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A Phosphatidylinositol 3-Kinase/p70 Ribosomal S6 Protein Kinase Pathway Is Required for the Regulation by Insulin of the p85α Regulatory Subunit of Phosphatidylinositol 3-Kinase Gene Expression in Human Muscle Cells*

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Insulin acutely up-regulates p85α phosphatidylinositol 3-kinase (p85αPI 3-K) mRNA levels in human skeletal muscle (Laville, M., Auboeuf, D., Khalfallah, Y., Vega, N., Riou, J. P., and Vidal, H. (1996) *J. Clin. Invest.* 98, 43–49). In the present work, we attempted to elucidate the mechanism of action of insulin in primary cultures of human muscle cells. Insulin (10^{-7} M, 6 h of incubation) induced a 2-fold increase in p85αPI 3-K mRNA abundances (118 ± 12 *versus* 233 ± 35 amol/μg total RNA, $n = 5$, $p < 0.01$) without changing the expression levels of insulin receptor, IRS-1, glycogen synthase, and Glut 4 mRNAs in differentiated myotubes from healthy subjects. The effect is most probably due to a transcriptional activation of the p85αPI 3-K gene because the half-life of the mRNA was not affected by insulin treatment (4.0 ± 0.8 *versus* 3.1 ± 0.4 h). PD98059 (50 μM) did not modify the insulin response but increased p85αPI 3-K mRNA levels in the absence of insulin, suggesting that the mitogen-activated protein kinase pathway exerts a negative effect on p85αPI 3-K mRNA expression in the absence of the hormone. On the other hand, the insulin effect was totally abolished by LY294002 (10 μM) and rapamycin (50 nM). In addition, overexpression of a constitutively active protein kinase B increased p85αPI 3-K mRNA levels. These results indicate that the phosphatidylinositol 3-kinase/PKB/p70S6 kinase pathway is required for the stimulation by insulin of p85αPI 3-K gene expression in human muscle cells.

Phosphatidylinositol (PI)[1] 3-kinase (EC 2.7.1.67) is one of the key components of insulin signaling (1, 2). This lipid kinase is activated when the SH2 domains of its p85 regulatory subunit bind to the insulin receptor substrates (IRS) on specific tyrosine-phosphorylated sites (1–3). PI 3-kinase phosphorylates the D-3 position of the inositol ring of phosphoinositides (1, 2), generating potential second messengers that participate in the activation of protein kinase B (PKB) and p70 S6 kinase (p70S6K) (4, 5). Mainly using inhibitors, like LY294002 and wortmannin (inhibitors of PI 3-kinase) and rapamycin (inhibitor of p70S6K) (6–8), the PI 3-kinase pathway was demonstrated to participate in a variety of insulin effects. These include (but are not limited to) the regulation of glucose uptake, the activation of glycogen synthesis, the inhibition of lipolysis, the control of protein synthesis, the inhibition of apoptosis, and the insulin-induced membrane ruffling (1, 2).

The PI 3-kinase involved in insulin actions belongs to the class 1a of heterodimeric p85/p110 PI 3-kinases (9). The p85 regulatory subunit is an adaptator protein that links the p110 catalytic subunit to upstream signaling molecules. Two different genes coding highly homologous p85 (α and β) have been identified in mammalians, but the p85α regulatory subunit (p85αPI 3-K) has been studied in more detail (2, 9). In cultured cells, expression of mutants of p85αPI 3-K dramatically altered the response to insulin, supporting a major role of this subunit in the transduction of the insulin signal (2, 10, 11). In humans, it was recently demonstrated that the phosphorylation and activation of PI 3-kinase in response to insulin were significantly reduced in isolated muscle strips from insulin resistant obese patients (12). Part of this defect could be accounted for by a noticeable decrease in the expression level of p85αPI 3-K protein in the skeletal muscle of these subjects (12). In type 2 (non-insulin-dependent) diabetes mellitus, the PI 3-kinase pathway is also altered in skeletal muscle (13), although the involvement of the p85αPI 3-K has not yet been demonstrated (14). We have recently found that insulin acutely increases the mRNA levels of p85αPI 3-K in skeletal muscle of healthy lean volunteers during a hyperinsulinemic euglycemic clamp study (15). Futhermore, this regulation by insulin is defective in muscle of type 2 diabetic patients (16). In control subjects, the amplitude (2-fold increase) and the kinetics (3 h of insulin infusion are sufficient) of the effect of insulin suggested that the gene encoding the p85αPI 3-K could be a target gene of insulin in human skeletal muscle (15).

The list of the potential target genes of insulin is growing (17); however, in skeletal muscle, one of the main insulin sensitive tissues, only hexokinase II has been clearly demonstrated to be regulated by insulin at the gene level (18). Using the rat L6 myotube cell line, Osawa *et al.* (18) have demonstrated that the transcriptional regulation by insulin of hexokinase II gene requires the PI 3-kinase/p70S6K pathway. In human skeletal muscle, the *in vivo* effect of insulin on the expression of important genes of insulin action has been reported in few studies (15, 19–21), but the mechanism involved was not studied.

In the present work, we investigated the regulation of insulin receptor, IRS-1, p85αPI 3-K, and glycogen synthase mRNA

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[1] The abbreviations used are: PI, phosphatidylinositol; PKB, protein kinase B; p70S6K, p70 ribosomal S6 protein kinase; p85αPI 3-K, p85α regulatory subunit of PI 3-kinase; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; IGF-1, insulin-like growth factor 1; PBS, phosphate-buffered saline; Glut 4, glucose transporter 4; PCR, polymerase chain reaction; EGF, epidermal growth factor.

expression by insulin in primary cultures of human skeletal muscle cells. In agreement with what we have previously observed during a hyperinsulinemic clamp (15), we found that insulin up-regulates the expression of the p85αPI 3-K gene in cultured cells and that the PI 3-kinase/PKB/p70S6K pathway is involved in this effect.

EXPERIMENTAL PROCEDURES

Materials—Culture media were from Life Technologies, Inc. Recombinant human insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) were purchased from Prepor Tech Inc. (Rocky Hill, NJ). Porcine insulin, LY294002, rapamycin, PD 98059, and actinomycin D were from Sigma. Monoclonal mouse anti-rabbit skeletal myosin fast (heavy chain) antibody was obtained from Sigma, and fluorescein isothiocyanate-conjugated anti-mouse IgG was obtained from Zymed Laboratories Inc. (Montrouge, France). pSG5-Gag-PKB expression vector were a generous gift of Dr. P. Coffer (Utrecht, The Netherlands).

Culture of Human Skeletal Muscle Cells—Muscle biopsies (about 1 g) from the lumbar mass *(erector spinae)* were obtained, with the consent of the patient, during surgical procedures. The subjects (age = 49 ± 5 years, body mass index = 26 ± 3 kg/m^2) did not suffer from pathologies known to affect the sensitivity to insulin such as type-2 diabetes mellitus, dyslipidemia, or hypertension. The experimental protocol was approved by the Ethics Committee of the Hospices Civils de Lyon.

Muscle samples were collected in ice-cold Ham's F-10 medium supplemented with 20% fetal calf serum, 1% chicken embryo extract, 1% antibiotics (100 units/ml penicillin/100 μg/ml streptomycin) and processed immediately according to the method described by Hurel *et al.* (22) with minor modifications. Briefly, tissue sample was washed in complete Ham's F-10 medium, and all visible connective and fat tissues were removed. Muscle was then minced and placed (about 500 mg) in sterile flasks containing 15 ml of a trypsine/EDTA solution (Life Technologies, Inc.). After stirring gently at 37 °C during 20 min, debris was allowed to settle. The supernatant was collected and centrifugated for 5 min at 150 × *g*, and the pellet was resuspended in 1 ml of complete Ham's F-10 medium. The remaining debris was submitted to other rounds (generally two or three rounds) of digestion in trypsin/EDTA. All the pellets were then pooled and washed in complete medium. Cells were seeded and grown to subconfluence in 25-cm^2 Primaria culture flasks (Falcon) coated with 0.1% gelatin and were then transferred to 75-cm^2 culture flasks. Medium was changed every 2 days. At confluence, myoblasts were allowed to differentiate into myotubes in α-minimum essential medium containing 2% fetal calf serum and 1% antibiotics. Fusion and multinucleation of the cells were observed after about 1 week. Myotubes of human skeletal muscle were always used 12–16 days after induction of the differentiation process.

To assess the differentiated state, myotubes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, blocked with 2% bovine serum albumin for 30 min, and washed three times for 5 min in PBS. Cells were incubated with the primary antibodies (monoclonal anti-skeletal myosin-fast/heavy chain or monoclonal anti-sarcomeric α-actin) for 120 min at room temperature. After washing, they were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody for 60 min at room temperature. Finally, cells were mounted in Fluoprep (BioMerieux, France) and observed with a Leitz fluorescent miscroscope.

2-Deoxyglucose Transport—Human myoblasts were grown in 12-well dishes and differentiated at confluence. Myotubes were preincubated during 5 h in serum-free α-minimum essential medium (5.5 mM glucose) and then incubated in fresh serum-free medium containing different concentrations of insulin for 90 min, in 95% air/5% CO_2 at 37 °C. At the end of the incubation, cells were washed four times with prewarmed transport buffer (150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 10 mM Hepes, pH 7.4). Glucose uptake was then performed in triplicate by the addition of 10 μl of [^{3}H]2-deoxyglucose (50 μM, 0, 4 μCi/well). Nonspecific transport was determined in presence of 5 μM of cytochalasin B (Sigma). After 10 min at ambient temperature, incubation medium was aspirated, and cells were washed five times with ice-cold PBS. Cells were solubilized in 500 μl of 0.1 N NaOH. An aliquot (100 μl) was used for protein content determination according to the method of Bradford (23). 400 μl of the suspension were neutralized with 1 N HCl, added to scintillation vials, and counted in Ultima Gold scintillation liquid (Packard Instrument, Rungis, France).

Effects of Insulin on Gene Expression—Myotubes were cultured for 12–16 days in 6-well plates, serum-starved overnight, and then left untreated or treated with 10^{-7} M of insulin for 6 h at 37 °C. Inhibitors (LY294002, rapamycin, PD98059, or actinomycin D) were added in dimethyl sulfoxide (final concentration, 0.1%). Control cells were treated with equal amount of vehicle. At the end of the incubation, cells were observed under microscope and scrapped in the presence of the lysis buffer from the RNeasy kit for total RNA preparation (Qiagen, Courtaboeuf, France). Total RNA was further purified following the instruction of the manufacturer, resuspended in 40 μl of RNase free water, and stored at −80 °C until quantification of specific mRNAs. Concentration and purity of each sample were assessed by absorbance measurement at 260 nm and by the 260 nm/280 nm ratio, respectively. Integrity of total RNA preparations was verified on agarose gel.

Insulin receptor, IRS-1, p85αPI 3-K, glycogen synthase, and Glut 4 mRNA levels were determined by quantitative reverse transcription-competitive polymerase chain reaction (PCR). The method relies on the addition of known amounts of a competitor DNA molecule in the PCR to standardize the amplification process (24, 25). The construction of the competitors, the validation of the assays and the conditions of the RT-PCR reactions have been described in details previously (15, 24). During the PCR, we used sense primers that were 5′-end labeled with Cy-5 fluorescent dye (Eurogentec, Seraing, Belgium). Their sequences were identical to those reported previously (15). The use of these primers allowed the synthesis of fluorescent PCR products that were analyzed with an automated laser fluorescence DNA sequencer (ALFexpress, Pharmacia, Upsala, Sweden) in 4% denaturating polyacrylamide gels. The initial concentration of target mRNA was determined at the competition equivalence point as described previously (24).

Quantification of p85αPI-3K Protein by Western Blotting—Myotubes were homogenized in PBS lysis buffer containing 1% Nonidet P-40 (Sigma), 0.5% sodium desoxycholate (Sigma), 0.1% SDS (Sigma) and supplemented with a freshly prepared mixture of protease inhibitors (ICN Pharmaceuticals, Orsay, France). Proteins (5 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidine difluoride membrane (Millipore, France), and blocked overnight at 4 °C in blocking buffer (PBS, 0.1% Tween-20, 1% skim milk, and 1% bovine serum albumin). P85αPI-3K was identified using a rabbit polyclonal antibody that recognizes human, mouse, and rat p85α subunit of the PI 3-kinase (Upstate Biotechnology, New York, NY). Membranes were incubated for 1 h at room temperature with the antibody (1:1000) and next developed with an anti-rabbit IgG peroxidase conjugate (Sigma) diluted at 1:10,000. The complex was visualized using a chimioluminescent kit (Specichrom, Lyon, France).

Transient Expression of PKB in Human Myotubes—Expression of a constitutively active PKB was performed by transient transfection of human muscle cells with the expression vector pSG5-Gag-PKB initially constructed by Burgering and Coffer (26). This plasmid has been successfully used to demonstrate the role of PKB in the PI 3-kinase/p70S6K pathway (26) and in insulin-induced translocation of Glut 4 to the plasma membrane of adipocytes (27). The empty pSG5 plasmid (Stragtagene, La Jolla, CA) was used as a control. The plasmid DNAs were obtained using a maxi kit (Qiagen), and their concentrations were determined by measuring the absorbance at 260 nm.

Transfections were done with Fugene-6 (Roche Diagnostics, Meylan, France) according to the manufacturer. Briefly, cells in 6-well plates were incubated for 72 h in 2 ml of culture medium containing 6 μl of Fugene-6 and 1 μg of plasmid DNA. Because transfection of fully differentiated primary cells is generally difficult to perform, we used myoblasts at different stages. In a first condition, myoblasts at about 50% confluence were transfected and incubated for 72 h in Ham's F-10 medium supplemented with 20% fetal calf serum and 1% antibiotics. In a second condition, myoblasts at confluence were incubated in the differentiation medium (α-minimum essential medium containing 2% fetal calf serum and 1% antibiotics) containing Fugene-6, and the plasmid DNA and were incubated for 72 h. At the end of the incubation period, cells were scrapped in the presence of the lysis buffer from the RNeasy kit for total RNA preparation (Qiagen), and p85αPI 3-K mRNA levels were determined by reverse transcription-competitive PCR.

RESULTS

Characterization of the Cultured Human Muscle Cells—After trypsin digestion, satellite cells from human muscle grew in an elongated fiber-like configuration. Orientation, fusion, and differentiation of confluent myoblasts into myotubes were initiated by changing the proliferation medium into α-minimum essential medium containing 2% fetal calf serum. Experiments were performed 12–16 days after initiation of the differentia-

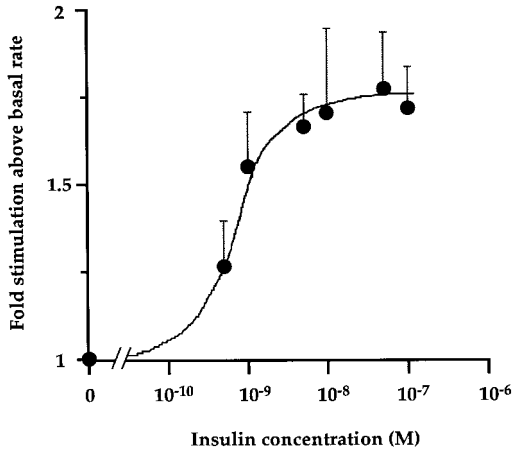

FIG. 1. **Insulin stimulation of 2-deoxyglucose uptake in cultured human myotubes.** Myotubes were incubated as described under "Experimental Procedures." Data are the means ± S.E. with cell preparations from six different subjects. In each experiment, points were determined in duplicate.

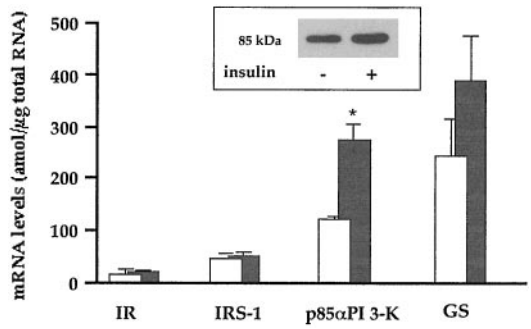

FIG. 2. **Levels of insulin receptor (*IR*), IRS-1, p85*α*PI 3-K, and glycogen synthase (*GS*) mRNAs in cultured human myotubes.** Specific mRNA levels were determined by reverse transcription-competitive PCR after 6 h of incubation in the presence or absence of 10^{-7} M insulin, as described under "Experimental Procedures." The *open boxes* represent the values without insulin, and the *closed boxes* represent the values with insulin. Results are the means ± S.E. with cells preparations from four different subjects (except for p85*α*PI 3-K mRNA, $n = 5$). Statistical significance was assessed with a Student's t test for paired series. *, $p \leq 0.01$ in the presence *versus* in the absence of insulin. The *inset* present the p85*α*PI 3-K protein levels measured by Western blotting after 12 h of incubation without or with insulin.

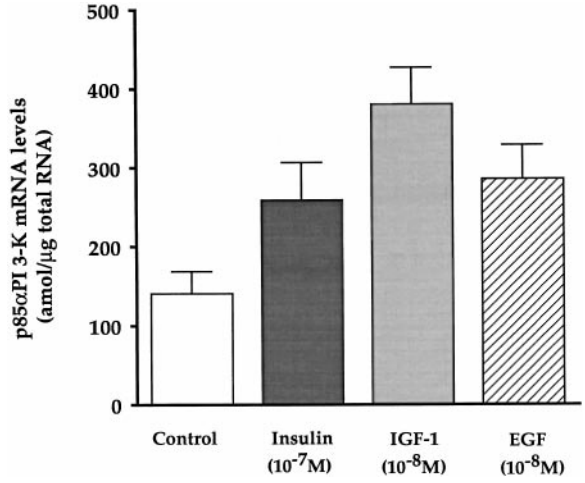

FIG. 3. **Effect of IGF-1 and EGF on p85*α*PI 3-K mRNA levels.** Myotubes were incubated for 6 h either insulin, IGF-1, or EGF at the indicated concentrations. Data are the means ± S.E. with cell preparations from four different subjects.

tion. At this stage, most cells showed a multinucleated status that characterizes mature myotubes (28). Similarly to what was observed by Henry *et al.* (28), immunocytofluorescence studies demonstrated a significant expression of myosin and of the striated muscle-specific sarcomeric α-actin in human myotubes (not shown). Creatine phosphokinase activity was about 5-fold higher in myotubes than in nonfused myoblasts (90 ± 23 *versus* 19 ± 5 milliunits/mg of protein).

To ensure that human myotubes were responsive to insulin, we performed 2-deoxyglucose uptake experiments. After verification that glucose transport rates were linear for at least 20 min at room temperature (data not shown), the experiments were carried out during 10 min of incubation to be sure that studies were performed in the initial rate of transport. Fig. 1 shows the effect of insulin on glucose uptake in different preparations of myotubes. Basal specific glucose uptake rates were 48 ± 11 pmol/min/mg protein. In the presence of cytochalasin B, the nonspecific transport represented less than 30%. Insulin induced an about 2-fold increase in glucose uptake, with a maximal stimulation at 50 nM of insulin. Half-maximal stimulation occurred at the concentration of 1 ± 0.3 nM insulin (Fig. 1), a value that was in the same range as the IC_{50} for insulin binding (0.8 nM) reported by Henry *et al.* in cultured human myotubes (28).

Basal and Insulin-stimulated Expression of Target mRNAs in Human Myotubes—Fig. 2 shows the mRNA levels of insulin receptor, IRS-1, p85*α*PI 3-K, and glycogen synthase in cultured human myotubes. The expression levels of these different target mRNAs were similar to what was previously found in skeletal muscle biopsies (15, 16). In contrast to the muscle tissue, human myotubes in culture expressed very low levels of Glut 4 mRNA (0.8 ± 0.5 amol/μg of total RNA). Attempts to increase Glut 4 expression by changing the incubation conditions and/or time after differentiation were unsuccessful (data not shown). Treatment of the cells with 10^{-7} M insulin for 6 h produced a significant 2-fold increase in p85*α*PI 3-K mRNA levels (Fig. 2). Furthermore, a significant increase in the p85*α*PI 3-K protein level was observed after 12 h of incubation with insulin (Fig. 2, *inset*). The concentration of the mRNA encoding glycogen synthase tended also to increase in the presence of insulin, but the effect did not reach significance. Insulin receptor and IRS-1 mRNA levels were not affected by insulin (Fig. 2). We did not find any effect of insulin on Glut 4 mRNA levels, even after 24 h of incubation with 10^{-7} M insulin (data not shown). Fig. 3 shows that the induction of p85*α*PI 3-K mRNA levels could also be produced by IGF-1 (10^{-8} M) and EGF (10^{-8} M), indicating that the effect was not specific to insulin.

Effects of Actinomycin D on Insulin-induced p85αPI 3-K mRNA Expression—To verify whether the induction of p85*α*PI 3-K mRNA by insulin reflected a transcriptional activation of the p85*α*PI 3-K gene or/and post-transcriptional stabilization of the mRNA, we investigated the effect of actinomycin D, a potent inhibitor of RNA polymerase II. Myotubes were preincubated for 6 h with or without 10^{-7} M insulin before addition of 10^{-6} M actinomycin D. Fig. 4 shows that the kinetics of the decrease of the p85*α*PI 3-K mRNA levels were similar under the two conditions. This experiment was reproduced in three independent preparations of human myotubes, and the obtained results clearly demonstrated that insulin did not affect the half-life of p85*α*PI 3-K mRNA (3.1 ± 0.4 *versus* 4.0 ± 0.8 h with *versus* without insulin, respectively). This strongly suggested that the action of insulin mainly occurs at the transcriptional level.

Effects of LY294002, Rapamycin, and PD98059—To dissect in more detail the signaling pathway involved in the action of insulin, we investigated the effects of commonly used inhibi-

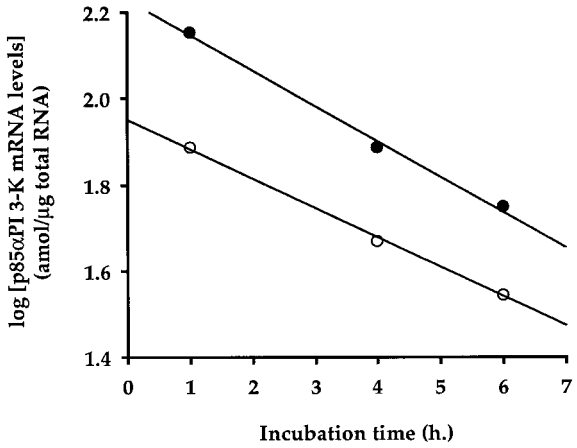

FIG. 4. **Effect of insulin on p85αPI 3-K mRNA stability.** Myotubes were preincubated with 10^{-7} M insulin for 6 h before addition of 10^{-6} M actinomycin D. The mRNA levels of p85αPI 3-K were determined by reverse transcription-competitive PCR during the following 6 h. Each point was determined in duplicate.

tors. Fig. 5 shows that the induction by insulin of p85αPI 3-K mRNA expression was completely abolished in the presence of 5×10^{-5} M LY294002 (*panel A*) and of 10^{-8} M rapamycin (*panel B*). A lower concentration of LY294002 (10^{-5} M) and of rapamycin (10^{-9} M) produced the same effects (data not shown). These two inhibitors did not affect the basal levels of p85αPI 3-K mRNA. To test the involvement of the MAPK pathway, myotubes were incubated with the MAPK kinase inhibitor, PD 98059. Fig. 5*C* shows that addition of 5×10^{-5} M of PD 98059 did not prevent the induction by insulin of p85αPI 3-K mRNA. Interestingly, PD 98059 alone increased by more than 2-fold the mRNA levels of p85αPI 3-K, reaching the same levels as in the presence of insulin. Taken together, these results strongly suggested that insulin stimulation of p85αPI 3-K gene expression requires the PI 3-kinase/p70S6K pathway.

Induction of p85αPI 3-K mRNA Expression by Transient Expression of PKB—PKB is a downstream kinase of PI 3-kinase and is involved, at least in part, in the stimulation of p70S6K (26). To further demonstrate that the PI 3-kinase/p70S6K pathway is implicated in the effect of insulin on p85αPI 3-K gene expression, human muscle cells were transiently transfected with an expression vector of a constitutively active PKB (26, 27). Fig. 6 shows that transfection with pSG5-Gag-PKB increased the mRNA levels of p85αPI 3-K both in undifferentiated myoblasts and in myoblasts that were incubated with the differentiation medium for 72 h.

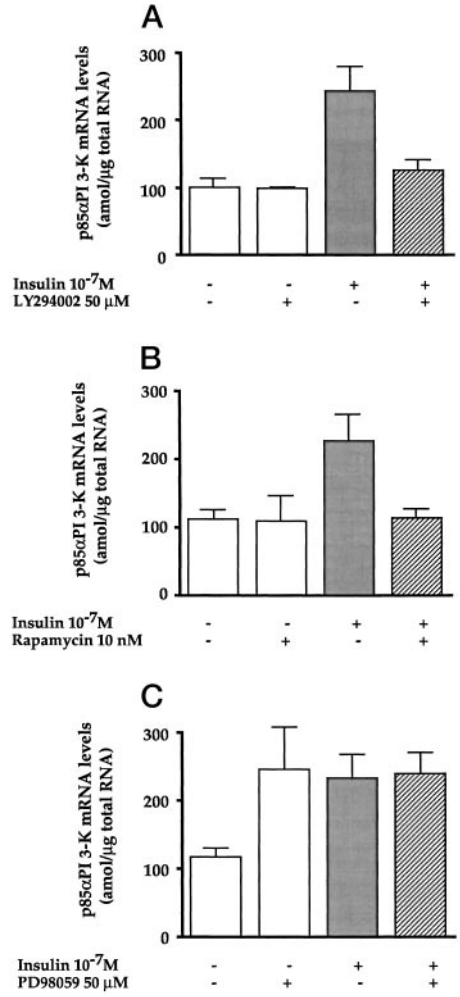

FIG. 5. **Effects of LY294002 (*A*), rapamycin (*B*), and PD98059 (*C*) on basal and insulin-induced p85αPI 3-K mRNA.** Myotubes were treated for 6 h with or without insulin and LY294002, rapamycin, or PD98059 at the indicated concentrations. Results are the means ± S.E. with cells preparations from three different subjects.

DISCUSSION

Insulin modulates cell metabolism by altering the activity or the intracellular localization of critical enzymes and by changing their expression levels. Insulin can control specific protein amount, in part by acting at the level of mRNA translation and mainly at the level of their gene expression (17). This last action is certainly a major effect of insulin and the list of insulin-regulated genes is rapidly growing (17). Insulin has been clearly shown to participate in the regulation of the expression of genes coding key enzymes of glucose and lipid metabolisms (17, 18, 29–32), structural proteins (17, 33), and some *trans*-acting factors (17, 34). However, and in contrast to the regulation of the activity of pre-existing cellular proteins, the mechanism of action of insulin on gene expression is still poorly understood (1, 17). In hepatoma cell lines and in primary rat hepatocytes, the transcriptional stimulation of phosphoenol pyruvate carboxykinase (29) and glucose-6-phosphate dehydrogenase (30) was shown to require the PI 3-kinase/p70S6K pathway. The inhibitory effect of insulin on glucose-6-phosphatase gene expression was found to involve activation of PI 3-kinase but not the downstream kinases PKB and p70S6K (31). In rat L6 cell line differentiated into myotubes, Osawa *et al.* (18) have shown that the PI 3-kinase/p70S6K pathway is required for insulin stimulation of hexokinase II gene transcription. Interestingly, the above results indicated that the regulation by insulin of the gene coding metabolic enzymes mainly involves an activation of PI-3-kinase without recruitment of the MAPK pathway. It should be mentioned that the regulation of Glut 3 expression by chronic insulin treatment in L6 muscle cells was reported to require the $p21^{ras}$/MAPK pathway, whereas the regulation of Glut 1 was mainly dependent on the stimulation of the p70S6K pathway (35). A role of the MAPK in insulin-induced regulation of gene transcription has been also suggested in the expression of the early responsive genes c-*fos* and c-*jun* (36).

Skeletal muscle is the primary site of insulin-stimulated glucose uptake and utilization (37). *In vivo* studies have demonstrated that insulin could increase the mRNA expression levels of hexokinase II, glycogen synthase, p85αPI 3-K, Glut 4, and Rad mRNAs in human skeletal muscle (15, 19–21). To study the mechanism of action of insulin on gene expression in a human muscle cell model, we have used primary cultures of differentiated myotubes. This cell model has been previously utilized by Henry and co-workers (28, 38–40) to investigate the regulation of glucose transport and glycogen synthesis in cells

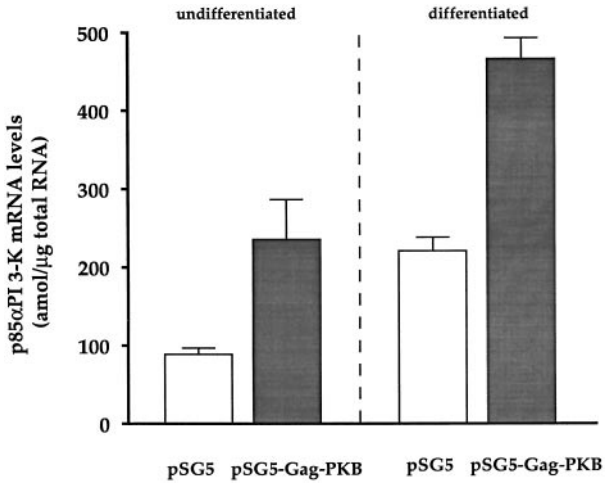

FIG. 6. **Up-regulation of p85αPI 3-K mRNA levels by transient transfection of human myoblasts with a constitutively active PKB.** Cells were transfected with pSG5 plasmid as control or pSG5-Gag-PKB using Fugene-6. The conditions of transfection are described under "Experimental Procedures." Undifferentiated refers to myoblasts at about 50% confluence incubated for 72 h after transfection. Differentiated refers to confluent cells transfected in the differentiation medium. Experiments were done in triplicate from cell preparations from two different subjects.

derived from healthy lean subjects and from type 2 diabetic patients. In agreement with these previous reports, we obtained human myotubes that exhibited a multinucleated aspect after orientation and fusion of undifferentiated myoblasts. They expressed structural proteins that are characteristic of striated muscle. The relative mRNA expression levels of key proteins of insulin action (insulin receptor, IRS-1, p85αPI 3-K, and glycogen synthase) were in the same range as what we have previously observed in human muscle biopsies (15, 16). However, Glut 4 mRNA expression was extremely low in cultured myotubes. This observation was in agreement with the results of Henry *et al.* (28), who did not detect Glut 4 mRNA by RNase protection assay in untreated human myotubes. However, significant amounts of Glut 4 protein could be visualized by Western blotting in these cells (28), suggesting that insulin might stimulate glucose uptake through this transporter.

In the present study, we demonstrated, in human primary culture of muscle cells, that insulin increases p85αPI 3-K mRNA and protein expression, most probably by acting at the transcriptional level. The expression levels of insulin receptor, IRS-1, glycogen synthase, and Glut 4 mRNAs were not significantly affected under our experimental conditions. IGF-1 and EGF also increased p85αPI 3-K mRNA levels, indicating that the effect was not specific to insulin but that other growth factors can also control the expression of p85αPI 3-K gene. In addition, we found that the positive effect of insulin on p85αPI 3-K mRNA levels is not limited to the muscle cells but was also found in HepG2 and 3T3-L1 cell lines (data not shown), as well as in human adipose tissue *in vivo* (16), thus reinforcing the conclusion that p85αPI 3-K is a target gene of insulin.

The effect of insulin on p85αPI 3-K gene expression in human myotubes was completely blocked by inhibitors of PI 3-kinase and p70S6K activity but not by an inhibitor of the MAPK pathway. These results strongly suggested that the PI 3-K/p70S6K pathway is the main pathway required for the action of insulin, a situation that is reminiscent of the reported results on the action of insulin on the expression of genes coding metabolic enzymes (18, 29–32). The effect of IGF-1 was also prevented by rapamycin and decreased by about 50% by LY294002 (data not shown), indicating that the effect of IGF-1 was probably mediated by the same pathway as the effect of insulin. Involvement of the PI 3-K/p70S6K pathway was further confirmed by the demonstration that transient expression of a constitutively active PKB increased p85αPI 3-K mRNA levels in human myoblasts. Therefore, the PI 3-K/PKB/p70S6K pathway appears to mediate the effect of insulin on p85αPI 3-K gene expression in human muscle cells, as is the case for the regulation of hexokinase II in the L6 cell line (18). A surprising result was the observation that PD 98059 increased p85αPI 3-K mRNA in human myotubes, suggesting a possible negative role of the MAPK pathway in the absence of insulin on the expression of the p85αPI 3-K gene. This conclusion, however, requires additional investigations to clearly understand the precise role of the MAPK pathway in the regulation of p85αPI 3-K mRNA and/or gene expression.

It is now accepted that the insulin-induced regulation of gene transcription is mediated by interactions of *trans*-acting factors with *cis*-responsive DNA sequences (17). *Cis*-acting elements, referred as insulin response elements, have been already identified in the promoter region of some insulin-regulated genes (17). However, in contrast to other hormone response elements, no insulin response element consensus sequence has been identified to date (17). To our knowledge, the promoter sequence of the human p85αPI 3-K gene has never been reported. Cloning of this promoter is thus now required to further study the regulation of the p85αPI 3-K gene and to identify *trans*-acting factors that link the PI 3-kinase/p70S6K insulin signaling pathway to the transcriptional machinery within the nucleus.

Defective regulation of the PI 3-kinase pathway has already been reported in the skeletal muscle of insulin resistant patients (12–14). This pathway plays a crucial role in the transcriptional control of several key genes (18, 29–32, 36), including the p85αPI 3-K gene (the present work). Therefore, altered transmission of the insulin signal to the promoter of specific genes could contribute to the development of pathologies with insulin resistance like type 2 diabetes or obesity (17). Supporting this hypothesis, an impaired regulation of hexokinase II (41) and Glut 4 (21) gene expression has been already reported in skeletal muscle of diabetic patients. We have also recently demonstrated that the acute regulation by insulin of p85αPI 3-K mRNA expression was altered in muscle and in adipose tissue of type 2 diabetic patients (16). Investigations of the mechanism of action of insulin in primary cultured of human myotubes from diabetic subjects should get more insight into the possible molecular defect(s) that can lead to the development of this pathology.

In summary, the data presented in this work demonstrate that insulin up-regulates p85αPI 3-K gene transcription in human myotubes. The PI 3-kinase/PKB/p70S6K pathway is required to transduce the insulin signal. These results provide additional support to the emerging concept that the insulin signal involved in the regulation of the transcription of metabolic genes is mainly conveyed by the PI 3-kinase pathway. In addition to the genes encoding key enzymes of glucose and lipid metabolisms (16, 17, 26–29, 33), the PI 3-kinase cascade participates in the transcriptional regulation of the p85α regulatory subunit of PI 3-kinase, an essential component of its own pathway.

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