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Growth hormone regulation of metabolic gene expression in muscle: a microarray study in hypopituitary men

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Sjögren K, Leung K-C, Kaplan W, Gardiner-Garden M, Gibney J, Ho KK. Growth hormone regulation of metabolic gene expression in muscle: a microarray study in hypopituitary men. *Am J Physiol Endocrinol Metab* 293: E364–E371, 2007. First published April 24, 2007; doi:10.1152/ajpendo.00054.2007.— Muscle is a target of growth hormone (GH) action and a major contributor to whole body metabolism. Little is known about how GH regulates metabolic processes in muscle or the extent to which muscle contributes to changes in whole body substrate metabolism during GH treatment. To identify GH-responsive genes that regulate substrate metabolism in muscle, we studied six hypopituitary men who underwent whole body metabolic measurement and skeletal muscle biopsies before and after 2 wk of GH treatment (0.5 mg/day). Transcript profiles of four subjects were analyzed using Affymetrix GeneChips. Serum insulin-like growth factor I (IGF-I) and procollagens I and III were measured by RIA. GH increased serum IGF-I and procollagens I and III, enhanced whole body lipid oxidation, reduced carbohydrate oxidation, and stimulated protein synthesis. It induced gene expression of IGF-I and collagens in muscle. GH reduced expression of several enzymes regulating lipid oxidation and energy production. It reduced calpain 3, increased ribosomal protein L38 expression, and displayed mixed effects on genes encoding myofibrillar proteins. It increased expression of circadian gene CLOCK, and reduced that of PERIOD. In summary, GH exerted concordant effects on muscle expression and blood levels of IGF-I and collagens. It induced changes in genes regulating protein metabolism in parallel with a whole body anabolic effect. The discordance between muscle gene expression profiles and metabolic responses suggests that muscle is unlikely to contribute to GH-induced stimulation of whole body energy and lipid metabolism. GH may regulate circadian function in skeletal muscle by modulating circadian gene expression with possible metabolic consequences.

substrate metabolism; metabolic genes; transcript profiling

GROWTH HORMONE (GH) is an important regulator of substrate metabolism and body composition (24, 26). GH deficiency in adults causes metabolic abnormalities that result in a phenotype of increased adiposity, diminished lean tissue, and impaired physical function, which are reversed with replacement therapy.

Energy and substrate metabolism of the body is almost entirely determined by the lean body mass. Muscle is an important contributor to whole body metabolism, as it is a major component of the lean body mass. Muscle is a target tissue of GH action, as reflected by the development of reduced muscle mass and strength in GH deficiency. GH treatment of hypopituitary adults increases whole body resting energy expenditure and lipid oxidation, reduces carbohydrate utilization, and stimulates anabolism (17, 39). Regional amino acid balance studies show that GH acutely stimulates protein synthesis in forearm muscle, providing strong evidence that muscle contributes to whole body measures of anabolism in response to GH (14, 15).

Muscle is also a major site of fatty acid metabolism. It is widely assumed that the increase in whole body fat oxidation in hypopituitary subjects during GH treatment reflects the stimulation of β-oxidation by GH in muscle. However, recent studies in rodents have reported that GH reduces the expression of fat oxidation genes in skeletal muscle but increases the expression of these genes in liver (12, 36, 37). Although metabolic studies were not performed, these findings question assumptions about the metabolic role of GH in muscle. There is a paucity of information on the regulation of metabolic genes by GH in skeletal muscle in humans and the contribution of muscle to whole body substrate metabolism.

The aim of this study was to identify GH-responsive genes that regulate the metabolic process in skeletal muscle. We tested the hypothesis that changes in whole body substrate metabolism might reflect the transcriptional effect of GH on genes regulating the metabolic process in muscle. To this end, we undertook a combined whole body metabolic and muscle gene expression study in hypopituitary subjects replaced with GH. We used the microarray technology to quantify the expression of genes involved in fuel and protein metabolism in skeletal muscle biopsies obtained from hypopituitary men before and after GH therapy and related the gene expression profiles to changes in fuel oxidation and whole body protein turnover (17, 22).

MATERIALS AND METHODS

Patients

Six hypopituitary men were recruited from the Endocrine Outpatient Clinic at St. Vincent's Hospital, Sydney, Australia. This study was part of a larger study investigating the single and combined effects of GH and testosterone on substrate, protein, and sodium homeostasis, some results of which have been published (17, 22).

GH deficiency was confirmed by a peak GH response to insulin-induced hypoglycemia of <3 ng/ml, with duration of hypopituitarism for at least 1 yr. All subjects received stable hormone replacement for thyroid and adrenal deficiencies throughout the study. The Research

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Ethics Committee of St. Vincent's Hospital, Sydney, approved the studies. Written informed consent was obtained from all subjects.

All subjects underwent a 6-wk run-in period when GH and testosterone were withdrawn. During this time and throughout the studies, they were instructed to follow their usual diet and habitual activity. Subjects were studied before and at the end of a 2-wk treatment with GH (0.5 mg/day Humatrope; Lilly Australia, Sydney, NSW, Australia) in an open-labeled study. One subject did not comply with the protocol, stopping GH only 2 wk before the baseline study. The metabolic and microarray from this subject were excluded. The mean age of the remaining five subjects was 53 yr (range 34–76).

Study Protocol

All subjects presented after an overnight fast at 8 AM to the Clinical Research Facility, the Garvan Institute of Medical Research for the following procedures: *1*) blood samples for measurement of insulin-like growth factor I (IGF-I), procollagens I (PINP), and III (PIIINP); *2*) indirect calorimetry for quantification of resting energy expenditure and fat oxidation; *3*) a whole body leucine turnover study; and *4*) a muscle biopsy of the vastus lateralis. The entire protocol lasted 4 h with blood samples taken at the start and the muscle biopsy undertaken on completion of the whole body leucine turnover study. O_2 consumption and CO_2 production rates were measured with an open circuit, ventilated-hood system (Deltatrac monitor; Datex Instrumentation, Helsinki, Finland) for quantifying resting energy expenditure and fat oxidation using standard equations (Ferannini) as previously described (29). CVs for energy expenditure and substrate oxidation were 4.2 and 4%, respectively. Details of the whole body leucine turnover study have been published (17, 19). Rates of leucine appearance (an index of proteolysis), nonoxidative leucine disposal (an index of protein synthesis), and leucine oxidation (an index of protein oxidation) were calculated using the reciprocal pool technique. Fat and leucine oxidation were expressed as milligrams per minute.

Serum Assays

Serum IGF-I was measured by RIA after acid-ethanol extraction as described (7), with intra-assay CVs of 9.4, 8.3, and 10.3% at 48, 254, and 1510 μg/l, respectively. Testosterone was measured by a solid-phase chemiluminescent enzyme immunoassay (Immulite 2000; DPC, Los Angeles, CA), with an intra-assay CV of 9.6% at 6.7 nmol/l. The NH_2-terminal peptide of PINP and that of PIIINP were measured by RIAs (Orion Diagnositcs, Espoo, Finland), with intra-assay CVs of <7 and <5%, respectively (28).

Muscle Biopsy

Local anesthetic (1% lignocaine) was injected ~2 cm above the patella over the vastus lateralis, usually of the right leg. A small piece of muscle was obtained with a 6-mm university hospital needle with suction assistance. Specimens were immediately blotted for blood, frozen in liquid nitrogen, and stored at −80°C. The muscle biopsies were obtained at ~12 noon.

Preparation of RNA and Microarray Hybridization

Total RNA was isolated from the snap-frozen muscle biopsies with Tri Reagent (Sigma-Aldrich, Sydney, NSW, Australia) according to the manufacturer's instructions. The quality of total RNA, cDNA synthesis, cRNA amplification, and cRNA fragmentation was checked by electrophoresis on a 1% agarose gel stained with ethidium bromide. Recombinant RNA was prepared as previously described (6). Briefly, cDNA was transcribed from 2 μg of mRNA using a poly(T) nucleotide primer containing a T7 RNA polymerase promoter (Sigma Genosys, Sydney, NSW, Australia). Biotinylated antisense target cRNA was subsequently synthesized by in vitro transcription using the BioArray High Yield RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY). Fifteen micrograms of biotin-labeled target cRNA were then fragmented and added to a hybridization mixture containing probe array controls and blocking agents. Hybridization to individual Affymetrix GeneChip Human Genome U133A oligonucleotide arrays (HG-U133A chips; Affymetrix, Santa Clara, CA), which contained 22,000 probe sets representing ~14,500 genes, was conducted according to the manufacturer's protocols. Washing and staining of the hybridized probe arrays were performed on an automated fluidics station. Hybridization signals were visualized using phycoerythrin-conjugated streptavidin (Molecular Probes, Eugene, OR). The stained probe array was scanned using the Agilent GeneArray Laser Scanner, and the resultant image was captured as a data image (.CEL) file. The signal intensities for the β-actin and the GAPDH genes were used as internal quality controls. The ratio of fluorescent intensities for the 5′ and the 3′ ends of those housekeeping genes were <3.

The hybridization signals from one subject were low and excluded from analysis. However, the RNA sample from this subject was included in the quantitative real-time PCR (qRT-PCR) verification (see below).

Bioinformatics

To correct for variation between GeneChips, the signal data of .CEL files of Affymetrix HG-U133A chips were quantile normalized, with probe set intensities calculated using the Robust multiarray average method (20), as implemented in the Affy package of Bioconductor (16). After conversion from log values to original intensities, the data were filtered to remove probe sets with mean intensities below 100 in both the GH-treated and baseline control groups. For each gene, a 2×2 factorial ANOVA, with moderated standard errors of the log fold change estimates, was used to estimate the effect of GH after adjustment for patient ID [ebayes function of the *limma* package (34) of Bioconductor]. A gene was considered regulated if it demonstrated a fold change of ≥ 1.4 or ≤ 0.7, and $P \leq 0.005$ in response to GH treatment. These criteria are similar to those published for similar studies (12, 38). An estimate of the false discovery rate was calculated as the ratio of median number of genes satisfying P value and fold change cutoffs in 500 randomly permuted data sets compared with the number observed in the original data set.

The HG-U133A annotation file dated April 11, 2006 was downloaded from Affymetrix, from which the Gene Title, Gene Symbol, Gene Ontology (GO) Biological Process, GO Cellular Component, and GO Molecular Functions of the regulated genes were identified. The genes were then grouped by their unique GO classifications (Biological Process, Cellular Component and Molecular Function). Normalized intensities of the regulated genes were scaled and clustered using the Hierarchical Clustering tool in GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA).

qRT-PCR Verification

The microarray findings were validated by qRT-PCR using mainly the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Stockholm, Sweden). Samples were run in triplicate, the assay was repeated twice, and similar results were obtained. The cDNA was amplified under the following conditions: 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The mRNA abundance of each gene was calculated using the "standard curve method" (*User Bulletin 2*; PE Applied Biosystems) and adjusted for the expression of β-actin. The use of β-actin as an internal control in skeletal muscle has been validated in several studies (21, 25, 27). Furthermore, semiquantitative analysis of β-actin expression in our samples did not show any regulation by GH. Predesigned TaqMan gene expression assays (PE Applied Biosystems) were used, with the assay IDs as follows: β-actin (4326315E), STAT1 (Hs00234829_m1), COL3A1 (Hs00164103_m1), CPT-IB (Hs_00993895_gl), MYH7B (Hs00293096_m1), and CLOCK (Hs00231857_ml).

The transcript abundance of IGF-I was quantified by qRT-PCR with LightCycler-Fast Start Reaction Mix SYBR Green I in a LightCycler (Roche Molecular Biochemicals, Indianapolis, IN). The sequences for the forward and reverse primers for human IGF-I were 5′-CAAACAAAAAGCCTGTCCACCC-3′ and 5′-TATTCTAAGCCTTCTCCCAAGTGC-3′, respectively, and the PCR product had an expected length of 138 nucleotides. The PCR program started with an initial denaturation step at 94°C for 12 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, with ramping rates at 20°C/s. Values of crossing point (the first turning point) for standards were used to construct a calibration curve, from which copy numbers for the samples were estimated.

Statistical Analysis

As stated above, not all the data could be used for analysis as one subject did not comply with the protocol, and in another subject the hybridization signals were unacceptably low. Analyses of GH-responsive proteins in blood and of whole body metabolism were performed in five subjects, excluding the subject who violated the study protocol. Results for samples before and after GH treatment were analyzed using Student's paired *t*-test. The validation by qRT-PCR was undertaken in five subjects, excluding the noncompliant subject. After logarithmic transformation, the results for samples before and after GH treatment were analyzed using paired Student's *t*-test. Microarray data were analyzed in four subjects. The statistical analysis for the microarray data is described in the *Bioinformatics*.

RESULTS

Biochemical and Metabolic Changes

The mean (±SE) baseline IGF-I (8.4 ± 1.8 nmol/l; $n = 5$; normal range 25–45 nmol/l) and testosterone (1.5 ± 0.5 nmol/l; normal range 12–30 nmol/l) were subnormal, indicating compliance with withdrawal from replacement treatment. GH administration resulted in a significant increase in circulating IGF-I, PINP, and PIIINP (Table 1). During GH treatment, both resting energy expenditure and lipid oxidation increased, whereas carbohydrate oxidation fell. This was accompanied by a reduction in whole body leucine oxidation and a corresponding increase in nonoxidative leucine disposal, an index of protein synthesis (Table 1). The rate of leucine appearance as an index of proteolysis did not change with GH treatment (Table 1).

GH-Responsive Genes in Muscle

GH induced differential expression of 117 genes in muscle, 23 of which were upregulated and 94 downregulated, with a false discovery rate of 17.8% (European Bioinformatics Institute database accession no. E-TABM-206). To confirm the microarray results, the expression of six genes was studied by qRT-PCR. Hierarchical clustering analysis was used to visualize the transcript profiles of GH-treated and baseline samples. The baseline and the GH-treated samples fall into two separate clusters as expected when a GH-responsive tissue is exposed to GH after deficiency (Fig. 1).

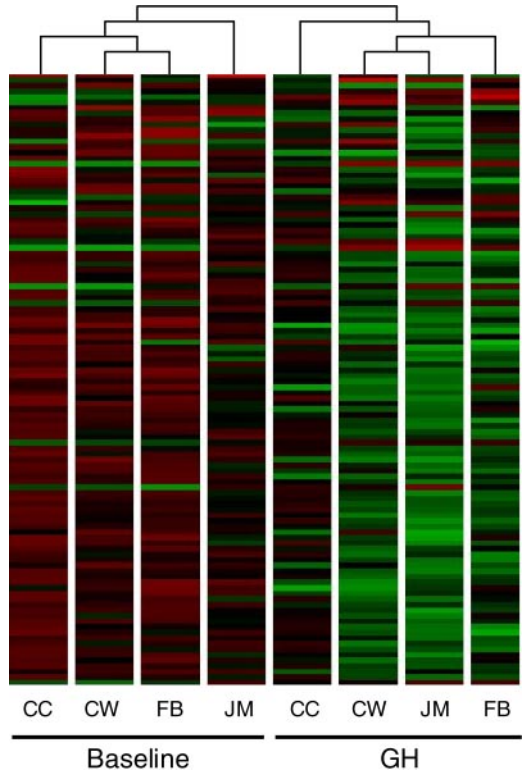

Fig. 1. Hierarchical cluster analysis was performed on 117 genes differentially expressed in skeletal muscle from hypogonadal men on growth hormone (GH) treatment. Letters at the bottom of the figure indicate patient identities. Up- and downregulated genes are indicated in red and green, respectively.

Table 1. *Biochemical and metabolic results*

	Baseline	GH
IGF-I, nmol/l	8.4±1.8	33.4±6.2*
PINP, μg/l	27.0±6.7	34.9±6.1*
PIIINP, μg/l	2.4±0.3	4.3±0.5*
Resting energy expenditure, kJ/24 h	1640±116	1743±188*
Lipid oxidation, mg/min	69.4±8.5	80.9±17.0*
Carbohydrate oxidation, mg/min	46.8±14.5	35.5±17.3*
Leucine oxidation, mg/min	32.8±4.7	21.2±8.6*
Nonoxidative leucine disposal, mg/min	120.5±10.5	138.0±29.7*
Leucine appearance, mg/min	153.3±14.9	159.2±21.4

Results are means ± SE. PINP and PIIINP, procollagens I and III. By Student's paired *t*-test, *$P < 0.05$ vs. baseline.

The GH-responsive genes were assigned to several functional categories (Table 2). A complete catalog of these genes is attached separately as supplementary data (Supplementary Table). Although a treatment period of 2 wk would mean that several of these genes may reflect secondary effects of GH treatment, we refer to them as GH-responsive genes for simplicity.

IGF-I and collagens. The responses of some known GH-regulated genes were first studied, particularly those that were increased in blood (i.e., IGF-I and procollagens I and III). GH increased the IGF-I transcript level more than twofold (Table 3). GH also increased the expression levels of collagen genes COL1A2 and -3A1 around threefold, respectively (Table 3).

Fuel metabolism. As shown in Table 3, GH altered the expression of a wide range of metabolic genes in muscle. It reduced significantly the expression of a large group of genes involved in lipid metabolism, including fatty acid-binding protein-3 (FABP-3) and several key enzymes mediating mitochondrial fatty acid β-oxidation. GH also reduced expression of long-chain acyl-CoA synthetase-1 (ACSL1), which activates cytosolic fatty acids to acyl-CoA and to acyl-CoA thio-

Table 2. *Functional classification of GH-responsive genes in muscle*

Classification	GO ID*	No. of Responsive Genes		
		Total	Upregulated	Downregulated
Transcription	0016350	16	5	11
Signal transduction	0007165	5	2	3
Substrate metabolism	0008152/0006629	15	1	14
Mitochondrial respiratory chain	0006118/0006811	4	0	4
Protein metabolism				
Synthesis	0006412	4	1	3
Degradation	0006508	1	0	1
Posttranslation modification	0008284	9	1	8
Intracellular transport	0006886	3	1	2
Ion/substrate transport	0006811	9	0	9
Cell proliferation	0008283	2	1	1
Apoptosis	0006915	6	1	5
Cell adhesion	0007155	5	3	2
DNA/RNA processing	0006364	3	0	3
Muscle development/function	0007517	9	0	9
Skeletal development	0001501	1	1	0
Neurogenesis/organogenesis/morphogenesis	0007399/0009887/0009653	6	2	4
Immune response	0006955	2	0	2
Unknown function		10	1	9
Unknown identity		7	3	4
Total responsive genes		117	23	94

*GO ID, Gene Ontology biological process identity nos.

esterase 2 (ACOT2), which converts acyl-CoA back to fatty acids in peroxisomes.

In contrast to lipid metabolism, the expression of only three enzymes in carbohydrate metabolism was significantly affected by GH (Table 3). There was a reduction in pyruvate dehydrogenase kinase-4 expression (a negative regulator of pyruvate oxidation) and in glycogen synthase 1 expression (the enzyme responsible for glucose storage in muscle). GH also reduced the expression of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2, which catalyzes the syn-

Table 3. *GH-responsive genes: IGF-I and members in collagen family, fuel and protein metabolism*

Gene Title	Gene Symbol	Fold Change*	*P* Value
Insulin-like growth factor I	**IGF-I**	**2.32**	**0.002**
Collagen family			
Type 1, α2	COL1A2	2.79	0.002
Type 3, α1	COL3A1	3.09	<0.001
Lipid metabolism			
Fatty acid-binding protein-3	FABP3	0.58	<0.001
Acyl-CoA synthetase, long-chain family member 1	ACSL1	0.63	0.003
Carnitine palmitoyltransferase IB (muscle)	**CPT-IB**	**0.50**	**<0.001**
Acyl-CoA dehydrogenase, family member 8	ACAD8	0.62	0.004
Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase	HADHA	0.63	0.002
3-Hydroxybutyrate dehydrogenase (heart, mitochondrial)	BDH	0.67	0.004
Acyl-CoA thioesterase 2	ACOT2	0.67	0.004
Carbohydrate metabolism			
Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	0.36	0.002
Glycogen synthase 1 (muscle)	GYS1	0.68	0.004
6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	PFKFB2	0.42	0.001
Tricarboxylic acid cycle			
Oxoglutarate dehydrogenase	OGDH	0.58	0.001
Succinate dehydrogenase complex, subunit B	SDHB	0.67	0.003
Mitochondrial respiratory chain			
Oxidase (cytochrome *c*) assembly 1-like	OXA1L	0.67	0.004
NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa (NADH-coenzyme Q reductase)	NDUFS3	0.65	0.003
ATP synthase, H^+ transporting, mitochondrial F_0 complex, subunit c (subunit 9), isoform 2	ATP5G2	0.60	0.003
ATP synthase, H^+ transporting, mitochondrial F_0 complex, subunit g	ATP5L	0.65	0.004
Protein synthesis			
Ribosomal protein L38	RPL38	2.33	0.004
Mitochondrial ribosomal protein S2	MRPS2	0.61	0.003
Mitochondrial ribosomal protein S7	MRPS7	0.67	0.003
Mitochondrial ribosomal protein S34	MRPS34	0.61	0.003
Protein degradation			
Calpain 3	CAPN3	0.63	0.004

*GH-treated relative to baseline samples; genes in boldface were verified by qRT-PCR and shown in Figure 2.

thesis and degradation of fructose 2,6-biphosphate (the enzyme responsible for directional flux of substrate in glycolysis). Taken together, GH reduced the expression of genes promoting lipid oxidation. It also suppressed the expression of genes that promote glycogen storage and downregulate glucose utilization.

The effects of GH on genes involved in energy production were investigated next (Table 3). GH significantly reduced the transcript levels of oxoglutarate dehydrogenase and succinate dehydrogenase complex subunit B in the tricarboxylic acid (TCA) cycle. There was also a significant reduction in the expression of four enzymes in the mitochondrial respiratory chain (one cytochrome *c* oxidase, one NADH-coenzyme Q reductase, and two ATP synthase subunits in the mitochondrial F_0 complex). Together, these changes suggest that GH reduced substrate utilization for energy production in muscle.

Protein metabolism. GH exerted diverse effects on genes involved in protein synthesis and degradation (Table 3). Among the genes catalyzing protein synthesis, the transcript level of cytoplasmic ribosomal protein L38, a component of the 60S ribosomal subunit, was increased over twofold, whereas those of three mitochondrial ribosomal proteins fell. The level expression of calpain 3, a cysteine protease, was reduced. The expression of genes involved in protein oxidation, such as aminotransferases, was unaffected by GH treatment (data not shown).

Other genes. STAT. The signal transducers and activators of transcription (STATs) are a family of transcription factors activated by numerous cytokines and growth factors. STAT1 expression was upregulated more than twofold on the microarray after GH administration ($P = 0.002$; Supplementary Table). No significant changes in expression of STAT3 or -5 occurred following GH treatment.

MUSCLE DEVELOPMENT AND FUNCTION. GH downregulated nine genes that participate in muscle development and function (0.5- to 0.67-fold; Supplementary Table). Among these were four muscle structural proteins: tropomyosin 2 (TPM2), myosin light-chain 1 slow a (MLC1SA), myosin heavy polypeptide 7β (MYH7B), and troponin I type 1 (TNNI1). As these findings stand in contrast to the positive regulatory effect of GH on muscle mass in hypopituitary subjects, we investigated the effects on the expression of myofibrillar proteins (8). One of the genes with the greatest fold change on the microarray was myosin heavy polypeptide 8, although it did not meet our selection criteria (3.3-fold, $P = 0.05$). Indeed, studies using qRT-PCR showed a marked upregulation of MYH8 in muscle after GH (14.4 ± 7.9-fold, $P = 0.04$). These data suggest that GH exerts a mixed effect on the expression of myofibrillar protein in muscle.

CIRCADIAN. In addition to the metabolic genes, an interesting finding of this microarray study was the effect of GH on two core circadian genes (CLOCK and PERIOD) in muscle. Circadian genes encode proteins that control daily rhythmic expression of functional proteins (1, 4). GH increased the transcript level of CLOCK 1.65-fold ($P = 0.001$) and reduced that of PERIOD 1 (PER1) to 0.57-fold ($P = 0.002$; Supplementary Table).

Verification of Microarray Results

To verify the microarray data, the transcript levels of six genes (IGF-I, COL3A1, CPT-IB, CLOCK, STAT1, and MYH7B) were quantified by qRT-PCR. Analysis was performed on individual samples, with β-actin included as an internal control. As shown in Fig. 2, the regulation of IGF-I, COL3A1, CLOCK, and STAT1 gene expression was confirmed by qRT-PCR, whereas the changes for CPT-IB and MYH7B did not reach statistical significance ($P = 0.06$ and 0.13, respectively).

DISCUSSION

The aims of this study were to identify GH-responsive genes regulating substrate and energy metabolism in skeletal muscle and to relate their expression as a means of deducing muscle

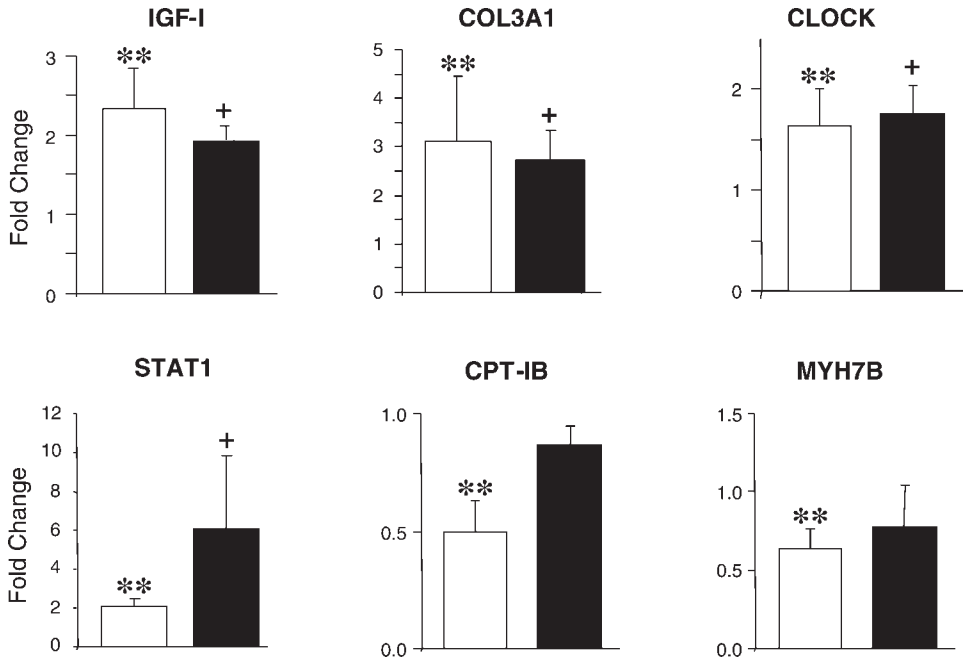

Fig. 2. Microarray results (open bars) and quantitative RT-PCR verification (filled bars) of selected GH-responsive genes. Data are fold changes of GH-treated samples above baseline samples, and presented as means ± SE. By ANOVA, $**P \leq 0.01$ vs. baseline; by Student's *t*-test, $+P \leq 0.05$ vs. baseline.

contribution to whole body metabolism. One of the strength of this study is the concomitant quantification of gene expression and metabolism in a group of well-characterized hypopituitary men. GH treatment at replacement doses increased blood levels of recognised GH-responsive proteins, such as IGF-I and procollagens I and III, and stimulated whole body metabolic rate, fat oxidation, and protein synthesis while reducing carbohydrate oxidation. All of these responses are expected biological effects of GH. In skeletal muscle, GH treatment increased the expression of IGF-I and several collagen genes, reduced the expression of genes promoting fat oxidation and energy production. It exerted mixed effects on genes regulating protein synthesis, and reduced the transcript level of calpain 3, a protease. These findings revealed a concordance in circulating IGF-I and procollagens and the expression of these genes in skeletal muscle with GH treatment. However, there was discordance between the effects of GH on whole body energy expenditure, fat and glucose oxidation, and on expression of muscle genes regulating these processes that were studied here. This study also provides the first evidence that GH regulates peripheral circadian function by affecting gene expression of CLOCK and PERIOD.

Fuel Metabolism

A major finding in this study was the striking difference between whole body metabolic and muscle gene expression data. GH significantly stimulated whole body resting energy expenditure and fat oxidation and reduced carbohydrate oxidation. However, GH induced changes in gene expression opposite to those regulating the above metabolic processes.

Lipids are a major source of energy for skeletal muscle. Fatty acids dissociated from lipids are first activated by acyl-CoA synthetases (10) and then undergo β-oxidation in mitochondria and, to a lesser extent, in peroxisomes. Carnitine palmitoyltransferase I (CPT I) controls fatty acid translocation into mitochondria, which is the rate-limiting step for β-oxidation (33). GH suppressed multiple genes regulating fatty acid utilization in muscle (Table 3), including FABP-3, which transports fatty acids to mitochondria for oxidation (18), and ACSL1. The expression levels of several enzymes mediating fatty acid oxidation fell, indicating an extensive repression of mitochondrial β-oxidation. The expression of CPT-IB was significantly downregulated on the microarray. Verification by qRT-PCR showed a strong trend ($P = 0.06$), although the change did not reach statistical significance.

Peroxisomes are alternative sites for fatty acid β-oxidation (33). Contrary to its effects in mitochondria, GH did not affect expression of peroxisomal β-oxidation enzymes (data not shown), except for reducing peroxisomal long-chain acyl-CoA thioesterase (ACOT2), which catalyzes the hydrolysis of acyl-CoA to fatty acids for exporting out of peroxisomes (11). The collective data suggest that GH suppresses expression of genes mediating fatty acid oxidation in mitochondria but not in peroxisomes.

GH suppressed the expression of glycogen synthetase 1, the key enzyme regulating glycogenesis, predictably reducing glycogen storage and increasing the provision of glucose for oxidation. Previous clinical studies have reported that GH suppresses glycogen synthase activity in muscle both in normal humans (3) and in GH-deficient subjects (9). This was associated with decreased glycogen content and increased glucose concentrations in muscle (9). Our result indicates that the suppression is, at least in part, at the transcriptional level. Furthermore, GH reduced the transcript level of pyruvate dehydrogenase kinase-4, which phosphorylates and inactivates the pyruvate dehydrogenase complex, the key enzyme converting pyruvate to acetyl-CoA for oxidation in the TCA cycle (35). As the expression level of pyruvate dehydrogenase kinase-4 was decreased by GH, less of pyruvate dehydrogenase would be inactivated through phosphorylation, leading to an increased capacity for mediating the conversion of pyruvate to acetyl-CoA for oxidation. Collectively, these findings suggest that GH diverts glucose from storage to utilization, along with a switch from lipid to carbohydrate utilization in muscle.

GH suppressed the expression of several genes encoding components of the TCA cycle and mitochondrial respiratory chain (Table 3). It reduced the expression levels of oxoglutarate dehydrogenase and succinate dehydrogenase complex subunit B in the TCA cycle, indicating decreased pathway flux. Most notably, GH suppressed the expression of four enzymes in the mitochondrial respiratory chain. A downregulation of those gene transcripts suggests a reduction of energy production capacity in skeletal muscle by GH.

These gene expression changes in human skeletal muscle are supported by findings in animals that GH reduces expression of genes promoting lipid utilization and energy production while increasing those promoting glucose oxidation in muscle (37). The discordance between transcriptional and metabolic findings questions whether skeletal muscle is a major contributor to GH-induced changes in whole body substrate metabolism. The possibility that liver rather than muscle is the major organ contributing to whole body substrate and energy metabolism is supported by studies in animals. In the rat liver, GH stimulates expression of genes encoding enzymes, enhancing lipid oxidation (37) and the mitochondrial respiratory chain (12, 36) and inhibits those in glucose oxidation (37). The gene expression findings, however, do not exclude the possibility that GH upregulates lipid oxidation and energy production in muscle at the posttranscriptional level. However, we are unaware of any data reporting dissociation between expression and the translation or activities of the enzymes induced by GH in muscle.

Protein Metabolism

In the clinical studies, GH reduced whole body rates of leucine oxidation, an index of protein oxidation and enhanced nonoxidative disposal of leucine, an index of protein synthesis without affecting leucine appearance, an index of protein breakdown. Protein metabolism is a complex process involving amino acid transfer from muscle and other peripheral tissues and organs to the liver, where amino acids may enter a number of pathways, including the urea cycle, gluconeogenesis, oxidation, or incorporation into hepatic proteins.

Caution should be exercised in assuming that the above-mentioned changes represent activity in muscle, since muscle accounts for about 40% of the body protein mass. However, there is strong evidence from arteriovenous balance studies across forearm muscle that GH acutely stimulates protein synthesis (14, 15) in parallel with changes in whole body protein metabolism similar to those observed in the present study (13).

From the skeletal muscle biopsy studies, GH exerted no detectable effect on genes regulating protein oxidation, such as aminotransferases (data not shown). It reduced the expression of calpain 3, a nonlysosomal cysteine protease that has been implicated in muscle development (23). Among those involved in protein synthesis (Table 3), ribosomal protein L38 expression was upregulated more than twofold, suggesting increased protein synthesis, which is in concordance with the whole body protein turnover findings (Table 1).

Circadian Genes

Circadian genes encode proteins that control daily rhythmic expression of functional proteins, orchestrating physiological and behavioral processes in response to cyclic changes in the environment (4). In mammals, circadian systems are present in the hypothalamic suprachiasmatic nucleus (SCN) of the brain and in peripheral tissues (1). The SCN governs the peripheral circadian clocks through actions of endocrine factors such as glucocorticoids (5, 40).

Among the circadian genes identified, CLOCK is a pivotal regulator (41). CLOCK induces transcription of PERIOD and CRYPTOCHROME, which feed back to repress its activity. CLOCK also stimulates the expression of peroxisome proliferator-activated receptor-α and ubiquitin-specific protease 2 (30–32), inducing rhythmic control of lipid and protein metabolism in peripheral tissues.

GH is a potential mediator for the SCN's regulation of peripheral circadian clocks. Pituitary secretion of GH is diurnal and under the control of somatostatin from the hypothalamus (2). It may act as an endocrine factor delivering temporal signals from the SCN to peripheral tissues. In line with this proposal, we show that GH regulated the expression of CLOCK and PER1 in muscle. Most interestingly, GH caused a dissociation of the CLOCK control by PERIOD; it induced the expression of CLOCK and suppressed that of PER1. These data suggest that GH may augment CLOCK action by stimulating the CLOCK expression and simultaneously attenuating inhibition by PERIOD.

In summary, GH regulates a large number of metabolic genes in skeletal muscle. GH exerted concordant effects on the expression of IGF-I and collagens in muscle and their respective levels in blood. It induced discordance between the whole body substrate metabolism and metabolic gene expression in muscle. Assuming that the transcriptional changes also reflect effects on protein expression, we conclude that muscle is unlikely to contribute to GH-induced changes in substrate and energy metabolism. Finally, we provide the first evidence that GH regulates the muscle circadian clock, an effect that may have significant implication in regulation of muscle function.

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