Acute Hyperglycemia Induces a Global Downregulation of Gene Expression in Adipose Tissue and Skeletal Muscle of Healthy Subjects

Emmanuelle Meugnier,[1] May Faraj,[2] Sophie Rome,[1] Geneviève Beauregard,[2] Angélique Michaut,[1] Véronique Pelloux,[3] Jean-Louis Chiasson,[4] Martine Laville,[1] Karine Clement,[3] Hubert Vidal,[1] and Rémi Rabasa-Lhoret[2,4]

To define the effects of acute hyperglycemia per se (i.e., without the confounding effect of hyperinsulinemia) in human tissues in vivo, we performed global gene expression analysis using microarrays in vastus lateralis muscle and subcutaneous abdominal adipose tissue of seven healthy men during a hyperglycemic-euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release. We found that doubling fasting blood glucose values while maintaining plasma insulin in the fasting range modifies the expression of 316 genes in skeletal muscle and 336 genes in adipose tissue. More than 80% of them were downregulated during the clamp, indicating a drastic effect of acute high glucose, in the absence of insulin, on mRNA levels in human fat and muscle tissues. Almost all the biological pathways were affected, suggesting a generalized effect of hyperglycemia. The induction of genes from the metallothionein family, related to detoxification and free radical scavenging, indicated that hyperglycemia-induced oxidative stress could be involved in the observed modifications. Because the duration and the concentration of the experimental hyperglycemia were close to what is observed during a postprandial glucose excursion in diabetic patients, these data suggest that modifications of gene expression could be an additional effect of glucose toxicity in vivo. *Diabetes* 56:992–999, 2007

Altered glycemic control in individuals with type 1 and type 2 diabetes is associated with increased risk of micro- and macrovascular complications (1). The mechanisms of the deleterious effects of hyperglycemia, which is referred to as glucotoxicity, have been largely investigated. It is accepted that oxidative stress induced by hyperglycemia could be the main cause of the different pathways leading to diabetes complications (2,3). Importantly, acute glucose fluctuations exhibit a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia (4). Furthermore, acute hyperglycemia induces deleterious effects in various tissues and, from epidemiological studies, the harmful effect of hyperglycemia for cardiovascular complications appears to be mainly related to postprandial glucose excursion (5).

On the other hand, excess carbohydrate leads to the activation of several genes that promote storage of glycogen and triglycerides in liver, skeletal muscle, and adipose tissue (6). Although these effects are generally produced through a combined action with insulin, the identification of a glucose-responsive transcription factor named ChREBP (carbohydrate responsive element binding protein) (7,8) has recently shed new light on the mechanisms whereby glucose could directly affect gene transcription.

Until now, the effects of high glucose concentrations have mostly been studied in cell culture experiments and using animal models, and little is known about the in vivo molecular mechanisms of hyperglycemia in human tissues. The development of microarray technology offers powerful tools for characterizing the consequences of experimental hyperglycemia at the level of the transcriptome in accessible tissues such as skeletal muscle and subcutaneous adipose tissue. To study the effects of hyperglycemia without the confounding effect of insulin, a group of healthy volunteers was submitted to a 3-h hyperglycemic-euinsulinemic clamp using somatostatin infusion to block endogenous insulin release (9). The short duration of the experimental hyperglycemia should reduce possible secondary effects due to metabolic modifications and was chosen to simulate, at least in part, a postprandial glucose excursion.

RESEARCH DESIGN AND METHODS

The volunteers gave their written consent after being informed of the nature, purpose, and possible risks of the study. The experimental protocol was approved by the ethics committee of University of Montreal. The characteristics of the subjects are presented in Table 1. None had a familial or personal history of diabetes, obesity, dyslipidemia, or hypertension, and they were not taking medications.

The study was conducted in the postabsorptive state after a 12-h fast. Upon arrival, each subject was submitted to anthropometric measurements, and fasting serum samples (preclamp samples) and preclamp adipose tissue and skeletal muscle biopsies were taken from one side of the abdomen and one leg. One hour after the first blood sampling, the 3-h hyperglycemic-euinsulinemic clamp was started. In the last 30 min of the clamp, blood samples were collected (postclamp samples) at 10-min intervals, after which postclamp

From the [1]Institut National de la Santé et de la Recherche Médicale (INSERM) Unit 870, Institut National de la Rechereche Agronomique Unit 1235, Claude Bernard University, R. Laennec Faculty of Medicine, Lyon, France; the [2]Metabolic Dysfunctions Laboratory, Department of Nutrition, University of Montreal, Montreal, Quebec, Canada; [3]INSERM Unit 755 Nutriomique, University Pierre and Marie Curie–Paris 6, Faculty of Medicine, Les Cordeliers, France; and the [4]Research Center, Centre Hospitalier de l'Université de Montréal (CHUM), Montreal, Quebec, Canada.

Address correspondence and reprint requests to Hubert Vidal, UMR INSERM870/INRA1235, Faculté de Médecine R. Laennec, Rue G. Paradin, F-69372 Lyon Cedex 08, France. E-mail: vidal@sante.univ-lyon1.fr.

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E.M. and M.F. contributed equally to this work.

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ChoRE, carbohydrate response element; ChREBP, carbohydrate responsive element binding protein; SP-1, specificity protein 1.

TABLE 1
Baseline characteristics of the lean healthy men ($n = 7$)

Age (years)	21.7 ± 1.5
Weight (kg)	65.9 ± 5.2
BMI (kg/m^2)	22.1 ± 1.4
Total cholesterol (mmol/l)	4.0 ± 0.8
LDL cholesterol (mmol/l)	2.0 ± 0.4
HDL cholesterol (mmol/l)	1.5 ± 0.7
Nonesterified fatty acids (mmol/l)	0.71 ± 0.14
Triglyceride (mmol/l)	1.2 ± 0.3
Fasting glucose (mmol/l)	5.1 ± 0.3
Fasting insulin (mU/l)	9.4 ± 0.9
Fasting C-peptide (μg/l)	2.1 ± 0.4

Data are means ± SD.

adipose tissue and skeletal muscle biopsies were taken from the other side of the body.

Hyperglycemic-euinsulinemic clamp. The hyperglycemic-euinsulinemic clamp was a modification of the method used by Del Prato et al. (10). The objective was to increase plasma glucose 5.5 mmol/l above fasting level (11) by infusing 20% dextrose in two phases: *1*) bolus dose to increase glycemia to the desired target and *2*) continuous infusion dose adjusted every 5–10 min according to measured plasma glucose to maintain glycemia at the desired target. To maintain euinsulinemia, endogenous insulin secretion was inhibited using somatostatin (Sandosatin; Novartis Pharma, Dorval, Quebec, Canada). Sandostatin was infused in two phases: *1*) a bolus dose of 25 μg over 1 min given 5 min before the bolus of glucose and *2*) a continuous maintenance dose of 1.0 μg/min (10). Inhibition of endogenous insulin secretion was verified by measurement of plasma C-peptide. Insulin replacement was started at the same time of glucose infusion to maintain fasting insulin levels (continuous insulin infusion rate 3.45 mU/m^2 per min or 0.1 mU · kg^{-1} · min^{-1}). Four subjects agreed to repeat a clamp experiment without hyperglycemia but with similar infusion of somatostatin as a control study.

Adipose tissue and skeletal muscle biopsies. Biopsies were performed under local anesthesia (3 ml of 20 mg/ml Xylocaine). Subcutaneous abdominal white adipose tissue samples were obtained from the peri-umbilical level by needle biopsy. Percutaneous biopsies were obtained from the vastus lateralis muscle with a Weil Blakesley plier, as previously described (12,13). Postclamp adipose tissue and skeletal muscle samples were collected at the end of the clamp when serum glucose and insulin concentrations were still maintained at the target levels. Tissue samples were immediately frozen in liquid nitrogen and then stored at −80°C for later extraction of RNA.

Measurements of serum parameters. Glucose concentrations were measured with a glucose analyzer (Beckman Glucose, Mississauga, Ontario, Canada). Serum triglyceride and total and LDL cholesterol were measured using an automated analyzer (Beckman-Coulter, Brea, CA). Serum insulin and C-peptide were measured in duplicates with a commercial radioimmunoassay kit (Linco Research, St. Charles, MO).

Total RNA preparation, amplification, and microarray hybridization. Tissue samples were ground in liquid nitrogen, and total RNA was extracted using either the guanidinium thiocyanate–alcohol precipitation method for the skeletal muscle biopsies or the RNeasy total RNA mini kit (Qiagen, Courtaboeuf, France) for the fat tissue, as previously described (13). RNA concentrations and integrity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Because of low quality of the RNA preparation from the adipose tissue of one subject, the microarray study included seven subjects for the skeletal muscle and six subjects for the adipose tissue.

For both tissues, 500 ng total RNA were amplified using the MessageAmp aRNA kit (Ambion, Austin, TX). This amplification procedure is well validated, and it has been demonstrated that it does not distort the relative abundance of individual mRNAs within a RNA population (14,15). Amplified RNA (10 μg) from basal and hyperglycemic conditions of each subject was used to generate aminoallyl cDNA using Superscript II (Invitrogen, Eragny, France) and then chemically coupled with cyanine 3 (Cy3) or Cy5 dyes (GE Healthcare Biosciences, Orsay, France). They were hybridized overnight to the cDNA microarray slides according to the protocol of P. Brown's laboratory (http://cmgm.stanford.edu/pbrown/protocols/index.html), as previously described (12,16). The cDNA microarrays were obtained from the Microarray Core Facility of the Stanford School of Medicine (Stanford, CA) and consisted of 41,760 spots of amplified cDNAs (39,728 IMAGE clones corresponding to 19,186 UniGene clusters) printed on glass slides.

Analysis of microarray data. After hybridization, the microarray slides were scanned with a GenePix 4000A microarray scanner (Axon Instruments, Union City, CA) and the images were analyzed using GenePix Pro 3.0 software. Data files were entered into the Stanford Microarray Database. A uniform scale factor was applied to normalize signal intensities between Cy5 and Cy3 using linear regression analysis. Flagged spots and spots with fluorescence intensities below 2.5-fold above the background for both dyes were not taken into account. The $\log_2$(Cy5/Cy3) ratios of the other spots were calculated for each slide. To compare results from the different subjects, data from each slide were normalized in log-space to have a mean of zero using Cluster 3.0 software. Only spots with recorded data on all the slides were selected for further analysis. With these selection criteria, 35,187 spots were retrieved, corresponding to 33,953 IMAGE clones and 17,260 distinct UniGene clusters for skeletal muscle, and 34,806 spots, corresponding to 33,607 IMAGE clones and 17,023 distinct UniGene clusters for adipose tissue. Genes with a significantly different expression level during the hyperglycemic clamp were identified using the significant analysis of microarrays (SAM) procedure (17). The lists of regulated genes were established using a false discovery rate of 5% and taking into account the genes with a fold change higher than 1.4 (or −1.4).

Quantitation of mRNAs using real-time RT-PCR. First-strand cDNAs were synthesized from 500 ng total RNA in the presence of 100 units of Superscript II (Invitrogen, Eragny, France) using a mixture of random hexamers and oligo (dT) primers (Promega, Charbonnières, France). Real-time PCR assays were performed using a LightCycler (Roche Diagnostics, Meylan, France) as previously described (18). The list of the PCR primers is available on request (meugnier@univ-lyon1.fr).

Analysis of gene promoter sequences. The promoter sequences of the genes with significant changes in mRNA levels during the clamp (1,000 bp upstream of the transcription starting site) were retrieved from TRASER and analyzed using MatInspector from the Genomatix software package (Genomatix Suite release 3.0, München, Germany). A statistical method based on a z test (comparison of two proportions) was used to calculate the enrichment of the transcription factor binding sites in the promoter datasets of the genes of interest by comparison to their occurrence in sets of gene promoter sequences of the same size, randomly drawn in the list of genes present on the microarray (i.e., with fluorescence signal of their probes higher than 2.5-fold the background after the normalization procedures described above). A z value ≥1.98 was considered significant with an α error $P < 0.05$.

RESULTS

Baseline characteristics of the seven healthy lean young volunteers are presented in Table 1. During the hyperglycemic-euinsulinemic clamp, serum glucose was roughly doubled (preclamp: 5.1 ± 0.3; postclamp: 9.8 ± 1.2 mmol/l, $P < 0.0001$). Endogenous insulin secretion was inhibited by somatostatin infusion, as assessed by unchanged serum C-peptide level during the clamp (2.1 ± 0.4 vs. 2.7 ± 1.6 μg/l, $P = 0.255$). The low rate of exogenous insulin infusion to maintain basal fasting insulin level resulted in a slight, but significant, increased in serum insulin at the end of the clamp (preclamp: 9.4 ± 0.9; postclamp: 15.5 ± 4.0 μU/l, $P = 0.008$). Concomitantly, the plasma concentration of nonesterified fatty acids significantly decreased during the clamp (preclamp: 0.71 ± 0.14; postclamp: 0.27 ± 0.12 mmol/l, $P = 0.0013$).

Using cDNA microarrays, the global changes in gene expression induced during the clamp were analyzed in both skeletal muscle and subcutaneous adipose tissue. Our selection procedure sorted out a list of 316 significantly regulated genes in skeletal muscle and 336 in adipose tissue (see Tables 1S and 2S, respectively, in the online appendix available at http://dx.doi.org/10.2337/db06-1242). More than 80% of these genes were downregulated during the clamp in both tissues (266 out of 316 in muscle and 274 out of 336 in adipose tissue). The top 15 up- and downregulated genes in the two tissues, when taking into account their fold change during the hyperglycemic clamp, are presented in Table 2. To validate the microarray results, changes in mRNA expression of 17 genes regulated during hyperglycemic clamp (both up- and

TABLE 2
List of the top 15 up- and downregulated genes in skeletal muscle and adipose tissue during the hyperglycemic-euinsulinemic clamp

	Name	Symbol	Fold change	Cytoband
Skeletal muscle (UG cluster)				
Hs.517581	Heme oxygenase (decycling) 1	HMOX1	3.80	22q12
Hs.432132	Putative lymphocyte G0/G1 switch gene	G0S2	2.84	1q32.2-q41
Hs.374950	Metallothionein 1X	MT1X	2.20	16q13
Hs.516105	Actin, gamma 2, smooth muscle, enteric	ACTG2	1.70	2p13.1
Hs.460867	Metallothionein 1B (functional)	MT1B	1.70	16q13
Hs.591761	Homer homolog 1 (Drosophila)	HOMER1	1.69	5q14.2
Hs.507916	TSC22 domain family, member 1	TSC22D1	1.67	13q14
Hs.591319	Ubiquitin specific protease 13 (isopeptidase T-3)	USP13	1.63	3q26.2-q26.3
Hs.513490	Aldolase A, fructose-bisphosphate	ALDOA	1.62	16q22-q24
Hs.584744	Calcyphosine	CAPS	1.60	19p13.3
Hs.148778	Oxidation resistance 1	OXR1	1.60	8q23
Hs.475353	LIM and cysteine-rich domains 1	LMCD1	1.58	3p26-p24
Hs.478275	Tumor necrosis factor (ligand) super family, member 10	TNFSF10	1.58	3q26
Hs.334347	Creatine kinase, muscle	CKM	1.55	19q13.2-q13.3
Hs.154084	Phosphorylase, glycogen; muscle	PYGM	1.51	11q12-q13.2
Hs.8364	Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	−2.88	7q21.3-q22.1
Hs.272499	Dehydrogenase/reductase (SDR family) member 2	DHRS2	−2.34	14q11.2
Hs.98255	Receptor tyrosine kinase-like orphan receptor 2	ROR2	−2.28	9q22
Hs.506663	Huntingtin interacting protein E	HYPE	−2.26	12q24.1
Hs.591337	V-myb myeloblastosis viral oncogene homolog (avian)	MYB	−2.21	6q22-q23
Hs.420269	Collagen, type VI, alpha 2	COL6A2	−2.14	21q22.3
Hs.567612	Major facilitator super family domain containing 7	MFSD7	−2.10	4p16.3
Hs.106019	Protein phosphatase 1, regulatory subunit 10	PPP1R10	−2.05	6p21.3
Hs.483238	Rho GTPase activating protein 29	ARHGAP29	−1.96	1p22.1
Hs.591091	Paired box gene 5 (B-cell lineage specific activator)	PAX5	−1.96	9p13
Hs.511748	Semaphorin 4D	SEMA4D	−1.93	9q22-q31
Hs.204238	Lipocalin 2 (oncogene 24p3)	LCN2	−1.93	9q34
Hs.131431	Eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	−1.93	2p22-p21
Hs.459952	Stannin	SNN	−1.92	16p13
Hs.160264	Hyperpolarization activated cyclic nucleotide-gated potassium channel 4	HCN4	−1.92	15q24-q25
Adipose tissue (UG cluster)				
Hs.293274	Adenomatosis polyposis coli downregulated 1	APCDD1	1.69	18p11.22
Hs.591588	Hexokinase 2	HK2	1.65	2p13
Hs.443750	Vacuolar protein sorting 45A (yeast)	VPS45A	1.58	1q21-q22
Hs.34114	ATPase, Na^+/K^+ transporting, alpha 2 (+) polypeptide	ATP1A2	1.58	1q21-q23
Hs.499205	Iroquois homeobox protein 3	IRX3	1.57	16q12.2
Hs.154652	Chromosome 8 open reading frame 72	C8orf72	1.55	8q12.1
Hs.179260	Chromosome 14 open reading frame 4	C14orf4	1.52	14q24.3
Hs.67201	5′,3′-nucleotidase, cytosolic	NT5C	1.48	17q25.1
Hs.524579	Lysozyme (renal amyloidosis)	LYZ	1.48	12q15
Hs.520554	Tubulin tyrosine ligase-like family, member 2	TTLL2	1.48	6q27
Hs.6136	Rho GTPase activating protein 20	ARHGAP20	1.47	11q22.3-q23.1
Hs.154433	Cyclic nucleotide gated channel beta 3	CNGB3	1.46	8q21-q22
Hs.369592	Thyroid adenoma associated	THADA	1.46	2p21
Hs.164267	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	DYRK3	1.46	1q32.1
Hs.122038	Acyl-CoA thioesterase 6	ACOT6	1.46	14q24.3
Hs.466743	Mitogen-activated protein kinase kinase kinase 10	MAP3K10	−3.06	19q13.2
Hs.280987	MutS homolog 3 (*E. coli*)	MSH3	−2.89	5q11-q12
Hs.590891	Phosphoglucomutase 3	PGM3	−2.69	6q14.1-q15
Hs.569908	Transcription factor 4	TCF4	−2.63	18q21.1
Hs.159525	Cell growth regulator with EF-hand domain 1	CGREF1	−2.62	2p23.3
Hs.495731	BMX non-receptor tyrosine kinase	BMX	−2.53	Xp22.2
Hs.24485	Chondroitin sulfate proteoglycan 6 (bamacan)	CSPG6	−2.53	10q25
Hs.2561	Nerve growth factor, beta polypeptide	NGFB	−2.46	1p13.1
Hs.541273	V-akt murine thymoma viral oncogene homolog 2	AKT2	−2.46	19q13.1-q13.2
Hs.463466	Carbonic anhydrase X	CA10	−2.32	17q21
Hs.86492	Small muscle protein, X-linked	SMPX	−2.31	Xp22.1
Hs.431417	Arylalkylamine N-acetyltransferase	AANAT	−2.30	17q25
Hs.478199	Protein kinase C, iota	PRKCI	−2.28	3q26.3
Hs.521482	Src homology 2 domain containing adaptor protein B	SHB	−2.25	9p12-p11
Hs.276925	GTP binding protein 1	GTPBP1	−2.24	22q13.1

TABLE 3
Comparison of microarray results with real-time RT-PCR

Transcript symbol	Unigene cluster	Skeletal muscle		Adipose tissue	
		Microarray (fold change)	qPCR (fold change)	Microarray (fold change)	qPCR (fold change)
GSTP1	Hs0.523836	−1.74	−1.45 ± 0.08*	−1.54	−1.41 ± 0.08*
PDK4	Hs0.8364	−2.88	−4.09 ± 0.99*	−2.17	−2.49 ± 0.36*
PER1	Hs0.445534	−1.66	−1.75 ± 0.20*	−1.60	−1.55 ± 0.17*
PGM3	Hs0.590891	−1.40	−1.03 ± 0.10	−2.69	−1.66 ± 0.14*
TCEB3	Hs0.584806	−1.73	−1.47 ± 0.03*	−1.85	−1.41 ± 0.10*
TNFSF10	Hs0.478275	1.58	1.47 ± 0.21*	1.41	1.76 ± 0.23*
ACACB	Hs0.234898	1.40	1.36 ± 0.15*	NR	1.09 ± 0.18
FOXO1A	Hs0.370666	1.47	1.96 ± 0.25*	NR	1.15 ± 0.14
G0S2	Hs0.432132	2.84	2.01 ± 0.48*	NR	1.12 ± 0.16
IRS1	Hs0.471508	−1.51	−1.55 ± 0.13*	NR	1.05 ± 0.21
JAM2	Hs0.517227	−1.67	−1.53 ± 0.17*	NR	−1.02 ± 0.09
MT1X	Hs0.374950	2.2	1.54 ± 0.13*	NR	1.53 ± 0.14*
MT1F	Hs0.513626	1.47	ND	NR	2.34 ± 0.46*
MT2A	Hs0.534330	1.45	1.41 ± 0.14*	NR	1.37 ± 0.12*
AGTRL1	Hs0.438311	NR	1.29 ± 0.10	−1.53	−1.33 ± 0.13*
HK2	Hs0.591588	NR	1.51 ± 0.20*	1.65	1.52 ± 0.24*
PSMA4	Hs0.251531	NR	−1.05 ± 0.06	1.40	1.43 ± 0.21*
SREBP1c	Hs0.592123	NR	1.18 ± 0.19	NR	1.49 ± 0.23*
ChREBP	Hs0.520943	NR	1.21 ± 0.16	NR	1.08 ± 0.12
MTF1	Hs0.591505	NR	1.01 ± 0.12	NR	−1.12 ± 0.16

The fold changes in mRNA levels during the hyperglycemic clamp were measured on tissue samples from seven subjects for skeletal muscle and six subjects for adipose tissue. RT-qPCR was performed as indicated in RESEARCH DESIGN AND METHODS. Values were corrected by the mRNA level of hypoxanthine phosphoribosyl transferase used as a housekeeping gene (18). *$P < 0.05$ after vs. before clamp, using the Student's paired t test. Transcripts found to be not regulated during the clamp using microarrays (i.e., fold change <1.4) are indicated with NR (not regulated). ND, not detectable in a reliable manner using RT-qPCR.

downregulated) were verified using real-time PCR (Table 3).

Four volunteers accepted to repeat a control study with infusion of somatostatin for 3 h, at the same rate as in the hyperglycemic-euinsulinemic clamps. Under these experimental conditions, insulinemia decreased (11.5 ± 2.5 vs. 6.2 ± 1.6 μU/l, $P = 0.005$) and glycemia slightly increased (5.0 ± 0.1 vs. 7.1 ± 1.1 mmol/l, $P = 0.029$). Skeletal muscle and adipose tissue samples before and after somatostatin infusion were analyzed by real-time PCR. As shown in the Table 3S, the variations in the mRNA levels observed during the hyperglycemic clamp were not found in the somatostatin control study, and opposite regulation was observed for AGTRL1 in skeletal muscle, GSTP1 in adipose tissue, and PDK4 in both tissues. These data suggest therefore a minor contribution of somatostatin infusion to the overall change in gene expression observed during the hyperglycemic clamp.

Using gene ontology (www.geneontology.org) and manual assignment based on SOURCE (http://smd-www.stanford.edu/cgi-bin/source/sourceSearch), OMIM, and PubMed, the genes were classified into 13 functional groups (Tables 1S and 2S): 12 functional groups encompassing most of the biological processes and a group of hypothetical proteins and expressed sequence tag (EST). The number of up- and downregulated genes in each of these groups is presented in Fig. 1. The changes in gene expression during the clamp displayed very similar patterns in the two tissues. Most of the functional groups were characterized by a profound downregulation in gene expression, during the clamp, in both tissues (Fig. 1). Interestingly, the genes classified under the terms "enzymes" and "response to stress" appeared to be less affected and presented a higher proportion of upregulated genes than the others. For this latter category, the difference was mostly evident in the muscle (Fig. 1). When taking into account the two tissues, 16 genes out of 48 (33%) for "enzymes" and 8 out of 16 (50%) for "response to stress" were upregulated during the clamp. Regarding the genes coding for enzymatic proteins, it was noticeable that the expression of several key genes involved in glucose utilization and metabolism (such as hexokinase 2, phosphofructokinase, aldolase, and acetyl-CoA carboxylase β) were increased (Tables 1S and 2S). In parallel, the expression of pyruvate dehydrogenase kinase 4 (PDK4), which negatively controls the rate of glucose oxidation, was strongly decreased in both tissues. Regarding the genes related to the response to stress, seven genes were upregulated in the skeletal muscle, including several members of the metallothionein family. They are also present in the list of the most upregulated genes during the clamp (Table 2), and the variation of the expression of MT1X and MT2A mRNAs was confirmed by quantitative PCR (Table 3). Interestingly, significant upregulation was also found in adipose tissue using PCR, whereas the changes did not reach the selection criteria in the microarray experiments (fold change of 1.32 for MT1X, 1.38 for MT1F, and 1.12 for MT2A).

To get more insight into the transcriptional mechanisms that may contribute to the changes in gene expression during the hyperglycemic clamp, we analyzed the promoter sequences of the affected genes. Using TRASER, we were able to retrieve the promoter sequences of 90% of the 605 genes regulated during the hyperglycemic clamp.

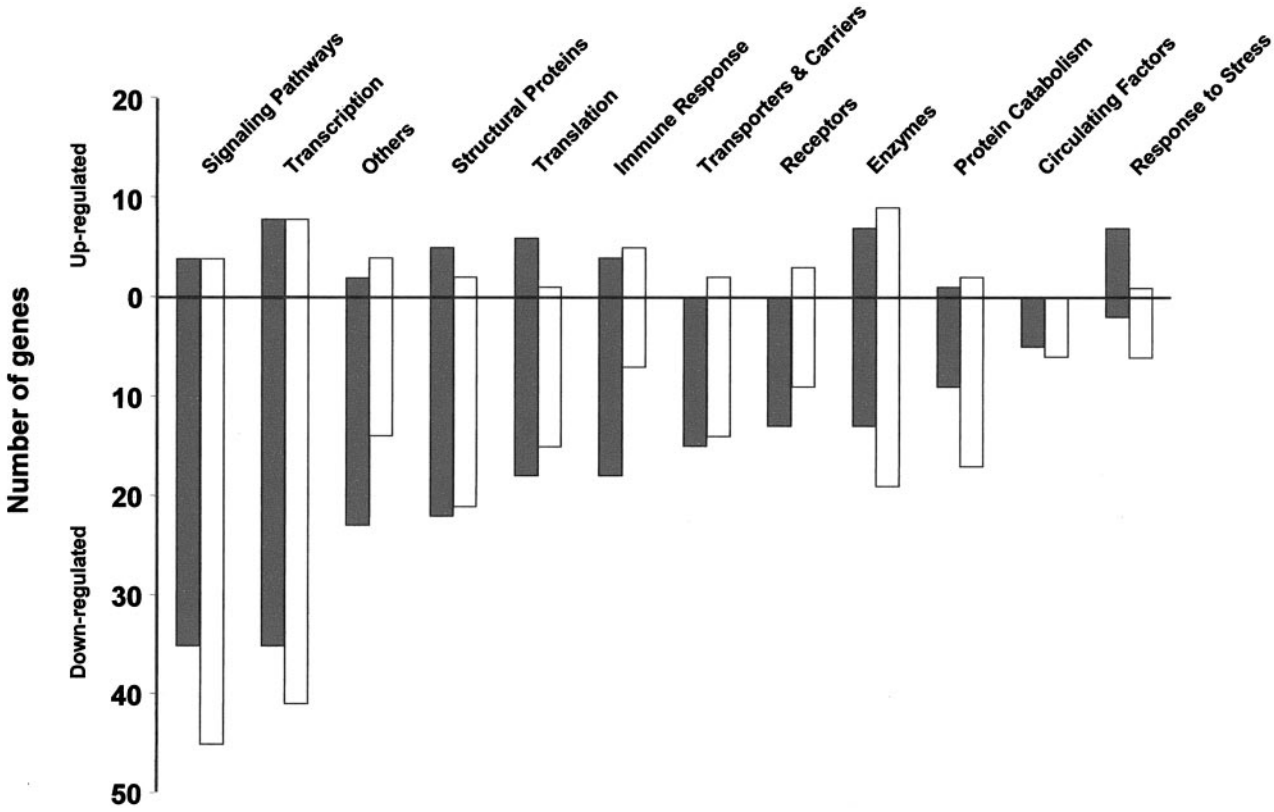

FIG. 1. Functional group distribution of the up- and downregulated genes during the hyperglycemic clamp in skeletal muscle and adipose tissue. ■, skeletal muscle; □, adipose tissue.

Putative binding sites for transcription factors were searched for using MatInspector software, and their frequency in the sets of regulated genes was statistically compared toward sets of randomly drawn genes using a z test. Table 4 shows the transcription factor matrices with significantly different occurrence between the two sets (z score >1.98). Regarding the upregulated genes, four transcription factor matrices were more frequently found. Of interest, 44% of the upregulated genes have a metal responsive element consensus sequence for MTF1 (metal transcription factor 1), which is involved in the regulation of the metallothioneins. Nine matrices were found to be enriched in the set of downregulated genes. NRSF (neuron-restricted silencer factor) and FKHD (fork head domain factors) are known repressors of transcription. However, the difference from the set of nonregulated genes appears too small to support a critical contribution of these factors in the observed massive downregulation of gene expression (Table 4).

We also looked more specifically at the presence of the carbohydrate response element (ChoRE). ChoRE is recognized by the transcription factor ChREBP, which was recently proposed as a mediator of the positive transcriptional effects of glucose in the liver (8). A total of 77 genes were found to contain a ChoRE in the set of regulated genes during the clamp. However, its prevalence was not different in the regulated genes or in sets of randomly drawn genes (Table 4).

DISCUSSION

The aim of the present study was to gain more insight into the molecular mechanisms contributing to the effects of acute hyperglycemia per se (i.e., without confounding effect of hyperinsulinemia) in human skeletal muscle and adipose tissue. Using cDNA microarrays, we performed a global analysis of the changes in gene expression in vastus lateralis muscle and subcutaneous abdominal adipose tissue of healthy control subjects during a hyperglycemic-euinsulinemic clamp with infusion of somatostatin to inhibit endogenous insulin release. This method has already been used to study the effect of hyperglycemia per se on glucose metabolism in normal subjects (9,10). Blood glucose concentration was maintained for 3 h in the range classically observed during postprandial glucose excursions in impaired glucose-tolerant subjects and in ~40% of the type 2 diabetic patients (19). Although exogenous insulin was infused at a low rate, a modest increase in insulinemia was observed. However, this variation remained within the fasting physiological range and was negligible when compared with the concentrations reached during the postprandial state in healthy individuals (20).

Using microarray analysis, we found that doubling fasting plasma glucose values while maintaining plasma insulin in the fasting range modifies the expression of 316 genes in skeletal muscle and 336 in adipose tissue. Because we analyzed crude tissue biopsies, it should be taken into account that these changes could reflect regulation in different cell types that are present in the tissues, such as endothelial cells or fibroblasts. More than 80% of the affected genes were downregulated at the end of the hyperglycemic clamp. This indicates a drastic effect of acute high glucose, in the absence of insulin, on mRNA levels in human fat and muscle tissues.

Multiple mechanisms have been described to explain the effects of chronic hyperglycemia (2). Oxidative stress

TABLE 4
Gene promoter analysis

Transcription factor matrix	Upregulated genes (n = 84)	Randomly drawn genes (n = 84)	z score
HICF	51.2	34.0	3.099
XBBF	71.4	52.4	2.542
FAST	25.0	42.9	2.444
MTF1	44.0	28.6	2.086
PRDF	48.8	33.3	2.039
CHRE	11.9	13.1	0.233
NFκB	66.7	73.8	1.012
SP-1F	92.8	90.1	0.806
	Downregulated genes (n = 472)	Randomly drawn genes (n = 472)	
SF-1F	49.4	38.8	3.278
EREF	41.3	32.6	2.764
NRSF	56.8	48.5	2.542
MINI	50.0	42.4	2.350
MOKF	65.3	57.8	2.342
HEN1	27.5	21.2	2.275
FKHD	78.6	72.2	2.268
OAZF	37.7	30.7	2.264
FAST	43.4	36.9	2.058
ChoRE	14.2	16.7	1.080
NFKB	70.1	66.7	1.120
SP-1F	84.4	82.2	1.325

Data are percentages unless otherwise indicated. The promoter sequences (1,000 bp upstream from the transcription start site) of the genes upregulated (n = 84) and downregulated (n = 472) during the clamp were analyzed for the presence of transcription factor matrices using MatInspector from Genomatix. The table shows the matrices displaying a significantly different frequency in the sets of regulated genes when compared with the same size sets of randomly drawn genes using a z test. A significant difference (α error <5%) corresponds to a z score >1.98. The score of three transcription factor matrices that did not reach significance and that are discussed in the text (ChoRE, nuclear factor κB [NFκB], and SP-1) are also presented.

is proposed as a unifying paradigm (2,21). Importantly, there is growing evidence that not only chronic, but also acute increases in glycemia can generate an oxidative stress. This was clearly demonstrated in vivo in type 2 diabetic patients (4). Interestingly, a significant increase in the plasma level of nitrotyrosine, a marker of oxidative stress, has been observed during a 2-h hyperglycemic clamp in healthy subjects (22). In the present study, we observed a marked induction of the mRNA expression of several genes belonging to the metallothioneins, which are cysteine-rich proteins acting as scavengers of free radicals. They are induced in response to reactive oxygen species production (23,24). Interestingly, 44% of the upregulated genes during the hyperglycemic clamp contain a consensus sequence for MTF1, a transcription factor known to activate metallothionein gene expression and that is potentially involved in the response to oxidative stress (25). A more thorough analysis of the function of these genes demonstrates that most of them (14 of 35) have been previously shown to be regulated in response to various biological stresses and that five were implicated in the regulation of apoptosis (Table 4S). We also found a significant upregulation of FOXO1A mRNA in skeletal muscle. FOXO members have been implicated in cellular protection against oxidative stress (26). These data strongly suggested that an oxidative stress and probably the production of reactive oxygen species in skeletal muscle and adipose tissue were induced during the hyperglycemic-euinsulinemic clamp. It should be noticed, however, that we did not observe a systemic induction of oxidative stress during the clamp when measuring markers such as oxidized LDL, nitrotyrosine, or total antioxidant capacity in plasma samples (data not shown). This suggests that the effects of 3 h of hyperglycemia were mostly at the tissue level, as supported by a recent report showing that hyperglycemic clamp in rodents induces oxidative stress locally in adipose tissue (27).

The modification of the activity of specific transcription factors by oxidative stress could be a possible mechanism to explain the observed global downregulation of gene expression during the hyperglycemic clamp. Hyperglycemia and reactive oxygen species overproduction have been shown to promote O-linked glycosylation of the transcription factor specificity protein 1 (SP-1) in cultured endothelial cells (28). However, this is unlikely to explain the present observation since long-term incubations with high glucose concentration were required for this effect to occur, and, more importantly, SP-1 modification increases its transcriptional activity (28). Moreover, the frequency of the potential SP-1 binding site in the promoter region of the downregulated genes is not different from what can be found in a set of randomly selected genes (Table 4). Another potential candidate is the transcription factor nuclear factor κB. Its activation by high glucose concentration and oxidative stress is well demonstrated in various cell types (29,30). This generally leads to the induction of genes related to apoptosis and inflammatory response. However, currently there are no data showing generalized downregulation of gene expression in response to nuclear factor κB activation. The search for the DNA motif recognized by nuclear factor κB in the sequences of the gene promoters indicated that 70% of the downregulated genes

could be potential targets of this transcription factor. However, the same frequency (67%) was also found in nonregulated genes (Table 4). Regarding other transcription factors, the analysis of the promoter sequences did not point out new candidates to explain the downregulation of gene expression during the clamp. In addition to transcriptional regulation, it is important to take into account that the changes in mRNA levels during the hyperglycemic clamp may also be the result of modifications of mRNA stability. Although less information is available regarding the regulatory mechanisms in vivo (31), it is likely that they could play a role in the marked downregulation of gene expression observed in a few hours only in muscle and adipose tissue.

Whereas >80% of the genes were downregulated during the hyperglycemic clamp, it should not be neglected that there was a positive effect on the mRNA expression of ~100 genes. Among them, we found an increase in the mRNA levels of several genes of the glycolytic and glucose oxidation pathways, such as hexokinase 2 (HK2), phosphofructokinase (PFKP), and acetyl-CoA carboxylase β (ACACB), which may suggest activation of glucose metabolism during the hyperglycemic clamp. This concerted induction could be a consequence of the slight rise in insulinemia during the clamp. Nevertheless, under similar experimental conditions, it has been reported that hyperglycemia per se is able to induce glucose utilization in normal subjects (9). In rodents, a positive effect of acute hyperglycemia on glycolytic and lipogenic genes is also classically observed in liver, both in vivo and in vitro (6). The transcription factor ChREBP has been proposed to mediate this effect (7,8). Using RT-qPCR, we found that ChREBP mRNA is expressed in human adipose tissue and skeletal muscle (31.5 ± 6.6 and 3.9 ± 0.5 amol/μg total RNA, respectively). Its expression was not regulated during the hyperglycemic clamp (Table 3). The gene promoter analyses revealed that the frequency of a ChoRE motif is not different in sets of regulated or randomly drawn genes, suggesting that ChREBP may not play a major role in the changes observed during the hyperglycemic clamp in adipose tissue and skeletal muscle.

Glucose excursion, particularly during the postprandial period, is now regarded as an important risk factor for diabetes complications (5,32,33). It is well demonstrated that repetition of hyperglycemia over time produces the strongest deleterious effects in diabetic patients (4,34,35). In the present study, we found that a single spike of hyperglycemia for 3 h in healthy subjects is able to produce a marked reduction in the expression of genes coding proteins involved in almost all the biological processes. It is likely that this effect occurred because insulinemia was maintained at a basal level during the hyperglycemic clamp. Indeed, insulin promotes efficient glucose metabolism and various anabolic effects in most tissues. We have demonstrated that hyperinsulinemia induces a strong regulation of gene expression in human skeletal muscle with upregulation of >500 genes (12). However, it could not be excluded that other factors related to the experimental procedure may have contributed to the observed effects. Somatostatin inhibits not only insulin release, but also glucagon and growth hormone production. However, the data from the somatostatin control study did not support a major contribution of somatostatin infusion per se in the observed changes in gene expression during the hyperglycemic clamp. Changes in other metabolic parameters, such as nonesterified fatty acids that decreased during the clamp, could also eventually affect gene expression in peripheral tissues.

In summary, the present study demonstrates that 3 h of hyperglycemia while maintaining basal fasting insulinemia in healthy subjects provokes a marked reduction in the mRNA levels of about 500 genes in skeletal muscle and adipose tissue. Almost all the biological pathways appear to be affected. In parallel, the induction of a number of genes related to detoxification and free radical scavenging indicates that hyperglycemia-induced oxidative stress could be involved. Because the duration and the concentration of the experimental hyperglycemia could simulate a postprandial glucose excursion in diabetic patients with limited or no insulin production, these data suggest that modifications of gene expression could be a novel mechanism taking place in the pathological processes of hyperglycemia.

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