

Insulin-Regulated Mitochondrial Gene Expression Is Associated With Glucose Flux in Human Skeletal Muscle

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To identify abnormally expressed genes contributing to muscle insulin resistance in type 2 diabetes, we screened the mRNA populations from normal and diabetic human skeletal muscle using cDNA differential display and isolated abnormally expressed cDNA clones of mitochondrial-encoded NADH dehydrogenase 1 (ND1), cytochrome oxidase 1, tRNA^{leu}, and displacement loop. We then measured mRNA expression of these mitochondrial genes using a relative quantitative polymerase chain reaction method in biopsies taken before and after an insulin clamp in 12 monozygotic twin pairs discordant for type 2 diabetes and 12 matched control subjects and in muscle biopsies taken after an insulin clamp from 13 subjects with type 2 diabetes, 15 subjects with impaired glucose tolerance, and 14 subjects with normal glucose tolerance. Insulin infusion increased mRNA expression of ND1 from 1.02 ± 0.04 to 2.55 ± 0.30 relative units ($P < 0.001$) and of cytochrome oxidase 1 from 0.80 ± 0.01 to 1.24 ± 0.10 relative units ($P < 0.001$). The ND1 response to insulin correlated with glucose uptake ($r = 0.46$, $P = 0.002$). Although the rate of insulin-mediated glucose uptake was decreased in the diabetic versus the nondiabetic twins (5.2 ± 0.7 vs. 8.5 ± 0.8 mg · kg⁻¹ fat-free mass · min⁻¹, $P < 0.01$), insulin-stimulated ND1 expression was not significantly different between them (2.4 ± 0.5 vs. 2.7 ± 0.5 relative units). Neither was there any significant intrapair correlation of ND1 expression between the monozygotic twins ($r = -0.15$, NS). We conclude that insulin upregulates mitochondrial-encoded gene expression in skeletal muscle. Given the positive correlation between ND1 expression and glucose uptake and the lack of intrapair correlation between monozygotic twins, mitochondrial gene expression may represent an adaptation to intracellular glucose flux rather than an inherited trait. *Diabetes* 48:1508–1514, 1999

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COX1, cytochrome oxidase 1; D-loop, displacement loop; FBG, fasting blood glucose; FFM, fat-free mass; H-strand, heavy strand; IGT, impaired glucose tolerance; IRE, insulin response element; ND1, NADH dehydrogenase 1; RT-PCR, reverse transcription-polymerase chain reaction.

Skeletal muscle insulin resistance is a characteristic feature of type 2 diabetic patients and is observed in their nondiabetic offspring (1). However, the mechanisms responsible for skeletal muscle insulin resistance in type 2 diabetes remain incompletely understood (2). Reduced glucose-induced thermogenesis and reduced maximal aerobic capacity have been demonstrated in type 2 diabetic patients (3,4) and their nondiabetic offspring (5). As mitochondrial DNA defects have been described only in a small group of diabetic patients, usually in combination with hearing loss (6,7), it is possible that defects in mitochondrial mRNA expression might be involved in the development of insulin resistance in type 2 diabetes. In support of this view, overexpression of mitochondrial-encoded genes, including the NADH dehydrogenase gene, was recently reported in muscle of type 2 diabetic patients (8).

In an effort to identify abnormally expressed genes responsible for muscle insulin resistance in type 2 diabetes, we screened the mRNA populations from normal and diabetic human skeletal muscle using polymerase chain reaction (PCR) differential display (9) and isolated from the diabetic cDNA panels the abnormally expressed mitochondrial heavy chain-encoded cDNA clones of NADH dehydrogenase 1 (ND1), 4, and 5; cytochrome oxidase 1 (COX1); tRNA^{leu}; and a displacement-loop (D-loop) fragment. Among these clones, ND1 and D-loop were only identified in cDNA panel of diabetes; the other clones were also identified in the control panel. Because transcription of most of the mitochondrial heavy chain-encoded genes is carried out on a primary transcript followed by extensive posttranscriptional processing (10), and changes in mitochondrial gene expression have been associated with diabetes (8), we selected four mitochondrial heavy chain-encoded genes—ND1, D-loop, COX1, and tRNA^{leu}—to examine whether mitochondrial gene expression is related to insulin resistance and, further, whether their expression is under genetic control. To accomplish this, we examined mitochondrial gene expression in muscle biopsies taken before and after an insulin clamp from 12 monozygotic twin pairs discordant for type 2 diabetes and from 12 control subjects (11,12) and in muscle biopsies taken after an insulin clamp from 42 individuals with various degrees of insulin sensitivity and glucose tolerance.

RESEARCH DESIGN AND METHODS

cDNA differential display. cDNA differential display was performed as previously described (9), with some modifications. Briefly, muscle biopsies were

TABLE 1

Clinical characteristics of the monozygotic twins discordant for type 2 diabetes and the control subjects in protocol 1

	Type 2 diabetic twins	Nondiabetic co-twins	Control subjects
<i>n</i> (F/M)	12 (5/7)	12 (5/7)	12 (5/7)
Age (years)	64 ± 3	64 ± 3	61 ± 2
BMI (kg/m ²)	30.1 ± 1.3	27.5 ± 1.3	26.0 ± 1.0
Fasting plasma glucose (mmol/l)	11.4 ± 1.1*	6.0 ± 0.2†	5.4 ± 0.2
Glucose uptake (mg · kg ⁻¹ FFM · min ⁻¹)	5.2 ± 0.7*	8.5 ± 0.8†	11.4 ± 0.9
Glucose storage (mg · kg ⁻¹ FFM · min ⁻¹)	2.8 ± 0.6*	4.8 ± 0.6‡	7.6 ± 0.9
Glucose oxidation (mg · kg ⁻¹ FFM · min ⁻¹)	2.4 ± 0.2*	3.7 ± 0.2	3.8 ± 0.2
Insulin (μU/ml)			
Baseline	9.7 ± 2.3	7.1 ± 0.9	6.4 ± 0.7
Clamp	73.6 ± 7.2	69.4 ± 5.7	78.0 ± 4.5

Data are means ± SE. **P* < 0.01, †*P* < 0.05, ‡*P* < 0.02 vs. control subjects.

obtained from two pairs of diabetic and healthy control male subjects after overnight fasting (diabetic patients: age 45–57 years, BMI 24.7–27.7 kg/m², fasting blood glucose [FBG] 11.8–13.4 mmol/l; control subjects: age 49–53 years; BMI 24.5–29.2 kg/m², FBG 5.8–5.7 mmol/l). Total RNA was isolated by the acid guanidinium thiocyanate method (13). Total RNA (200 ng) was reverse-transcribed in 20 μl reaction with 2.5 μmol/l of each of the T₁₂VA, T₁₂VC, T₁₂VG, T₁₂VT (V = ACG) primers in the presence of 200 U Superscript II reverse transcriptase (RT) (Life Technologies, Glasgow, Scotland) and 25 μmol/l dNTP for 60 min at 37°C. After heat inactivation of the RT at 95°C for 5 min, 2 μl of the RT reaction was added to 18 μl of PCR mix containing 1× PCR buffer, 12.5 μmol/l dNTP, 5 μCi [³⁵S]dATP, 2.5 μmol/l of each respective T12VN primer, 2.5 μmol/l of one of the 20 arbitrary primers (Operon Technologies, Alameda, CA) and 1 U Amplitaq DNA polymerase (Perkin Elmer, Branchburg, NJ). PCR was run for 40 cycles (94°C, 30 s; 40°C, 90 s; 72°C, 30 s). PCR product (4.0 μl) was analyzed on a 5% sequencing gel. Any band visible in the cDNA panel from the type 2 diabetic patients but absent from the control panel or vice versa was selected for further evaluation. The recovery of corresponding cDNAs was achieved by elution of the DNA bands from the sequencing gel and PCR reamplification (9). The recovered cDNAs were subcloned using the T-Cloning Kit (MBI Fermentas, Vilnius, Lithuania), sequenced using Sequenase (version 2.0; USB, Cleveland, OH), and compared with sequences in GenBank (GenBank 104; DNASTAR, Madison, WI).

Quantitation of mitochondrial gene expression

Subjects in protocol 1. A total of 12 monozygotic twin pairs discordant for type 2 diabetes and 12 healthy subjects without family history of diabetes participated in this study. All subjects were Caucasians. Type 2 diabetes had been diagnosed after the age of 40 years based on a standardized 75-g oral glucose tolerance test (14). The control subjects were matched to nondiabetic twins for age, sex, and BMI (Table 1). Monozygosity of the twins was confirmed by genetic and biochemical markers (12). Insulin sensitivity was measured by a 3-h euglycemic-hyperinsulinemic clamp (11,12). Muscle biopsies were obtained in the basal state (0 min) and at the end of the clamp (180 min), frozen immediately in liquid nitrogen, and stored at –80°C until analyzed.

Subjects in protocol 2. A total of 13 patients with type 2 diabetes, 15 patients with impaired glucose tolerance (IGT), and 14 sex-, age-, and BMI-matched

healthy control subjects without family history of diabetes participated in the study (Table 2). All subjects were Caucasians. Diagnosis of diabetes was based on the 1985 World Health Organization criteria using a 75-g oral glucose tolerance test. All subjects participated in a 2-h euglycemic-insulin clamp combined with indirect calorimetry to estimate insulin-stimulated glucose disposal and glucose oxidation (1). Glucose storage was defined as the difference between glucose disposal and glucose oxidation. Muscle biopsies were taken immediately after the insulin clamp, put into liquid nitrogen, and stored at –80°C until analyzed.

Informed consent was obtained from all subjects. The protocol was approved by the regional ethics committee, and the procedures were performed according to the principles of the Declaration of Helsinki.

RT-PCR quantitation of gene expression. The RNA expression of mitochondrial ND1 was examined using a modified “primer-dropping” RT-PCR method (15). Total RNA was extracted from muscle biopsies from protocols 1 and 2 and reverse-transcribed as described above except that 5 μmol/l oligo (dT)₁₈ was used as the primer instead of T₁₂VN (N = A, C, G, or T). Total cDNA was then subjected to PCR coamplification of one of the mitochondrial target genes together with cyclophilin as a reference gene. The mitochondrial gene primer pairs (from 5' to 3') were GAGGTCAGAAAGTAGGGTCTT (ND1F), TATCTCCACACTAGCA GAGAC (ND1R); AGGGAGGTAAGAGTCAGAAG (COX1F), GACCGTTGAC TATTCTCTAC (COX1R); AGATGGCAGAGCCCGGTAAT (tRNA^{leu}F), AGAA GAGCGATGGTGAGAGC (tRNA^{leu}R); CACCATCCTCCGTGAAATCA (D-loopF), and AATGGGATGAGGCAGGAATC (D-loopR). The cyclophilin primer pairs (cycloF-cycloR or cycloF-cycloR2) were GTCTCCTTTGAGCTGTTTG (cycloF), TGGCTCCACAATATTCATGC (cycloR), and CTGGGAACCAATTTGTGTTGG (cycloR2). The primer sequences of cyclophilin defined the intron-exon borders of the gene structure (GenBank accession no. X52851) for cDNA amplification. For ND1 measurement, the PCR condition for protocol 1 was 20 μl PCR reaction containing 2 μl of RT reaction, 1× PCR buffer, 200 μmol/l dNTP, 2% (vol/vol) formamide, 0.5 U Taq DNA polymerase (Perkin Elmer), 0.4 μmol/l cyclophilin primers (cycloF and cycloR), and 0.1 μmol/l ND1 primers. The PCR was run for 28 cycles (94°C, 60 s; 58°C, 60 s; 72°C, 60 s) and followed by a final extension at 72°C for 10 min. The PCR condition for protocol 2 was the same as for protocol 1 except that the ND1 primer concentration was 0.05 μmol/l and the number of cycles was 30. PCR coamplification was maintained within the exponential phase

TABLE 2

Clinical characteristics of the type 2 diabetic, IGT, and control subjects in protocol 2

	Type 2 diabetes	IGT	Control
<i>n</i> (male)	13	15	14
Age (years)	65 ± 2	66 ± 1	66 ± 1
BMI (kg/m ²)	28.4 ± 1.0	27.1 ± 1.0	27.6 ± 0.8
FBG (mmol/l)	11.2 ± 1.0	5.3 ± 0.2†	4.6 ± 0.1
Glucose uptake (mg · kg ⁻¹ · min ⁻¹)	2.6 ± 0.4*	4.0 ± 0.4†	5.9 ± 0.6
Glucose storage (mg · kg ⁻¹ · min ⁻¹)	1.2 ± 0.3*	2.1 ± 0.4†	3.8 ± 0.5
Glucose oxidation (mg · kg ⁻¹ · min ⁻¹)	1.4 ± 0.2*	2.0 ± 0.1	2.1 ± 0.2
Insulin (μU/ml)			
Baseline	10.5 ± 2.1‡	7.8 ± 1.7	4.7 ± 0.9
Clamp	91.9 ± 5.6	89.4 ± 4.4	84.8 ± 6.6

Data are means ± SE. **P* < 0.01, †*P* < 0.03, ‡*P* < 0.05 vs. control subjects.

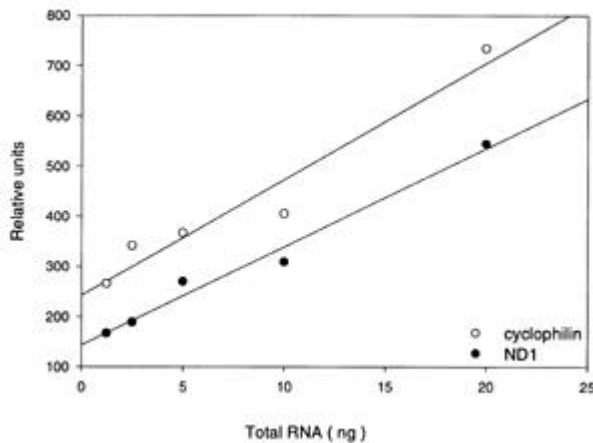


FIG. 1. Linearity of PCR coamplification of ND1 and cyclophilin. Total RNA was reverse-transcribed using oligo (dT)₁₈. Serial twofold dilutions of reverse-transcribed total RNA (20, 10, 5, 2.5, and 1.25 ng) were prepared and then subjected to PCR coamplification (94°C, 60 s; 58°C, 60 s; 72°C, 60 s) of both ND1 and cyclophilin for optimal 28 cycles. The PCR products were analyzed on 2% agarose gel containing ethidium bromide and quantitated using scanning densitometry. *r* = 1 for both ND1 and cyclophilin.

(*r* = 1 for both ND1 and cyclophilin) under the assay conditions as demonstrated (Fig. 1). Similar PCR reactions as in protocol 2 were conducted for quantitation of COX1 coamplified with cycloF and cycloR and for tRNA^{leu} and D-loop coamplified with cycloF and cycloR2. PCR products were separated on a 2% agarose gel containing ethidium bromide, photographed with UPP-110HA printing paper (Sony, Tokyo), and quantitated using Personal Densitometer SI scanner together with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The mRNA signals were expressed relative to that of cyclophilin. The intra-assay variation of the relative quantitative RT-PCR was 11.6%; the interassay variation was 10.4%.

Ribonuclease protection assay. To validate the finding, ND1 gene expression was also quantitated with the ribonuclease protection assay. Total RNA was obtained from muscle biopsies of five healthy individuals before and after an insulin clamp as described in protocol 1. PCR products of ND1 (with ND1F and ND1R primers) and β-actin were cloned into pGem4Z and pGem-T in vitro transcription vectors (Promega, Madison, WI), respectively. The PCR primers for β-actin were (5' to 3') GCGAGAAGATGACCCAGATCATGT and AGGACTCCATGCCAGGAAGGAAG. We chose β-actin as an internal reference in the ribonuclease protection assay because the expression level of cyclophilin is relatively low in skeletal muscle and was hardly detectable using the limited amount of RNA in this assay. The α-[³²P]CTP-labeled antisense RNA probes of ND1 and β-actin were made using Riboprobe System-SP6/T7 (Promega). Both ND1 and β-actin probes were then mixed with 5 μg total RNA, and ribonuclease protection assay was performed using RPA III kit (Ambion, Austin, TX) according to the manu-

facturer's instructions. The protected bands were separated with 5% sequencing gel and analyzed with the phosphorImager (Molecular Dynamics, Sunnyvale, CA). The ND1 mRNA level was expressed relative to β-actin.

Mitochondrial DNA content. Quantitation of mitochondrial DNA content was performed in muscle biopsies taken in the basal state from four patients with type 2 diabetes (age 51 ± 2 years, FBG 10.7 ± 2.1 mmol/l, BMI 24.1 ± 1.2 kg/m²) and four control subjects (age 45 ± 4 years, FBG 5.8 ± 0.1 mmol/l, BMI 28.0 ± 2.5 kg/m²). In addition, mitochondrial DNA content was measured in muscle biopsies taken in both the basal and insulin-stimulated states from two diabetic patients and two control subjects. Total genomic DNA was isolated from 10–20 mg muscle biopsies using a proteinase K digestion method (16). The mitochondrial ND1 DNA level was measured by relative quantitative PCR and related to the amount of genomic β-actin. The primers for ND1 were the same as described above. The primers for genomic β-actin (GenBank accession no. M10277) were 5'-AGGCCAACCGCGA GAAGATG-3' and 5'-CAGCCTGGATAGCAACGTAC-3'. PCRs were run within the range of linear amplification according to the primer dropping method (15) as follows: 20 μl PCR reaction contained 12 ng genomic DNA, 1× PCR buffer, 200 μmol/l dNTP, 2% (vol/vol) formamide, 2% (vol/vol) DMSO, 0.5 U Taq DNA polymerase (Perkin Elmer), and 0.2 μmol/l β-actin primers (as the starter primer pair). The PCR was run for 30 cycles (94°C, 60 s; 56°C, 60 s; 72°C, 60 s) followed by a final extension at 72°C for 10 min. The ND1 primer pair (as the second primer pair) was added to the final concentration of 0.1 μmol/l during PCR and run for 21 cycles. The PCR products were analyzed and quantitated as described above. The ND1 DNA level was expressed relative to β-actin.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using NCSS 6.0.21 statistical package (NCSS Statistical Software, Kaysville, UT). The significance of difference within or between groups was tested by Wilcoxon or Mann-Whitney rank tests. The relationship between various variables was analyzed by Spearman correlations. Correlations between ND1 expression and insulin-mediated glucose metabolism was adjusted for BMI using Kendall partial variable correction among the subjects in protocol 1, since ND1 expression was correlated with BMI (*r* = 0.43, *P* < 0.01). This was not necessary in the subjects in protocol 2, who had similar BMI values.

RESULTS

cDNA differential display. Of >4,000 cDNA species screened, 54 differentially expressed cDNA bands with >200 bp were selected; 31 of them were present preferentially in the cDNA panel of the type 2 diabetic patients, the other 23 in that of the control subjects. Sequencing analysis of the clones isolated from these bands revealed 66 distinct sequence signals; 43 of them matched with >95% sequence identity to genes or expressed sequence tags in the GenBank, and 23 sequences showed no match with any known genes (Table 3). Among the signals matched to known genes, 56% (24 of 43) showed sequence similarity with mitochondrial heavy chain-encoded genes. Twenty signals with matches

TABLE 3
Differentially expressed candidate clones

Clones from diabetic subjects	Best match in GenBank	Clones from control subjects	Best match in GenBank
D11, D12, D31, D32 D7, D9	ND1(Mt) Myosin	C47 C43, C57	Kiaa0093 L-3-phosphoserine Phosphatase homolog
D8	α-actin	C56	Carbonic anhydrase III, exon7
D10	Ubiquitin hydrolase	C62	ND2(Mt)
D16, D17	D-loop(Mt)	C54	16s rRNA(Mt)
D20, D24	ND5(Mt)	C44	ND5(Mt)
D21	Ribosomal protein S15a	C46	Ribosomal protein S18
D25, D27, D28	Titin	C42, C58, C59, C66	Titin
D26	tRNA ^{leu} (Mt)	C66	tRNA ^{leu} (Mt)
D29, D30	COX1(Mt)	C60, C61	COX1(Mt)
D3	Germinal center kinase related kinase	C33	FXR1
D6, D14, D15	ND4(Mt)	C35, C36, C38, C40	ND4(Mt)
Nine clones	No match	Fourteen clones	No match

FXR1, human fragile X mental retardation protein 1 homolog; Mt, mitochondrial-encoded.

to known genes (ND1, D-loop, germinal center kinase related kinase, carbonic anhydrase, L-3-phosphoserine phosphatase) were observed in only one of the panels (Table 3). Signals corresponding to COX1, ND4, ND5, tRNA^{leu}, and titin genes appeared in both panels as different bands defined by different screening primer combinations. The ND1 clones were isolated only from the type 2 diabetes cDNA panel, and subsequent quantitative RT-PCR analysis of the ND1 mRNA level in the two pairs of fasting muscle biopsies used for cDNA differential display also showed increased ND1 gene expression in the type 2 diabetic patients (1.70–7.26 relative units) compared with control subjects (1.50–2.68 relative units).

RT-PCR quantitation in protocol 1

Insulin effect on mitochondrial gene expression. Insulin infusion increased gene expression of ND1 (baseline versus clamp in relative units, 1.02 ± 0.04 vs. 2.55 ± 0.30 , $P < 0.001$), COX1 (0.80 ± 0.01 vs. 1.24 ± 0.10 , $P < 0.001$), and D-loop (0.80 ± 0.02 vs. 1.27 ± 0.17 , $P < 0.01$) (Fig. 2). The stimulatory effect of insulin on ND1 and COX1 expression was observed in both control subjects (ND1, 1.10 ± 0.10 vs. 2.58 ± 0.52 , $P < 0.01$;

COX1, 0.80 ± 0.02 vs. 1.37 ± 0.23 , $P < 0.01$) and diabetic twins (ND1, 1.00 ± 0.05 vs. 2.36 ± 0.55 , $P < 0.01$; COX1, 0.80 ± 0.02 vs. 1.08 ± 0.10 , $P < 0.01$) (Fig. 2). The effect of insulin on ND1 (0.99 ± 0.07 vs. 2.72 ± 0.52 , $P < 0.01$) and COX1 (0.81 ± 0.03 vs. 1.28 ± 0.15 , $P < 0.01$) gene expression in nondiabetic twins was similar to that seen in the control subjects. Insulin stimulated D-loop gene expression in control subjects (0.86 ± 0.02 vs. 1.56 ± 0.38 , $P < 0.01$) with no significant effect in diabetic twins (0.76 ± 0.03 vs. 1.03 ± 0.15 , NS). Insulin had no significant effect on tRNA^{leu} gene expression in control subjects (0.76 ± 0.02 vs. 1.66 ± 0.58 , NS) or diabetic patients (0.72 ± 0.01 vs. 1.08 ± 0.24 , NS).

Analysis with the ribonuclease protection assay also showed that insulin infusion increased ND1 gene expression relative to β -actin from 0.37 ± 0.13 to 0.61 ± 0.11 relative units ($n = 5$, $P < 0.02$) (Fig. 3), which was compatible with the stimulatory effect of insulin on mitochondrial gene expression analyzed by quantitative RT-PCR. In keeping with the findings in diabetic and control subjects, insulin had no effect on the mitochondrial DNA copy number (baseline, 2.06 ± 0.59 ; clamp, 2.17 ± 0.26 relative units, NS); therefore, the observed effect reflected a true influence of insulin on gene expression rather than on mitochondrial DNA synthesis.

Relationship between mitochondrial gene expression and insulin sensitivity. Diabetic twins had lower rates of insulin-stimulated glucose uptake, glucose storage, and glucose oxidation compared with control subjects (Table 1). Nondiabetic monozygotic co-twins also had lower insulin-stimulated glucose uptake and glucose storage rates than control subjects (8.5 ± 0.8 vs. 11.4 ± 0.9 mg \cdot kg⁻¹ fat-free mass [FFM] \cdot min⁻¹, $P < 0.03$, and 4.8 ± 0.6 vs. 7.6 ± 0.9 mg \cdot kg⁻¹ FFM \cdot min⁻¹, $P < 0.02$, respectively) (Table 1). However, nondiabetic co-twins had similar rates of glucose oxidation compared with control subjects (3.7 ± 0.2 vs. 3.8 ± 0.2 mg \cdot kg⁻¹ FFM \cdot min⁻¹, NS) (Table 1). The postclamp ND1 mRNA levels correlated significantly with glucose uptake ($r = 0.45$, $P = 0.01$) and glucose storage ($r = 0.47$, $P < 0.01$). Although the postclamp mRNA levels of COX1 ($r = 0.30$, $P = 0.08$), D-loop ($r = 0.28$, $P = 0.10$), and tRNA^{leu} ($r = 0.22$, $P = 0.21$) did not correlate significantly with glucose uptake, the postclamp

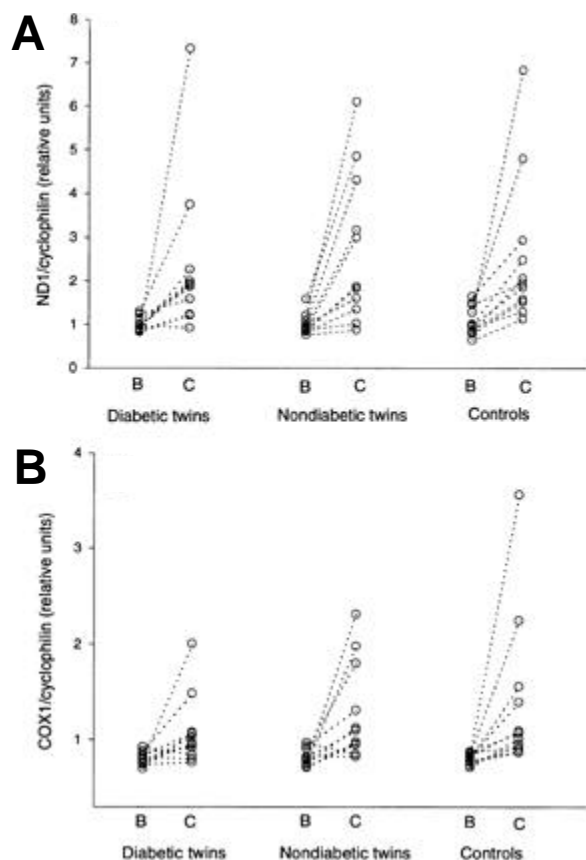


FIG. 2. Effect of insulin on mitochondrial gene expression. Total RNA was extracted from muscle biopsies taken from the subjects before and after a 3-h euglycemic-insulin clamp. Mitochondrial gene expression was then measured by relative RT-PCR and expressed relative to cyclophilin. **A:** Effect of insulin on ND1 mRNA. Insulin increased ND1 mRNA similarly in each study group (baseline vs. clamp, diabetic twins: 1.00 ± 0.05 vs. 2.36 ± 0.55 ; nondiabetic twins: 0.99 ± 0.07 vs. 2.72 ± 0.52 ; controls: 1.10 ± 0.10 vs. 2.58 ± 0.52 relative units, $P < 0.001$ for each group). **B:** Effect of insulin on COX1 mRNA. Insulin increased COX1 mRNA similarly in each study group (diabetic twins: 0.80 ± 0.02 vs. 1.08 ± 0.10 ; nondiabetic twins: 0.81 ± 0.03 vs. 1.28 ± 0.15 ; controls: 0.80 ± 0.02 vs. 1.37 ± 0.23 , $P < 0.01$ for each group).

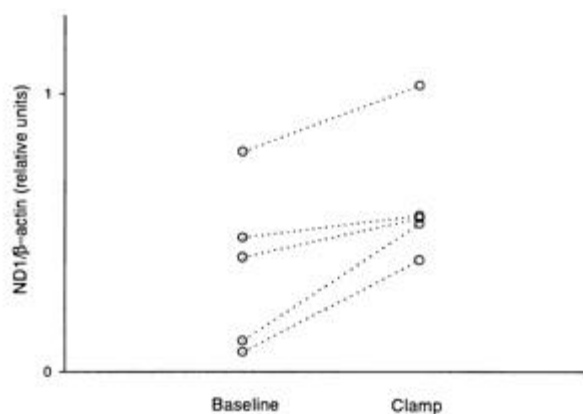


FIG. 3. Effect of insulin on ND1 gene expression analyzed with the ribonuclease protection assay. Total RNA was obtained from muscle biopsies of five healthy individuals before and after a 3-h insulin clamp. Total RNA (5 μ g) was analyzed with ND1 and β -actin antisense RNA probes in the ribonuclease protection assay. The protected bands were separated with 5% sequencing gel and quantitated with the phosphorImager. Insulin infusion increased ND1 gene expression relative to β -actin from 0.37 ± 0.13 to 0.61 ± 0.11 relative units ($P < 0.02$).

mRNA expression of these genes correlated with that of ND1 ($r = 0.85, 0.65, \text{ and } 0.53$, respectively; $P < 0.001$).

Intra-twin pair relationship of ND1 expression. In contrast to decreased insulin-mediated glucose metabolism in twins discordant for diabetes (Table 1), insulin-stimulated ND1 expression did not differ significantly between the diabetic and nondiabetic twins (2.36 ± 0.54 vs. 2.72 ± 0.52 relative units, NS) (Fig. 2) or between the nondiabetic twins and the control subjects (2.72 ± 0.52 vs. 2.58 ± 0.52 relative units, NS) (Fig. 2). There was no significant intrapair correlation for ND1 expression between the discordant monozygotic twins in the basal ($r = -0.15$, NS) or insulin-stimulated ($r = -0.28$, NS) state (Fig. 4).

RT-PCR quantitation in protocol 2

Relationship between ND1 expression and insulin sensitivity. Insulin-stimulated glucose uptake was significantly impaired in patients with type 2 diabetes and IGT compared with that in control subjects (1.4 ± 0.2 and 4.0 ± 0.45 vs. 5.9 ± 0.6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $P = 0.02$ and < 0.01 , respectively) (Table 2). The reduction in glucose uptake in patients with type 2 diabetes and IGT was mainly due to a reduction in glucose storage (1.2 ± 0.3 and 2.1 ± 0.4 vs. 3.8 ± 0.5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; $P < 0.02$ and < 0.01 , respectively) (Table 2). Type 2 diabetic patients had impaired glucose oxidation compared with control subjects (1.4 ± 0.2 vs. 2.1 ± 0.2 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $P < 0.01$) (Table 2). Insulin-stimulated ND1 expression correlated significantly with insulin-stimulated glucose uptake ($r = 0.46$, $P = 0.002$) (Fig. 5), glucose storage ($r = 0.44$, $P = 0.003$), and glucose oxidation ($r = 0.35$, $P = 0.02$). Insulin-stimulated ND1 expression in relation to FBG concentration is shown in Fig. 6. Although there was a great variation in ND1 gene expression, particularly in individuals with nondiabetic fasting glucose levels, ND1 expression tended to be lower in those with FBG concentrations > 8.3 mmol/l than in those with FBG concentrations < 8.3 mmol/l (0.69 ± 0.03 vs. 0.81 ± 0.04 relative units, $P = 0.11$). Among the patients with FBG concentrations > 8.3 mmol/l ($n = 9$), ND1 expression correlated with FBG levels ($r = 0.70$, $P = 0.03$), and glucose uptake also tended to increase with increasing FBG in these patients.

DISCUSSION

We isolated a total of 66 cDNA sequences as candidate differential signals by screening diabetic and control muscle

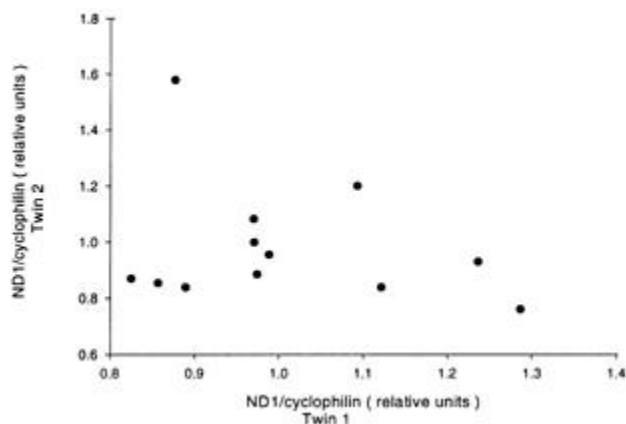


FIG. 4. Lack of intrapair correlation for ND1 expression between 24 monozygotic twins. ND1 expression was measured in basal muscle biopsies by relative RT-PCR and expressed relative to cyclophilin.

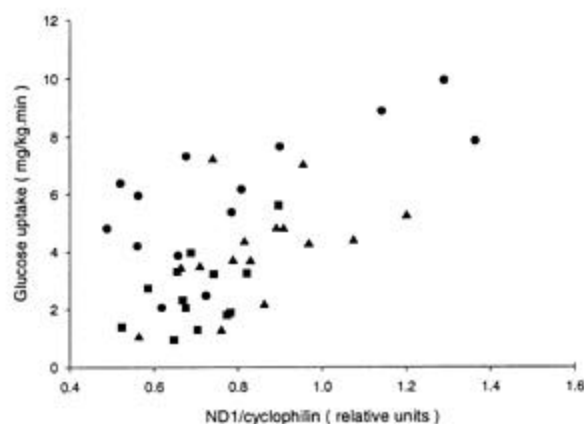


FIG. 5. Relationships between ND1 expression and insulin-stimulated glucose uptake measured during a 2-h euglycemic-hyperinsulinemic clamp. ND1 expression was measured by relative RT-PCR in muscle biopsies taken from subjects after the clamp and expressed relative to cyclophilin. ND1 expression correlated significantly with insulin-stimulated glucose uptake ($r = 0.46$, $P = 0.002$, $n = 42$). ■, type 2 diabetes; ▲, IGT; ●, control.

cDNA populations using cDNA differential display. These sequence signals represent genes involved in various cell functions, such as mitochondrial oxidative phosphorylation (ND1, COX1), cellular carbon dioxide/oxygen transport (carbonic anhydrase), protein targeting (ubiquitin hydrolase), and transcriptional regulation (germinal center kinase related kinase). Half of the identified signals represent mitochondrial-encoded genes, reflecting the relative abundance of mitochondrial gene expression in muscle. Most of the signals with matches to known genes were observed in the diabetic or control cDNA panel. Signals corresponding to COX1, ND4, ND5, tRNA^{leu}, and titin genes, however, were observed in both diabetic and control cDNA panels even though these signals in each case appeared as different bands defined by different screening primer combinations, suggesting a certain degree of sequence redundancy, probably due to the low stringent conditions during PCR display. The presence of sequence redundancy could to some extent lead to a dis-

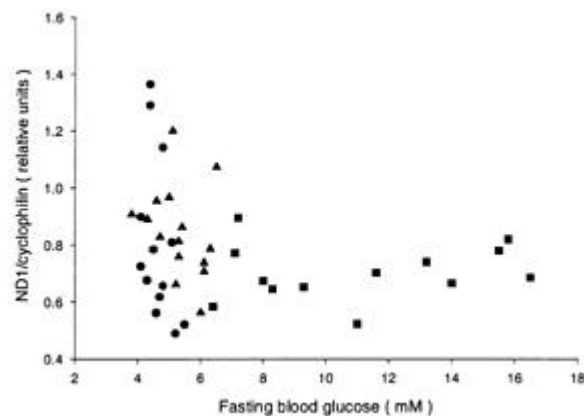


FIG. 6. Relationship between insulin-stimulated ND1 expression and FBG. ND1 expression showed a tendency of decrease with increasing FBG levels. The expression levels were somewhat lower at FBG > 8.3 mmol/l compared with < 8.3 mmol/l (0.69 ± 0.03 vs. 0.81 ± 0.04 , $P = 0.10$). Among the patients with FBG > 8.3 mmol/l , there was a slight increase in ND1 expression ($r = 0.70$, $n = 9$, $P < 0.05$). ■, type 2 diabetes; ▲, IGT; ●, control.

torted signal output. This may apply to signals corresponding to myosin, α -actin, and titin genes, since these genes encode muscle structural proteins and would be expressed constitutively rather than differentially. The mitochondrial ND1 signal, however, was observed only in the diabetic cDNA panel; this finding was compatible with the result of increased expression of ND1 analyzed with quantitative PCR in the same fasting diabetic muscle samples as used in the PCR display.

The mitochondrion is the major site for substrate oxidation and energy production in eukaryotes. Because impaired glucose metabolism and oxidative capacity have been observed in diabetic patients and their first-degree relatives (1,3–5), mitochondrial genes might play a role in the development of insulin resistance characteristic of the diabetic state. A number of human mitochondrial genes are encoded by the mitochondrial genome, which is a closed circular, double-stranded DNA of 16,569 bp. The heavy strand (H-strand) functions as the template for 12s and 16s mitochondrial rRNAs, 14 tRNAs, and 12 mRNAs including ND1, COX1, and tRNA^{leu} genes, while the light strand is the template for one mRNA and eight tRNAs. In this study, we demonstrated that insulin infusion increased muscle ND1, COX1, and D-loop mRNA expression during the insulin clamp. Further, the ND1 response correlated significantly with insulin-stimulated glucose uptake.

Insulin plays a key regulatory role in glucose uptake and metabolism. It has been shown that insulin regulates expression of nuclear-encoded genes involved in glucose metabolism and insulin action such as PEPCK, Rad, p85 α of PI3-kinase, GLUT4, and hexokinase II (17–19). Relatively little is known about the effect of insulin on expression of mitochondrial-encoded genes. Mitochondrial gene expression is regulated by the nuclear-encoded mitochondrial transcription factor A (20,21) and environmental factors such as hypoxia (22) and exercise (23). Insulin can now be added to this list, since insulin increased gene expression of ND1 and COX1 in muscle. This finding is in accordance with those from studies in rats in which insulin increased not only mitochondrial RNA expression in the liver (24) but also mitochondrial protein synthesis in the heart (25). The mechanisms by which insulin influences mitochondrial gene expression are unknown. By searching sequence similarity with known insulin response elements (IRE), we observed that a locus in the mitochondrial genome (positions 413–446, GenBank accession no. D38112) has 83% matched sequence similarity (24 of 29) to the 29-bp IRE of mouse amylase (26), and this locus also contains the IRE sequence motif T(G/A) TTTTG (27). Therefore, this locus near the 5' end of the 12s rRNA gene might be an IRE mediating the effect of insulin on mitochondrial gene expression. Glucose also induces transcription of a number of genes in liver (28) and muscle (29). Therefore, it is possible that the observed effect of insulin on mitochondrial gene expression could be mediated through glucose metabolism or insulin action.

Changes in mitochondrial gene expression could also be the result of changes in mitochondrial DNA copy number, since each mitochondrion contains 5–10 copies of DNA and each cell contains several hundreds of mitochondria. Differential expression of mitochondrial genes in different muscle fiber types has been correlated with the mitochondrial DNA copy number (30). In addition, exercise-induced changes in mito-

chondrial gene expression are also correlated with changes in mitochondrial DNA copy number (31). The changes in mitochondrial DNA copy number cannot explain the stimulatory effect of insulin on mitochondrial gene expression in this study, however, since mitochondrial DNA copy number was not affected by insulin. In accordance, insulin increased mitochondrial gene expression in the liver of rats without altering the mitochondrial DNA content (24). Also, thyroid hormone has been shown to increase mitochondrial gene expression in rat skeletal muscle and liver without affecting mitochondrial DNA content (32). It therefore seems likely that insulin stimulates mitochondrial ND1 gene expression through a pre-translational mechanism rather than through the effect on mitochondrial DNA synthesis. Mapping of mitochondrial gene transcripts has demonstrated that transcription of mitochondrial H-strand yields two primary transcripts, I and II (10). Since the ND1 gene is located on primary transcript II, which covers nearly the full length of the H-strand (10), it is likely that the regulatory effect of insulin may also include other genes on the mitochondrial H-strand. In support of this, ND1 gene expression correlated significantly with expression of some other mitochondrial genes, including COX1, tRNA^{leu}, and a D-loop region. In accordance, the coordinate changes in expression of four other mitochondrial H-strand genes (cytochrome oxidase 1 and 3, ND4, and 12s rRNA) have also been reported in muscle of patients with diabetes (8).

Because insulin resistance is observed already in nondiabetic offspring of type 2 diabetic patients (1), it has been regarded as an inherited trait. Insulin sensitivity correlated significantly with ND1 gene expression and partially with other mitochondrial gene expression, suggesting that ND1 gene expression could contribute to the inherited insulin resistance. This is less likely, however, since there was no intrapair correlation in ND1 expression between the monozygotic twins. Some caution is warranted in the interpretation of the twin data, since the twins did not show any intrapair correlation for insulin-stimulated glucose uptake, perhaps because they were discordant for diabetes and secondary changes in insulin sensitivity could have occurred as a consequence of glucose toxicity. Preliminary data from monozygotic and dizygotic twins suggest that insulin sensitivity is more influenced by environmental factors than insulin secretion (33). The correlation between ND1 gene expression and insulin-stimulated glucose uptake may represent the consequence rather than the cause of insulin resistance. In Fig. 6, a sharp decrease in ND1 gene expression is shown when FBG exceeds 8 mmol/l. The curve is almost superimposable on the curve of insulin-stimulated glucose uptake, which also decreases with increasing FBG. We therefore advance the hypothesis that ND1 gene expression is at least partially regulated by glucose flux into the cell; that is, when glucose flux decreases, mitochondrial gene expression decreases. This is further supported by the finding of a correlation between ND1 gene expression and FBG in the diabetic patients with glucose values >8.3 mmol/l. In these patients, we also observed a slight increase in glucose uptake with increasing FBG concentrations.

Antonetti et al. (8) reported increased basal mitochondrial H-strand-encoded gene expression in muscle of diabetic patients undergoing surgery. It is possible that the increase in mitochondrial gene expression could have been due to elevated insulin levels or mass action of glucose in poorly con-

trolled diabetic patients. To circumvent these problems, we studied patients and control subjects under similar hyperinsulinemic conditions during the insulin clamp. We observed no significant difference in insulin-stimulated ND1 expression between diabetic and control subjects in spite of a correlation between insulin-stimulated ND1 expression and insulin sensitivity. It was possible that a potential decrease in insulin-stimulated ND1 expression in the diabetic subjects was counteracted by hyperglycemia, since insulin-stimulated ND1 expression correlated with glucose uptake in those patients with FBG >8.3 mmol/l. This possibility is also compatible with the PCR display results of increased basal ND1 expression in the two diabetic patients with elevated FBG.

In summary, insulin infusion acutely increased mitochondrial-encoded ND1, COX1, and D-loop gene expression. The ND1 response to insulin correlated with insulin-stimulated glucose metabolism. This correlation may represent the consequence rather than the cause of insulin resistance, suggesting that glucose flux into the cell may regulate mitochondrial gene expression.

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REFERENCES

- Eriksson J, Franssila Kallunki A, Ekstrand A, Saloranta C, Widen E, Schalin C, Groop L: Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. *N Engl J Med* 321:337-343, 1989
- DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318-368, 1992
- Gumbiner B, Thorburn AW, Henry RR: Reduced glucose-induced thermogenesis is present in noninsulin-dependent diabetes mellitus without obesity. *J Clin Endocrinol Metab* 72:801-807, 1991
- Eriksson KF, Lindgarde F: Poor physical fitness, and impaired early insulin response but late hyperinsulinaemia, as predictors of NIDDM in middle-aged Swedish men. *Diabetologia* 39:573-579, 1996
- Groop L, Forsblom C, Lehtovirta M, Tuomi T, Karanko S, Nissen M, Ehrnstrom BO, Forsen B, Isomaa B, Snickars B, Taskinen MR: Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* 45:1585-1593, 1996
- van den Ouweland JM, Lemkes HH, Ruitenbeek W, Sandkuijl LA, de Vijlder MF, Struyvenberg PA, van de Kamp JJ, Maassen JA: Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nat Genet* 1:368-371, 1992
- Ballinger SW, Shoffner JM, Hedaya EV, Trounce I, Polak MA, Koontz DA, Wallace DC: Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion. *Nat Genet* 1:11-15, 1992
- Antonetti DA, Reynet C, Kahn CR: Increased expression of mitochondrial-encoded genes in skeletal muscle of humans with diabetes mellitus. *J Clin Invest* 95:1383-1388, 1995
- Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971, 1992
- Montoya J, Gaines GL, Attardi G: The pattern of transcription of the human mitochondrial rRNA genes reveals two overlapping transcription units. *Cell* 34:151-159, 1983
- Vaag A, Alford F, Beck Nielsen H: Intracellular glucose and fat metabolism in identical twins discordant for non-insulin-dependent diabetes mellitus (NIDDM): acquired versus genetic metabolic defects? *Diabet Med* 13:806-815, 1996
- Vaag A, Henriksen JE, Madsbad S, Holm N, Beck Nielsen H: Insulin secretion, insulin action, and hepatic glucose production in identical twins discordant for non-insulin-dependent diabetes mellitus. *J Clin Invest* 95:690-698, 1995
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159, 1987
- World Health Organization: *Diabetes Mellitus: Report of a WHO Study Group*. Geneva, World Health Org., 1985 (Tech. Rep. Ser. no., 727)
- Wong H, Anderson WD, Cheng T, Riabowol KT: Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. *Anal Biochem* 223:251-258, 1994
- Strauss WM: Preparation of genomic DNA from mammalian tissue. In *Current Protocols in Molecular Biology*. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, Eds. New York, Wiley, 1995, p. 2.2.1-2.2.3
- Hansson RW, Reshef L: Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* 66:581-611, 1997
- Laville M, Auboeuf D, Khalfallah Y, Vega N, Riou JP, Vidal H: Acute regulation by insulin of phosphatidylinositol-3-kinase, Rad, Glut 4, and lipoprotein lipase mRNA levels in human muscle. *J Clin Invest* 98:43-49, 1996
- Osawa H, Sutherland C, Robey RB, Printz RL, Granner DK: Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin. *J Biol Chem* 271:16690-16694, 1996
- Virbasius JW, Scarpulla RC: Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. *Proc Natl Acad Sci USA* 91:1309-1313, 1994
- Tiranti V, Rossi E, Ruiz Carrillo A, Rossi G, Rocchi M, DiDonato S, Zuffardi O, Zeviani M: Chromosomal localization of mitochondrial transcription factor A (TCF6), single-stranded DNA-binding protein (SSBP), and endonuclease G (ENDOG), three human housekeeping genes involved in mitochondrial biogenesis. *Genomics* 25:559-564, 1995
- Webster KA, Gunning P, Hardeman E, Wallace DC, Kedes L: Coordinate reciprocal trends in glycolytic and mitochondrial transcript accumulations during the in vitro differentiation of human myoblasts. *J Cell Physiol* 142:566-573, 1990
- Murakami T, Shimomura Y, Fujitsuka N, Nakai N, Sugiyama S, Ozawa T, Sokabe M, Horai S, Tokuyama K, Suzuki M: Enzymatic and genetic adaptation of soleus muscle mitochondria to physical training in rats. *Am J Physiol* 267:E388-E395, 1994
- Germanyuk YL, Minchenko AG: Effect of insulin on RNA and protein biosynthesis in liver mitochondria from normal and alloxan diabetic rats. *Endocrinol Exp* 12:233-243, 1978
- McKee EE, Grier BL: Insulin stimulates mitochondrial protein synthesis and respiration in isolated perfused rat heart. *Am J Physiol* 259:E413-E421, 1990
- Johnson TM, Rosenberg MP, Meisler MH: An insulin-responsive element in the pancreatic enhancer of the amylase gene. *J Biol Chem* 268:464-468, 1993
- O'Brien RM, Noisin EL, Suwanichkul A, Yamasaki T, Lucas PC, Wang JC, Powell DR, Granner DK: Hepatic nuclear factor 3- and hormone-regulated expression of the phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein 1 genes. *Mol Cell Biol* 15:1747-1758, 1995
- Towle HC: Metabolic regulation of gene transcription in mammals. *J Biol Chem* 270:23235-23238, 1995
- Tsao TS, Burcelin R, Charron MJ: Regulation of hexokinase II gene expression by glucose flux in skeletal muscle. *J Biol Chem* 271:14959-14963, 1996
- Williams RS: Mitochondrial gene expression in mammalian striated muscle: evidence that variation in gene dosage is the major regulatory event. *J Biol Chem* 261:12390-12394, 1986
- Williams RS, Salmons S, Newsholme EA, Kaufman RE, Mellor J: Regulation of nuclear and mitochondrial gene expression by contractile activity in skeletal muscle. *J Biol Chem* 261:376-380, 1986
- Wiesner RJ, Kurowski TT, Zak R: Regulation by thyroid hormone of nuclear and mitochondrial genes encoding subunits of cytochrome-c oxidase in rat liver and skeletal muscle. *Mol Endocrinol* 6:1458-1467, 1992
- Lehtovirta M, Kaprio J, Forsblom C, Tuomilehto J, Groop L: Insulin secretion and insulin sensitivity in monozygotic and dizygotic twin pairs (Abstract). *Diabetologia* 40:170A, 1997