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ORIGINAL PAPER

The effect of insulin on expression of genes and biochemical pathways in human skeletal muscle

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Abstract To study the insulin effects on gene expression in skeletal muscle, muscle biopsies were obtained from 20 insulin sensitive individuals before and after euglycemic hyperinsulinemic clamps. Using microarray analysis, we identified 779 insulin-responsive genes. Particularly noteworthy were effects on 70 transcription factors, and an extensive influence on genes involved in both protein synthesis and degradation. The genetic program in skeletal muscle also included effects on signal transduction, vesicular traffic and cytoskeletal function, and fuel metabolic pathways. Unexpected observations were the pervasive effects of insulin on genes involved in interacting pathways for polyamine and S-adenoslymethionine metabolism and genes involved in muscle development. We further confirmed that four insulin-responsive genes, RRAD, IGFBP5, INSIG1, and NGFI-B (NR4A1), were significantly up-regulated by insulin in cultured L6 skeletal muscle cells. Interestingly, insulin caused an accumulation of NGFI-B (NR4A1) protein in the nucleus where it functions as a transcription factor, without translocation to the cytoplasm to promote apoptosis. The role of NGFI-B (NR4A1) as a new potential mediator of insulin action highlights the need for greater understanding of nuclear transcription factors in insulin action.

Keywords Insulin · Differential gene expression · Human skeletal muscle

Abbreviations

IRS	Intracellular insulin receptor substrate
LBM	Lean body mass
OGTTs	Oral glucose tolerance tests
DEPC water	Diethyl pyrocarbonated-treated water
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
RRAD	Ras-related associated with diabetes
INSIG1	Insulin induced gene 1
EGR3	Early growth response protein 3
IGFBP5	Insulin-like growth factor binding protein 5
NR1D1	Nuclear receptor subfamily 1, group D, member 1

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NR4A1 (NGFI-B)	Nuclear receptor subfamily 4, group A, member 1
NR4A2 (NURR1)	Nuclear receptor subfamily 4, group A, member 2
NRIP1	Nuclear receptor interacting protein 1
PPP2R4 (PR53)	Protein phosphatase 2A, regulatory subunit B
UQCRC1	Ubiquinol-cytochrome c reductase core protein 1
PPARD	Peroxisome proliferator-activated receptor-δ
NDUFV1	NADH-ubiquinone oxidoreductase flavoprotein 1
MYOG	Myogenin
MYF6	Myogenic factor 6
PI 3-kinase	Phosphoinositide-3-kinase
SREBF1	4′-6-Diamidino-2-phenylindole, sterol regulatory element binding transcription factor 1
MINOR	NR4A3
MEF2B	Myocyte enhancer factor 2B
PPARγ	Peroxisome proliferative activated receptor γ
HNF1	Transcription factor 1 hepatic
HNF2	Transcription factor 2 hepatic
THRA	Thyroid hormone receptor α
NR1D1, Rev-Erbα	Nuclear receptor subfamily 1, group D, member 1
NR1D2	Nuclear receptor subfamily 1, group D, member 2
MT1F	Metallothionein 1F (functional)
MT1H	Metallothionein 1H
MT1X	Metallothionein 1X
MT2A	Metallothionein 2A
EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1
ETF1	Eukaryotic translation termination factor 1
GC20	Translation factor sui1-homolog
MAN2C1	Mannosidase, alpha, class 2C, member 1
TGM5	Transglutaminase 5
ITPKC	Inositol 1,4,5-trisphosphate 3-kinase C
SORBS1	Sorbin and SH3 domain containing 1
SOCS7	Suppressor of cytokine signaling 7
PTEN	Phosphatase and tensin homolog
PRKAR1A	cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)
PPP1R3C	Protein phosphatase 1, regulatory (inhibitor) subunit 3C
PPP2R5A	Protein phosphatase 2, regulatory subunit B (B56), alpha isoform
ART3	ADP-ribosyltransferase 3
G3BP	Ras-GTPase-activating protein SH3-domain-binding protein
G3BP2	GTPase activating protein (SH3 domain) binding protein 2
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3
ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4
ARHGEF12	Rho guanine nucleotide exchange factor (GEF) 12
DNCL1	Dynein, cytoplasmic, light polypeptide 1
DCTN6	Dynactin 6, transcript variant 1
MAT2A	Methionine adenosyltransferase II, alpha
AHCYL1	S-adenosylhomocysteine hydrolase-like 1
ADM1	Adrenomedullin-1
SLC7A5	Solute carrier family 7, member 5
4′-6-Diamidino-2-phenylindole	
qRT-PCR	Real-time quantity reverse transcript-PCR
IGF	Insulin-like growth factor
TPM2	β-tropomyosin
SREBP	Sterol regulatory element binding protein
SCAP	Sterol regulatory element binding protein cleavage-activating protein
bFGF	Basic fibroblast growth factor
TPA	Tetradecanoyl phorbol acetate
FOXO1A	Forkhead box O1A
JUNB	Jun B proto-oncogene
IER2	Immediate early response 2
FOXG1A	Forkhead box G1A
FNTA	Farnesyltransferase alpha-subunit
FUT6	Fucosyltransferase 6
KYNU	Kynureninase
p85 alpha	PI-3-kinase regulatory subunit, polypeptide 1
CDC42EP3	CDC42 effector protein 3
ACTG1	Actin gamma 1
ACTR3	Actin-related protein 3
TUBB	β-tubulin
TBCC	Tubulin-specific chaperone C

Introduction

Insulin is a potent pleiotropic hormone that affects processes such as cellular growth, differentiation, apoptosis,

ion flux, energy expenditure, and carbohydrate, lipid, and protein metabolism. These diverse actions are initiated by specific binding to high-affinity receptors on the plasma membrane of target cells [1, 2], which then activate both a metabolic signaling pathway through PI-3 kinase and a mitogenic pathway through the ras/MAPK cascade. The cascade of the insulin signal into the cell has been extensively studied as it relates to early events in signal transduction. However, we lack an understanding of more distal events in insulin signaling that engage various effector systems, and of the integrated effects on gene expression that underlie the hormone's multiple actions. A comprehensive assessment of differential expression in response to insulin is now possible using cDNA microarray technology. This knowledge could enhance our understanding of insulin action and how responses are integrated to mediate the full spectrum of hormonal effects.

Skeletal muscle is the main site for insulin-dependent glucose disposal in humans [3, 4]. Insulin stimulates glucose uptake and glucose use in oxidative and storage pathways. Approximately 80% of insulin-responsive glucose uptake involves skeletal muscles and this tissue is the major site of glycogen storage, lipid oxidation, protein turnover, and thermogenesis. Insulin resistance involving skeletal muscle is also central to the pathogenesis of human diseases, including the Metabolic Syndrome and Type 2 Diabetes which cause a large and increasing burden on public health. The molecular mechanisms, by which insulin regulates muscle metabolism, and the underlying defects causing insulin resistance have not been fully elucidated.

In order to better understand the mechanisms of insulin action in skeletal muscle, we have used the hyperinsulinemic euglycemic clamp technique and obtained skeletal muscle biopsies under basal and insulin-stimulated conditions in insulin sensitive individuals. By employing a relatively large sample compared with studies published to date, our analyses could address some of these issues relevant to the study of gene expression in human tissues. We demonstrated that insulin regulates gene expression for a large number of transcription factors, metabolic pathways, and biocellular functions. Analyses of microarray data combined with studies in L6 muscle cells identified a key role for NGFI-B (NR4A1), linking insulin function in the skeletal muscle.

Materials and methods

Human subjects

Microarray studies were performed in 20 insulin-sensitive individuals (11 males/nine females; 13 European-Americans, six African-Americans and one Hispanic-American). Participants were consecutively identified on the basis of having a maximal insulin-stimulated glucose disposal rate (GDR) of >13.9 mg/kg lean body mass (LBM)/min during a hyperinsulinemic euglycemic clamp procedure; the mean GDR value in study subjects was 16.3 ± 1.8 mg/kg LBM/min. RT-PCR studies were performed for validation in separate group of six insulin-sensitive subjects (five females/one male; four European-Americans, two African-Americans). All subjects were normoglycemic and were not taking medications that would affect metabolism or glucose homeostasis. Clinical characteristics are shown in Table 1. These subjects are heterogeneous for the ethnic (European-Americans, African-Americans and Hispanic-American) and the BMI (20.2–35.5). Each volunteer had a normal physical examination, an unremarkable medical history, and was verified by blood chemistry examination to have normal hepatic, renal, and thyroid functions. All study volunteers provided written informed consent and the study was approved by the Institutional Review Board.

Euglycemic-hyperinsulinemic clamp procedure

Subjects were studied on a metabolic research ward where they remained active. The subjects were allowed to equilibrate for 2 days on a weight-maintenance diet (28–32 kcal/kg/day) consisting of 50% carbohydrate, 35% fat

Table 1 Clinical characteristics

	Age (years)	Body fat (%)	Fasting insulin (μU/ml)	Fasting glucose (mg/dl)	Body mass index (kg/m^2)	Glucose disposal rate (mg/kg/min)
Insulin-sensitive individuals used for microarray analysis (n = 20)						
Min-max	21–54	33.2–57.6	1.3–7.8	71–95	20.2–35.5	14–21.9
Mean	37.4	30.1	4.5	81	26.6	16.3
STD deviation	8.8	10.6	1.9	10	4.4	1.8
Insulin-sensitive individuals used for qRT-PCR validation study (n = 6)						
Min-max	27–50	18.6–49.2	1.7–8.9	82–94	22–32	15.1–23.2
Mean	37.5	31.3	4.3	86.7	25.4	18.7
STD deviation	9.6	11.3	2.7	5.1	4.2	3.6

and 15% protein. Standard 75 g oral glucose tolerance tests were performed to assure normal glucose tolerance. Peripheral insulin sensitivity was assessed using the hyperinsulinemic euglycemic glucose clamp technique as previously described [5, 6]. The in vivo glucose disposal rate was calculated on the basis of the glucose infusion rate corrected for changes in glucose pool size, assuming a distribution volume of 19% body weight and pool fraction of 0.65. During the clamp, regular insulin (Humulin, Eli Lily & Co., Indianapolis, IN) was administered at a constant rate of 200 mU/m^2/min through the brachial vein. This infusion rate produced a mean steady-state serum insulin level of 3,363 ± 64 pmol/l, sufficient to completely suppress hepatic glucose output [5]. During the steady state period, the serum glucose was maintained at 90 mg/dl with a coefficient of variation <4% by monitoring serum glucose levels at 5 min intervals and adjusting the infusion rate of a 20% dextrose solution. Glucose uptake rates were assessed over the last hour of the 3 h clamp study, and were normalized for lean body mass (excluding bone mass) determined by whole-body dual energy x-ray absorptiometry scanning.

Muscle biopsies

Percutaneous needles biopsies of the vastus lateralis were performed as previously described [7, 8]. In each subject, one biopsy was performed under basal conditions after an overnight fast prior to the clamp study, and a second was performed in the contralateral leg after 3 h of steady-state hyperinsulinemia. To perform the biopsy, a side-cutting needle (Poppler and Sons, New Hyde Park, NY) was inserted through a small incision in locally anesthetized skin (2% lidocaine), ~10–15 cm above the patella on the anterolateral thigh, and ~400 mg muscle tissue was retrieved. This tissue was then blotted on sterile cloth, placed immediately into liquid N_2, and then transferred to a –80°C freezer for storage. The time between the actual biopsy and snap-freezing of the tissue in liquid N_2 was 5–10 sec. Tissues obtained under these conditions have yielded high quality RNA for measurement of gene expression in previous studies [8–10].

RNA preparation

RNA extraction and cDNA preparation: RNA was isolated by phenol/chloroform extraction using the Ultraspec RNA isolation Kit from Biotecx Laboratories, Inc (Houston, TX). Human muscle biopsy specimens were homogenized in Ultraspec solution using a polytron followed by glass tube/Teflon pestle. RNA quality from each biopsy was examined by the A260/A280 absorbance ratio and by electrophoresis of RNA on agarose formaldehyde gels. SuperScript II Choice System (Invitrogen Corp., Carlsbad, CA) was used to convert total RNA to double-stranded cDNA. In vitro transcription was accomplished using double-stranded cDNA and the MEGAscript T7 System (Ambion, Inc., Houston, TX) in the presence of biotinylated UTP and CTP to generate labeled antisense RNA. cRNA was separated from unincorporated nucleotides using RNeasy spin columns from Qiagen, Inc. (Valencia, CA). The cRNA was then resupsended in diethyl pyrocarbonated-treated (DEPC) water for microarray study. For real-time RT-PCR assays, the RNA was treated with 1 unit of amplification grade deoxyribonuclease I (Promega) per microgram of RNA to remove genomic DNA according to the manufacturer's instructions before RT performance. A negative control containing all of the RT reagents in the absence of RT enzyme (no RT control) was also routinely performed.

Microarray analyses

To study insulin's ability to regulate gene expression, we carried out microarray analysis using the Affymetrix Hu95A chip of human skeletal muscle biopsies. Biotinylated cRNA from paired basal and insulin-stimulated muscle biopsies were hybridized to the chips according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA). The labeled cRNA was fragmented in fragmentation buffer (200 mM Tris–acetate (pH 8.1), 50 mM KOAc, 150 mM MgOAc) and hybridized to the microarrays in 200 ml of hybridization solution containing 10 mg labeled target in Mes buffer (0.1 M Mes (pH 6.7), 1 M NaCl, 0.01% Triton X-100) and 0.1 mg/ml herring sperm DNA. Arrays were placed on a rotisserie and rotated at 60 rpm for 16 h at 45°C. Following hybridization, the arrays were washed with 63 SSPE-T (0.9 M NaCl, 60 mM NaH_2PO_4 (pH 7.6), 6 mM EDTA, 0.005% Triton X-100) at 22°C on a fluidics station (Affymetrix, Inc., Santa Clara, CA) for 10 × 2 wash cycles, and subsequently with 0.1 M Mes at 45°C for 30 min. The arrays were then stained with a streptavidin–phycoerythrin conjugate (Molecular Probes), followed by two sets of 10 wash cycles. To enhance the signals, the arrays were further stained with ant-streptavidin antibody for 30 min followed by a 15 min staining with a streptavidin–phycoerythrin conjugate (Molecular Probes). After 10 × 2 additional wash cycles, the arrays were scanned at a resolution of 3 mm, using a specifically design confocal scanner (Affymetrix).

Cell culture and differentiation

L6 muscle cells, derived from rat thigh muscle tissue, were originally obtained from ATCC (Manassas, VA) and cultured as previously described [11]. The muscle myoblasts

were maintained in medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5 g/l L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 100 units/ml penicillin, and 100 mg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. At 60% confluence, all of the cells were then incubated for an additional 24 h in serum-free medium and then treated with either no additions as a control, or with 5 nM insulin or 10 nM insulin for 6 h or 16 h.

Quality assessment, management, and statistical analysis of microarray data

Biotinylated cRNA from basal and insulin-stimulated muscle biopsies were hybridized to the Affymetrix Hu95A oligonucleotide arrays according to the manufacturer's instructions (Affymetrix, Inc., Santa Clara, CA). We used Affymetrix annotation (Human Genome U95Av2 Array). We applied a number of conventional and novel statistical techniques to the data as previously reported [12–15]. We used the High Dimension Biology Statistical Analysis (HDBStat!) software for details see http://www.ssg.uab.edu/hdbstat/. A Welch-corrected version of Student's *t* test (two tailed; allowing for unequal variances) was applied. False discovery rates were calculated as described [16]. We used the standardized mean difference (δ) as both a measure of effect size and to indicate whether a gene was up-regulated or down-regulated in the insulin stimulated group: positive numbers for up-regulated and negative numbers for down-regulated. This standardized mean difference is defined as the difference between two means divided by the pooled within group standard deviation (SD). It is calculated as $\frac{\text{mean}_1 - \text{mean}_2}{\sqrt{(\text{SD}_1^2 + \text{SD}_2^2)/2}}$ where mean_i and SD_i are the mean and SD for group i, for $i = 1, 2$ [17]. This is a well established method of effect size. Detailed description for the formula and conception are available at http://en.wikipedia.org/wiki/Effect_size. We initially tested for differentially-expressed genes without filtering because there is ambiguity in the field as whether the process of data filtering enhances the veracity of results [18, 19]. Moreover, gene filtering seems to be less valuable as sample size increases [20]. Nevertheless, we subsequently conducted filtering based on the MAS5.0 present/absent calls and indicated in the tables for each gene whether its mRNA expression would be classified as ''expressed above background (present)''. The present/absent calls for each chip were generated using the MAS5.0 algorithm implemented in the *affy* package in Bioconductor with default settings (http://www.bioconductor.org/). The present or absent call for each gene was determined using a 25% fraction present rule, which deems a gene as present when there are at least 25% of chips in one group are called at present by MAS5.0 [20]. We include results for both filtered and unfiltered genes leaving the reader the choice as to considering or not those genes that would have been filtered.

Quantitative reverse transcription-polymerase chain reaction analysis (qRT-PCR)

For quantitative PCR, reverse transcribed were quantified by real time PCR on a MX3000 apparatus (Stratagene, La Jolla, CA) as previously described [21], using specific oligonucleotide primers indicated as follow: for human β-actin, 5′-AGCATTGCTTTCGTGTAAAT-3′ (5′ primer) and 5′-AGACCAAAAGCCTTCATACA-3′ (3′ primer); for human GAPD, 5′-GAAGGTGAAGGTCGGAGTC-3′ (5′ primer) and 5′-GAAGATGGTGATGGGATTTC-3′ (3′ primer); for human 18S, 5′-CGGCTACCACATCCAAGG AA-3′ (5′ primer) and 5′-GCTGGAATTACCGCGGCT-3′ (3′ primer); for human RRAD, 5′-TGTACTCAGTG ACGGACAAG-3′ (5′ primer) and 5′-CGCACGACACC TTCAAACAG-3′ (3′ primer); for human INSIG1, 5′-CCT TTGGTGGACATTTGATCGT-3′ (5′ primer) and 5′-GCG TAGCTAGAAAAGCTATGGTGAT-3′ (3′ primer); for human EGR3, 5′-CCATGATTCCTGACTACAACCTC-3′ (5′ primer) and 5′-GTGGATCTGCTTGTCTTTGAATG-3′ (3′ primer); for human IGFBP5, 5′-ATGGATTTGAGAGG AAAGAGAGG-3′ (5′ primer) and 5′-AGCACCCTCCT AAGGTTACTCAC-3′ (3′ primer); for human NR1D1, 5′-GGATTTGAGACAGGAACAGA-3′ (5′ primer) and 5′-ACAGAATCGAACTCTGCACT-3′ (3′ primer); for human NR4A1, 5′-GATAGATGCCCTGTATCCAA-3′ (5′ primer) and 5′-GGTGTCAAACTCTCCTGTGT-3′ (3′ primer); for human NR4A2, 5′-CCAAGAGAGTGGAAG AACTG-3′ (5′ primer) and 5′-AGGTAGAAAATGCGC TGTAG-3′ (3′ primer); for human NRIP1, 5′-TGAGCA CTCCACCTTTACTT-3′ (5′ primer) and 5′-GCTTTTTGT AAGGTCCATTG-3′ (3′ primer); for human PPP2R4, 5′-A TGGACGACAAGGCGTTCA-3′ (5′ primer) and 5′-TTA TAGGAAGTAGTCTGGGGTG-3′ (3′ primer); for human UQCRC1, 5′-TGTCTCGTGCAGACTTGACC-3′ (5′ primer) and 5′-GAAGCGGCATGGAGTAAGAG-3′ (3′ primer); for human PPARD, 5′-TCTTCCTCAACGACCAGG TT-3′ (5′ primer) and 5′-GCGTTGAACTTGACA GCA AA-3′ (3′ primer); for human NDUFV1, 5′-TTACGCCA TGATCCTCACAA-3′ (5′ primer) and 5′-ATAGCCAGA GCCACAAGCAT-3′ (3′ primer); for rat 18S, 5′-CACCA CCACCCACGGAATCG-3′ (5′ primer) and 5′-ACGGAA GGGCACCACCAGGA-3′ (3′ primer); for rat RRAD, 5′-T GATGGGCACATCTGTCTGT-3′ (5′ primer) and 5′-A AGACGGACCTGAAGCAGAA-3′ (3′ primer); for rat INSIG1, 5′-GATAGGGCTGCCAGAGTCAG-3′ (5′ primer) and 5′-GACGAGGTGATAGCCACCAT-3′ (3′ pri-

mer); for rat IGFBP5, 5′-CTGCTTTCTCTTGTAG AATCCTT-3′ (5′ primer) and 5′-GTTTGCCTCAACGA AAAGAGCT-3′ (3′ primer); for rat NGFI-B, 5′-GGAT CTTTTCCGCCCACTTC-3′ (5′ primer) and 5′-ACTTG CTCTCTGGCTCCCTG-3′ (3′ primer); for rat MYOG, 5′-T ACCTTCCTGTCCACCTTCA-3′ (5′ primer) and 5′-G GCCTCATTCACTTTCTTGA-3′ (3′ primer); and for rat MYF6, 5′-AGGCCTTGAAGCGTAGAACT-3′ (5′ primer) and 5′-GGAATGATCCGAAACACTTG-3′ (3′ primer). PCR amplification was performed in a volume of 20 µl containing 100 nM of each primer and the Brilliant Quantitative PCR Master Mix Kit as recommended by the manufacturer (Stratagene, La Jolla, CA). For each primer pair, a melting curve was used to identify a temperature at which only the ampicon, and not the primer dimers, accounted for SYBR green-bound fluorescence. mRNA levels were normalized to 18S rRNA and results were expressed as arbitrary mRNA units. Amplification products using SYBR Green detection were routinely checked by gel electrophoresis on a 2% agarose gel then visualized under UV light following staining with 0.05% ethidium bromide to confirm the size of the DNA fragment and that only one product was formed.

Immunofluorescence analyses

To visualize the actin cytoskeleton and NGFI-B (NR4A1) protein expression, L6 cells were grown on coverslips, then fixed with 4% paraformaldehyde and permeabilized at –20°C with 100% acetone. Fixed cells were incubated with NGFI-B (NR4A1) antibody at a 1:800 dilution overnight and subsequently with Alexa goat anti-rabbit secondary antibody for 1 h (1:100, Molecular Probe). The slides were washed three times with PBS and then incubated with 4′,6-Diamidino-2-phenylindole (DAPI) for 10 min. Coverslips were mounted in 0.2% *n*-propyl gallate in PBS:glycerol.

Immunoblot analysis

Briefly, cells in six-well plates were incubated as indicated, and then disrupted on ice with lysis buffer (Sigma, St. Louis, MO). Lysates were heated for 10 min at 100°C. 30 µg aliquots of total protein were resolved by SDS-PAGE to detect NGFI-B (NR4A1). Anti-NGFI-B (NR4A1) antibody (Santa Cruz Biotech) was used at a 1:200 dilution. Goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase was used as secondary antibody at a 1:10,000 dilution. Proteins were detected by the Enhanced Chemiluminescence's method according to the manufacturer's instruction. Immunoblots were exposed to x-ray film to produce bands, and intensity of immunofluorescent images was quantified by QuantityOne analysis software (BioRad).

Statistical analysis

The qRT-PCR results in basal and insulin stimulated muscle biopsies are expressed as means ± SE, and reproducibility was confirmed in three separate experiments for the cultured cell studies. Statistical comparisons were performed with a Wilcoxon signed rank test for paired values or Mann–Whitney *U*-test for unpaired samples. The threshold for significance was set at $P < 0.05$ (two-tailed).

Results

Identification of insulin-regulated genes

Statistical significance for differential expression in response to insulin was assessed using a two-sided paired *t* test comparing expression under basal conditions to that in insulin stimulated tissues as published previously [12]. While it is difficult to assign a level of statistical significance for rigorous data interpretation, we have chosen to identify genes that are both differentially expressed in response to insulin at the $P < 0.01$ level and a false discovery rate <15%. Using these criteria, we compared Affymetrix oligonucleotide arrays hybridized with cDNA prepared from basal and insulin-stimulated human skeletal muscle. Hyperinsulinemia under conditions of the euglycemic clamp led to significant up-regulation of 346 different genes and a down-regulation of 433 genes. The complete list of insulin-regulated genes are shown under supporting information on the journal web site (Supplemental Table 1). Of the 779 genes found to be regulated by insulin in insulin-sensitive individuals, 232 (69 up- and 123 down-regulated) were ESTs or hypothetical proteins. Of the 635 genes with known functions (Table 2), the majority corresponded to genes coding for proteins involved in the CHO, lipid, glucose metabolism (3.33%), protein turnover and processing (5.65%), post-translation protein modification (1.16%), regulation of gene expression (12.84%), vesicle trafficking (2.31%), polyamine metabolism and methyltransferease activity (1.41%), muscle fiber/contraction and myogenesis (2.95%), metallothioneins (0.51%), cell cycle/apoptosis/oncogenes/tumor suppression (6.93%), the cytoskeleton/microtubules (2.57%), mitochondria (1.80%), receptor functions (3.85%), channel/carrier activity (2.57%), signal transduction (7.70%), and cytokines/secreted proteins/extracellular proteins/immune response (8.34%). These categories included genes that are known to influence IGF-1 receptor and PI-3 kinase activity in signal transduction; methionine adenosyltransferase, ubiquitin-protein ligase, and co-chaperone activity in protein processing and turnover, and PPARγ and lipin 1 that could help determine intramyocellular lipid content and turnover.

Table 2 Functional classification of insulin-regulated genes in skeletal muscle from 20 insulin sensitive individuals

Functional classification	Down-regulation[a]	Up-regulation[a]
CHO, fat, lipid, glucose metabolism	17	9
Protein metabolism		
Protein synthesis and ribosomes	5	11
Protein folding	2	2
Protein catabolism and proteosomes	5	19
Post-translation protein modification	8	1
Gene expression regulation		
Transcription factors, regulation of transcription	32	39
Polymerase activity	5	4
RNA processing	2	18
Vesicle trafficking		
Small molecular weight GTP binding proteins	5	13
Polyamine metabolism and methyltransferease activity	3	8
Muscle fiber/contraction	18	5
Metallothioneins	0	4
Cell cycle/apoptosis/oncogenes/tumor suppressors	33	21
Cytoskeleton/microtubules	14	6
Mitochondria	2	12
Receptors	24	6
Channels/carriers	13	7
Signal transduction	31	29
Cytokines/secreted proteins/extracellular proteins	39	26
Miscellaneous	175	106
Total	433	346

[a] Significant expression in insulin-stimulated muscle compared with basal muscle based on $P < 0.01$, and false discovery rate $< 15\%$. The full list of all 779 differentially expressed genes is included as supplementary information available at the website

Confirmation studies using qRT-PCR

To assess validity of the microarray results using alternative technology, expression of 14 genes was also measured by quantitative RT-PCR in muscle biopsies obtained before (basal) and during hyperinsulinemic euglycemic clamps. These studies involved a separate set of 6 insulin-sensitive individuals not participating in the microarray studies. As shown in Table 3, the qRT-PCR measurements confirmed that expression of eight out of 10 genes, affected by insulin in the microarray studies, were also regulated by insulin in the qRT-PCR study, including β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPD), ras-related associated with diabetes (RRAD), insulin-like growth factor binding protein 5 (IGFBP5), NR4A1 (NGFI-B), nuclear receptor interacting protein 1 (NRIP1), early growth response 3 (EGR3), and insulin induced gene 1 (INSIG1). The remaining two out of 10 genes differentially expressed on microarrays, nuclear receptor subfamily 1, group D, member 1 (NR1D1, Rev-Erbα) and protein phosphatase 2A regulatory subunit B (PPP2R4, PR53) exhibited a change in the same direction when assessed by qRT-PCR, but these differences did not achieve statistical significance. mRNA levels of four genes, which were not affected by insulin in the microarray study, were also confirmed to be non-responsive to insulin by qRT-PCR, including nuclear receptor subfamily 4, group A, member 2 (NURR1), ubiquinol-cytochrome c reductase core protein 1 (UQCRC1), peroxisome proliferator-activated receptor-δ (PPARD), and NADH-ubiquinone oxidoreductase flavoprotein 1 (NDUFV1). Thus, microarray and qRT-PCR yielded similar results for 12 out of 14 genes in two separate groups of insulin-sensitive subjects.

Effects of insulin on key genes in cultured skeletal muscle cells

We further pursued validation of insulin's regulatory effects in human muscle by studying cultured L6 myotubes. Given the fact that insulin prominently affected genes involving muscle development, two key transcription factors for myogenesis were examined, MYOG and MYF6. In cell culture, proliferating L6 myoblasts were first fully differentiated into post-mitotic multinucleated myotubes, and then exposed to both 5 nM and 10 nM insulin for 16 h. As shown in Fig. 1, both insulin concentrations significantly increased expression of MYOG and concomitantly reduced expression of MYF6 mRNA when measured by qRT-PCR after 16 h in culture. The up-regulation of MYOG and down-regulation of MYF6 in L6 cultured

Table 3 Effect of insulin on gene expression in skeletal muscle assessed by quantitative RT-PCR

Gene name	Basal*	Insulin-stimulated*	*P* value*
Genes regulated by insulin in the microarray study			
β-actin	1.07E-04 ± 2.28E-05	1.22E-03 ± 3.89E-04†	0.031
GAPD	1.03E-04 ± 9.95E-05	2.28E-04 ± 2.21E-04†	0.016
RRAD	1.59E-06 ± 9.17E-07	1.41E-05 ± 7.27E-06†	0.047
IGFBP5	1.69E-07 ± 4.29E-08	3.29E-07 ± 7.04E-08†	0.031
NGFI-B	4.69E-10 ± 2.93E-10	6.09E-06 ± 6.08E-06†	0.016
NRIP1	7.09E-10 ± 2.17E-10	2.54E-09 ± 9.00E-10†	0.047
EGR3	1.45E-10 ± 9.25E-11	1.26E-09 ± 5.24E-10†	0.031
INSIG1	9.13E-07 ± 5.68E-07	2.50E-06 ± 1.02E-06†	0.046
NR1D1	1.57E-06 ± 1.3E-06	6.18E-07 ± 4.63E-07	0.219
PPP2R4	1.74E-04 ± 1.72E-04	4.77E-07 ± 1.89E-07	0.156
Genes not regulated by insulin in the microarray study			
NURR1	2.95E-10 ± 1.67E-10	5.18E-05 ± 5.18E-05	0.078
UQCRC1	2.19E-03 ± 1.25E-03	3.28E-03 ± 2.24E-03	0.410
PPARD	2.28E-14 ± 2.26E-14	3.02E-15 ± 2.89E-15	0.125
NDUFV1	7.19E-06 ± 4.09E-06	8.67E-06 ± 4.60E-06	0.527

* mRNA results for individual genes were normalized to the mRNA level of the constitutively expressed 18S RNA. The data are presented as mean ± standard error (SE)

P value comparing basal and insulin stimulated values were calculated by Wilcoxon signed rank test

† $P < 0.05$ compared with basal level

The measurements before (basal) and during hyperinsulinemic clamps involved a separate set of six insulin-sensitive subjects who did not participate in the microarray study. A total of 10 selected genes were significantly regulated by insulin on the microarray study and four genes were non-responsive to insulin

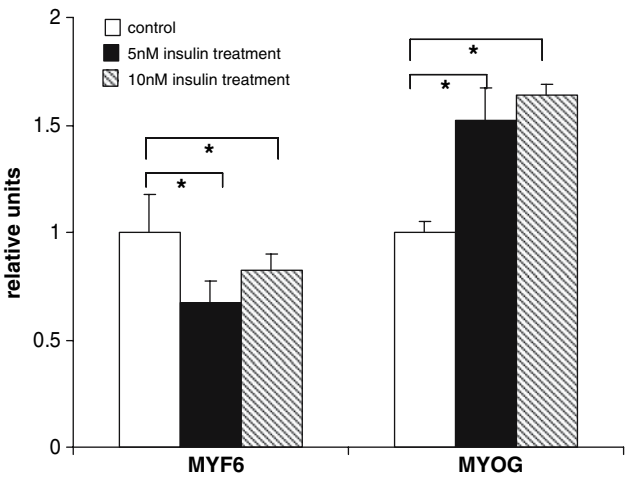

Fig. 1 Effects of insulin of MYOG and MYF6 mRNA levels in cultured L6 myotubes. Panel A: Fully-differentiated L6 myotubes were incubated for 24 h in serum-free medium and then treated in the absence (control) or presence of 5 nM or 10 nM insulin for 16 h. Levels of MYF6 and MYOG mRNA were measured by qRT-PCR, and normalized as number of target transcripts per 18S RNA transcript. Values are presented relative to the respective mRNA level in control samples, and represent the mean of three independent experiments. Significance was determined by Mann–Whitney *U*-test. *$P < 0.05$ versus control

myotubes recapitulated the effects of insulin in human muscle. Since these same inverse changes in MYF6 and MYOG gene expression are required for differentiation of myoblasts into myotubes [22, 23], this action of insulin could help maintain expression of the fully differentiated functional phenotype for skeletal muscle in vivo.

We explored this idea by testing for effects on additional genes, which were observed to be insulin-responsive in the microarray study. As shown in Fig. 2A–D, we confirmed that insulin significantly increased mRNA expression for RRAD, IGFBP5, NGFI-B (NR4A1) and INSIG1 in L6 myotubes at concentrations of 5 and 10 nM insulin after 16 h. Insulin-mediated increments in gene expression were observed as early as 1 h after insulin addition for RRAD, IGFBP5, and INSIG1, and at 6 h for NGFI-B (NR4A1) (data not shown).

Insulin regulation and subcellular localization of NGFI-B (NR4A1) protein expression

We determined the effect of insulin on expression of NGFI-B (or NR4A1) protein, in order to better gauge the physiologically significance of the NGFI-B (NR4A1) mRNA response. Cellular content of NGFI-B (NR4A1) protein was assessed by Western blot as shown in Fig. 3.

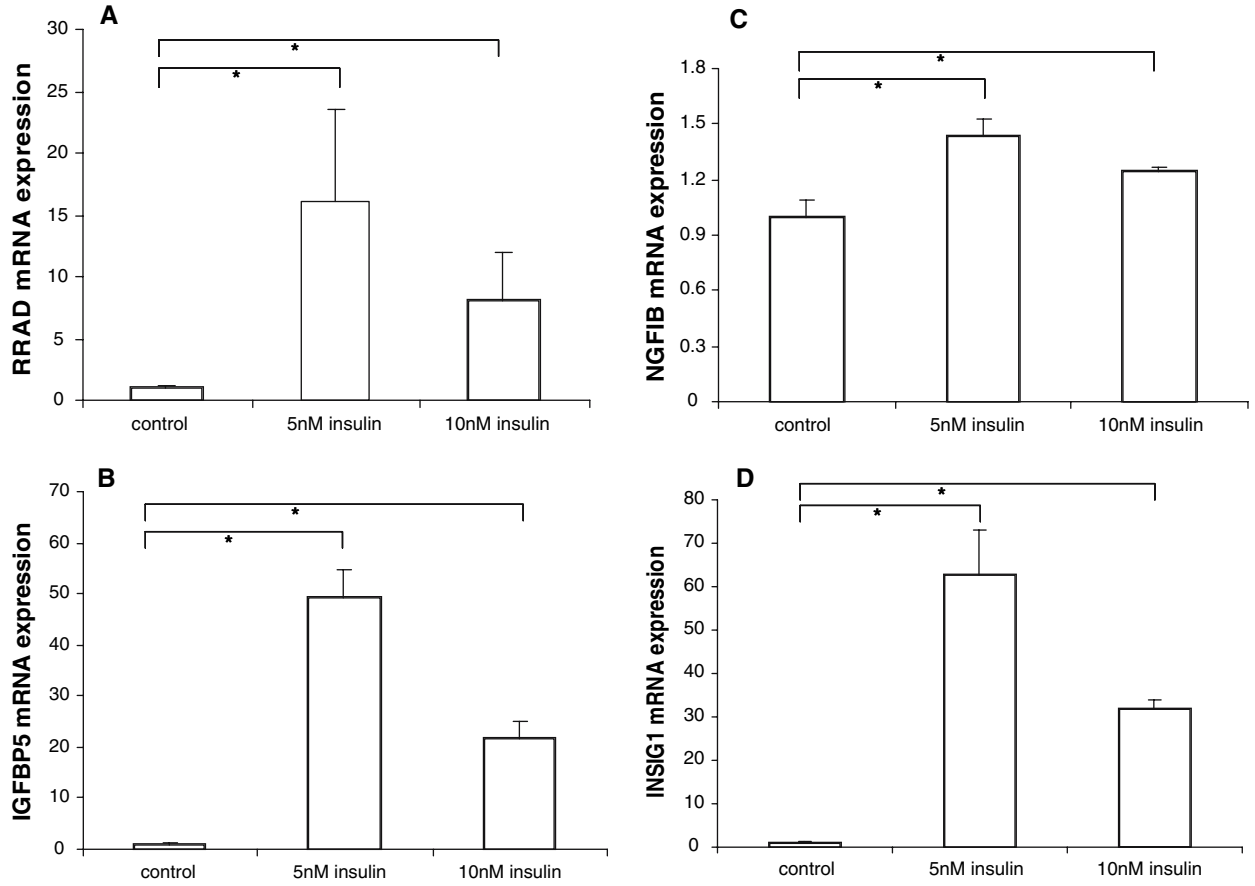

Fig. 2 Insulin regulation and signaling pathways for key genes in muscle development and function. Fully-differentiated L6 myotubes were incubated for 24 h in serum-free medium and then treated in the absence (control) or presence of 5 nM or 10 nM insulin for 16 h. mRNA levels of RRAD (**A**), IGFBP5 (**B**), NGFI-B (NR4A1) (**C**) and INSIG1 (**D**) were measured by qRT-PCR, and normalized as number of target transcripts per 18S RNA transcript. Values are presented relative to the respective mRNA level in control samples, and represent the mean of three independent experiments. Significance was determined by Mann–Whitney *U*-test. $*P < 0.05$ versus control

Treatment of cells with 5 nM or 10 nM insulin significantly increased NGFI-B (NR4A1) protein levels by 2–3 fold above basal. Furthermore, the subcellular localization of NGFI-B (NR4A1) protein, after its induction by insulin, was assessed by immunofluorescence microscopy. As shown in Fig. 4, 5 nM insulin for 16 h resulted in a modest increase in NGFI-B (NR4A1) protein (panels d, e, f); whereas, treatment with 10 nM insulin resulted in more marked increments in NGFI-B (NR4A1) at both 8 h (Panels g, h, i) and 16 h (Panels j, k, l). Importantly, Fig. 4 demonstrates that all newly synthesized NGFI-B (NR4A1) protein was completely localized within the nucleus, and was, therefore, in a position to act as a *trans* factor in regulating insulin's effects on gene transcription.

Discussion

We have studied insulin's effects on gene expression in human skeletal muscle using microarray technology. These studies involved muscle biopsies taken before and during euglycemic hyperinsulinemic clamp studies in a group of healthy insulin-sensitive subjects. Insulin had an extensive capability to regulate gene expression. Generally, these effects were not confined to genes encoding rate-limiting proteins for isolated pathways, but involved multiple components of interacting pathways. These effects on multiple genes constitute the basis for systematic and orchestrated regulation of cellular functions in response to insulin.

Gene expression and protein turnover

Insulin displayed a remarkable and extensive ability to impact the regulated processes controlling gene and protein expression. These effects were noted on all stages from transcription, to mRNA processing, translation, post-translational protein modification, and protein degradation. We observed that 70 transcription factors were differentially expressed in response to insulin; examples included

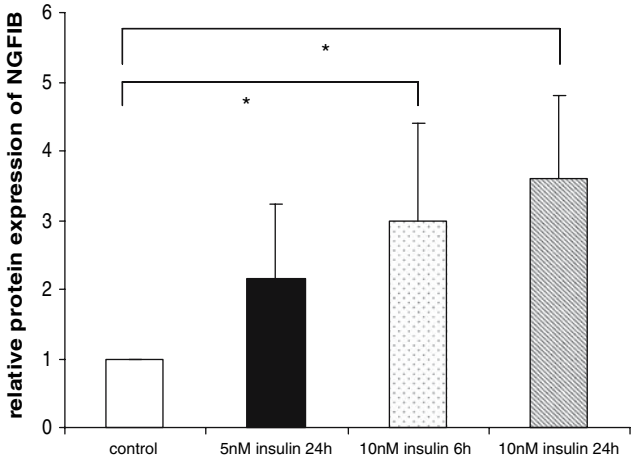

Fig. 3 Effects of insulin on NGFI-B (NR4A1) protein expression in L6 myotubes. Fully-differentiated L6 myotubes were incubated for 24 h in serum-free medium and then treated in the absence (basal) or presence of 5 nM insulin, or 10 nM insulin for 6 or 16 h. Cross-reacting NGFI-B (NR4A1) proteins on immunoblots were quantified by autoradiography and densitometry. The graph shows data in relative units with the mean value in basal cells set equal to 1.0. $^*P < 0.05$ versus control. The data are mean ± SE of three separate experiments

up-regulation of forkhead box O1A (FOXO1A), MYOG, sterol regulatory element binding transcription factor 1 (SREBF1), NR4A3 (MINOR), jun B proto-oncogene (JUNB) and Immediate early response 2 (IER2), and down-regulation of forkhead box G1A (FOXG1A), MYF6, myocyte enhancer factor 2B (MEF2B), peroxisome proliferative activated receptor γ (PPARγ), transcription factor 1 and 2 hepatic, (HNF1, HNF2), thyroid hormone receptor α (THRA), and NR1D1 and NR1D2. Many of the insulin-responsive transcription factors contain zinc fingers. It is interesting in this regard that insulin increased expression of multiple metallothionein genes (MT1F, MT1H, MT1X, and MT2A), which sequester zinc for zinc-fingered transcription factors. For this reason, zinc and metallothionein are known to localize into the nucleus during myoblast differentiation in response to IGF-1 [24, 25].

Insulin altered expression of genes involved in chromatin regulation and polymerase activity, as well as 22 genes involved in mRNA processing (19 up-regulated, three down-regulated). Insulin also regulated genes participating in translation of mRNA transcripts (e.g., EIF4A1, ETF1, and GC20), as well as in the processing and folding,

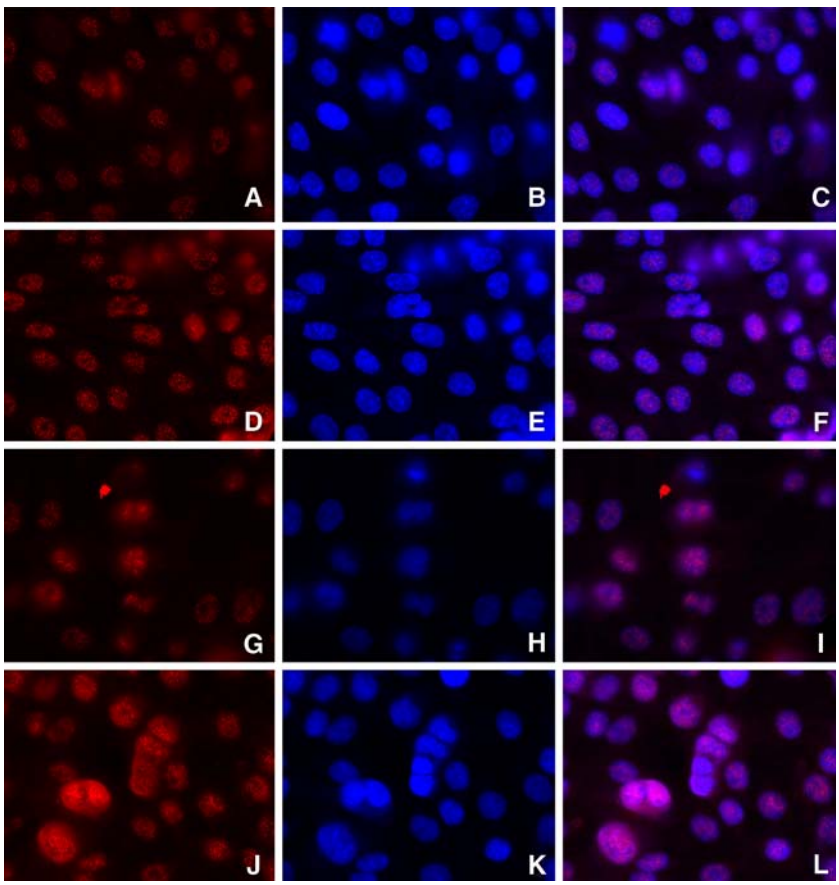

Fig. 4 Immunofluorescence microscopy of NFGI-B in L6 myotubes. Fully-differentiated L6 myotubes were incubated for 24 h in serum-free medium and then treated in the absence (basal) or presence of 5 nM insulin, or 10 nM insulin for 8 or 16 h as indicated. NGFI-B (NR4A1) is labeled in red (**a**, **d**, **g**, **j**), and nuclei are labeled in blue (**b**, **e**, **h**, **k**) by staining with DAPI. Purple color indicates co-localization of NGFI-B (NR4A1)and nuclei (**c**, **f**, **i**, **l**). Panels a, b, c: L6 skeletal muscle cells maintained in basal condition. Panels d, e, f: L6 skeletal muscle cells cultured with 5 nM insulin for 16 h. Panels g, h, i: L6 skeletal muscle cells cultured with 10 nM insulin for 6 h. Panels j, k, l: L6 skeletal muscle cells cultured with 10 nM insulin for 16 h. Magnification: ×1000

post-translational modification, and degradation of proteins. These insulin-regulated genes encode several heat shock proteins, other chaperonins (e.g., DnaJ family homologues), several stress response proteins (e.g., SERP1), and enzymes that mediate post-translational protein modifications with farnesyltransferase (FNTA), mannosidase (MAN2C1), fucosyltransferase (FUT6), kynureninase (KYNU), and transglutaminase (TGM5) activities. Finally, insulin is known to have pronounced effects on protein turnover in skeletal muscle, and, accordingly, influenced protein degradation as well as synthesis. For example, insulin regulated expression of 12 genes involved in ubiquitin-mediated protein degradation, including ubiquitin conjugating enzymes, protein ligases, and proteases that target proteins for proteosomes.

Signal transduction

Tyrosine phosphorylation of IRS-1 and subsequent docking and activation of PI 3-kinase is an early committed step in insulin activation of metabolic signal transduction pathways. We found that insulin down-regulated expression of IRS-1 and up-regulated PI-3-kinase regulatory subunit, polypeptide 1 (p85 alpha) (PIK3R1). These observations support previous findings in human muscle [26] showing two fold reduction in IRS-1 and an increase in the p85 alpha subunit of PI 3-kinase in response to insulin [27]. Other important insulin-regulated genes that participate in signal transduction include inositol 1,4,5-triphosphate 3-kinase C (ITPKC), sorbin and SH3 domain containing 1 (SORBS1), suppressor of cytokine signaling 7 (SOCS7), phosphatase and tensin homolog (PTEN), cAMP-dependent protein kinase regulatory subunit type 1 alpha (PRKAR1A), and subunits of protein phosphatases 1 (PPP1R3C) and 2 (PPP2R5A).

Vesicle traffic

Insulin stimulates glucose transport in muscle by recruiting intracellular vesicles containing GLUT4 glucose transporters to the cell membrane. Vesicle trafficking is also central to other metabolic processes in muscle, and examples of vesicle cargo include insulin-like growth factor II/mannose 6-phosphate receptor, transferrin, caveolin, and other channels and receptors. In the current study, a major insulin action in human skeletal muscle was increased expression of genes related to vesicle trafficking. Small molecular weight GTP-binding proteins up-regulated by insulin include ADP-ribosylation factors (ARF4 and ARF6), RAB1A, RAB14, RAB23B, RRAD, as well as related regulatory proteins ADP-ribosyltransferase (ART3), Ras-GTPase activating protein SH3 domain-binding proteins (G3BP and G3BP2), CDC42 effector protein 3 (CDC42EP3), and Rho guanine nucleotide exchange factors (ARHGEF4 and ARHGEF12). Vesicle traffic also requires an intact cytoskeleton, microtubules, and molecular motors. Insulin increased gene expression for actin gamma 1 (ACTG1), actin-related protein 3 (ACTR3), the essential structural protein β-tubulin (TUBB), tubulin-specific chaperone C (TBCC), and two dynein motor-related proteins (DNCL1 and DCTN6). Thus, insulin has the ability to augment expression of the cytoskeletal machinery for vesicle trafficking together with small molecular weight GTP binding proteins needed to direct this traffic.

Methyl transfer and polyamine metabolism

An unexpected effect of insulin was its ability to regulate genes participating in methyl transfer and polyamine metabolism. Insulin increased expression of the methionine adenosyltransferase (MAT2A) gene that catalyzes the biosynthesis of S-adenosylmethionine from methionine and ATP. S-adenosylmethionine is the methyl donor for at least 140 enzymes (KEGG encyclopedia). S-adenosylmethionine is converted to S-adenosylhomocysteine, which is then catabolized to adenosine and homocysteine by S-adenosylhomocysteine hydrolase-like 1 (AHCYL1), another gene up-regulated by insulin. This increase in AHCYL1 gene expression would predictably increase homocysteine production by muscle. In addition, S-adenosylmethionine is decarboxylated by adenosylmethionine decarboxylase 1 (ADM1), another gene up-regulated by insulin, and then serves as a propylamine group donor in the biosynthesis of the polyamines (i.e., putrescine, spermidine, and spermine). Polyamines are synthesized from arginine, which enters cells through the cationic amino acid transporter solute carrier family 7 member 5 (SLC7A5), a gene that was also up-regulated by insulin. Therefore, insulin action will result in increased availability of arginine and S-adenosylmethionine together with increased expression of rate-limiting enzyme, ADM1, required for polyamine biosynthesis.

Validation studies

Insulin-mediated induction of RRAD, IGFBP5, INSIG1, and NGFI-B (NR4A1) was further confirmed by qRT-PCR both in muscle biopsies from a second sample of human subjects and in differentiated L6 cell myotubes. RRAD expression has previously been shown to be enhanced in some individuals with Type 2 diabetes, and muscle RRAD protein content has been linked to body composition and the regulation of energy expenditure [28–30]. The GDP-bound form of RRAD preferentially interacts with calmodulin and calmodulin-dependent protein kinase II [29]

and with β-tropomyosin (TPM2) as a putative facilitator of cytoskeletal organization during the transition from myoblast to myocyte [31]. IGFBP5 plays a role in myoblast differentiation in response to insulin or insulin-like growth factor (IGF) stimulation [24, 25, 32]. IGFBP5 is expressed in muscle cells in the developing embryo and during the terminal differentiation of several myogenic cell lines [33]. INSIG-1 blocks processing of sterol regulatory element-binding proteins (SREBPs) and is an essential regulator of sterol metabolism [34, 35]. It is believed that INSIG1 expression increases concomitant with SREBP-1 activation as a counterbalance to prevent excessive SREBP-1 action [36]. Furthermore, INSIG-1 expression restricts lipogenesis in mature adipocytes and blocks differentiation in preadipocytes [37, 38]. In the context of the current study, these data suggest that INSIG1 could play a role in the regulation of lipid metabolism by insulin in human skeletal muscle.

A novel finding of this study is that NGFI-B (NR4A1) is an insulin responsive gene; the difference in expression in basal versus insulin-stimulated muscle was highly significant in the microarray study (P = 1.61E-07, FDR = 4.06E-04). We also observed that insulin directly increased NGFI-B (NR4A1) expression in L6 myotubes, and has previously been shown to be induced by serum growth factors and membrane depolarization as an immediate-early response gene in neuronal cells [39, 40]. In the nucleus, it functions as a transcription factor regulating gene expression, but, when targeted to the mitochondria, NGFI-B (NR4A1) takes on a novel role as a mediator of apoptosis [41]. The current data indicate that insulin increases NGFI-B (NR4A1) protein, and that the NGFI-B (NR4A1) remains localized in the cell nucleus consistent with a role in mediating insulin's cellular actions rather than promoting apoptosis. In a recent report, reduced expression of NGFI-B (NR4A1) mRNA in C2C12 cells, produced by stable expression of NGFI-B-specific siRNA, affected expression of both muscle development genes (e.g., MYOG, troponin 1 and 2) and genes involved in fuel metabolism including GLUT4, CD36, UCP3, AMP-activated protein kinase-γ3, adiponectin receptor 2, and caveolin-3 [42]. These observations led these authors [42] to conclude that NGFI-B (NR4A1) was involved in energy expenditure and adaptive thermogenesis. The exact role of NGFI-B (NR4A1) in these pathways and in muscle development will require further study. The observations that NGFI-B (NR4A1) is insulin-responsive and induces genes involved in fuel metabolism suggests that NGFI-B (NR4A1), and other insulin-responsive members of the NR4A family (e.g., NR4A3 or MINOR), could be attractive potential targets for research in insulin resistance, the Metabolic Syndrome, and Type 2 Diabetes.

Most reports regarding insulin's regulatory effects on gene expression in muscle have involved only single genes or small numbers of genes with a limited focus [10, 28, 43–47]. One previous study by Rome et al. [48] employed microarray to analyze changes in mRNA levels during euglycemic hyperinsulinemic clamps in skeletal muscle from healthy subjects. Surprisingly, our results showed limited overlap with the insulin-regulated genes identified in this previous study. There were 263 genes that were differentially expressed on the chips used by Rome et al. and also present on the chips used in the current study. Out of these 263 genes, we observed that only 41 (16%) were significantly regulated by insulin in the current study. The discrepancy in these two studies could due to: (i) utilization of different types of chips (cDNA element microarrays versus Affymetrix oligonucleotides); (ii) metabolic differences in the research volunteers since the current study included only insulin sensitive subjects while Rome et al. did not measure insulin sensitivity and would have recruited subjects over a broader range of insulin sensitivity/resistance; (iii) use of different criteria to determine significance levels; and (iv) most importantly, the different sample sizes involved in the studies. Rome et al. studied six individuals compared with 20 in the current study. Pavlidis et al. [49] assessed replicability of microarray results for detecting differential expression, and indicated that, for most studies, 10–15 replicates are necessary to yield stable results [49], in general agreement with studies by Hwang et al. and Pan et al. [50, 51]. These considerations highlight the need for adequate power in microarray studies.

In interpreting the current data, it is important to keep several caveats in mind. The clamp technique has allowed us to assess the effects of steady state hyperinsulinemia over 3 h, compared with gene expression under fasting conditions, while glucose is maintained at euglycemia. The extensive changes in transcription factor gene expression may not yet have resulted in altered expression of genes regulated by these factors, which could occur at later time points. Some effects on gene expression may be due to other factors not directly related to insulin since during the clamp there is increased flux and metabolism of glucose, a decline in circulating free fatty acids, and an increase in norepinephrine concentrations. Also, the skeletal muscle biopsies will contain blood vessels, fibroblasts, and adipocytes as well as muscle cells. Thus, while muscle cells are predominant, other cell types could contribute to gene expression assessed on the microarrays. Even so, the study of a relatively large sample population (n = 20) of individuals, all classified as insulin sensitive, allowed us to identify insulin-regulated genes using rigorous statistical analysis. Furthermore, insulin responsiveness of genes was confirmed using qRT-PCR in a second group of subjects and by studying insulin effects in cultured L6 myotubes.

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