

## Microarray Profiling of Human Skeletal Muscle Reveals That Insulin Regulates ~800 Genes during a Hyperinsulinemic Clamp\*<sup>§</sup>

Received for publication, January 10, 2003, and in revised form, February 20, 2003  
Published, JBC Papers in Press, March 5, 2003, DOI 10.1074/jbc.M300293200

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**Insulin action in target tissues involved precise regulation of gene expression. To define the set of insulin-regulated genes in human skeletal muscle, we analyzed the global changes in mRNA levels during a 3-h hyperinsulinemic euglycemic clamp in vastus lateralis muscle of six healthy subjects. Using 29,308 cDNA element microarrays, we found that the mRNA expression of 762 genes, including 353 expressed sequence tags, was significantly modified during insulin infusion. 478 were up-regulated and 284 down-regulated. Most of the genes with known function are novel targets of insulin. They are involved in the transcriptional and translational regulation (29%), intermediary and energy metabolisms (14%), intracellular signaling (12%), and cytoskeleton and vesicle traffic (9%). Other categories consisted of genes coding for receptors, carriers, and transporters (8%), components of the ubiquitin/proteasome pathways (7%) and elements of the immune response (5.5%). These results thus define a transcriptional signature of insulin action in human skeletal muscle. They will help to better define the mechanisms involved in the reduction of insulin effectiveness in pathologies such as type 2 diabetes mellitus, a disease characterized by defective regulation of gene expression in response to insulin.**

Insulin is an anabolic hormone that exerts a wide spectrum of effects and modulates a variety of biological processes and metabolic pathways such as glucose and lipid metabolism, protein synthesis and degradation, cell growth and differentiation, and DNA synthesis. To perform these actions insulin modifies the activity and the subcellular location of key regulatory enzymes and proteins, often by affecting their phosphorylation state (1). Furthermore, insulin also controls the amount of numerous proteins, in part by acting at the level of mRNA translation and mainly at the level of their gene expression (2). Over the past 15 years, the regulation of gene expression by

insulin has been recognized as a major effect of the hormone, and about 150 insulin-regulated genes have been reported (2). However, the identification of insulin-regulated genes has been mostly performed in cell culture experiments and using animal models (2). Little is known about the transcriptional changes induced *in vivo* by insulin in human tissues, particularly in skeletal muscle, the main site of insulin-dependent glucose disposal and the major site of insulin resistance in type 2 diabetes mellitus. Although it has been demonstrated that insulin can modulate the mRNA levels of some important genes in human skeletal muscle, such as hexokinase II, Glut4, p85 $\alpha$  phosphatidylinositol 3-kinase, or lipoprotein lipase (3–5), most of these studies were focused on a small number of selected genes involved in glucose and lipid metabolism (5, 6) and did not give a global view of insulin action on gene expression.

It is likely that insulin coordinates a complex program of transcriptional changes in target tissues, which represents the molecular basis of its action. Hence, the characterization of this global pattern of modifications in skeletal muscle is an important step for a better understanding of the mechanism of action of insulin and of its defects in situations of insulin resistance. The development of microarray technology offers powerful tools for characterizing such large scale changes in transcript levels. Recently, this methodology was applied to investigate the effects of intensive insulin treatment for 10 days on the mRNA profile in skeletal muscle of type 2 diabetic patients (7). Using Affymetrix oligonucleotide microarrays allowing the analysis of 6800 mRNAs, the expression of about 100 genes was found to be modified during insulin therapy (7). With a similar methodology, it was also demonstrated that 3 days of insulin treatment induced changes in the abundance of about 100 mRNAs in streptozotocin diabetic mice, suggesting a coordinated regulation of gene expression by insulin in skeletal muscle (8). However, it was difficult to conclude on the direct role of insulin on gene expression in these two studies because the observed modifications could be the consequences of the metabolic changes that occurred during the treatment with insulin.

To determine the global transcriptional modifications directly produced by insulin in human skeletal muscle, we used cDNA microarrays (9) to analyze the changes in the mRNA levels of 29,308 genes and expressed sequence tags (ESTs)<sup>1</sup> induced by 3 h of euglycemic hyperinsulinemic clamp in the vastus lateralis muscle of healthy lean subjects. It is well accepted that the hyperinsulinemic clamp method allows the

\* This work was supported in part by INSERM Action Thématique concertée Nutrition Grant 4NU10G and by grants from the Institut de Recherche Servier, Région Rhône-Alpes, and Claude Bernard Fondation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental table.

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<sup>1</sup> The abbreviations used are: EST, expressed sequence tag; RT, reverse transcription; CREBBP, cAMP-response element-binding protein (CREB)-binding protein; SNARE, soluble N-ethylmaleimide factor attachment protein receptor; IRS, insulin receptor substrate.

TABLE I  
Sequences of the primers used for mRNA quantitation by real-time RT-PCR

Transcript name	UniGene cluster	Sense primer	Antisense primer
PPP2R3A	Hs.28219	ACGCTTGTTCAGAGGAATC	TCCAAATTCAGAGGGAGAGG
PDE4D	Hs.172081	GACCTCTCTCCAAAGTCTATG	TGGATGGTTGGTTGCACATGG
CREBBP	Hs.23592	GCCGTTTACCATGAGATCC	CAAACGCCTTGTCCAGCATC
RYBP	Hs.7910	TCTGAAAGATCCTCCTAGTG	CTGTGATAATGACGGTGACG
NR5A2	Hs.183123	CCAGGAGTAGTATCTTCTTC	GTACCACTTGTCCGTAATG
FACL2	Hs.154890	CCATTTGAACAGGTCAAAGGC	AGGTACTTATCTGCGACCT
FAT/CD36	Hs.75614	TGATGATGAACAGCAGCAACAT	GAACCTGTGTTGTCCAGCG
HK2	Hs.198427	ATCCCTGAGGACGACATCATGCC	ATGTCGATGTGGCGCATCTC
PIK3R1	Hs.6241	TGACGCTTTCAAACGCTATC	CAGAGAGTACTCTTGCAATC

individual effect of insulin to be studied, and the short duration of the insulin infusion was chosen to limit possible secondary effects due to metabolic modifications. Rapid changes in the mRNA levels of candidate genes have been previously reported in human skeletal muscle during similar euglycemic hyperinsulinemic clamp conditions (5, 6). We report here that insulin directly modulates the mRNA levels of about 800 genes in human muscle. Most of them are novel targets of the hormone and belong to functional classes that can account for most of the biological and metabolic effects of insulin in human skeletal muscle.

#### EXPERIMENTAL PROCEDURES

**Subjects**—Twelve lean healthy Caucasian volunteers (4 men and 9 women) with a mean ( $\pm$ S.E.) age of  $32 \pm 4$  years and a body mass index of  $22.2 \pm 0.6$  kg/m<sup>2</sup> participated in the study. None had a familial or personal history of diabetes, obesity, dyslipidemia, or hypertension, and they were not taking medication except for oral contraceptives. They were on their usual diet before the study, and none was engaged in heavy exercise. All subjects gave 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 (France).

**Study Design**—All studies were performed in the postabsorptive state after an overnight fast. The volunteers were submitted to a 3-h euglycemic hyperinsulinemic clamp with an insulin infusion rate of 2 milliunits·min<sup>-1</sup>·kg<sup>-1</sup>, as described previously (5). Percutaneous biopsy of the vastus lateralis muscle was obtained with a Weil Blakesley plier before and after 3 h of insulin infusion (5). Average muscle samples of  $63 \pm 4$  mg (wet weight) were obtained, immediately frozen in liquid nitrogen, and stored at  $-80$  °C until total RNA preparation.

**Total RNA Preparation and Amplification**—Frozen tissue samples were crushed in liquid nitrogen, and total RNA was extracted with the guanidinium thiocyanate method (5). RNA concentrations and integrity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). The mean yield of total RNA was  $0.28 \pm 2$   $\mu$ g/mg of muscle (wet weight) and was not different for the biopsies taken before and after the clamp.

RNA preparations from 6 subjects (2 men, 4 women) were amplified using the MessageAmp antisense RNA kit (Ambion, Austin, TX) in order to generate probe for hybridization to the cDNA microarrays. Briefly, the procedure consists of reverse transcription of 1  $\mu$ g of total RNA with an oligo(dT) primer bearing a T7 promoter sequence followed by *in vitro* transcription of the resulting DNA with T7 RNA polymerase to generate antisense RNA copies of each mRNA (10). This amplification procedure, now largely accepted, has been validated before, and it has been demonstrated that it does not distort the relative abundance of individual mRNAs within an RNA population (10, 11).

**Probe Labeling and Hybridization**—Ten  $\mu$ g of antisense RNA from basal and insulin-treated conditions of each of the six subjects were labeled with cyanine (Cy) 3 or Cy5 dyes during a random-primed reverse transcription reaction using the CyScribe First-Strand cDNA labeling kit (Amersham Biosciences). They were hybridized overnight at 65 °C to the cDNA microarray according to the protocol recommended by the Stanford Functional Genomics Facility ([www.microarray.org/sfg7/jsp/home.jsp](http://www.microarray.org/sfg7/jsp/home.jsp)). The cDNA microarrays consisted of PCR-amplified cDNAs printed on glass slides with 42,557 spots representing 29,308 UniGene clusters.

**Analysis of Microarray Data**—The six slides were scanned with a GenePix 4000A microarray scanner (Axon Instruments, Union City, CA), and the images were analyzed using Genepix pro 3 software. Data files were entered into the Stanford Microarray Data base ([\[www5.stanford.edu/MicroArray/SMD\]\(http://www5.stanford.edu/MicroArray/SMD\)\). A uniform scale factor was applied to normalized signal intensities between Cy5 and Cy3. Flagged spots and spots with an average intensity below 2.5-fold above the background were not retained for further analysis. The  \$\log\_2\(\text{Cy5/Cy3}\)\$  ratio of the other spots was calculated for each slide. To compare the results from the different subjects, data from each slide were normalized in log space to have a mean of 0 and a S.D. of 1 by using the Cluster program \(46\). Only cDNAs with recorded data on the six slides were then selected for further analysis. At this stage, 16,140 spots were recovered. Genes with significant changes in mRNA levels in response to insulin were identified using the Significant Analysis of Microarrays \(SAM\) procedure \(12\), a validated statistical technique for identifying differentially expressed genes across high density microarrays. This procedure provides a list of "significant" genes and an estimate of the false discovery rate, which represents the percentage of genes that could be identified by chance \(9, 12\).](http://genome-</a></p>
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**Quantitation of mRNAs Using Real-time RT-PCR**—First-strand cDNAs were first synthesized from 500 ng of total RNA in the presence of 100 units of Superscript II (Invitrogen) using both random hexamers and oligo(dT) primers (Promega, Charbonnières, France). The real-time PCR was performed using a LightCycler (Roche Diagnostics) in a final volume of 20  $\mu$ l containing 5  $\mu$ l of a 60-fold dilution of the RT reaction and 15  $\mu$ l of reaction buffer from the FastStart DNA Master SYBR Green kit (Roche Diagnostics) with 3 mM MgCl<sub>2</sub> and the specific forward and reverse primers (Eurogentec, Seraing, Belgium). The list of the primers is available in Table I. After amplification, a melting curve analysis was performed to verify the specificity of the reaction. For quantification, a standard curve was systematically generated with six different amounts (150–30,000 molecules/tube) of purified target cDNA cloned in the pGEM plasmid (Promega). The analysis was performed using the LightCycler software (Roche Diagnostics).

#### RESULTS AND DISCUSSION

**Euglycemic Hyperinsulinemic Clamp**—To investigate the effect of insulin on gene expression, healthy lean volunteers were submitted to a 3-h euglycemic hyperinsulinemic clamp to achieve supra-physiological plasma insulin concentrations (5, 6). Insulinemia increased from  $41 \pm 3$  pM in the basal state to  $858 \pm 52$  pM during the last hour of the clamp. Plasma free fatty acid concentration decreased from  $463 \pm 64$  to  $34 \pm 4$  mM. The glycemia was clamped at  $4.6 \pm 0.1$  mM. The rate of glucose infusion required to maintain euglycemia during the last hour of the clamp was  $11.1 \pm 0.6$  mg·kg<sup>-1</sup>·min<sup>-1</sup>, indicating a normal effect of insulin on whole body glucose disposal rate (5, 6). Glucose oxidation rate, as determined by indirect calorimetry, rose from  $1.3 \pm 0.2$  mg·kg<sup>-1</sup>·min<sup>-1</sup> before insulin infusion to  $3.5 \pm 0.2$  mg·kg<sup>-1</sup>·min<sup>-1</sup> at the end of the clamp ( $p < 0.001$ ). Glucose storage, estimated from the non-oxidative glucose disposal rate, was  $7.6 \pm 0.6$  mg·kg<sup>-1</sup>·min<sup>-1</sup> during the last hour of insulin infusion. Skeletal muscle biopsies were taken before and at the end of the hyperinsulinemic clamp, and total RNA was prepared from each tissue samples.

**Analysis of cDNA Microarray Data**—For cost issue, the cDNA microarray experiments were performed with the RNA preparations from 6 subjects (2 men and 4 women) of the 12 involved in the study. Their characteristics and their metabolic responses during the clamp did not differ from the data of the whole group (data not shown). The cDNA microarrays used in

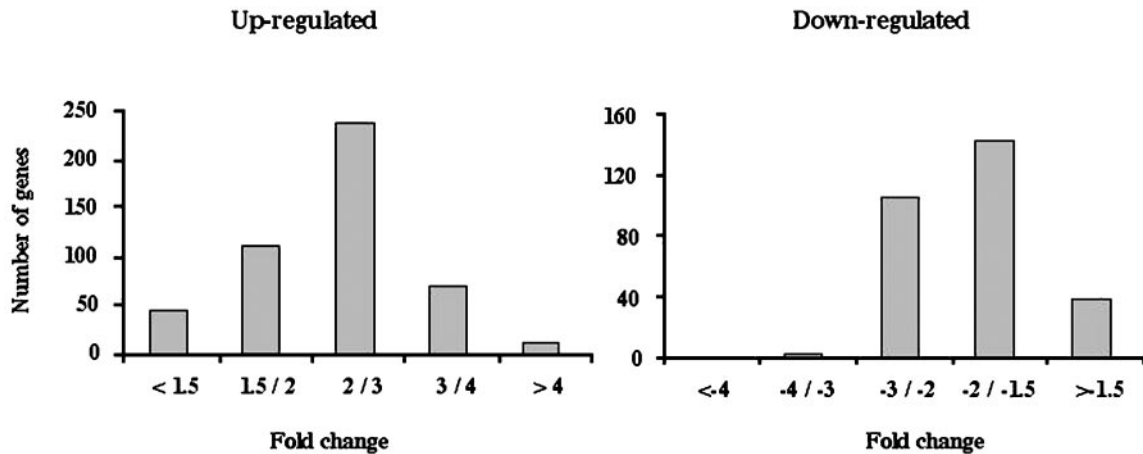


FIG. 1. Distribution of the -fold changes in mRNA levels induced by insulin in human skeletal muscle.

the present work allowed analysis of the changes in mRNA levels of 29,308 genes and ESTs. The SAM procedure (9, 12) was performed on 16,140 spots for which signals were recovered in the six experiments. With an estimated false discovery rate of 4%, we found 1,065 cDNAs that were differentially regulated by insulin. As shown in the supplemental table, this procedure selected cDNAs for which the  $\log_2(\text{Cy5}/\text{Cy3})$  ratio changed in the same direction on the six slides. This indicated that the corresponding genes were modulated by insulin in the same way in the six subjects. Because of replications of some cDNAs on the microarray, the 1065 cDNAs corresponded to 762 different genes (a list is available in the supplemental table). Of these 762 genes, 478 were up-regulated, and 284 were down-regulated during the 3 h of the hyperinsulinemic clamp. Fig. 1 shows that the majority ( $n = 322$ ) of the up-regulated genes displayed a more than 2-fold change in mRNA levels, as estimated by the mean Cy5 on Cy3 intensity ratio of the 6 experiments. Only 13 increased 4-fold or more. Among the down-regulated genes, 104 displayed a 2-fold or more decrease in expression level.

To test the validity of the microarray experiments and the SAM procedure, the changes in mRNA levels of 9 genes were quantified using real-time RT-PCR in muscles of the whole group of subjects who underwent the 3-h hyperinsulinemic clamp. These genes displayed wide differences in their response to insulin on the microarray (from  $-1.95$  to  $5.04$ -fold change) and belong to the 3 main functional categories that were defined to classify the insulin-regulated genes (see "Functional Classification of the Regulated Genes"). As shown in Table II, the changes measured by real-time RT-PCR confirmed the microarray data for all genes except one. The down-regulation of the nuclear receptor NR5A2 observed by microarray was not reproduced by RT-PCR (Table II). However, it should be mentioned that the absolute mRNA level of NR5A2 (about  $0.05 \text{ amol} \cdot \mu\text{g}^{-1}$  total RNA) was closed to the lower limit of the RT-PCR assay.

We further verified that the procedures used in the microarray experiment did not select only genes with high expression in muscle. Using the Stanford Online Universal Resource for Clones and ESTs (SOURCE at source.stanford.edu) and the published reconstruction of the human skeletal muscle transcriptional profile (13–15), we found that 32 of the 762 insulin-regulated genes have been reported to be more expressed in the muscle than in other tissues, with only 6 belonging to the 400 genes considered as highly expressed in skeletal muscle (13, 15). Conversely, about 150 genes, mostly ESTs with unknown functions, had never been found to be expressed in the skeletal muscle. This suggested that the procedures used in the study

TABLE II

Comparison of microarray results with quantitative RT-PCR

The -fold changes in mRNA levels of 10 selected genes were determined by real-time RT-PCR using a LightCycler on total RNA preparations from 12 subjects. Data are the means  $\pm$  S.E. Microarray experiments were performed on amplified RNA from six subjects.

Transcript name	UniGene cluster	-Fold changes	
		cDNA microarrays	RT-PCR
PPP2R3A	Hs.28219	2.27	$1.47 \pm 0.07^a$
PDE4D	Hs.172081	2.04	$1.58 \pm 0.08^a$
CREBBP	Hs.23592	-1.83	$-1.24 \pm 0.07^b$
RYBP	Hs.7910	1.58	$1.62 \pm 0.08^a$
NR5A2	Hs.183123	-1.95	$1.62 \pm 0.16$
FACL2	Hs.154890	4.11	$1.59 \pm 0.09^b$
FAT/CD36	Hs.75614	5.04	$1.82 \pm 0.16^a$
HK2	Hs.198427	3.02	$2.92 \pm 0.37^a$
PIK3R1	Hs.6241	1.8	$1.97 \pm 0.11^a$

<sup>a</sup>  $p < 0.005$ , using paired  $t$  test.

<sup>b</sup>  $p < 0.05$ , using paired  $t$  test.

did not select genes according to their mRNA expression levels and, thus, allowed to analyze the effect of insulin on the global muscle transcriptome. However, because the analysis was performed in tissue biopsies, it could not be excluded that regulation of gene expression in other cell types than in muscle cells might have contributed to the detected changes.

Of the 762 genes that showed a regulation during the hyperinsulinemic clamp, 627 have a known chromosomal location in the data bases (see the supplemental table). No gene was found on chromosome Y due to the fact that women participated in the study and the procedure to select the regulated genes excluded those that did not give a signal in the six experiments. Analysis of the chromosomal location revealed that the insulin-regulated genes are widely distributed among the chromosomes and the number of genes on each chromosome is grossly proportional to the length of the chromosome (e.g. 56 genes located on chromosome 1 and 10 on chromosome 21). It could be noted that 6 regions (5q31, 6p21.3, 12q24, 16p13.3, 17p13.1, and 22q13.2) are characterized by the co-localization of five or more regulated genes. None of these loci has been reported to be linked with insulin resistance or type 2 diabetes in genetic studies. However, four of these loci (5q31, 6p21.3, 12q24, 17p13.1) have been linked to physical performance and health-related fitness phenotypes (16).

**Functional Classification of the Regulated Genes**—Gene ontology annotations, SOURCE and PubMed (www.ncbi.nlm.nih.gov) were used to assign the regulated genes into functional categories (see the supplemental table for a complete list of the genes and their classification). Of the 762 genes found to be

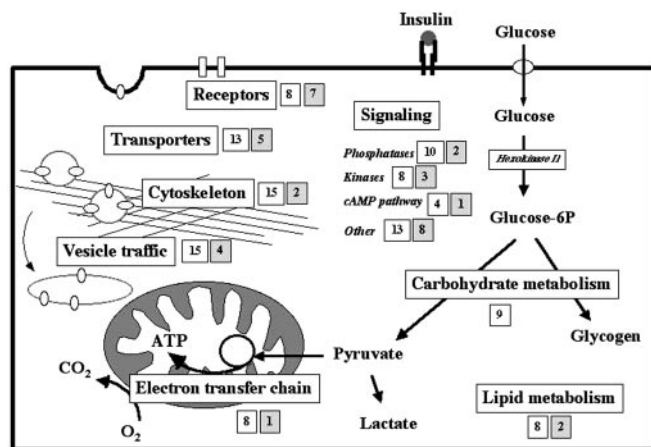


FIG. 2. Schematic representations of the effects of insulin on gene expression in skeletal muscle. The main functional categories of the regulated mRNAs are shown. The number of up-regulated genes is indicated with white boxes, and the number of down-regulated genes is indicated with gray boxes.

regulated by insulin, 353 (163 up and 190 down) corresponded to ESTs or hypothetical proteins. Among them, 16.5% displayed a more than 2.5-fold increased expression during the clamp and 20.2% displayed a more than 2-fold decreased, suggesting that a number of new insulin-regulated genes will probably emerge from the completion of the genome annotation. Of the 409 genes with known functions, the majority corresponded to genes coding for proteins involved either in the transcriptional and translational regulation (29%), intermediary and energy metabolisms (14%), or in intracellular signaling (12%). Other functional categories consisted of genes involved in cytoskeleton and vesicle traffic (9%), receptors, carriers, and transporters (8%), components of the ubiquitin/proteasome pathways (7%), and the immune response (5.5%). In addition, 63 genes (42 up, 21 down) did not correspond to one of these major categories. Figs. 2 and 3 are schematic representations of these main metabolic and functional pathways with indications of the number of up- and down-regulated genes. The different functional groups are discussed in more details below.

**Transcriptional and Translational Regulation**—One of the most remarkable biological effects of insulin is its profound impact on the turnover of cellular proteins mainly through an increase of gene transcription and a stimulation of mRNA translation (2). It is, thus, not surprising that the majority of the genes found to be regulated during an acute insulin infusion codes for transcription factors (such as basic transcription factor 3, general transcription factor 2A, polymerase II transcription cofactor 4) and for proteins involved in RNA transport, processing, and translation (splicing factors 1 and 10, karyopherins 1 and 3, eukaryotic translation initiation factors 2 and 3, eukaryotic translation elongation factor 1). In addition, the mRNA expression of 14 ribosomal proteins, including 4 of the mitochondria, is markedly induced during the hyperinsulinemic clamp. Insulin also affects the expression of 20 genes coding for chaperones, heat-shock proteins, or enzymes involved in post-translational modifications, hence contributing to the complete process of protein synthesis that generally includes proper protein folding and a number of post-translational modifications.

**Intermediary and Energy Metabolism**—In skeletal muscle, insulin increases the uptake of glucose, fatty acids, and amino acids and directs their metabolic fates into specific pathways. The storage of glucose into glycogen and fatty acid into triglycerides is enhanced by insulin, whereas amino acids are mostly

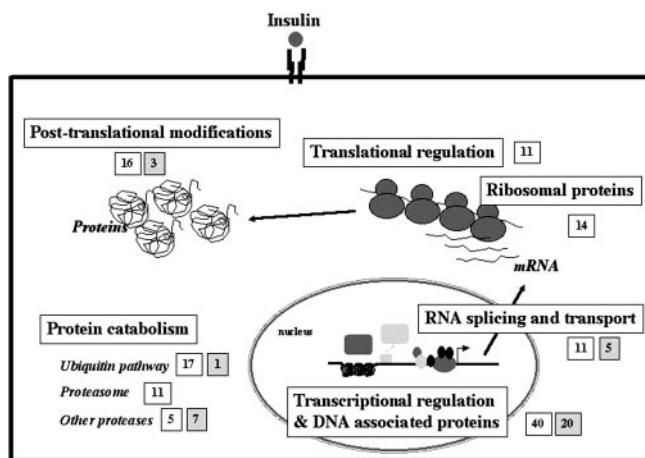


FIG. 3. Schematic representations of the effects of insulin on gene expression in skeletal muscle. The main functional categories of the regulated mRNAs are shown. The number of up-regulated genes is indicated with white boxes, and the number of down-regulated genes is indicated with gray boxes.

used for protein synthesis. Insulin also increases energy metabolism and ATP synthesis mainly by stimulating glucose oxidation, whereas fatty acid oxidation is almost completely inhibited (17). The observed changes in the gene expression pattern during the hyperinsulinemic clamp are in agreement with these metabolic effects.

Hexokinase II catalyzes the phosphorylation of glucose into glucose 6-phosphate after its entry into the muscle cells. Changes in the level and the activity of this key enzyme of glucose metabolism would markedly affect both the glycolytic and the glycogenic fluxes. Up-regulation of hexokinase II expression by insulin has been previously reported in human muscle (3, 6). In addition to hexokinase II, the increased expression of glycogenin and the catalytic subunit of protein phosphatase 1 (PPP1G) is in agreement with the stimulation by insulin of glycogen synthesis. Glycogenin is a self-glucosylating protein involved in the initiation of glycogen synthesis (18), and protein phosphatase 1G is one of the regulatory enzymes controlling glycogen synthase activity (19).

As for glucose, insulin increases fatty acid uptake and triglyceride storage in skeletal muscle (20). It has been recently demonstrated that part of the mechanism of this effect involved the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane in muscle cells (21). Both the microarray and the quantitative RT-PCR data provide evidence that insulin increases FAT/CD36 and fatty acid CoA ligase (FACL2) expression in human skeletal muscle. In addition, the mRNA expression of the adipose differentiation-related protein (ADRP, also called adipophilin) is increased during the hyperinsulinemic clamp. ADRP is a lipid droplet-associated protein involved in the package of neutral lipids in most mammalian cells (22). All these effects may, therefore, contribute to higher rates of entry, esterification, and storage of long chain fatty acids in muscle cells in response to insulin stimulation.

Insulin increases oxygen consumption and ATP synthesis in most cells. The generation of ATP and the coupling between oxidation and phosphorylation mostly rely on the activity of the electron transport chain in the inner mitochondrial membrane. We found that the mRNA levels of seven proteins from the different enzymatic complexes of the electron transport chain were increased by insulin. Interestingly, it was recently reported that the expression of a number of these proteins is reduced in a streptozotocin diabetic rats and that the treatment with insulin leads to correction of this defect (8). All these data thus support a direct and an important role of insulin in

the control of the mitochondrial electron transport chain at the transcriptional level.

**Intracellular Signaling**—Insulin binding to its membrane receptor triggers the activation of inter-related signaling cascades mostly through protein-protein interactions and phosphorylation/dephosphorylation mechanisms (1, 23). However, little is known regarding the transcriptional regulation of the key proteins of this signaling network in response to insulin. We found previously that the mRNA levels of the p85 $\alpha$  regulatory subunit of phosphatidylinositol 3-kinase is increased during a hyperinsulinemic clamp in healthy volunteers (5). Using cDNA microarray, we confirmed this observation, and more importantly, we identified at least 49 other insulin-target genes coding for proteins potentially involved in signaling. Not surprising with respect to the important role of protein phosphatases both in insulin action and in insulin signaling regulation (24), 12 of these 49 genes code for phosphatases. In addition, insulin induces the mRNAs of 2 phosphodiesterases (PDE4D and PDE7A) and of the protein kinase A anchor protein 6 (AKAP6), that may participate in the counter-regulatory effect of insulin on the cAMP pathway. This well described anti-cAMP effect of insulin is corroborated by the decreased expression of CREBBP mRNA, a cofactor that participates in the transcriptional regulation of the cAMP-responsive genes (25). Importantly, we also found that insulin strongly increases the mRNA level of CAP, the CBL-associated protein (SH3D5). The adaptor protein CAP is involved in the interaction of CBL with insulin receptor, and it has been demonstrated that the CAP-CBL complex dissociates from the insulin receptor and moves to lipid raft upon insulin stimulation, generating a pathway that is crucial in the regulation of glucose transport (26).

**Cytoskeleton and Vesicle Traffic**—The mRNA levels of 19 genes encoding for proteins involved in the vesicle traffic and 17 genes for cytoskeleton proteins were changed by the insulin treatment. In muscle cells in culture, insulin induces cytoskeleton reorganization associated with membrane ruffling and localized accumulation of pinocytotic/endocytotic vesicles (27). It is also well described that one of the major actions of insulin is to promote glucose uptake in skeletal muscle through the translocation of the Glut4 facilitative glucose transporter from an intracellular vesicle pool to the plasma membrane (28). This effect is dependent upon both microtubule- and actin-based cytoskeletal structures (29). The microtubule network and the cytoskeleton are also implicated in the proper subcellular localization of signaling molecule, such as the IRS-1-phosphatidylinositol 3-kinase complex (27). We found that several important components of the actin network are target genes of insulin *in vivo* in human skeletal muscle. The mRNA levels of actin-b, cofilin 2, vinculin, and supervillin among others are markedly increased after 3 h of insulin infusion. Furthermore, insulin also regulated the mRNA levels of genes involved in the microtubule network like the microtubule-based motors dynein and kinesin (PIN, KIF5B, KIF1C, KIFAP3). In addition to actin- and microtubule-based cytoskeletal structures, the translocation of Glut4 requires a number of proteins involved in the docking and fusion process of the vesicles with the plasma membrane. This includes members of the v-SNARE and t-SNARE complexes and small GTP-binding proteins involved in regulating membrane traffic. Among them, we found that insulin regulates the expression of the GTP binding Rab5 that has been recently shown to interact with the motor protein dynein and to participate in Glut4 internalization (30).

**Receptors, Carriers, and Transporters**—Insulin modulates the mRNA levels of 33 genes coding membrane receptors and cellular transporters and carriers. Among them, we found that insulin up-regulates Glut8 (also called GlutX1 or SLC2A8), a

recently discovered novel glucose transporter (31) that has been shown to be involved in insulin-stimulated glucose uptake in different cell models (31, 32). Previous studies demonstrate that the expression of Glut4 is induced in human skeletal muscle during an hyperinsulinemic clamp (4–6). Unfortunately, this could not be shown in the present study because the cDNA probe corresponding to Glut4 (SLC2A4) was not spotted on the microarrays. Among the membrane receptors found to be modulated by insulin during the hyperinsulinemic clamp, four belong to the cytokine receptor family, suggesting an effect of insulin on the expression of genes involved in the immune response.

**Immune Response and Cytokine Actions**—During the last decade, growing number of evidence was accumulated, demonstrating that cross-talks occur between insulin and cytokine signaling pathways (33). Moreover, diseases with insulin resistance (*e.g.* type 2 diabetes, obesity) are often associated with increased levels of inflammation markers (34). We found that hyperinsulinemia induces significant changes in the expression levels of 24 genes involved in cytokine action and immune response in human skeletal muscle, including interleukin 17D, transforming growth factor  $\beta$ 2, interleukin 13 receptor, and interferon  $\gamma$  receptor 2. Some of these genes may represent novel candidates for the link between insulin action and inflammation (34).

**Ubiquitin-Proteasome Pathway**—It is currently recognized that insulin promotes net skeletal muscle protein synthesis and that inhibition of protein breakdown plays an important role in this process (35). Rather unexpectedly, we found that insulin increases the expression level of 17 mRNAs of the ubiquitin-conjugating enzymes and 11 mRNAs of the proteasome components. This may suggest increased degradation of peculiar proteins after insulin infusion in human skeletal muscle. Indeed, it is well documented that controlled degradation of specific proteins by the ubiquitin-proteasome system plays an important role in the execution of various biological events, such as the regulation of different signal transduction pathways, including insulin action. Indeed, the proteasome has been directly involved in the selective degradation of the insulin receptor substrates IRS-1 and -2 (36) during prolonged insulin stimulation. Moreover, direct interaction of the glucose transporters Glut1 and Glut4 with members of the ubiquitin family has been evidenced and proposed to play a role in the control of glucose uptake (37).

The ubiquitin-proteasome system is also involved in the regulation of transcription (38). We found that insulin increases the mRNA of USP16, which participates in the deubiquitination of histones H2A and H2B (39), a process that has been correlated with transcriptionally active DNA. This is consistent with the general activation of gene transcription upon insulin stimulation. In addition, the ubiquitin-proteasome pathway has been implicated in the control of the amount of specific transcription factors such as nuclear factor  $\kappa$ B1, retinoid X receptors  $\alpha$ , peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$ , thyroid hormone receptor, or sterol regulatory element-binding proteins (38, 40–43). Most of these transcription factors have been directly implicated in or related to insulin action (44, 45). All together, these data and our observation of a marked impact of insulin on the expression of members of the ubiquitin-proteasome system strongly suggest that this pathway may play an important role in insulin action in human muscle.

**Summary and Conclusion**—The present data demonstrate that insulin infusion for 3 h during a hyperinsulinemic euglycemic clamp results in profound changes in the mRNA levels of about 800 genes in human skeletal muscle. About half of them are ESTs with still unknown functions. The others could be

classified into functional categories that can support most of the biological and metabolic effects of insulin. The microarray data were confirmed by measuring the mRNA levels of a subset of genes using real-time RT-PCR. Moreover, some of these genes, such as hexokinase II and p85 $\alpha$  phosphatidylinositol 3-kinase, have been previously reported to be regulated by insulin in separated studies. Therefore, our data markedly increased the list of the 120–150 target genes of insulin that was previously established, mostly on the basis of experiments with animal models. Among them, the ubiquitin-proteasome pathway emerged as an important component of insulin action in human muscle. A prominent issue now is to understand how insulin orchestrates the coordinated regulation of all these target genes. The mechanism of action of insulin on gene expression in skeletal muscle is still largely unknown. The identification of common insulin response elements in the promoter sequences of group of genes will help the discovery of the transcription factors linking the effect of insulin on multiple genes simultaneously. In addition, because accumulating data indicate that defects in basal and in insulin-regulated gene expression may be involved in the etiology of insulin resistance and type 2 diabetes mellitus (2, 6), certain of the 800 genes identified in the present work are potential novel candidates in pathologies with altered insulin responsiveness.

**Acknowledgments**—We thank C. Urbain, J. Peyrat, and V. Peloux for excellent technical assistance. We thank D. Langin for critical reading of the manuscript.

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