Acute Regulation by Insulin of Phosphatidylinositol-3-kinase, Rad, Glut 4, and Lipoprotein Lipase mRNA Levels in Human Muscle

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Abstract

We have investigated the acute regulation by insulin of the mRNA levels of nine genes involved in insulin action, in muscle biopsies obtained before and at the end of a 3-h euglycemic hyperinsulinemic clamp. Using reverse transcription-competitive PCR, we have measured the mRNAs encoding the two insulin receptor variants, the insulin receptor substrate-1, the p85α subunit of phosphatidylinositol-3-kinase, Ras associated to diabetes (Rad), the glucose transporter Glut 4, glycogen synthase, 6-phosphofructo-1-kinase, lipoprotein lipase, and the hormone-sensitive lipase. Insulin infusion induced a significant increase in the mRNA level of Glut 4 ($+56\pm13\%$), Rad ($+96\pm25\%$), the p85α subunit of phosphatidylinositol-3-kinase ($+92\pm18\%$) and a decrease in the lipoprotein lipase mRNA level ($-49\pm5\%$), while the abundance of the other mRNAs was unaffected. The relative expression of the two insulin receptor variants was not modified. These results demonstrate an acute coordinated regulation by insulin of the expression of genes coding key proteins involved in its action in human skeletal muscle and suggest that Rad and the p85α regulatory subunit of phosphatidylinositol-3-kinase can be added to the list of genes controlled by insulin. (*J. Clin. Invest.* 1996. 98:43–49.) Key words: reverse transcription–competitive polymerase chain reaction • gene expression • euglycemic hyperinsulinemic clamp

Introduction

A major action of insulin is the regulation of metabolic pathways of protein, glycogen, and fat synthesis. These anabolic effects are initiated by the stimulation of the insulin receptor tyrosine kinase after insulin binding to cell membranes and are dependent on the regulation of key controlling enzymes (1). The action of insulin involves modifications of the activity of these critical enzymes and changes in their expression. 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 (2). This last action is certainly a major effect of insulin, and the list of insulin-regulated genes is rapidly growing (2–4).

Skeletal muscle is the main site for insulin-dependent glucose disposal in humans (5). Insulin stimulates glucose uptake and glucose use in oxidative and storage pathways. While insulin action on the activity of the main enzymes that control metabolism in muscle has been well investigated, its role on the expression of genes involved in insulin metabolic action has received little attention. Moreover, when gene expression has been investigated in vivo, the studies generally involve diabetic, insulin-treated, or diet-controlled patients, and therefore brought only indirect evidence on the role of insulin. In pathologies with insulin resistance, the expression of only a few genes has been found altered in skeletal muscle. The expression of the protein Ras associated to diabetes (Rad)[1] was enhanced (6) and the expression of hexokinase II (7) and glycogen synthase (GS) (8) was decreased in patients with non–insulin-dependent diabetes mellitus (NIDDM). On the other hand, the expression of the genes coding Glut 4 (9) and 6-phosphofructo-1-kinase (PFK-1) (8) seemed unaltered in obese or NIDDM patients, and intensive insulin treatment did not modify the expression of the genes for GS and PFK-1 in type I diabetic subjects (10). Using the euglycemic hyperinsulinic glucose clamp, a method that allows the individual effect of insulin to be studied, it was found that Glut 4, GS, and hexokinase I mRNA levels were unaltered in normal muscle; whereas, hexokinase II mRNA increased three times in response to insulin (11). At higher insulin levels, other workers found that Glut 4 mRNA increased in skeletal muscle of normal subjects, but not in muscle from type I or type II diabetic patients (12, 13).

All these data suggest that some important genes involved in glucose metabolism could be regulated by insulin in human muscle. Until now, the study of the specific effects of insulin on the coordinated expression of genes has been limited by the difficulty of measuring the abundance of several mRNAs in percutaneous muscle biopsies due to the small amount of ma-

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1. *Abbreviations used in this paper:* β-μglob, beta-2-microglobulin; GS, glycogen synthase; HSL, hormone-sensitive lipase; IR Ex11, insulin receptor mRNA variant with exon 11; IRS-1, insulin receptor substrate-1; LPL, lipoprotein lipase; NIDDM, non–insulin-dependent diabetes mellitus; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase; PI-3K, phosphatidylinositol-3-kinase; Rad, Ras associated to diabetes; RT, reverse transcription.

terial. Using a sensitive and quantitative method of reverse transcription (RT) reaction, followed by competitive polymerase chain reaction (RT-competitive PCR), we investigated the acute regulation by insulin of the mRNA level of nine key genes involved in the metabolic pathways and in insulin action. The mRNA abundances of the target genes were determined in normal human muscle biopsies, obtained before and at the end of a 3-h euglycemic hyperinsulinic clamp. We have developed a multispecific internal standard (14, 15) that allowed us to measure, by RT-competitive PCR, the levels of the mRNAs encoding the insulin receptors (total mRNA and mRNA variant with exon 11), the insulin receptor substrate-1 (IRS-1), the p85 α regulatory subunit of phosphatidylinositol-3-kinase (PI-3K), Glut 4, Rad, GS, PFK-1, lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), and a reference mRNA encoding the constitutively-expressed beta-2-microglobulin (β-μglob) protein. The choice of these genes was made according to the role of their respective products in the metabolic action of insulin, but was limited by the availability of the human cDNA sequences at the time we constructed the multispecific standard. Using this approach, we have revealed the expression profile of the nine target genes and investigated their acute coordinate regulation by insulin in normal human muscle.

Methods

Subjects

Ten lean healthy volunteers (six men/four women) with a mean (±SEM) age of 24±1 yr and a body mass index of 21.4±0.5 kg/m^2 participated in the study. None of the subjects had a familial or personal history of diabetes, obesity, dyslipidemia, or hypertension or was taking any medication except for oral contraceptives. They were on their usual diet before the study and none were engaged in heavy exercise. All subjects gave their written consent after being informed of the nature, purpose, and possible risks of the study. The experimental protocol was approved by the ethics committee of Hospices Civils de Lyon and performed according to the French legislation (Huriet law).

Study design

All studies were performed in the postabsorptive state at least 12 hours after the last meal and initiated between 0800 and 0900 hours, after at least 30 min of bed rest. Intravenous catheters were inserted into veins of one forearm for insulin and glucose infusions. To obtain arterialized blood samples, another catheter was inserted, in a retrograde manner, in a vein of the opposite hand kept at 55°C in a warming blanket.

Muscle biopsy. A percutaneous biopsy of the vastus lateralis muscle was obtained with Weil Blakesley pliers. Approximately 5 ml of xylocaïne (1%) without epinephrine was injected into the skin and superficial tissue before the biopsy. The procedure involved a small incision (5 mm) through the skin and muscle sheath 15–20 cm above the knee. Muscle biopsies were taken at the beginning of the experiment and after 3 h of euglycemic hyperinsulinemic clamp. The clamp study began 2 h after the first biopsy to avoid the metabolic effect of biopsy-related stress. Average muscle samples of 57±4 mg wet wt (range 35–87) were obtained, immediately frozen in liquid nitrogen, and stored at −80°C until analyzed.

Euglycemic hyperinsulinemic clamp. Insulin (Actrapid; Novo, Copenhagen, Denmark) was infused at 12 pmol/kg per min for 3 h. The insulin infusion rate was primed according to Rizza et al. (16). Any decrease in blood glucose was prevented by the infusion of 20% glucose (Aguettant, Lyon, France) as described previously (17). After 3 h of euglycemic clamp, a second muscle biopsy was obtained from the opposite thigh. At this time, the insulin infusion was discontinued and the study ended.

RNA preparation

Muscle samples were pulverized in liquid nitrogen and total RNA was isolated using guanidium thiocyanate, phenol-chloroform extraction, and alcohol precipitation (18). The average yield of total RNA

Table I. Oligonucleotides Used to Quantify the Target mRNAs by RT-competitive PCR

			Size of PCR product	
mRNA species	Sense primers	Antisense primers	mRNA	Competitor
			bp	
Glut 4	5′-GCTACCTCTACATCATCCAG-3′	5′-TGTCTCGAAGATGCTGGTC-3′	266	306
PFK-2	5′-GAGGCAAGACCTATATCTCC-3′	5′-TGGTAGTGTTGGTGGCATC-3′	252	306
Rad	5′-TGTACTCAGTGACGGACAAG-3′	5′-CGCACGACACCTTCAAACAG-3′	244	306
GS	5′-GTGCTGACGTCTTTCTGGAG-3′	5′-CCAGCATCTTGTTCATGTCG-3′	249	306
IRS-1	5′-ACTTGAGCTACGGTGACGTG-3′	5′-GCGGAACTCATCACTCATGG-3′	353	306
HSL	5′-TCTTCTGCACCAGCCACAAC-3′	5′-AGATGGTCTGCAGGAATGGC-3′	252	306
PFK-1	5′-GGTGTACAAGCTTCTAGCTC-3′	5′-CAAGTTTAGAGCCACCTTGG-3′	254	306
β-μglob	5′-CCAGCAGAGAATGGAAAGTC-3′	5′-GATGCTGCTTACATGTCTCG-3′	269	306
PI-3K	5′-TGACGCTTTCAAACGCTATC-3′	5′-CAGAGAGTACTCTTGCATTC-3′	248	306
BZR	5′-TCTGGAAAGAGCTGGGAGG-3′	5′-CCGCCATACGCAGTAGTTG-3′	268	305
IR Ex11	5′-AACCTCTTCAGGCACTGGTG-3′	5′-TCAAGTGGACGACGTTGTTC-3′	374	326
total IR	5′-AACACTTCCTCGACCAGCG-3′	5′-TCAAGTGGACGACGTTGTTC-3′	265	306
LPL	5′-ACACAGCTGAGGACACTTGC-3′	5′-CACTGGGTAATGCTCCTGAG-3′	227	267

The primer sequences were selected as indicated in Methods. Glut 4, insulin-responsive glucose transporter (23); PFK-2, 6-phosphofructo-2-kinase (24); Rad, protein Ras associated to diabetes (6); GS, glycogen synthase (25); IRS-1, insulin receptor substrate-1 (22); HSL, hormone-sensitive lipase (26); PFK-1, 6-phosphofructo-1-kinase (27); β-μglob, β2-microglobulin (28); PI-3K, p85α regulatory subunit of phosphatidylinositol-3-kinase (29); BZR, peripheral benzodiazepine receptor (30); IR EX11, insulin receptor variant with exon 11 (31), total IR, total insulin receptor (31); LPL, lipoprotein lipase (32). The quantification of PFK-2 mRNA could not be performed because of a mutation in the sequence of the multispecific competitor. The reference BZR mRNA was not amplified efficiently from human muscle total RNA preparations and thus was not measured in the present study.
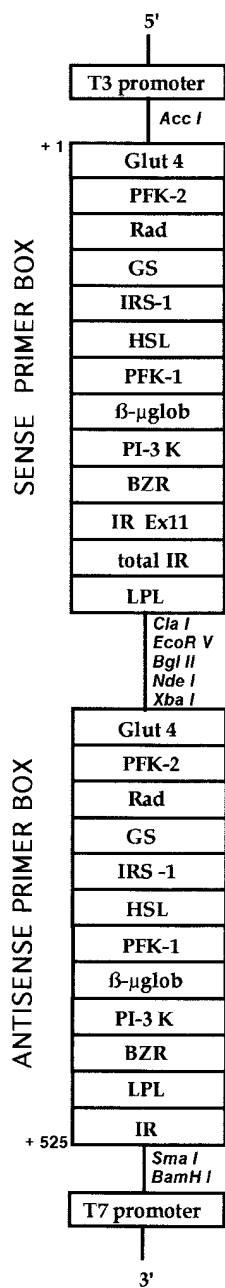
SENSE PRIMER BOX

5'

| T3 promoter |

Acc I

+1

| Glut 4 |
| PFK-2 |
| Rad |
| GS |
| IRS-1 |
| HSL |
| PFK-1 |
| ß-μglob |
| PI-3 K |
| BZR |
| IR Ex11 |
| total IR |
| LPL |

Cla I
EcoR V
Bgl II
Nde I
Xba I

ANTISENSE PRIMER BOX

| Glut 4 |
| PFK-2 |
| Rad |
| GS |
| IRS -1 |
| HSL |
| PFK-1 |
| ß-μglob |
| PI-3 K |
| BZR |
| LPL |
| IR |

+525

Sma I
BamH I

| T7 promoter |

3'

Figure 1. Organization of the multispecific internal standard. The construction of the multispecific standard is explained in Methods. The 525-bp-long standard was subcloned in the pBluescript KS+ phagemid. The T3 RNA polymerase promoter was located upstream of the sense primer box that corresponded to the juxtaposition of the 13 specific-sense primer sequences and was followed by the antisense primer box with the complementary sequences of the 12 antisense primers in the same order. The abbreviations used are defined in Table I.

was 0.27±0.03 and 0.31±0.02 μg/mg muscle (wet wt) for the biopsies taken before and at the end of the hyperinsulinemic clamp, respectively (no significant difference). The absorption ratio (260:280 nm) was between 1.7 and 2.0 for all preparations and the integrity of the RNA was verified on agarose gel colored with ethidium bromide. Total RNA was stored at −80°C until quantification of the target mRNAs.

Quantification of mRNAs

Specific mRNAs were quantified by reverse transcription reaction followed by RT-competitive PCR, which consists of the coamplification of a known amount of standard DNA with the target cDNA in the same tube. The standard is designed to use the same PCR primers as the target, but yields a PCR product of different size, so that the two amplicons can be separated by gel electrophoresis and quantified (19, 20).

Selection of the primers and construction of the multispecific standard. The standard, or competitor, was a 525-bp long synthetic gene, the sequence of which corresponded to the juxtaposition of 13 spe-

cific sense-primer sequences (sense-primer box), followed by the juxtaposition of the complementary sequences of 12 specific antisense primers in the same order (antisense primer box) (Fig. 1, Table I). Total and Ex11 insulin receptor mRNAs were measured with the same antisense primer (located in exon 13) and with specific sense primers (located in exons 12 and 11). The multispecific standard was constructed by a technique of oligonucleotide overlap extension and amplification by PCR (21), starting with four purified long oligonucleotides (139 to 151 bases; Eurogentec, Seraing, Belgium) that successively covered all the sequence of the standard, with small overlaps. A base substitution, at position + 309, appeared during the synthesis of the competitor so that we could not perform the competitive PCR assay of the 6-phosphofructokinase-2-kinase/fructose-2,6-bisphosphatase (PFK-2) mRNA. The phagemid containing the competitor cDNA was purified in large amount, quantified by absorption at 260 nm, and stored at −20°C as concentrated stock solution. Working solutions at defined concentrations (25 amol/μl to 10^{-3} amol/μl) were prepared by serial dilution in 10 mM Tris-HCl (pH 8.3), and 1 mM EDTA buffer, aliquoted under small volume and stored at −20°C.

Reverse transcription reaction. For each mRNA, a specific first-strand cDNA synthesis was performed from 0.5 to 1 μg of total RNA with 2.5 U of thermostable reverse transcriptase (*Tth* DNA polymerase; Promega Corp., Charbonnière, France) in 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 1 mM MnCl$_2$, 0.2 mM deoxynucleoside triphosphates, and 15 pmol of the specific antisense primer, in a final volume of 20 μl. The medium was overlaid with mineral oil and subjected to incubations for 3 min at 60°C followed by 15 min at 70°C in the thermal cycler (Omnigene; Hybaid, Teddington, UK). The reaction was stopped by heating at 99°C for 5 min. After chilling on ice, 4 μl of water was added to the RT medium, from which 20 μl was sampled for cDNA quantification by PCR.

Competitive PCR. The 20-μl sample of the RT reaction was added to 80 μl of PCR mix (10 mM Tris–HCl, pH 8.3, 100 mM KCl, 0.75 mM EGTA, 5% glycerol) containing 0.2 mM deoxynucleoside triphosphates, 5 U of *Taq* polymerase (Life Technologies, Cergy Pontoise, France), 45 pmol of the corresponding sense primer, and 30 pmol of the antisense primer. Four 20-μl aliquots were then transferred to microtubes containing 5 μl of defined working solutions of the competitor cDNA. The microtubes were kept in ice until the thermal cycler was up to 90°C. After 120 s at 94°C, the PCR mixtures were subjected to 40 cycles of PCR amplification with a cycle profile including denaturation for 40 s at 95°C, hybridation for 50 s at 55°C, and elongation for 50 s at 72°C.

Analysis of PCR products. The amplification products of 15 μl of each PCR were separated in a 3 or 4% agarose gel, stained with ethidium bromide, and photographed (665 film; Polaroid Corp., Cambridge, MA). The band densities were evaluated from the negative film with a Vernon photometer-integrator. After correction for the difference in nucleotide number, the logarithm of the density ratio of the competitor band to the target mRNA band was plotted vs the logarithm of the initial amount of competitor cDNA. The initial concentration of target cDNA in the PCR was determined at the competition equivalence point as previously described (20).

Preparation of RNA by in vitro translation. Parts of the coding sequence of Glut 4, the insulin receptor, and β-μglob mRNAs, which included the sequences targeted in the RT-competitive PCR assay, were amplified by RT-PCR from a total RNA preparation obtained from a normal human muscle biopsy, and subcloned in the phagemid pGEM-T (Promega Corp.). The three partial cDNAs were used to produce RNA by in vitro translation (Riboprobe System, Promega Corp.) and the synthetic RNAs were quantified by RT-competitive PCR as describe above.

Control experiments and the presentation of the results. The absence of contamination was verified periodically by control experiments that omitted RNA or cDNA in the reactions. For the mRNA of IRS-1, the coding sequence is contained within a single exon (22) and, in the cases of PI-3K and Rad mRNAs, the organization of the genes was

not reported when we selected the primer sequences, so that they could be located in the same exon. Therefore, when these mRNAs were measured, we checked for the absence of genomic DNA amplification by performing control RT-PCR without the reverse transcriptase in the RT step.

To accurately compare the target mRNA abundance between samples, total RNA from the two biopsies from the same subject (before and at the end of the clamp) were prepared at the same time. In addition, the different target mRNAs were always measured in parallel in the same run of PCR and with amounts of competitor taken from the same serial dilution so that the level of the different mRNAs could be compared. The results were then normalized and presented by reference to the mRNA level of the constitutively expressed β-μglob gene (15, 22). This presentation had the advantage of erasing errors in total RNA measurement between samples.

The results are expressed as means±SEM. Statistical comparisons were performed with a Wilcoxon nonparametric test for paired values. The threshold for significance was set at $P < 0.05$.

Results

Validation of the RT-competitive PCR assay. To validate the RT-competitive PCR assay, in vitro–synthesized RNAs (see Methods) were quantified in five mixtures that contained a constant amount of β-μglob RNA and different amounts of Glut 4 RNA (ranging from 0.1- to 10-fold the amount of β-μglob RNA) and insulin receptor RNA (ranging from 0.02- to 2-fold the amount of β-μglob RNA). Each mixture was prepared from the stock solutions of the synthetic RNAs and diluted to achieve a concentration of about 20 amol/μl (20 × 10^{-18} mol) of β-μglob RNA. The amount of β-μglob RNA was found to be 121.8±8.6 amol (mean±SD) in 5 μl of the different mixtures. Fig. 2 shows the RNA ratio (Glut 4 or insulin re-

ceptor RNA/β-μglob RNA) measured by RT-competitive PCR plotted against the initial RNA ratio in the mixtures. The slope of the standard curve was 1.18 for the ratio Glut 4/β-μglob ($r = 0.997$) and 1.05 for the ratio insulin receptor/β-μglob ($r = 0.999$). In addition, since the in vitro–synthesized insulin-receptor RNA contained the sequence of the exon 11, it could be measured with the primers specific for the insulin receptor mRNA variant with exon 11 (IR EX11). Similar results were obtained when the IR EX11 or the total IR primers were used (data not shown) and the slope of the standard curve for the ratio IR Ex11/β-μglob was 0.95 ($r = 0.999$). The standard curve (Fig. 2) shows that the RT-competitive PCR assay was linear from 2 to 1,000 amol of RNA in the RT reaction.

Euglycemic hyperinsulinemic clamp. Ten normal subjects were submitted to a 3-h euglycemic hyperinsulinemic clamp to achieve supraphysiological plasma insulin concentrations. Insulinemia increased from 42±3 pmol/liter in the basal state, to 816±28 pmol/liter during the last hour of insulin infusion. The fasting plasma glucose concentration was 4.4±0.1 mmol/liter and glycemia was clamped during the insulin infusion at 4.3±0.2 mmol/liter. The rate of glucose infusion required to maintain euglycemia during the last hour of insulin infusion was 51.1±2.7 mmol/kg per min.

Effect of insulin infusion on the abundance of the target mRNAs in muscle. Fig. 3 shows the mRNA level profile of the target genes in normal skeletal muscle, before and at the end of the hyperinsulinemic clamp. The results were expressed by reference to the level of β-μglob mRNA. The absolute values of β-μglob mRNA were 130±10 amol/μg of total RNA in the basal state and 123±8 amol/μg of total RNA at the end of the clamp (no significant difference). Before the clamp, the abundance of the different target mRNAs varied in a broad range from 2.4±0.5 (HSL) to 93.4±6.1% (Glut 4) of β-μglob
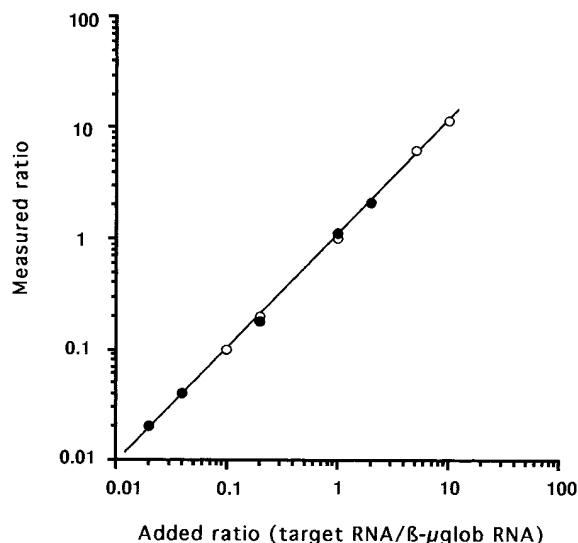

Figure 2. Validation of the RT-competitive PCR. In vitro–produced RNAs containing part of the coding sequence of β-μglob, Glut 4, and insulin receptor were measured in solutions containing a constant amount of β-μglob RNA and different amounts of Glut 4 and insulin receptor RNAs. The results were expressed as ratio Glut 4 (*open circles*) or insulin receptor (*filled circles*) RNA/β-μglob RNA. The initial ratio Glut4 RNA/β-μglob RNA in the solutions were 0.1, 0.2, 1, 5, and 10 and the initial ratio insulin receptor RNA/β-μglob RNA were 0.02, 0.04, 0.2, 1, and 2.
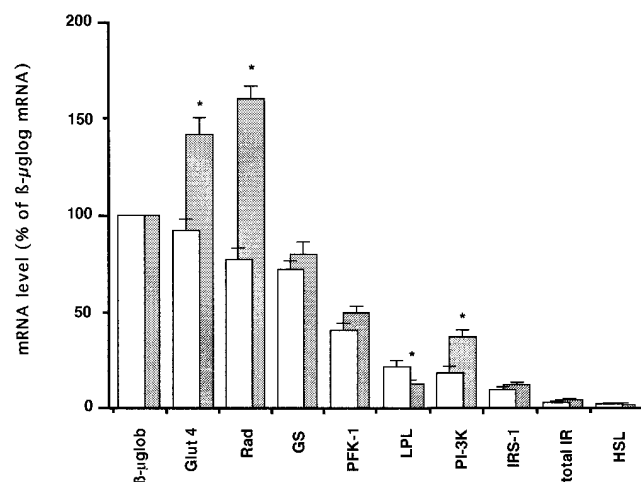
Figure 3. Profiles of the mRNA levels of the nine target genes in human muscle before and at the end of the euglycemic hyperinsulinemic clamp. Specific mRNA levels were determined by RT-competitive PCR and expressed as a percentage of the β-μglob mRNA level. The values measured before the euglycemic hyperinsulinemic clamp are represented by the open boxes, while the hatched boxes corresponded to the values obtained at the end of the 3-h clamp. Data are means±SEM ($n = 10$), *$P < 0.05$ using the nonparametric test of Wilcoxon for paired series.
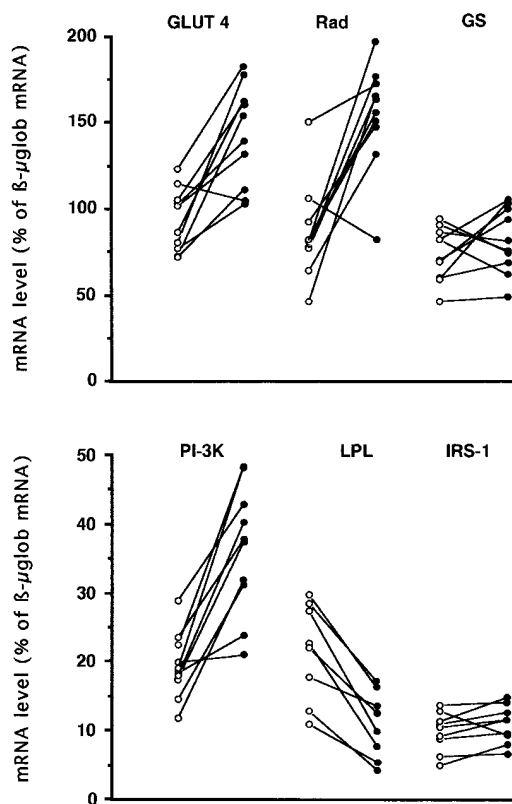
Figure 4. Effect of insulin infusion on mRNA levels of Glut 4, Rad, glycogen synthase, and p85α subunit of phosphatidylinositol-3-kinase. The mRNA levels, expressed as a percentage of β-μglob mRNA, were measured before (*open circles*) and at the end (*filled circles*) of the euglycemic hyperinsulinemic clamp in vastus lateralis muscle biopsies of the ten subjects. Individual variations of PFK-1, of the two variants of insulin receptor, and of HSL mRNA levels that did not change significantly during the clamp (Fig. 3), were not presented.

mRNA. With the exception of the mRNA for HSL, which is not a typical muscle enzyme, the least abundant mRNA encoded the insulin receptor (3.2±0.4% of β-μglob mRNA). The amount of the mRNA variant with exon 11 was 1.3±0.2% of β-μglob mRNA, thus representing 43±5% of the total insulin receptor mRNA. Rad mRNA was in the same range as the mRNAs for Glut 4 and GS.

After 3 h of hyperinsulinemia, the mRNAs encoding Glut 4, Rad, and PI-3K significantly increased; whereas, LPL mRNA decreased (Fig. 3). No change was observed in the level of the mRNAs for GS, PFK-1, IRS-1, insulin receptor, and HSL (Fig. 3). Fig. 4 shows the individual variations of the mRNA levels. Glut 4 mRNA increased by 56±13% ($P = 0.007$), Rad mRNA by 96±25% ($P = 0.093$) and PI-3K mRNA by 92±18% ($P = 0.005$), while LPL mRNA decreased by 49±5% ($P = 0.011$). Insulin infusion affected neither the amount (1.4±0.4% of β-μglob mRNA), nor the percentage (40±5% of total insulin-receptor mRNA) of the mRNA encoding the isoform of insulin receptor with exon 11. One subject had no response to insulin on both Glut 4 and PI-3K mRNA levels (Fig. 4). The two subjects with the highest level of Rad mRNA and no response to insulin were from the same family (brother and sister). None of these subjects presented abnormal glucose metabolism during the clamp.

Discussion

Investigation of the coordinated regulation of a number of target genes by insulin in human muscle was made possible by the development of a sensitive and reliable RT-competitive PCR method. In this study, we report the expression profile of several of the key genes that code proteins involved in the insulin-sensitive metabolic pathways and study their acute regulation by insulin. We demonstrate that insulin infusion changes the mRNA level of Glut 4, Rad, PI-3K, and LPL in normal human skeletal muscle, without affecting the level of GS, PFK-1, IRS-1, insulin receptor, or HSL mRNAs.

The RT-competitive PCR developed in this study is a powerful method of determining the absolute quantity of a given mRNA. However, due to possible imprecisions in total RNA concentration determinations and in the dilutions of the internal standard molecule, the results were presented by reference to the level of a reference mRNA that was measured using the same method (14, 15). We have validated this procedure using RNAs synthesized by in vitro translation. Nevertheless, the absolute values found by RT-competitive PCR in this study were in accordance with the mRNA levels already reported using other methods. We found that Glut 4 mRNA was the most abundant of the selected targets and varied from 77 to 146 amol/μg total RNA (72 to 115% of β-μglob mRNA), values that were similar to those reported by Schalin-Jäntti et al. (13) using a quantitative dot blot method (68 to 100 pg/μg total RNA corresponding to 78 to 115 amol/μg total RNA, taking 3.5 kb for Glut 4 mRNA, reference 23). Similarly, the level of insulin-receptor mRNA determined by solution hybridization method (33) ($\sim$ 2.5 amol/μg of total RNA, assuming that one copy corresponds to one molecule of mRNA and using the Avogadro constant) was in agreement with the values obtained by RT-competitive PCR in this study (3.2±0.4% of the β-μglob mRNA level that corresponded to values between 1.6 and 6.3 amol/μg of total RNA). Finally, we found that IRS-1 mRNA level was low in human muscle, but more abundant than the insulin-receptor mRNA, in agreement with a recent report using a semiquantitative PCR method (22). To our knowledge, quantitative data are not available for the other mRNAs. The use of a multispecific standard in the RT-competitive PCR assay allowed us to compare the levels of ten different mRNAs in a single muscle biopsy and thus to propose an expression profile of the mRNAs of nine genes involved in insulin action. This profile reveals large differences in the basal state, suggesting differences in gene expression. However, the variety of the mRNA levels could also be attributed to differences in mRNA stability.

Insulin acutely increased the mRNA level of Glut 4, Rad, and PI-3K and decreased the level of LPL mRNA. The most striking effects were the increases in Rad and PI-3K mRNAs. The Rad mRNA encodes a recently discovered protein related to the Ras/GTPase superfamily and it has been shown to be overexpressed in the muscle of NIDDM patients (6). Our finding that insulin induced a twofold increase in Rad mRNA level brings the first evidence that Rad expression is acutely regulated in vivo and argues for a role for Rad in the mechanism of action of insulin. It could also suggest that the overexpression of Rad in NIDDM muscle might be related to chronic hyperinsulinemia. One of the earliest postreceptor events in insulin signaling on glucose metabolism is the activation of phosphatidylinositol-3-kinase (1). This activation results from the bind-

ing of the p85α-regulatory subunit of phosphatidylinositol-3-kinase to specific phosphorylated tyrosine residues of IRS-1 (1). We show in this study that the mRNA level of the phosphatidylinositol-3-kinase regulatory subunit was increased by insulin, thus demonstrating that insulin acts not only on the activity of the enzyme, but also participates in the control of its expression.

Direct effects of insulin on human muscle Glut 4 mRNA have already been investigated. With an insulinemia maintained at around 390 pmol/liter, Mandarino et al. (11) did not observe change in Glut 4 mRNA level. However, using supraphysiological insulin concentrations ($\sim$ 750 pmol/liter), other investigators found a significant (35 to 50%) increase in Glut 4 mRNA (12, 13). This discrepancy probably results from the differences in insulin levels, since in our study with insulinemia maintained at 816±28 pmol/liter, Glut 4 mRNA increased by 56% at the end of the 3-h hyperinsulinemic clamp. For LPL, insulin infusion has been shown to decrease its enzyme activity by 35% during a 2-h clamp (34), which could be attributed to an insulin-induced inhibition of LPL expression. Indeed, we did find a decrease in LPL mRNA level in muscle under our conditions of hyperinsulinemia.

The abundance of the mRNAs for GS, PFK-1, IRS-1, and insulin receptor was not significantly affected during the clamp. The absence of alterations of GS and PFK-1 mRNAs in the presence of physiological levels of hyperinsulinemia has already been reported (8, 10, 11). The lack of change in the presence of high insulin concentrations suggests that the expression of these genes is not regulated by insulin. It cannot be excluded, however, that insulin effects on the expression of these genes require a longer time to become apparent. Alternative splicing of insulin receptor mRNA, leading to two receptor isoforms differing by the presence or the absence of 12 amino acids encoded by the exon 11, has been proposed to be regulated by insulin in vivo (35) and in vitro (36). Our results show that neither the total amount of insulin receptor nor the relative expression of the mRNA variants encoding the two isoforms varied during the hyperinsulinemic clamp. This is in agreement with our recent report suggesting that insulin is not the principal regulatory factor of insulin receptor mRNA splicing in rat tissues in vivo (20).

Recent reviews dealing with insulin action in liver pointed out that insulin effects on gene expression parallel its metabolic actions. Insulin produces a coordinate stimulation of the expression of genes coding key glycolytic enzymes and inhibition of the expression of genes coding key gluconeogenic enzymes (3, 37). Similarly, in the muscle, insulin increases Glut 4, PI-3K, and hexokinase II mRNAs (11), and decreases LPL mRNA, in agreement with the insulin stimulation of glucose uptake and use and the inhibition of fatty acid oxidation in this tissue.

As to the mechanism of the modification of the mRNA levels, insulin could affect the rate of the gene transcription and/or the stability of the mRNAs. Little is known about the hormonal regulation of the last process, but it is probably an important control step (2, 3). On the other hand, insulin could exert a transcriptional control of gene expression, either directly or through the stimulation of glucose metabolism in the cell (2–4, 37). Insulin response sequences and glucose response elements have been identified in the promoter sequence of several genes. Among the genes that seem to be regulated by insulin in our work, the promoter regions of the genes coding

Rad and the p85α subunit of phosphatidylinositol-3-kinase have not been characterized. In addition, putative insulin regulatory elements in the promoters of Glut 4 and LPL genes have not been clearly identified, while transcriptional effects of insulin on the Glut 4 gene have been demonstrated in rodent models and cell lines (38).

In conclusion, our work demonstrated an acute and coordinated regulation by insulin of the mRNA levels of key proteins involved in insulin action in the skeletal muscle of normal humans. We have identified two important genes, (namely, Rad and the p85 α regulatory subunit of phosphatidylinositol-3-kinase) that can be added to the growing list of the genes controlled by insulin. Since the products of these genes, in particular PI-3K, play a crucial role in the insulin mechanism of action, our findings strengthen the possibility that altered regulation of gene expression by insulin could result in insulin resistance (39). The methodology developed here allowing a wide screening of mRNA levels represents a valuable tool to test this hypothesis.

Acknowledgments

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