory domain, containing Ser^{473} [8]. Isoform-specific functions of Akt have been revealed from studies of knockout mice. Akt1 plays a critical role in embryonic development, postnatal survival and growth [9]. Akt2-deficient mice exhibit a diabetes-like syndrome [10]. Thus Akt2 is considered to be essential for the maintenance of glucose homeostasis and the control of insulin metabolic actions. This was recently confirmed by muscle-specific overexpression of constitutively active Akt2 in mice [11]. Finally, Akt3 plays a role in the development and organisation of the nervous system [12, 13]. Partially overlapping functions of the Akt isoforms have, however, been suggested by recent reports showing that Akt1, in addition to Akt2, plays a role in insulin action in adipocytes [14] and in lipid metabolism in skeletal muscle cells [15].

It is well established that insulin resistance in type 2 diabetic patients depends on defective insulin signalling in skeletal muscle. Reduced insulin-stimulated PI3K was observed in muscle biopsies [16–19] and in primary culture of human skeletal muscle established from type 2 diabetic patients [20, 21]. However, deregulation of Akt is more controversial, with studies reporting significant reductions of insulin-stimulated Ser^{473} or Thr^{308} phosphorylations [22, 23], and others showing no difference in phosphorylation or enzymatic activity of Akt between control participants and type 2 diabetic patients [18, 24]. A possible explanation for these discrepancies could be the fact that isoform-specific defects of Akt phosphorylation and activity have not been accounted for in these studies. Recently, it has been reported that the activities of Akt2 and Akt3, but not Akt1, are decreased in skeletal muscle biopsies from insulin-resistant morbidly obese participants [25].

To gain more insight on the contribution of each Akt isoform to insulin signalling defects in skeletal muscle in type 2 diabetes, we determined the phosphorylation status and catalytic activities of the three Akt isoforms in primary myotubes. We show that isoform-specific alterations of Akt phosphorylation do occur in myotubes from type 2 diabetic patients in response to insulin, with decreased Ser^{473} phosphorylation on Akt2 and decreased Thr^{308} phosphorylation on Akt1. Decreased Akt-Ser^{473} phosphorylation may be dependent on increased levels of the Akt2-specific phosphatase PH domain leucine-rich repeat protein phosphatase 1 (PHLPP1) in skeletal muscle from type 2 diabetic patients.

Methods

Participants All participants gave their written consent after being informed of the nature, purpose and possible risks of the study. Experimental protocols were approved by the Ethical Committees of the Hospices Civils de Lyon and performed according to French legislation (Huriet law).

Nine healthy lean volunteers and nine moderately obese type 2 diabetic patients were enrolled in the study. Their characteristics are presented in Table 1. Both men and women were recruited. No sex-related differences were observed in our measurements on biopsies or biopsy-derived myotubes. None of the control participants was taking medications or had impaired glucose tolerance, or a familial or personal history of diabetes, obesity, dyslipidaemia or hypertension. Type 2 diabetic patients were treated with oral hypoglycaemic agents (metformin and sulfonylurea). Participants were submitted to a 3 h hyperinsulinaemic–euglycaemic clamp with an insulin infusion rate of 2 mU kg^{-1} min^{-1} as described previously [26, 27]. Skeletal muscle (vastus lateralis) biopsies (wet weight about 80 mg) from five control participants and five type 2 diabetic patients were taken under local anaesthesia before and after the clamp [26] to be used for determination of Akt-Ser^{473} and -Thr^{308} phosphorylation in vivo. To establish primary cultures of skeletal muscle cells (myotubes), vastus lateralis muscle biopsies (wet weight about 200 mg) were taken under local anaesthesia in four control participants and four type 2 diabetic patients in the basal state.

Table 1 Characteristics of the participants

	Control participants (n=9)	Type 2 diabetes patients (n=9)
Sex (male/female)	4/5	6/3
Age (years)	44±3	51±3
BMI (kg/m^2)	22.9±0.5	30.1±0.7***
Fasting glucose (mmol/l)	5±0.2	7.8±0.4***
Fasting insulin (pmol/l)	42±6	84±12*
Plasma NEFA (μmol/l)	608±95	515±61
Plasma triacylglycerol (mmol/l)	690±104	1309±168*
Insulin-induced glucose disposal rate (mmol kg^{-1} min^{-1})	0.056±0.008	0.023±0.002***

* p<0.05, *** p<0.001 for type 2 diabetic patients vs control participants

Culture of human skeletal muscle cells Primary myoblasts were selected using a monoclonal antibody (5.1H11, produced from hybridoma DSHB; University of Iowa, Iowa City, IA, USA) combined with magnetic beads. Differentiated myotubes were prepared as previously described [20, 28]. Four days after initiation of the differentiation, cells showed polynucleated status and produced specific markers of human skeletal muscle. In agreement with previous studies [20, 29, 30], the rates of myoblasts' growth and fusion into myotubes were similar, and there was no morphological difference between cultured skeletal muscle cells from controls and type 2 diabetic patients.

Western blot analysis Myotubes and biopsies were homogenised at 4°C in 20 mmol/l Tris–HCl (pH 8.0), 138 mmol/l NaCl, 1% NP40 (*v*/*v*), 2.7 mmol/l KCl, 1 mmol/l $MgCl_2$, 5% glycerol (*v*/*v*), 5 mmol/l EDTA, 1 mmol/l Na_3VO_4, 20 mmol/l NaF, 1 mmol/l dithiothreitol (DTT) and protease inhibitor cocktail. Lysates were centrifuged (12,000×*g*, 10 min) and stored at −80°C before use. For Akt analysis in biopsies, 100 μg protein lysate was resolved on 10% SDS-PAGE (*w*/*v*), while 40 μg protein lysate was used when analysing myotubes. After transfer to polyvinylidene-fluoride (PVDF) membranes, Akt phosphorylations were detected using anti-phospho-Ser^{473} and anti-phospho-Thr^{308} antibodies (no. 9271 and no. 9275; Cell Signaling Technology, Danvers, MA, USA). According to the manufacturer, these phospho-antibodies detect the three Akt isoforms. In order to analyse specifically each Akt isoform, membranes were probed with isoform-specific antibodies (no. 2967 for Akt1, no. 2962 for Akt2 and no. 4059 for Akt3; Cell Signaling Technology). In order to normalise for equal protein loading, membranes were stripped and re-blotted with anti pan-Akt antibody (sc-1619; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an anti-α-tubulin antibody (sc-5286; Santa Cruz Biotechnology). For PDK1 analysis, anti-PDK1 and anti-phospho Ser^{241}-PDK1 antibodies (no. 3062 and no. 3061; Cell Signaling Technology) were used.

Akt immunoprecipitation To measure isoform-specific Ser^{473} and Thr^{308} phosphorylations, Akt1, Akt2 or Akt3 were immunoprecipitated from 400 μg protein lysates prepared from myotubes treated with or without insulin (100 nmol/l) for 20 min. Immunoprecipitations were performed overnight at 4°C, employing the same antibodies used for western blotting. Protein A (for Akt2 and 3) or protein G (for Akt1) sepharose was added and incubated for 3 hours at 4°C. After washing, immunoprecipitated proteins were resolved on 10% SDS-PAGE (*w*/*v*), transferred and immunodecorated with anti-phospho-Ser^{473} or anti-phospho-Thr^{308} antibodies. Detection was performed using rabbit IgG TrueBlot (no. 18-8816; eBioscience, San Diego, CA, USA), a secondary antibody recognising immunoprecipitated proteins without interfering with immunoprecipitated immunoglobulins. To normalise for equal protein amount, blots were stripped and re-probed with anti pan-Akt antibody.

Determination of Akt enzymatic activity After immunoprecipitation of Akt1, Akt2 or Akt3, sepharose beads were washed in the kinase buffer of the Akt kinase assay kit (no. 9840; Cell Signaling Technology). Akt activity was determined according to the manufacturer's procedure. Briefly, 0.2 mmol/l ATP and 1 μg glycogen synthase kinase-3 (GSK-3) fusion protein was added to the immunoprecipitates and incubated 30 min at 30°C. Supernatant fractions (30 μl) were loaded on 15% SDS-PAGE (*w*/*v*) and phosphorylated GSK-3 protein was detected using an anti-phospho-GSK-3α/β antibody. Normalisation of the blots was performed using anti pan-Akt antibody.

IRS-1-associated PI3K activity IRS-1 was immunoprecipitated using anti-IRS1 antibody (Upstate, Millipore, Billerica, MA, USA) at 4°C from 200 μg protein lysate from myotubes treated with or without insulin (100 nmol/l) for 20 min. PI3K-associated activity was measured in IRS-1 immunocomplexes using phosphatidylinositol (10 μg per reaction; Sigma-Aldrich, St Louis, MO, USA) and 10 μmol/l ATP (supplemented with ^{32}P-labelled γ-ATP, 185 kBq per reaction; Perkin Elmer, Waltham, MA, USA) as described [20, 31]. After the reaction, phosphoinositides were separated by thin-layer chromatography on silica plates (Merck, Darmstadt, Germany) and labelled products were visualised and quantified using Phosphor-Imager SI and Image Quant Software (Molecular Dynamics, Sunnyvale, CA, USA).

Measurement of glycogen synthesis Myotubes were treated for 90 min with or without 100 nmol/l insulin and then incubated for 3 h in 5 mmol/l glucose DMEM, supplemented with 12.5 mmol/l HEPES (pH 7.4) and containing

37 kBq/ml D-[U-^{14}C]glucose (PerkinElmer). After incubation, cells were washed twice with PBS and scraped in PBS/0.1% SDS (*w*/*v*). Lysates were assayed for protein content with the BioRad assay (BioRad, Marnes-la-Coquette, France). Glycogen was extracted as described [28] and the amount of [^{14}C]glucose incorporated into glycogen was determined by scintillation counting.

Quantification of PHLPP1/2 mRNAs *PHLPP1* and *PHLPP2* mRNA expression was measured by reverse transcription followed by real-time PCR, using a Light-Cycler (Roche Diagnostics, Meylan, France), in total RNA preparations from human skeletal muscle samples obtained in a previous study from control and type 2 diabetic patients with similar characteristics to those included in the present work [32]. First-strand cDNAs were synthesised from 500 ng total RNA in the presence of 100 units Superscript II (Invitrogen, Cergy Pontoise, France) using both random hexamers and oligo (dT) primers (Promega, Madison, WI, USA). Real-time PCR was performed in a 20 µl volume containing 5 µl of a 60-fold dilution of the RT reaction medium, 15 µl reaction buffer from the FastStart DNA Master SYBR Green kit (Roche Diagnostics) and 10.5 pmol of the forward/reverse primers (Eurobio, Les Ulis, France). Primers sequences are: *PHLPP1* forward, 5′-ACACCGTGATTGCTCACTCC-3′; reverse, 5′-TTCCAGTCAGGTCTAGCTCC-3′; *PHLPP2* forward, 5′-AGGTTCCTGAGCATCTCTTC-3′; reverse, 5′-GTTCAGGCCCTTCAGTTGAG-3′. Each assay was performed in duplicate and validation of the real-time PCR runs was assessed by evaluation of the melting point of the products and by the slope and error obtained with the standard curve. Analyses were performed using the Light-Cycler software (Roche Diagnostics). Results are presented as relative concentrations using hypoxanthine phosphoribosyltransferase (HPRT) mRNA levels as internal standard.

Statistical analysis Data are presented as means ± SEM. Statistical significance of the results was determined using a paired Student's *t* test when comparing the effect of insulin in culture myotubes or in muscle biopsies and unpaired Student's *t* test when comparing the data from control and type 2 diabetic patients. The threshold for significance was set at a p value$\leq$0.05.

Results

Insulin stimulation of Akt phosphorylation in human skeletal muscle biopsies The effects of insulin on Akt-Ser^{473} and -Thr^{308} phosphorylation was determined in muscle biopsies taken before and after a 3 h hyperinsulinaemic–euglycaemic clamp in five controls and five type 2 diabetic patients (Fig. 1). In control participants, insulin strongly increased Akt phosphorylation, both on Ser^{473} and Thr^{308} residues. In type 2 diabetic patients, basal Ser^{473} phosphorylation tended to be reduced, although the difference compared with control participants did not reach statistical significance (p=0.18). After the hyperinsulinaemic–euglycaemic clamp, Akt-Ser^{473} phosphorylation was significantly lower in the muscle of type 2 diabetic patients (p=0.022) compared with controls. Conversely, Thr^{308} phosphorylation did not differ in biopsies from controls versus type 2 diabetic patients, either in the basal or in the insulin-stimulated condition (Fig. 1). In agreement with previous studies [18, 21, 22], there was no alteration in the global amount of Akt in the skeletal muscle of type 2 diabetic patients compared with controls, nor did the 3 h clamp affect the total amount of Akt in human muscle (Fig. 1).

Isoform-specific Akt phosphorylation defects in myotubes from type 2 diabetic patients We next investigated the contribution of each Akt isoform to insulin signalling in primary myotubes. This cell model has been largely used to study the action of insulin and its defects in insulin resistance. In the present study, myotubes displayed reduced insulin-induced PI3K activity and glycogen synthesis when

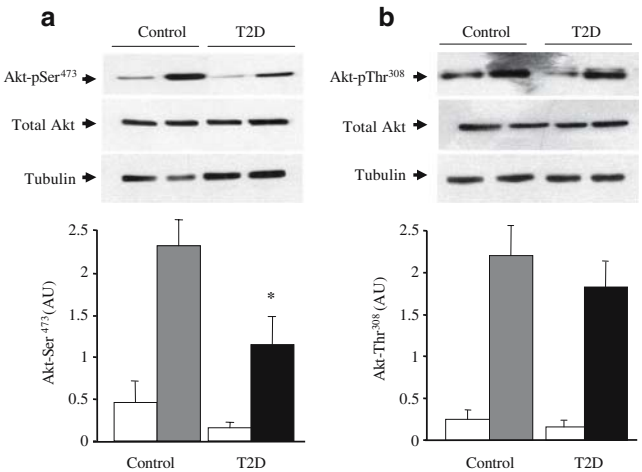

Fig. 1 Effects of hyperinsulinaemia on the phosphorylations of Ser^{473} and Thr^{308} of Akt in skeletal muscle of type 2 diabetic patients. Protein lysates (100 µg) from muscle biopsies taken before and at the end of a 3 h hyperinsulinaemic–euglycaemic clamp were separated on a 10% SDS-PAGE (*w*/*v*), transferred to polyvinylidenefluoride (PVDF) membranes and immunoblotted with antibodies directed to Akt-phospho (p)-Ser^{473} (**a**), Akt-pThr^{308} (**b**), total Akt and tubulin (to normalise for equal protein loading). The bars show the quantification of Akt-pSer^{473} and Akt-pThr^{308} signals obtained with muscle samples from five control and five type 2 diabetic (T2D) patients. White bars, biopsies taken before the clamp; grey and black bars, biopsies taken at the end of the clamp. *p<0.05 for Akt-Ser^{473} phosphorylation in control participants vs diabetic patients at the end of the hyperinsulinaemic clamp. AU, arbitrary units

prepared from the skeletal muscle of type 2 diabetic patients (Fig. 2).

As shown in Fig. 3, all Akt isoforms were detected in myotubes and the amount did not differ significantly in cells from controls and type 2 diabetic patients. However, Akt1 was increased, although in a non-significant manner, in type 2 diabetic patient-derived myotubes. This reflects a variability in Akt1 levels among diabetic patients, as already noticed in another study [22]. The phosphorylation status of each Akt isoform was then determined by specific immunoprecipitation on lysates from myotubes treated or not for 20 min with insulin (100 nmol/l). Ser^{473} and Thr^{308} phosphorylations were analysed in the immunoprecipitates by immunoblotting, and the blots were re-probed with a total Akt antibody to normalise for protein loading. Figure 4 shows the results of Ser^{473} phosphorylation. Insulin-stimulated Akt1 Ser^{473} phosphorylation was similar in myotubes from diabetic patients compared with control myotubes. On the contrary, there was a significant reduction in the ability of insulin to stimulate Akt2-Ser^{473} phosphorylation in myotubes from type 2 diabetic patients ($p=0.035$). Regarding Akt3, insulin-induced Ser^{473} phosphorylation was also diminished in myotubes from type 2 diabetic patients, but the decrease did not reach statistical significance ($p=0.229$) in comparison with the response in cells from controls.

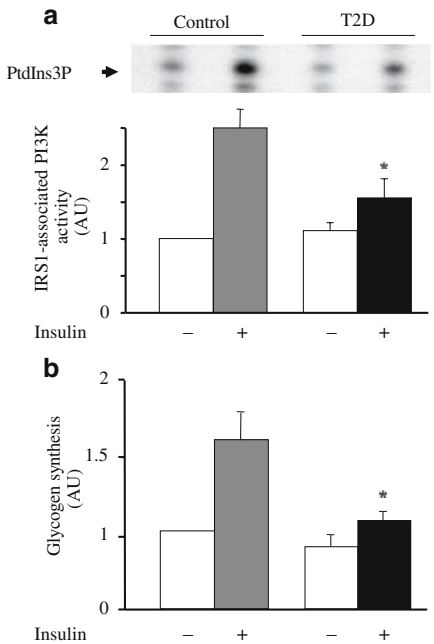

Fig. 2 Impaired insulin-stimulated IRS1-associated PI3K activity and glycogen synthesis in myotubes from type 2 diabetic patients. **a** Differentiated myotubes from controls and type 2 diabetic (T2D) patients were serum starved overnight and stimulated with 100 nmol/l insulin for 20 min. IRS1-associated PI3K activity was measured in IRS-1 immune complexes obtained from non-treated (–) or insulin-stimulated myotubes (+) as described in the Methods section. ^{32}P-labelled phosphatidylinositol 3-phosphate (PtdIns3P) was separated by thin-layer chromatography and quantified from three independent experiments. PI3K activity was set at 1 for the basal condition in myotubes from control participants. **b** Insulin-stimulated glycogen synthesis was measured in primary myotubes from control and T2D patients following a 3 h stimulation without (white bars) or with 100 nmol/l insulin (grey and black bars). Incorporation of [^{14}C]glucose into glycogen (corrected by the protein levels) was set at 1 for the basal condition in myotubes from control participants. *$p<0.05$ for insulin effect in cells from T2D patients vs control participants. AU, arbitrary units

In parallel to Ser^{473}, we also determined Thr^{308} phosphorylation. Figure 5 shows that insulin-stimulated phosphorylation of Akt1 on Thr^{308} was decreased twofold in myotubes from type 2 diabetic patients compared with myotubes from control participants (2.2±0.7 vs 4.1±1.1-fold increase over basal, respectively; $p=0.059$). Of note, Thr^{308} phosphorylation occurred mainly on a lower molecular mass form of Akt1 in cells from type 2 diabetic patients (Fig. 5). Regarding Akt2, insulin-induced Thr^{308} phosphorylation was similar in myotubes from control and diabetic patients (Fig. 5). Finally, analysis of Akt3-Thr^{308} phosphorylation was not possible due to poor reactivity of the phospho-Thr^{308} antibody towards Akt3 immunoprecipitates (data not shown).

PDK1 is the kinase mediating Akt-Thr^{308} phosphorylation. Figure 6 shows that neither the amount of protein nor Ser^{241} phosphorylation of PDK1 are altered in myotubes from type 2 diabetic patients, indicating that decreased Akt1-Thr^{308} phosphorylation is not due to faulty PDK1 activation.

Enzymatic activity of Akt isoforms is altered in myotubes from type 2 diabetic patients Since the phosphorylation state of the Akt isoforms differed between myotubes from control and diabetic patients, we next measured Akt kinase activities. An in vitro phosphorylation assay of GSK-3 was performed in isoform-specific immunoprecipitates of Akt1, Akt2 and Akt3 from myotubes treated or not with insulin for 20 min. As seen in Fig. 7, insulin stimulated the activity of all Akt isoforms in control myotubes, with a more pronounced effect on Akt1 and Akt3. Instead, we observed profound alterations in the response to insulin for each Akt isoform in myotubes from type 2 diabetic patients: basal activities of Akt1 and Akt2 were reduced and the response to insulin stimulation was suppressed for Akt1 and Akt2 and strongly reduced for Akt3 (Fig. 7).

mRNA expression of the Akt2-specific PHLPP1 protein serine phosphatase are altered in skeletal muscle from type 2 diabetic patients Control of Akt activity results from the balance between activatory phosphorylation and dephosphorylation-dependent inactivation. The recent discovery of two phosphatases, PHLPP1 and PHLPP2 [33], which terminate Akt signalling by dephosphorylating Ser^{473} in an isoform-specific manner, prompted us to evaluate their levels in skeletal muscle biopsies from control and type 2 diabetic patients (Fig. 8). PHLPP1 is the predominant isoform, with

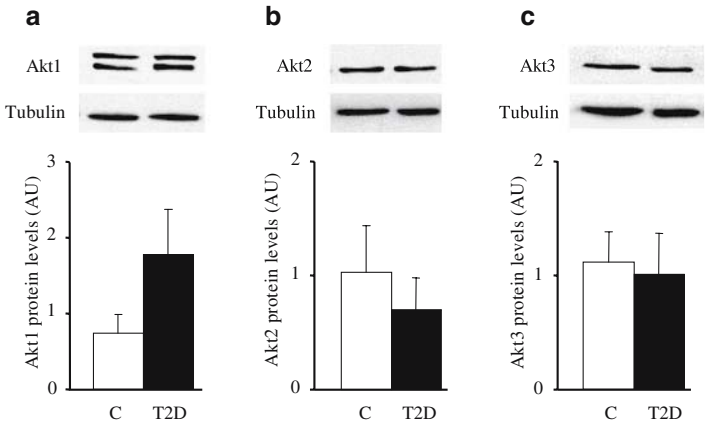

Fig. 3 Akt isoform levels in myotubes from control and type 2 diabetic participants. Protein lysates (40 μg) from control participants (white bars) or diabetic patients (black bars) were separated by SDS-PAGE and immunoblotted with specific antibodies to Akt1, Akt2 and Akt3. The blots are representative western blots for each isoform and immunoblotting with anti-tubulin antibodies to normalise for equal protein loading. The bar charts show quantification of Akt isoform levels in myotubes from control and type 2 diabetic patients, n=4 in each group. AU, arbitrary units

mRNA expression being about fivefold higher than that of PHLPP2. More importantly, mRNA expression of the Akt2-specific PHLPP1 was increased 1.5-fold in muscle from diabetic patients (p=0.024), while PHLPP2 levels were not different between groups (Fig. 8).

Discussion

It is widely accepted that Akt/PKB is required for the metabolic actions of insulin [34]. However, there have been contradictory results on whether the insulin-induced acti-

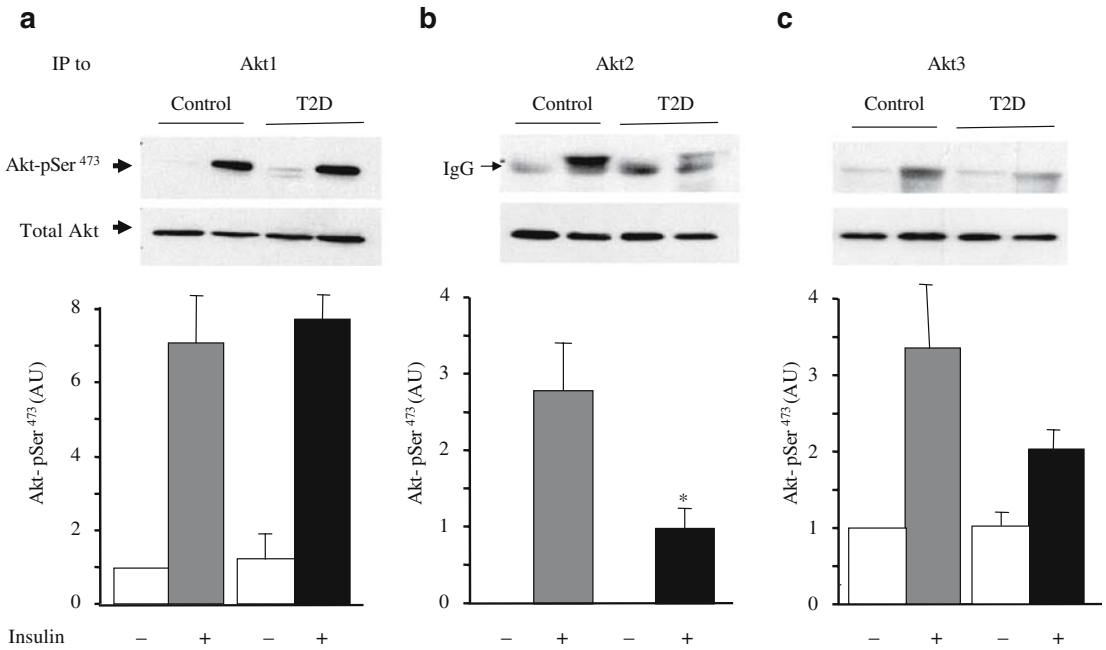

Fig. 4 Insulin-stimulated Ser^{473} phosphorylation of the specific Akt isoforms in myotubes from type 2 diabetic patients. Protein lysates (400 μg) from myotubes treated (grey and black bars) or not (white bars) with 100 nmol/l insulin for 20 min, were immunoprecipitated (IP) with specific antibodies to Akt1, Akt2 or Akt3. The immunoprecipitates were separated by SDS-PAGE and phosphorylation on Ser^{473} was detected with the Akt-phospho (p)-Ser^{473} antibody. Blots are representative western blots of Akt-Ser^{473} phosphorylation and total Akt amounts. The bar charts show quantification of Akt-Ser^{473} phosphorylation levels in 4 different experiments with myotubes from controls and type 2 diabetic (T2D) patients. For Akt1 and Akt3 data were set at 1 unit for the basal condition in myotubes from control participants. For Akt2, basal Ser^{473} phosphorylation was not detectable in human muscle cells from control or from diabetic patients. IgG represent the 55 kDa immunoglobulins. *p<0.05 for Akt-Ser^{473} phosphorylation after insulin stimulation in myotubes from type 2 diabetic patients vs cells from control participants. AU, arbitrary units

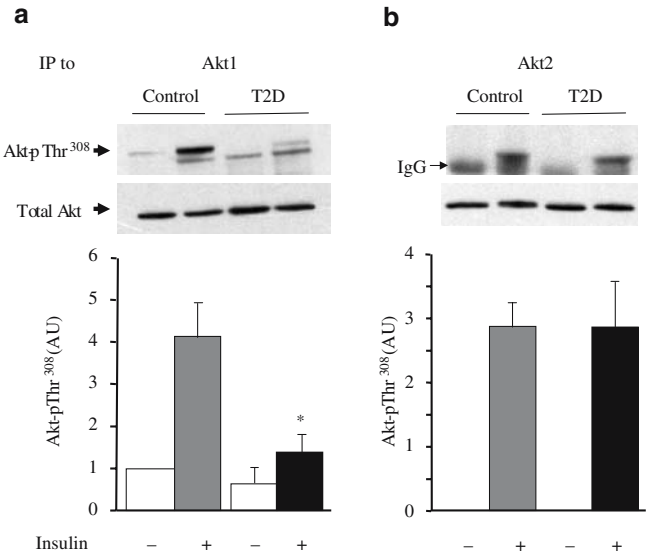

Fig. 5 Insulin-stimulated Thr^{308} phosphorylation of the specific Akt isoforms in myotubes from type 2 diabetic patients. Protein lysates (400 μg) from myotubes treated (grey and black bars) or not (white bars) with 100 nmol/l insulin for 20 min, were immunoprecipitated (IP) with specific antibodies to Akt isoforms. The immunoprecipitates were separated by SDS-PAGE and phosphorylation on Thr^{308} was detected with the Akt-phospho (p)-Thr^{308} antibody. Blots are representative western blots of Akt-Thr^{308} phosphorylation and total Akt amounts. Bar charts show quantification of Akt-Thr^{308} phosphorylation levels in four different experiments with myotubes from control and type 2 diabetic (T2D) patients. Accurate analysis of Akt3 Thr^{308} phosphorylation was not possible due to poor reactivity of the $pThr^{308}$ antibody towards Akt3 immunoprecipitates (data not shown). For Akt1 data were set at 1 unit for the basal condition in myotubes from control participants. For Akt2, basal Thr^{308} phosphorylation was not detectable in human muscle cells from control or from diabetic participants. IgG represents the 55 kDa immunoglobulins. *$p<0.05$ for Akt-Thr^{308} phosphorylation after insulin stimulation in myotubes from type 2 diabetic patients *vs* cells from control participants

vation of Akt in human skeletal muscle of type 2 diabetic patients is impaired [18, 21, 23]. In this study, we measured the phosphorylation and enzymatic activities of each Akt isoform in primary myotubes from human skeletal muscle. We found that the Akt phosphorylation pattern is altered in myotubes from type 2 diabetic patients in an isoform-specific manner.

Because of the limited amount of material available, it was not possible to immunoprecipitate each Akt isoform to analyse the specific phosphorylation pattern directly from biopsies. Thus, in muscle biopsies, we only evaluated overall Akt phosphorylation, which revealed decreased insulin-induced Ser^{473} phosphorylation in type 2 diabetic patients after the 3 h clamp, while Thr^{308} phosphorylation was unaffected (Fig. 1). Since Akt2 is the prominent isoform in skeletal muscle ([35]; D. Cozzone and H. Vidal, unpublished observation), overall Akt phosphorylation in biopsies probably reflects the phosphorylation state of Akt2. Subsequent use of primary myotubes overcame the problem of material availability. We observed that the three Akt isoforms are produced in myotubes (Fig. 3). This cell model displays several features of mature skeletal muscle, and myotubes derived from type 2 diabetic patients have consistently been shown to retain an insulin resistant phenotype with altered insulin-dependent glucose and lipid metabolism [29, 30, 36, 37] and defective insulin signalling [20, 36]. These defects include strong reduction in the activation of PI3K in response to insulin [20, 21].

Akt isoforms are downstream targets of PI3K. Full activation requires phosphorylation of Ser^{473} and Thr^{308}, with Thr^{308} phosphorylation only being sufficient to relay 15% of the maximal Akt activity [2, 4]. In myotubes from type 2 diabetic patients, we report a loss of efficacy of insulin to stimulate the enzymatic activity of all Akt isoforms (Fig. 7). This effect is consequential to the reduction of insulin-stimulated PI3K (Fig. 2). Furthermore, by evaluating the phosphorylations of each Akt isoform, we demonstrated the existence of isoform-specific alterations. Regarding Akt1, Thr^{308} phosphorylation is decreased without modification on Ser^{473} phosphorylation. The opposite is found for Akt2, with a specific alteration of Ser^{473} phosphorylation but not Thr^{308} phosphorylation. For Akt3, we could not analyse Thr^{308} phosphorylation, but Ser^{473} phosphorylation was decreased, although less than for Akt2.

By employing PDK-$1^{-/-}$ cells it was demonstrated that inhibition of Thr^{308} phosphorylation, independently from Ser^{473} phosphorylation, ablates Akt activity [38]. Defective stimulation of Thr^{308} phosphorylation in response to insulin could thus explain the marked reduction of Akt1 activity in myotubes from diabetic patients (Fig. 7). However, we did not observe alterations in the production or phosphorylation of PDK1 in myotubes from type 2 diabetic patients. Together with the fact that Akt2-Thr^{308} phosphorylation was unaffected, these data suggest that the defective Akt1-Thr^{308} phosphorylation is not due to a dysfunction of PDK-1.

Relative to Akt2, a specific defect of Ser^{473} phosphorylation was observed in myotubes from type 2 diabetic

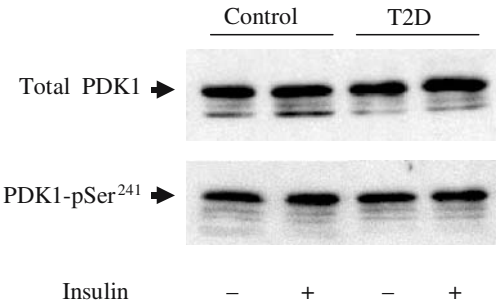

Fig. 6 PDK1 levels and phosphorylation in myotubes from type 2 diabetic patients. Protein lysates (40 μg) from myotubes from controls and type 2 diabetic (T2D) participants were separated by SDS-PAGE and immunoblotted with specific antibodies to phospho (p)-PDK1 and total PDK1. The western blots presented are representative of three different experiments

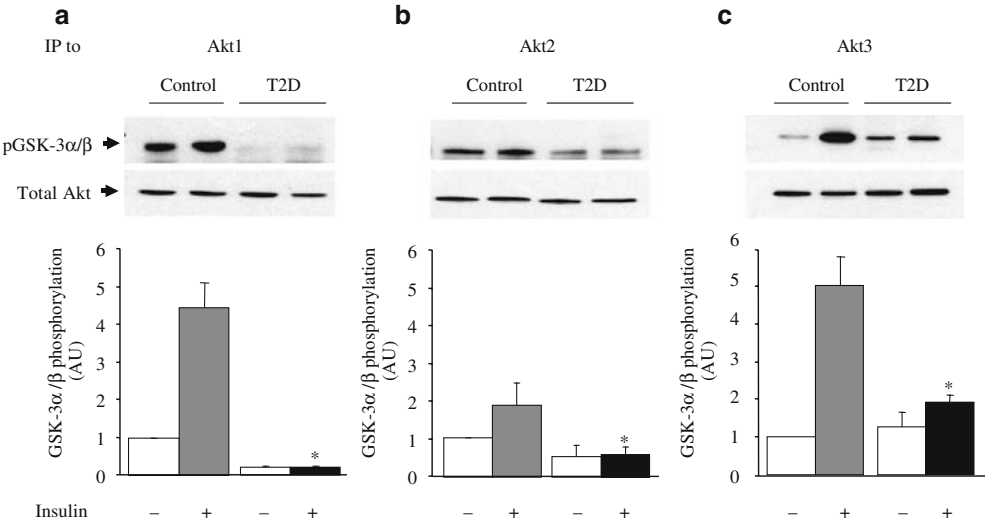

Fig. 7 Effect of insulin on Akt1, Akt2 and Akt3 kinase activities in myotubes from type 2 diabetic patients. Protein lysates (40 μg) from myotubes treated (grey and black bars) or not (white bars) with 100 nmol/l insulin for 20 min were immunoprecipitated with specific antibodies to Akt isoforms. Akt kinase activity was measured using a GSK-3 fusion protein as substrate, as indicated in the Methods section. The phosphorylation of the GSK-3 protein was measured by immunoblotting using anti-phospho GSK-3α/β antibody. Blots are representative western blots of GSK-3 phosphorylation by each immunoprecipitated (IP) Akt isoform, and total Akt amount to normalise for equal protein loading. The bar charts show quantification of GSK-3 protein phosphorylation levels in myotubes from control and type 2 diabetic (T2D) patients (n=4). For each Akt isoform, data were set at 1 unit for the basal condition in myotubes from control participants. *p<0.05 comparing insulin-stimulated Akt activity in muscle cells from control participants vs T2D patients. AU, arbitrary units

patients, without alteration of Thr^{308} phosphorylation. The Akt Ser473Ala mutant, phosphorylated solely on Thr^{308} upon insulin stimulation, retains a partial catalytic activity (15% of wild-type Akt [2]). Thus, normal Thr^{308} and decreased Ser^{473} phosphorylation might partially activate Akt2. However, we could not distinguish such partial activation from the basal state, considering that Akt2 activation in control myotubes was twofold. Several Akt-Ser^{473} kinases are known, including the rictor–mTOR complex [4]. Based on the fact that the Akt1-Ser^{473} phosphorylation is not altered, we assume that rictor-mTOR activity is not impaired and does not contribute to the specific defect of Akt2. An alternative mechanism could be the implication of isoform specific phosphatases. Indeed, PHLPP1 dephosphorylates the Ser^{473} residue of Akt2, whereas Akt1-Ser^{473} is specifically dephosphorylated by PHLPP2 [33]. Therefore we measured *PHLPP1* and *PHLPP2* mRNA expression in skeletal muscle biopsies. Our observation that *PHLPP1* mRNA is upregulated in type 2 diabetic patients (Fig. 8) provides an attractive hypothesis to explain the defect of Akt2-Ser^{473} phosphorylation. Likewise, the fact that *PHLPP2* mRNA expression is similar in control participants and type 2 diabetic patients is in keep with the unaltered Akt1-Ser^{473} phosphorylation. Further investigations determining the PHLPP1/2 protein levels and enzymatic activities are warranted to fully validate this hypothesis.

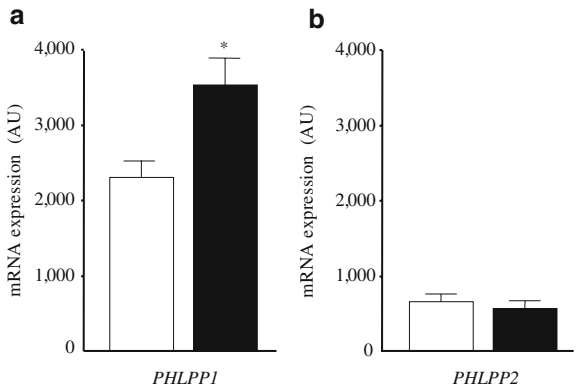

Fig. 8 Expresion of *PHLPP1* and *PHLPP2* mRNA in skeletal muscle biopsies from controls and type 2 diabetic subjects. The mRNA expression of (**a**) *PHLPP1* and (**b**) *PHLPP2* was determined by quantitative real-time PCR, as described in the Methods section. Vastus lateralis muscle samples were obtained from age-matched healthy control subjects (white bars) and type 2 diabetic patients (black bars) [32]. *p=0.024 comparing *PHLPP1* mRNA expression in biopsies from control subjects vs diabetic patients. AU, arbitrary units

In view of our data, it now appears important that a general consensus should be reached as to whether Akt action is impaired in skeletal muscle in type 2 diabetes. Krook et al. [22], using a non-commercial antibody recognising primarily (but perhaps not exclusively) Akt1, initially demonstrated defective insulin-induced activation of Akt1 in isolated muscle strips from biopsies from moderately obese type 2 diabetic patients. Soon after, Kim et al. [18], analysing muscle biopsies taken before and after an hyperinsulinaemic–euglycaemic clamp in type 2 diabetic patients, did not observe defective Akt1/2 activity nor phosphorylation in spite of decreased insulin-induced PI3K activation. This finding was further supported by the observation that total Akt phospho-

rylation was globally unchanged in muscle cell cultures and biopsies from controls versus type 2 diabetic patients [21, 23]. It should be noted, however, that these studies did not measure separately the phosphorylation of each Akt isoform, neither isoform-specific activity, since the antibodies used did not discriminate between Akt1 and Akt2. In another study, Brozinick et al. [25] showed that Akt1-Ser^{473} phosphorylation increases significantly upon insulin stimulation both in lean and, to a lesser extent, in obese participants. Yet, this study did not include type 2 diabetic patients, but morbidly insulin-resistant obese participants. Moreover, this study did not investigate the phosphorylations on Akt2 and Akt3 [25]. Finally, given the reduced Ser^{473} phosphorylation of Akt1 and IRS1-associated PI3K activity observed in obese participants, it is surprising that normal Akt1 activity was reported, while Akt2 and Akt3 activities were reduced [25]. No explanation was provided to address these discrepancies and, to our opinion, this underscores the importance of studying the phosphorylation of both Ser^{473} and Thr^{308}, as was done in our investigation. Another important issue is the origin of the muscle biopsies, which might influence the results. In our study, as in most studies [18, 21–23], biopsies were from vastus lateralis, while Brozinick et al. used strips from the rectus abdominal muscle [25]. Further methodological differences might include the use of antibodies from different sources, which could explain discrepancies in the results, especially regarding isoform levels, phosphorylation and activity.

The originality of our study, compared with the preceding ones, is that we provide data on both phosphorylation and activity for each Akt isoform, something that was investigated partly in Kim's and Brozinick's studies [18, 25]. Furthermore, we attempted to define possible mechanisms to explain the differences in Akt phosphorylation/activity between control participants and type 2 diabetic patients, including analysis of PDK1 and PHLPP.

In a recent report, siRNA-mediated Akt1 and Akt2 silencing in myotubes revealed Akt isoform-specific functions governing glucose and lipid metabolism [15]. An IRS-1/Akt2 pathway, requiring Ser^{473} phosphorylation, was involved in the regulation of glucose metabolism, while an IRS-2/Akt1 pathway, requiring Thr^{308} phosphorylation, mediated lipid uptake and metabolism [15]. Here, using a human myotubes model with naturally occurring insulin resistance, we demonstrate that the regulation by insulin of phosphorylation of both Akt2-Ser^{473} and Akt1-Thr^{308} are altered in myotubes from type 2 diabetic patients. Because defects in the regulation of glucose and lipid metabolism in skeletal muscle are hallmarks of type 2 diabetes mellitus, our findings, together with those of Bouzakri et al. [15], suggest that a combination of mechanisms, affecting the different pathways of Akt activation in response to insulin, contribute to insulin resistance. Studies are now needed to verify whether these alterations observed in cultured myotubes also occur *in vivo* in the skeletal muscle of type 2 diabetic patients.

In summary, we have demonstrated that myotubes from moderately obese type 2 diabetic patients are characterised by specific alterations in the phosphorylation of the different Akt isoforms, with defective Akt2-Ser^{473} phosphorylation and, to a lesser extent, Akt3-Ser^{473} phosphorylation and altered Thr^{308} phosphorylation in the case of Akt1. For each Akt isoform, profound inhibition of enzymatic activity was observed. These defects could be in part due to increased levels of PHLPP1 in the muscle of type 2 diabetic patients. Because specific biological roles of Akt isoforms have been proposed in skeletal muscle, our observations provide new clues to understand the defective action of insulin on different metabolic pathways in type 2 diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. DeFronzo RA, Ferrannini E, Sato Y, Felig P, Wahren J (1981) Synergistic interaction between exercise and insulin on peripheral glucose uptake. J Clin Invest 68:1468–1474
2. Alessi DR, Andjelkovic M, Caudwell B et al (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. Embo J 15:6541–6551
3. White MF (1997) The insulin signalling system and the IRS proteins. Diabetologia 40(Suppl 2):S2–S17
4. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101
5. Alessi DR, Deak M, Casamayor A et al (1997) 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. Curr Biol 7:776–789
6. Scheid MP, Marignani PA, Woodgett JR (2002) Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol 22:6247–6260
7. Fayard E, Tintignac LA, Baudry A, Hemmings BA (2005) Protein kinase B/Akt at a glance. J Cell Sci 118:5675–5678
8. Hanada M, Feng J, Hemmings BA (2004) Structure, regulation and function of PKB/AKT—a major therapeutic target. Biochim Biophys Acta 1697:3–16
9. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ (2001) Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 276:38349–38352
10. Cho H, Mu J, Kim JK et al (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292:1728–1731
11. Cleasby ME, Reinten TA, Cooney GJ, James DE, Kraegen EW (2007) Functional studies of Akt isoform specificity in skeletal

muscle in vivo; maintained insulin sensitivity despite reduced insulin receptor substrate-1 expression. Mol Endocrinol 21:215–228
12. Easton RM, Cho H, Roovers K et al (2005) Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Mol Cell Biol 25:1869–1878
13. Tschopp O, Yang ZZ, Brodbeck D et al (2005) Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. Development 132:2943–2954
14. Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP (2003) Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc Natl Acad Sci USA 100:7569–7574
15. Bouzakri K, Zachrisson A, Al-Khalili L et al (2006) siRNA-based gene silencing reveals specialized roles of IRS-1/Akt2 and IRS-2/Akt1 in glucose and lipid metabolism in human skeletal muscle. Cell Metab 4:89–96
16. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM (2005) Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. Diabetes 54:2351–2359
17. Beeson M, Sajan MP, Dizon M et al (2003) Activation of protein kinase C-zeta by insulin and phosphatidylinositol-3,4,5-(PO4)3 is defective in muscle in type 2 diabetes and impaired glucose tolerance: amelioration by rosiglitazone and exercise. Diabetes 52:1926–1934
18. Kim YB, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB (1999) Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. J Clin Invest 104:733–741
19. Krook A, Bjornholm M, Galuska D et al (2000) Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes 49:284–292
20. Bouzakri K, Roques M, Gual P et al (2003) Reduced activation of phosphatidylinositol-3 kinase and increased serine 636 phosphorylation of insulin receptor substrate-1 in primary culture of skeletal muscle cells from patients with type 2 diabetes. Diabetes 52:1319–1325
21. Nikoulina SE, Ciaraldi TP, Carter L, Mudaliar S, Park KS, Henry RR (2001) Impaired muscle glycogen synthase in type 2 diabetes is associated with diminished phosphatidylinositol 3-kinase activation. J Clin Endocrinol Metab 86:4307–4314
22. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H (1998) Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. Diabetes 47:1281–1286
23. Meyer MM, Levin K, Grimmsmann T, Beck-Nielsen H, Klein HH (2002) Insulin signalling in skeletal muscle of subjects with or without Type II-diabetes and first degree relatives of patients with the disease. Diabetologia 45:813–822
24. Cusi K, Maezono K, Osman A et al (2000) Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. J Clin Invest 105:311–320
25. Brozinick JT Jr, Roberts BR, Dohm GL (2003) Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle: potential role in insulin resistance. Diabetes 52:935–941
26. Ducluzeau PH, Perretti N, Laville M et al (2001) Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. Diabetes 50:1134–1142
27. Laville M, Auboeuf D, Khalfallah Y, Vega N, Riou JP, Vidal H (1996) Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4, and lipoprotein lipase mRNA levels in human muscle. J Clin Invest 98:43–49
28. Cozzone D, Debard C, Dif N et al (2006) Activation of liver X receptors promotes lipid accumulation but does not alter insulin action in human skeletal muscle cells. Diabetologia 49:990–999
29. Gaster M, Petersen I, Hojlund K, Poulsen P, Beck-Nielsen H (2002) The diabetic phenotype is conserved in myotubes established from diabetic subjects: evidence for primary defects in glucose transport and glycogen synthase activity. Diabetes 51:921–927
30. Henry RR, Ciaraldi TP, Abrams-Carter L, Mudaliar S, Park KS, Nikoulina SE (1996) Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. Biochemical and molecular mechanisms. J Clin Invest 98:1231–1236
31. Frojdo S, Cozzone D, Vidal H, Pirola L (2007) Resveratrol is a class IA phosphoinositide 3-kinase inhibitor. Biochem J 406:511–518
32. Debard C, Laville M, Berbe V et al (2004) Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of type 2 diabetic patients. Diabetologia 47:917–925
33. Brognard J, Sierecki E, Gao T, Newton AC (2007) PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. Mol Cell 25:917–931
34. Whiteman EL, Cho H, Birnbaum MJ (2002) Role of Akt/protein kinase B in metabolism. Trends Endocrinol Metab 13:444–451
35. Altomare DA, Lyons GE, Mitsuuchi Y, Cheng JQ, Testa JR (1998) Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. Oncogene 16:2407–2411
36. Henry RR, Abrams L, Nikoulina S, Ciaraldi TP (1995) Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects. Comparison using human skeletal muscle cell cultures. Diabetes 44:936–946
37. Jackson S, Bagstaff SM, Lynn S, Yeaman SJ, Turnbull DM, Walker M (2000) Decreased insulin responsiveness of glucose uptake in cultured human skeletal muscle cells from insulin-resistant nondiabetic relatives of type 2 diabetic families. Diabetes 49:1169–1177
38. Williams MR, Arthur JS, Balendran A et al (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr Biol 10:439–448