Insulin acutely up-regulates p85α phosphatidylinositol 3-kinase (PI3-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 p85α PI3-K mRNA abundances (118 ± 12 versus 233 ± 35 amol/mg 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 p85α PI3-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 p85α PI3-K mRNA levels in the absence of insulin, suggesting that the mitogen-activated protein kinase pathway exerts a negative effect on p85α PI3-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 p85α PI3-K mRNA levels. These results indicate that the phosphatidylinositol 3-kinase/PI3-K/PKB/p70S6 kinase pathway is required for the stimulation by insulin of p85α PI3-K gene expression in human muscle cells.

Phosphatidylinositol (PI)³ 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). PI3-K 3-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 PI3-Kinas (9). The p85 regulatory subunit is an adaptor 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 (p85α PI3-K) has been studied in more detail (2, 9). In cultured cells, expression of mutants of p85α PI3-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 p85α PI3-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 p85α PI3-K has not yet been demonstrated (14). We have recently found that insulin acutely increases the mRNA levels of p85α PI3-K in skeletal muscle of healthy lean volunteers during a hyperinsulinemic euglycemic clamp study (15). Furthermore, 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 p85α PI3-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, p85α PI3-K, and glycogen synthase mRNA...
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 p85αPI3-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 PreproTech Inc. (Rocky Hill, NJ). Recombinant IL-2, LL294002, rapamycin, PD188380, LY294002, and Fugene-6 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).

**Human Skeletal Muscle Cells—**Muscle biopsies (about 1 g) from the lumbar mass (rector spinae), were obtained with the consent of the patient, during surgical procedures. The subjects (age = 49 ± 5 years, body mass index = 26 ± 3 kg/m²) 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 Ethical Committee of the Hospitals Civils de Lyon.

Muscle samples were collected in ice-chilled Ham’s F-10 containing 20% fetal calf serum, 0.5% sodium desoxycholate, and 0.1% SDS (Sigma). Target mRNA was isolated using a kit for total RNA preparation (Qiagen, Hilden, Germany). Integrity of total RNA preparations was verified on agarose gel. Concentration and purity of each sample were assessed by absorbance measurement at 260 nm and by the 260/280 nm ratio, respectively. Integrity of total RNA preparations was verified on agarose gel.

**In vitro transfection**—Fetal myoblasts were harvested from the lumbar mass of adult Wistar rats weighing 250–300 g. Myoblasts were grown to subconfluence in 25-cm² Primaria culture flasks (Falcon) coated with 0.1% gelatin and were then transferred to 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, PD188380, 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 scraped in the presence of the lysis buffer from the RNaseasy 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 260/280 nm ratio, respectively. Integrity of total RNA preparations was verified on agarose gel.

**Fluorescence microscopy**—FluoPrep (BioMerieux, France) and observed with a Leitz fluorescent body for 60 min at room temperature. Finally, cells were mounted in fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody for 60 min at room temperature. After washing, they were incubated with 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, PD188380, 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 scraped in the presence of the lysis buffer from the RNaseasy 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 260/280 nm ratio, respectively. Integrity of total RNA preparations was verified on agarose gel.

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**2-Deoxyglucose Transport—**Human myoblasts were grown in 12-well dishes and differentiated at confluence. Myoblasts 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% CO₂ 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 MgSO₄, 1.2 mM CaCl₂, 2.5 mM NaH₂PO₄, 10 mM Hepes, pH 7.4). Glucose uptake was then performed in triplicate by the addition of 10 µl of [H]2-deoxyglucose (50 µM, 0, 4 µCi/well). Non-specific 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 three times with ice-cold PBS. Cells were then homogenized in 500 µl of 0.1 N NaOH. An aliquot (100 µl) was used for protein content determination according to the instruction of the manufacturer (Bradford). 200 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, PD188380, 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 scraped in the presence of the lysis buffer from the RNaseasy 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 260/280 nm ratio, respectively. Integrity of total RNA preparations was verified on agarose gel.

**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 differentiation...
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 sarcromeric 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 IC50 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, p85αPI 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 before addition of the half-life of p85αPI 3-K mRNA was 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 p85αPI 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-
Insulin Regulation of p85αPI-3 Kinase Gene Expression

**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^{-5} m actinomycin D. The mRNAs levels of p85αPI-3-K were determined by reverse transcription-competitive PCR during the following 6 h. Each point was determined in duplicate.

**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 cell preparations from three different subjects.

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 phosphoenolpyruvate 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 p21ras/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 *in vitro* 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.
Insulin Regulation of p85aPI 3-K Gene Expression

Involvement of the PI 3-K/p70S6K pathway was further confirmed by the demonstration that transient expression of a constitutively active PKB increased p85aPI 3-K mRNA levels in human myoblasts. Therefore, the PI 3-K/PKB/p70S6K pathway appears to mediate the effect of insulin on p85aPI 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 p85aPI 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 p85aPI 3-K gene. This conclusion, however, requires additional investigations to clearly understand the precise role of the MAPK pathway in the regulation of p85aPI 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 p85aPI 3-K gene has never been reported. Cloning of this promoter is thus now required to further study the regulation of the p85aPI 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 p85aPI 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 p85aPI 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 p85aPI 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 metabolism (16, 17, 26–29, 33), the PI 3-kinase cascade participates in the transcriptional regulation of the p85a regulatory subunit of PI 3-kinase, an essential component of its own pathway.

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