

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

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To understand better the defects in the proximal steps of insulin signaling during type 2 diabetes, we used differentiated human skeletal muscle cells in primary culture. When compared with cells from control subjects, myotubes established from patients with type 2 diabetes presented the same defects as those previously evidenced in vivo in muscle biopsies, including defective stimulation of phosphatidylinositol (PI) 3-kinase activity, decreased association of PI 3-kinase with insulin receptor substrate (IRS)-1 and reduced IRS-1 tyrosine phosphorylation during insulin stimulation. In contrast to IRS-1, the signaling through IRS-2 was not altered. Investigating the causes of the reduced tyrosine phosphorylation of IRS-1, we found a more than twofold increase in the basal phosphorylation of IRS-1 on serine 636 in myotubes from patients with diabetes. Concomitantly, there was a higher basal mitogen-activated protein kinase (MAPK) activity in these cells, and inhibition of the MAPKs with PD98059 strongly reduced the level of serine 636 phosphorylation. These results suggest that IRS-1 phosphorylation on serine 636 might be involved in the reduced phosphorylation of IRS-1 on tyrosine and in the subsequent alteration of insulin-induced PI 3-kinase activation. Moreover, increased MAPK activity seems to play a role in the phosphorylation of IRS-1 on serine residue in human muscle cells. *Diabetes* 52:1319–1325, 2003

Insulin resistance of skeletal muscle is the main defect associated with type 2 diabetes (1). Because all of the metabolic actions of insulin are impaired in diabetic muscle, including glucose transport, glycogen synthesis, glucose oxidation, and the regulation of gene expression, a defect in an early step of the insulin

signaling pathways could cause these abnormalities (1–3). A large body of evidence indicates that phosphatidylinositol (PI) 3-kinase is central to these different effects of insulin (2,4), and a reduced insulin-stimulation of PI 3-kinase activity has been demonstrated in patients with type 2 diabetes (5–9). It can therefore be assumed that defective activation of PI 3-kinase in muscle is one of the culprits of the impaired insulin action in skeletal muscle in type 2 diabetes. However, the causes of the reduced activation of PI 3-kinase during insulin stimulation are still not clearly defined. Several data suggest that it is related to a reduction in the association of PI 3-kinase with insulin receptor substrate (IRS)-1 as a consequence of a lower phosphorylation of IRS-1 on tyrosine residues (5–9). Although conflicting results have been reported (10–12), the reduced phosphorylation of IRS-1 on tyrosine residues could be due to a defective activity of the insulin receptor tyrosine kinase. Alternative mechanisms could be also proposed, such as an increase in specific tyrosine phosphatases or an increase in the phosphorylation of IRS-1 on serine/threonine residues. This latter possibility is a tempting hypothesis because phosphorylation of specific serine/threonine residues has been shown to prevent tyrosine phosphorylation of IRS-1, reducing its association with p85 α PI 3-kinase and the subsequent activation of PI 3-kinase (13–15).

The study of insulin signaling in vivo in human skeletal muscle is limited by the small amount of material available and by the fact that some metabolic parameters, such as glucose or free fatty acid plasma concentrations, differ among control subjects and patients with type 2 diabetes. An alternative and attractive approach is the utilization of primary culture of skeletal muscle cells. This cell model displays numerous features of mature skeletal muscle, and cells from different subjects can be studied under well-controlled experimental conditions (16–18). Importantly, myotubes established from patients with type 2 diabetes conserve the diabetic phenotype, including decreased insulin responsiveness of glucose uptake and glycogen synthase activation (16,19–23). Moreover, in agreement with the in vivo situation (5–9), altered insulin-stimulation of PI 3-kinase activity has been demonstrated in myotubes from patients with type 2 diabetes, and this defect seemed to be the consequence of a diminished association of PI 3-kinase

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ERK, extracellular signal-related kinase; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol.

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with IRS-1 (23). However, in another recent study, the altered activation of PI 3-kinase by insulin has been related to a reduced association of PI 3-kinase with IRS-2 rather than with IRS-1 in myotubes from obese subjects with impaired glucose tolerance (18). To get more insight into the mechanism that leads to altered insulin response in muscle of with patient diabetes, we investigated the possible causes of the altered regulation of PI 3-kinase activity by insulin in primary culture of skeletal muscle cells from patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Culture of human skeletal muscle cells. Muscle biopsies (~100 mg wet weight) were taken under local anesthesia from the vastus lateralis muscle of nine lean, healthy, control subjects (five men, four women; aged 42 ± 6 years, BMI 24 ± 1 kg/m², fasting plasma glucose 5.0 ± 0.2 mmol/l, and fasting plasma insulin 31 ± 4 pmol/l) with no familial or personal history of diabetes, dyslipidemia, or hypertension and from nine moderately obese patients with type 2 diabetes (four men, five women; aged 56 ± 4 years, BMI 32 ± 2 kg/m², fasting plasma glucose 9.6 ± 0.9 mmol/l, glycosylated HbA_{1c} $9.1 \pm 0.5\%$ and duration of diabetes 12 ± 3 years). None of the control subjects was taking medication except for oral contraceptive agents. The patients with type 2 diabetes were treated with diet alone ($n = 1$), oral hypoglycemic agents (metformin and sulfonylurea, $n = 3$), or insulin alone ($n = 5$). The experimental protocol was approved by the Ethics Committee of Hospices Civils de Lyon.

The satellite cells were isolated from the muscle biopsies by trypsin digestion and were grown in Ham's F10 medium supplemented with 20% FCS and antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml) as previously described in detail (17). Confluent myoblasts were allowed to fuse and to differentiate into myotubes in Dulbecco's modified Eagle's medium containing 2% FCS and antibiotics. Human myotubes were used 15 days after induction of the differentiation process. At this stage, most cells showed a multinucleated status and expressed specific markers of human skeletal muscle (17). In agreement with previous studies (16,20,22,23), the rates of myoblasts growing and fusion into myotubes were similar, and there was no apparent morphological difference among cultured skeletal muscle cells from control subjects and patients with type 2 diabetes.

Quantification of p85α PI 3-kinase and hexokinase II mRNA levels.

Total RNA from differentiated myotubes was prepared using the RNeasy kit (Qiagen, Courtaboeuf, France). The absolute concentrations of p85α PI 3-kinase and hexokinase II mRNAs were determined by RT-competitive PCR as previously described (17,24).

Determination of PI 3-kinase activity. Myotubes were lysed at 4°C in 20 mmol/l Tris-HCl (pH 7.4), 140 mmol/l NaCl, 10 mmol/l EDTA, 4 mmol/l NaVO₄, 100 mmol/l NaF, 10 mmol/l pyrophosphate, 1% Nonidet P-40, supplemented with a freshly prepared cocktail of protease inhibitors (ICN Pharmaceuticals, Orsay, France). Lysates were centrifuged (12,000g for 15 min), and 100 µg of supernatant proteins was used for immunoprecipitation either with a specific antibody directed against human p85α PI 3-kinase (Upstate Biotechnology, Lake Placid, NY) or with specific antibodies directed against human IRS-1 or IRS-2 (Upstate Biotechnology). After washing, PI 3-kinase activity was measured on the immunoprecipitates as previously described (25). The labeled phosphoinositides were visualized and quantified using a Phosphor-Imager SI and Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Determination of p85α PI 3-kinase and IRS-1 and IRS-2 protein amounts. Myotubes were homogenized in a PBS lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with freshly prepared cocktail of proteases inhibitors (ICN Pharmaceuticals). Proteins (100 µg) were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane. Quantification of p85α PI 3-kinase and IRS-1 and IRS-2 protein amounts was performed using specific human antibodies (Upstate Biotechnology).

Determination of IRS-1, IRS-2, and insulin receptor tyrosine phosphorylation. Cells were lysed in the buffer used for the measurement of PI 3-kinase activity. Insulin receptor, IRS-1, or IRS-2 was immunoprecipitated with specific antibodies (Upstate Biotechnology). Immunoprecipitates were separated by SDS-PAGE, and immunoblotting was performed with a specific antiphosphotyrosine antibody (PY 99; Santa Cruz Biotechnology, CA). After analysis, the membrane was stripped and blotted with the antibodies against insulin receptor β-subunit, IRS-1, or IRS-2 to normalize for protein amount.

Determination of IRS-1 phosphorylation on serine 636. Myotubes were lysed in the buffer used for the measurement of PI 3-kinase activity, supplemented with 10⁻⁷ M okadaic acid to inhibit serine/threonine phosphatases

(25). IRS-1 was immunoprecipitated from 500 µg of proteins and then separated by SDS-PAGE. After transfer, the membranes were probed with a polyclonal anti-phospho-Ser⁶³⁶ antibody (Eurogentec, Seraing, Belgium) raised against a synthetic peptide (GDYMPMPs⁶³⁶PKSVSAP, numbered according to the human IRS-1 sequence) that is conserved among mouse, rat, and human (26). After analysis, the blots were stripped and probed again with anti-IRS-1 antibody to normalize for equal protein amount.

The specificity of the anti-pSer⁶³⁶ antibody was verified using L6 myoblasts transfected either with the rat IRS-1 cDNA (gift from Dr. C.R. Kahn, Joslin Diabetes Center, Boston, MA) or with a mutated rat IRS-1 in which the serine 632 (corresponding to Ser⁶³⁶ in the human IRS-1 sequence) has been replaced by an alanine residue by site-directed mutagenesis (QuickChange Kit; Stratagene, La Jolla, CA). After insulin treatment, phosphorylation of wild-type IRS-1 was readily measurable, whereas the anti-pSer⁶³⁶ antibodies did not recognize the mutated form of IRS-1 (data not shown).

Determination of phosphorylated ERK-1 and ERK-2. Cells were lysed in the buffer used for the measurement of PI 3-kinase activity. Proteins were separated by SDS-PAGE, and phosphorylated mitogen-activated protein kinases (MAPK; extracellular signal-related kinase [ERK]-1 and -2) were detected using a human anti-phospho MAPK antibody (Upstate Biotechnology), as previously described (27).

RESULTS

Altered regulation of gene expression by insulin is conserved in primary culture of muscle cells from patients with type 2 diabetes. Previous studies have demonstrated that myotubes established from patients with type 2 diabetes conserve important characteristics of the diabetic phenotype, such as marked reduction in insulin-induced glucose uptake and glycogen synthesis (19–23). We first verified whether the action of insulin on glucose uptake was impaired in the myotubes from patients with type 2 diabetes prepared in the present study. Glucose uptake was measured as previously reported (17). In the absence of insulin, the rate of glucose uptake was 64 ± 15 pmol · min⁻¹ · mg protein⁻¹. Incubation for 30 min with 100 nmol/l insulin induced an average increase of ~4.5-fold (4.6 ± 0.6 ; $P = 0.001$; $n = 5$) in myotubes from control subjects. In contrast, there was no significant change in the rate of glucose uptake in muscle cells from patients with type 2 diabetes (1.2 ± 0.1 ; NS; $n = 4$). Then, we aimed to verify whether the regulation of p85α PI 3-kinase and hexokinase II gene expression, another action of insulin (24), was also altered in the muscle cells from patients with type 2 diabetes. Figure 1 shows that incubation with insulin (100 nmol/l for 6 h) induced a significant increase in the expression of both p85α PI 3-kinase and hexokinase II mRNAs in myotubes from the control subjects. Under the same conditions, there was no effect of insulin on the expression of these two genes in myotubes from patients with type 2 diabetes (Fig. 1). These data indicated thus that the primary cultures of muscle cells from patients with type 2 diabetes were characterized by a defective action of insulin not only on glucose uptake but also on the regulation of gene expression.

Reduced insulin stimulation of PI 3-kinase activity in muscle cells from patients with diabetes. Activation of PI 3-kinase is involved in the effect of insulin on glucose transport (2,3) and on the expression of p85α PI 3-kinase and hexokinase II (17). Figure 2 shows that basal PI 3-kinase activity was not different in myotubes from control subjects and patients with type 2 diabetes. The stimulation with insulin significantly increased PI 3-kinase activity in cells from control subjects but not in myotubes from patients with type 2 diabetes (Fig. 2). The lack of

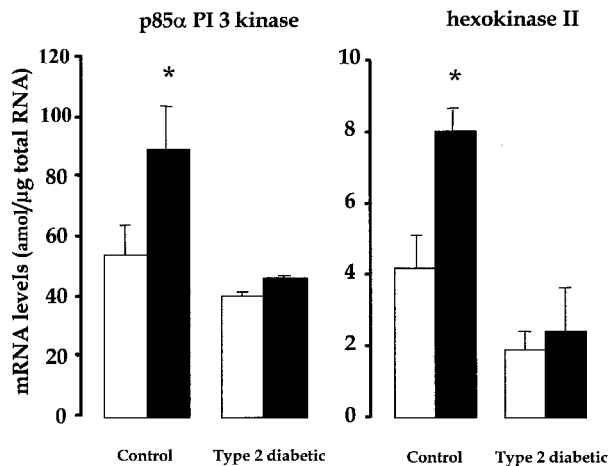


FIG. 1. The altered regulation of p85 α PI 3-kinase and hexokinase II gene expression by insulin is conserved in myotubes from patients with type 2 diabetes. Insulin-induced changes in p85 α PI 3-kinase and hexokinase II mRNA levels were determined by quantitative RT-PCR in myotubes from control subjects and patients with type 2 diabetes. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 6 h. Total RNA was prepared as indicated in RESEARCH DESIGN AND METHODS. □, untreated cells; ■, cells treated with insulin. The results are presented as absolute mRNA levels in amol/ μ g of total RNA. Data are the means \pm SE of five independent experiments made with myotubes from five different control subjects and five different patients with type 2 diabetes. * $P \leq 0.01$ in the presence versus in the absence of insulin using the paired Student's *t* test.

activation of PI 3-kinase activity was not due to a decrease in the protein (data not shown) or the mRNA expression levels of p85 α PI 3-kinase (Fig. 1) in cells from patients with diabetic. This defect was also found after 5, 10, 15, and 30 min of incubation with insulin, whereas a bell-shape curve, with maximal effect around 10 min, was observed in myotubes from control subjects (data not shown).

PI 3-kinase activity was then measured after specific immunoprecipitations with anti-IRS-1 or anti-IRS-2 antibodies. In the basal state, there was no difference in the PI 3-kinase activity associated with IRS-1 or IRS-2 in cells from control subjects and patients with diabetes (Fig. 3). Ten minutes of stimulation with insulin induced a robust association of PI 3-kinase activity with IRS-1 and IRS-2 in myotubes from control subjects. In contrast, in muscle cells from patients with type 2 diabetes, there was a dramatic reduction in the amount of PI 3-kinase activity associated with IRS-1 (7.3 ± 4.4 vs. 24.7 ± 4.2 , diabetic vs. control; $P = 0.001$), whereas IRS-2-associated PI 3-kinase activity was similar in cells from patients with diabetes and from control subjects (11.2 ± 4.4 and 11.5 ± 5.1 , respectively).

Decreased tyrosine phosphorylation of IRS-1 in muscle cells from patients with diabetes. The reduced activation of PI 3-kinase seemed thus to be mostly associated with a defective association of PI 3-kinase with IRS-1 in myotubes from patients with diabetes. This was not due to an altered amount of IRS-1 protein (Fig. 4A) but rather to a reduced tyrosine phosphorylation of IRS-1 in the presence of insulin (Fig. 4B). Insulin induced a 2.6 ± 0.3 -fold increase in the phosphorylation of IRS-1 on tyrosine residues in myotubes from control subjects but not in myotubes from patients with diabetes. In contrast to IRS-1, insulin induced an approximately twofold increase

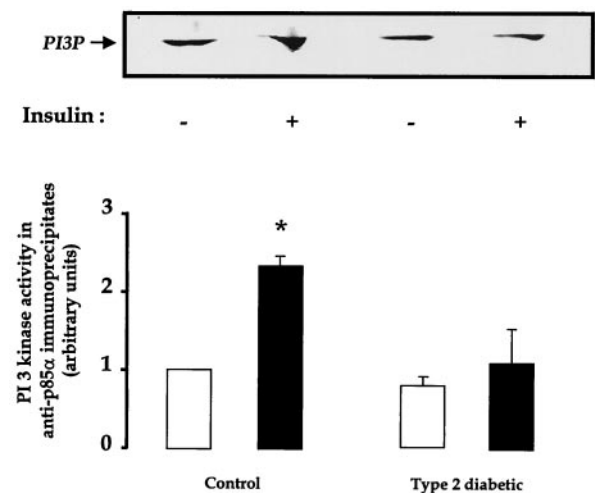


FIG. 2. PI 3-kinase activation in response to insulin. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 10 min. PI 3-kinase activity was determined in anti-p85 α antibody immunoprecipitates. □, untreated cells; ■, cells treated with insulin. The results are presented as arbitrary units taking basal PI 3-kinase activity of the cells from control subjects as one unit. Data are means \pm SE of five independent experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. * $P \leq 0.01$ in the presence vs. in the absence of insulin using the paired Student's *t* test.

in the tyrosine phosphorylation of IRS-2 in muscle cells from both the control subjects and the patients with type 2 diabetes (Fig. 4C). The protein amount of IRS-2 was similar in myotubes from the two groups (Fig. 4A).

We also verified whether the phosphorylation of insulin receptor on tyrosine residues, reflecting the tyrosine kinase activity of the receptor, was altered in muscle cells from patients with diabetic. Measured after 3 and 10 min of incubation with insulin, the phosphorylation of insulin receptor on tyrosine residues was similar in myotubes from control subjects and patients with diabetes (data not shown), indicating that the reduction in IRS-1 tyrosine phosphorylation during insulin stimulation was not due to a defective insulin receptor tyrosine kinase activity.

Increased phosphorylation of IRS-1 on serine 636 in muscle cells from patients with type 2 diabetes. Serine 636 is located in the close vicinity of one of the tyrosine-phosphorylated motifs of IRS-1 that are implicated in the interaction with PI 3-kinase (28). Using a specific anti phospho-Ser⁶³⁶ antibody (26), we found that the amount of IRS-1 phosphorylated on serine 636 was significantly higher in muscle cells from patients with diabetes than in cells from control subjects (Fig. 5). Further determinations ($n = 7$ experiments made with myotubes from four control subjects and four patients with type 2 diabetes) revealed that the phosphorylation of IRS-1 on serine 636 was 2.1 ± 0.3 -fold higher in myotubes from patients with type 2 diabetes than in cells from control subjects ($P < 0.005$). It is interesting that insulin stimulation induced a significant rise in the phosphorylation of IRS-1 on serine 636 in muscle cells from both groups (Fig. 5).

Phosphorylation of IRS-1 on serine 636 seems to be related to MAPK activity. Because serine 636 is located in a MAPK consensus phosphorylation site, we verified their possible involvement. In the basal state, the amount

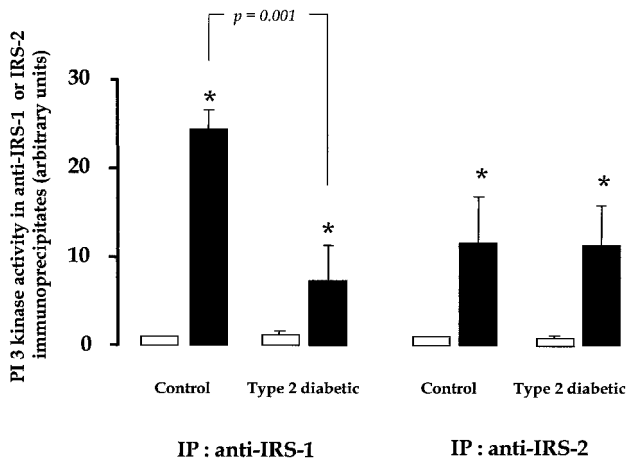


FIG. 3. Association of PI 3-kinase activity with IRS-1 and IRS-2 in response to insulin. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 10 min. Cells were lysed, and PI 3-kinase activity was determined in immunoprecipitates obtained with anti-IRS-1 and anti-IRS-2 antibodies. □, untreated cells; ■, cells treated with insulin. The results are presented as arbitrary units taking the basal IRS-associated PI 3-kinase activity of the cells from control subjects as one unit. Data are means \pm SE of five independent experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. * $P \leq 0.01$ in the presence vs. in the absence of insulin using the paired Student's *t* test. The difference in the insulin-induced PI 3-kinase activity associated with IRS-1 between cells from control and diabetic subjects is significant ($P = 0.001$) using the unpaired Student's *t* test.

of phosphorylated MAPKs (ERK-1 and -2) was significantly higher in muscle cells from patients with type 2 diabetes when compared with cells from control subjects (2.4 ± 0.2 -fold increase; $P < 0.001$; $n = 6$ subjects in each group). Figure 6 shows that insulin increased the phosphorylation of ERK-1 and -2 in myotubes from both control subjects and patients with diabetes, suggesting that the stimulation by insulin of the MAPK pathway is not impaired in cells from patients with diabetes.

For directly assessing the role of the MAPKs in the basal phosphorylation of IRS-1 on serine 636, the myotubes were incubated for 45 min with 10 μ mol/l PD98059, a potent inhibitor of the MAPK kinase. Incubation with PD98059 strongly reduced the phosphorylation of ERK-1 and ERK-2 (Fig. 7A). Under these conditions, the basal phosphorylation of IRS-1 on serine 636 was markedly reduced in muscle cells from the patients with diabetes (1.2 ± 0.4 vs. 2.5 ± 0.6 arbitrary units; $n = 3$ subjects in each group). A reduction in the phosphorylation of IRS-1 on serine 636 was also observed in the myotubes from control subjects (Fig. 7).

DISCUSSION

A number of recent studies have demonstrated the usefulness of human skeletal muscle cells in primary culture to investigate the action of insulin on glucose metabolism and on the regulation of gene expression (16–23). It has been consistently reported that myotubes from patients with type 2 diabetes display several metabolic defects that characterize in vivo insulin resistance of skeletal muscle, including reduced stimulation of glucose uptake and glycogen synthesis (16,19–23). We show here that the regulation by insulin of p85 α PI 3-kinase and hexokinase II gene expression is also altered in myotubes from patients with type 2 diabetes. The transcriptional regulation of

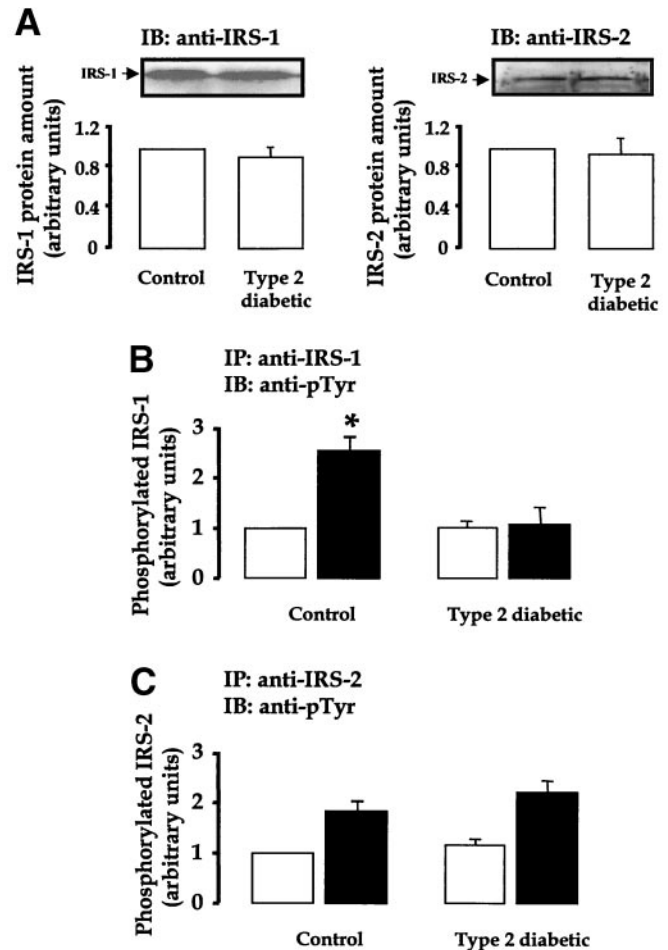


FIG. 4. IRS-1 protein expression and phosphorylation on tyrosine residues. **A:** Determination of IRS-1 protein amount by Western blotting. Bars represent the means \pm SE of five different experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. Data are expressed in arbitrary units taking the protein level in myotubes from control subjects as one unit. **B:** IRS-1 tyrosine phosphorylation. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 10 min. Cells were lysed, and IRS-1 protein was immunoprecipitated. After separation in SDS-PAGE, tyrosine phosphorylation was determined with a antiphosphotyrosine antibody. □, untreated cells; ■, cells treated with insulin. Data are means \pm SE of four independent experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. The results are presented as arbitrary units taking the basal phosphorylation of IRS-1 in cells from control subjects as one unit. * $P \leq 0.01$ in the presence vs. in the absence of insulin using the paired Student's *t* test. **C:** IRS-2 tyrosine phosphorylation. □, untreated cells; ■, cells treated with insulin. Data are means \pm SE of three independent experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. The results are presented as arbitrary units taking the basal phosphorylation of IRS-2 in cells from control subjects as one unit.

specific genes is one of the important biological actions of insulin (29). The expression of a number of genes has been found to be controlled by insulin in human skeletal muscle, and we recently reported that the regulation of p85 α PI 3-kinase and hexokinase II expression is altered in the muscle of patients with type 2 diabetes (24). Therefore, the persistence of all of these defects in vitro in myotubes makes this cell model a suitable and powerful tool to get more insight into the molecular causes of the defective action of insulin in human skeletal muscle.

Insulin signaling has been recently investigated in hu-

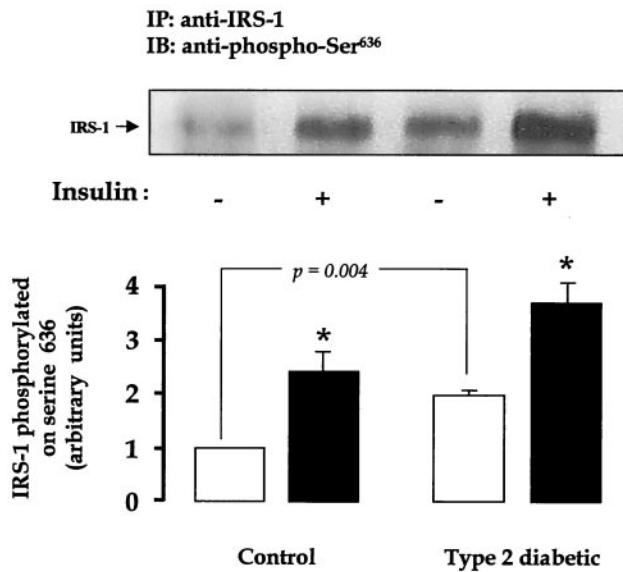


FIG. 5. Phosphorylation of IRS-1 on serine 636. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 5 min. Cells were lysed, and IRS-1 protein was immunoprecipitated. After separation in SDS-PAGE, the membranes were probed with a polyclonal anti-phospho-Ser⁶³⁶ antibody. □, untreated cells; ■, cells treated with insulin. Data are means ± SE of four independent experiments made with myotubes from four different control subjects and four different patients with type 2 diabetes. The results are presented as arbitrary units taking the basal serine 636 phosphorylation of IRS-1 in cells from control subjects as one unit. * $P \leq 0.01$ in the presence vs. in the absence of insulin using the paired Student's *t* test. The difference in the basal phosphorylation of IRS-1 on serine 636 between cells from control subjects and patients with diabetes was significant using the unpaired Student's *t* test.

man myotubes, and an altered insulin-stimulation of PI 3-kinase activity, related to a diminished association of PI 3-kinase with IRS-1, has been evidenced in cells from patients with type 2 diabetes (23). In another study,

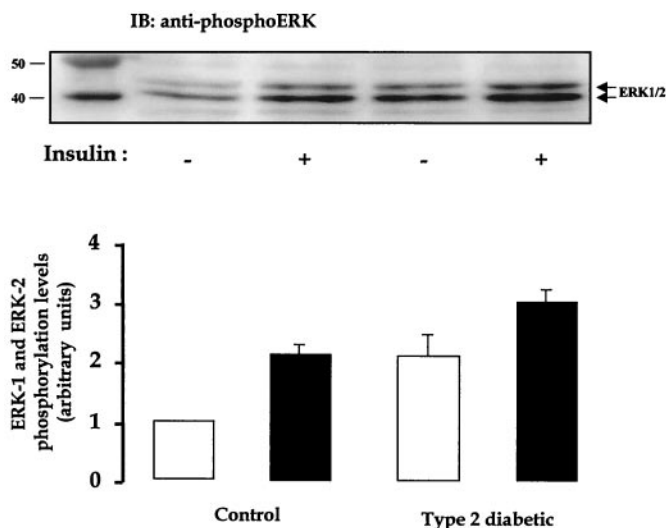


FIG. 6. Phosphorylation of ERK-1 and ERK-2. After overnight incubation without serum, myotubes were treated with insulin (100 nmol/l) for 30 min. After separation in SDS-PAGE, phosphorylated MAPKs (ERK-1 and ERK-2) were detected using a human anti-phospho MAPK antibody. □, untreated cells; ■, cells treated with insulin. Data are means ± SE of three independent experiments made with myotubes from three different control subjects and three different patients with type 2 diabetes. The results are presented as arbitrary units taking the total basal phosphorylation of ERK-1 and ERK-2 in cells from control subjects as one unit.

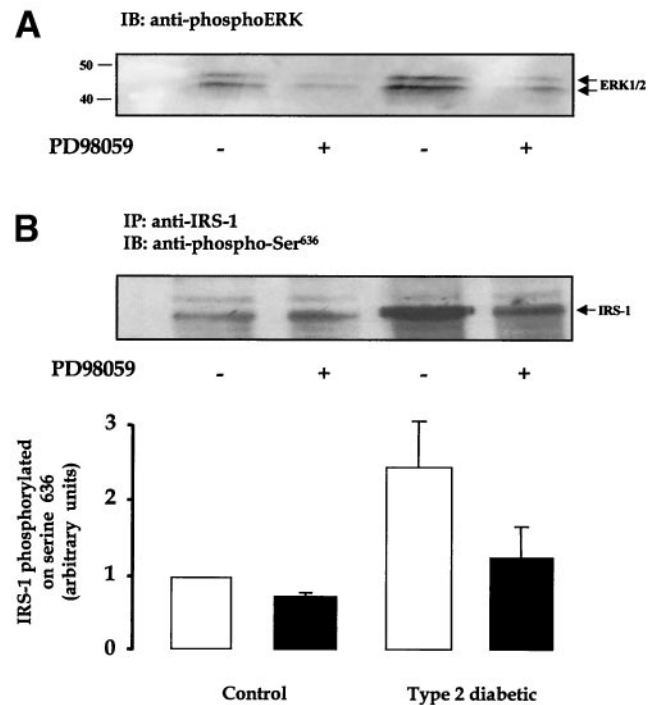


FIG. 7. Inhibition of MAPKs decreased phosphorylation of IRS-1 on serine 636. After overnight incubation without serum, the myotubes were treated with PD98059 (10 μ mol/l) for 45 min. Phosphorylation of MAPK (A) and phosphorylation of IRS-1 on serine 636 (B) were determined as indicated in the legends of Fig. 6 and Fig. 5, respectively. Bars represent the means ± SE of IRS-1 serine phosphorylation in myotubes from three different control subjects and three different patients with type 2 diabetes. □, untreated cells; ■, cells treated with PD98059. The results are presented as arbitrary units taking the basal serine 636 phosphorylation of IRS-1 in cells from control subjects as one unit.

however, the altered activation of PI 3-kinase by insulin seemed to be the consequence of a reduced association of PI 3-kinase with IRS-2 rather than with IRS-1 in myotubes from nondiabetic obese subjects with impaired glucose tolerance (18). Our data, obtained with myotubes from obese patients with type 2 diabetes, were in agreement with the data of Nikoulina et al. (23). Furthermore, we found that there was no change in IRS-1 protein amount but a marked reduction in the phosphorylation of IRS-1 on tyrosine residues that probably explains the decreased association of PI 3-kinase with IRS-1 in muscle cells from patients with diabetes.

In skeletal muscle, conflicting results have been reported regarding insulin receptor tyrosine kinase activity, showing either decreased (6,11) or normal (10,12) activation in type 2 diabetes. It has been suggested, however, that the differences observed among studies could result from secondary factors associated with diabetes, such as obesity, increased fatty acid concentration, or hyperglycemia (6,30). In the muscle cells in primary culture, maintained in a standard culture medium that avoided the influence of these secondary metabolic alterations, there was no alteration in the insulin-induced tyrosine phosphorylation of the β -subunit of insulin receptor. In agreement with a normal insulin receptor kinase activity, the phosphorylation of IRS-2 on tyrosine residues and the activation of the MAPKs in response to insulin stimulation were not altered in myotubes from patients with diabetes.

Taken together, these data strongly suggest that there is a specific defect in the transduction of the insulin signal through IRS-1 in myotubes from patients with type 2 diabetes.

An increasing body of evidence indicates that serine/threonine phosphorylation of IRS-1 can affect its phosphorylation on tyrosine residues, hence modulating downstream events of insulin signaling (13,14). Exposure of cultured cells to different factors, such as okadaic acid, tumor necrosis factor- α , or insulin, increases serine phosphorylation of IRS-1 and promotes insulin resistance (14,31,32). Several serine residues of IRS-1 have been reported to be phosphorylated (13,31,33–35), including serines 616 and 636, which are in the close vicinity of the tyrosines (tyrosines 612 and 632) involved in the binding of the SH2 domains of the regulatory subunits of PI 3-kinase (28). Moreover, using rodent IRS-1 protein with mutation of serine 632 (serine 636 in the human sequence), it has been suggested that this serine residue may play a role in the regulation of insulin-induced interaction between IRS-1 and PI 3-kinase (36). In the present work, we provide evidence for a significant higher phosphorylation of IRS-1 on serine 636 in the basal state in cells from patients with type 2 diabetes. It is thus possible that this phosphorylation prevents the tyrosine phosphorylation of IRS-1 during insulin stimulation and the subsequent association of IRS-1 with PI 3-kinase. Works are in progress to verify this hypothesis and to determine whether the phosphorylation of other serines of IRS-1 is also affected. In addition to the significantly higher basal phosphorylation level in myotubes from patients with diabetes, we found that the phosphorylation of IRS-1 on serine 636 is increased by insulin within minutes in the muscle cells. This effect of insulin was similar in myotubes from control subjects and patients with type 2 diabetes. The role of the insulin-induced phosphorylation of serine residues of IRS-1 and the involved kinases is presently unknown but may contribute to a negative feedback mechanism on the signaling pathway.

Serine 636 of IRS-1 is located in a consensus sequence for MAPK phosphorylation, and it has been reported that MAPKs are able to phosphorylate IRS-1 on serine 616 (33), which is in the same consensus motif as serine 636. We found a more than twofold increase in the basal MAPK activity, estimated by the phosphorylation of ERK-1 and ERK-2, in the myotubes of patients with type 2 diabetes. Moreover, inhibition of MAPK with PD98059 dramatically reduced the phosphorylation of IRS-1 on serine 636. These data indicate thus that the MAPKs are likely to play a role in the phosphorylation of serine 636 of IRS-1. It has been reported that the ability of insulin to stimulate the MAPK pathway is not altered in vivo in the skeletal muscle (6) and in vitro in myotubes of patients with type 2 diabetes (23). In agreement, we showed an increase in MAPK activity during insulin stimulation in myotubes from both the control subjects and the patients with diabetes. The activation of the MAPKs may thus participate in the increased phosphorylation of IRS-1 on serine 636 observed in response to insulin in muscle cells from the control subjects and the patients with type 2 diabetes.

Our data suggest that an increased level of MAPK activity in the basal state may be responsible for higher

serine 636 phosphorylation of IRS-1 and thus could participate in the defective activation of the PI 3-kinase by insulin in cells from patients with diabetes. This hypothesis remains to be confirmed as alteration in the basal activity of the MAPKs in the skeletal muscle of patients with diabetes has not been found in a recent study (6). However, in vitro studies have evidenced cross-talk between the MAPK and the PI 3-kinase signaling pathways in several cell types, with a number of data indicating down-regulatory effects of the MAPKs on PI 3-kinase activity (2,3).

In summary, we confirm in this work that skeletal muscle cells established from patients with type 2 diabetes present the same defects in the insulin signaling pathways than those previously evidenced in vivo in muscle biopsies, including impaired insulin-induced IRS-1 tyrosine phosphorylation, reduced association of IRS-1 with PI 3-kinase, and marked decrease in the stimulation of PI 3-kinase activity by insulin. These defects could explain, at least in part, the altered effect of insulin on glucose uptake and on the regulation of specific gene expression. We provide new data indicating that the IRS-2-dependent pathway is not affected in muscle cells from patients with diabetes. Furthermore, we demonstrate that skeletal muscle cells from moderately obese patients with type 2 diabetes are characterized by an increased basal phosphorylation level of IRS-1 on serine 636 and by a higher basal activity of the MAPKs. These new defects in the insulin signaling pathways may contribute to the impaired insulin action in the skeletal muscle in type 2 diabetes.

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