

**Acute hyperglycemia induces a global down-regulation of gene expression
in adipose tissue and in skeletal muscle of healthy subjects.**

*Received for publication on 5 September 2006 and accepted in revised form on
8 January 2007.*

**Emmanuelle MEUGNIER^{1,*}, May FARAJ^{2,*}, Sophie ROME¹, Geneviève
BEAUREGARD², Angélique MICHAUT¹, Véronique PELLOUX³, Jean-Louis
CHIASSON⁴, Martine LAVILLE¹, Karine CLEMENT³, Hubert VIDAL^{1,\$} and Rémi
RABASA-LHORET^{2,4}**

¹INSERM unit 449; INRA unit 1235; Claude Bernard University-Lyon 1, IFR 62, R. Laennec
Faculty of Medicine, Lyon, France.

²Metabolic Dysfunctions Laboratory, Department of Nutrition, University of Montreal,
Montreal, Canada

³INSERM unit 755 Nutriomique; University Pierre and Marie Curie-Paris 6, Faculty of
Medicine, Les Cordeliers, France

⁴Research Center, Centre hospitalier de l'Université de Montréal (CHUM), Montreal, Canada

* E. Meugnier and M. Faraj contributed equally to this work

Running title: Hyperglycemia down-regulates gene expression

^{\$} corresponding author :

Hubert VIDAL.
UMR INSERM449/INRA1235,
Faculté de Médecine R Laennec,
Rue G. Paradin,
F-69372 Lyon Cedex 08,
FRANCE.
Phone : 33 478 77 10 04
Fax : 33 478 77 87 62
E-mail : vidal@sante.univ-lyon1.fr

ABSTRACT

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 7 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 in adipose tissue. More than 80% of them were down-regulated 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 post-prandial glucose excursion in diabetic patients, these data suggest that modifications of gene expression could be an additional effect of glucose toxicity *in vivo*.

INTRODUCTION

Altered glycemic control in individuals with type 1 and type 2 diabetes mellitus 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 diabetic 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 hour hyperglycaemic-euinsulinemic

(HG-EI) 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

Subjects and study design. 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 post-absorptive state after a 12-hour fast. Upon arrival, each subject was submitted to anthropometric measurements, fasting serum samples (pre-clamp samples) and pre-clamp 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-hour hyperglycemia euinsulinemia (HGEI) clamp was started. In the last 30 minutes of the clamp, blood samples were collected (post-clamp samples) at 10-minute intervals after which a post-clamp adipose tissue and skeletal muscle biopsies were taken from the other side of the body.

Hyperglycemic euinsulinemic (HGEI) clamp. The HGEI 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 to 10 minutes according to measured plasma glucose to maintain glycemia at the desired target. In order to maintain euinsulinemia, endogenous insulin secretion was inhibited using somatostatin (Sandostatin, Novartis Pharma Inc, Dorval, 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/m²/min or 0.1 mU/kg/min). 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 periumbilical level by needle biopsy. Percutaneous biopsies were obtained from the *vastus lateralis* muscle with a Weil Blakesley plier, as previously described (12, 13). Post-clamp 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 analyzer, Mississauga, Canada). Serum triglyceride (TG) 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 guanidinium thiocyanate-alcohol precipitation method for the skeletal muscle biopsies or the RNeasy total RNA minikit (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). Due to 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 of 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). Ten μ g of amplified RNA from basal and hyperglycemic conditions of each subject was used to generate aminoallyl cDNA using Superscript II (Invitrogen, Eragny, France), 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 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 $\log_2(\text{Cy5/Cy3})$ 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 of 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 base pairs upstream of the transcription starting site) were retrieved from TRASER (<http://genome-www6.stanford.edu/cgi-bin/Traser/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 gene 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 alpha error $P < 0.05$.

RESULTS

Baseline characteristics of the seven healthy lean young volunteers are presented in Table 1. During the HGEI clamp serum glucose was roughly doubled (pre-clamp: 5.1 ± 0.3 and post-clamp: 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 $\mu\text{g/L}$, $p = 0.255$). The low rate of exogenous insulin infusion to maintain basal fasting insulin level resulted in a slight, but significant, increase in serum insulin at the end of the clamp (pre-clamp: 9.4 ± 0.9 and post-clamp: 15.5 ± 4.0 $\mu\text{U/L}$, $p = 0.008$). Concomitantly, the plasma concentration of non-esterified fatty acids significantly decreased during the clamp (pre-clamp: 0.71 ± 0.14 and post-clamp: 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 (supplementary Tables 1 and 2, respectively). More than 80% of these genes were down-regulated 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 down-regulated 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 down) were verified using real-time PCR (Table 3).

Four volunteers accepted to repeat a control study with infusion of somatostatin for 3 hours, 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 $\mu\text{U/L}$, $p = 0.005$) 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 supplementary Table 3, 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 for PDK4 in both tissues. These data suggested 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 (genome-www5.stanford.edu/cgi-bin/source), OMIM and PubMed, the genes were classified into 13 functional groups (supplementary Tables 1 and 2): 12 functional groups encompassing most of the biological processes and a group of hypothetical proteins and EST (expressed sequence tag). The number of up- and down-regulated genes in each of these groups is presented in Figure 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 down-regulation 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 up-regulated 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 up-regulated 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, acetyl-CoA carboxylase β) were increased (supplementary Tables 1 and 2). In parallel, the expression of pyruvate dehydrogenase kinase 4 (PDK4), that negatively controls the rate of glucose oxidation, was strongly decreased in both tissues. Regarding the

genes related to the response to stress, 7 genes were up-regulated in the skeletal muscle, including several members of the metallothionein family. They are also present in the list of the most up-regulated genes during the clamp (Table 2) and the variation of the expression of MT1X and MT2A mRNAs were confirmed by quantitative PCR (Table 3). Interestingly, significant up-regulation 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. Putative binding sites for transcription factors were searched for using MatInspector software, and their frequency in the sets of regulated genes was statistically compared towards sets of randomly drawn genes using a z-test. Table 4 shows the transcription factor matrices with significantly different occurrence between the 2 sets (z -score > 1.98). Regarding the up-regulated genes, 4 transcription factor matrices were more frequently found. Of interest, 44% of the up-regulated 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 down-regulated genes. NRSF (Neuron-Restricted Silencer Factor) and FKHD (Fork Head Domain Factors) are known repressors of transcription. However, the difference from the set of non-regulated genes appears too small to support a critical contribution of these factors in the observed massive down-regulation 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). Seventy seven 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 utilized to study the effect of hyperglycemia *per se* on glucose metabolism in normal subjects (9, 10). Blood glucose concentration was maintained for 3 hours in the range classically observed during post-prandial glucose excursions in impaired glucose tolerant subjects and in about 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 to 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 down-regulated 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 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 hour 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 ROS production (23, 24). Interestingly, 44% of the up-regulated genes during the hyperglycemic clamp contain a consensus sequence for MTF1, a transcription factor known to activate metallothionein gene expression, and which 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 5 were implicated in the regulation of apoptosis (supplementary Table 4). We also found a significant up-regulation 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 ROS 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 hours 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 down-regulation of gene expression during the hyperglycemic clamp. Hyperglycemia and ROS overproduction have been shown to promote O-linked glycosylation of the transcription factor 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 potential SP-1 binding site in the promoter region of the down-regulated 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 NF κ B. 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 is no data showing generalized down-regulation of gene expression in response to NF κ B activation. The search for the DNA motif recognized by NF κ B in the sequences of the gene promoters indicated that 70% of the down-regulated genes could be potential target of this transcription factor. However, the same frequency (67%) was also found in non-regulated genes (Table 4). Regarding other transcription factors, the analysis of the promoter sequences did not point out new candidate to explain the down-regulation 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 down-regulation of gene expression observed in few hours only in muscle and adipose tissue.

While more than 80% of the genes were down-regulated during the

hyperglycemic clamp, it should not be neglected that there was a positive effect on the mRNA expression of about one hundred 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 acetylCoA carboxylase β (ACACB), that 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/ μ g 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 in skeletal muscle.

Glucose excursion, particularly during the post-prandial period, is now regarded as an important risk factor for diabetic 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 hours 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 up-regulation of more than 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 non esterified fatty acids that decreased during the clamp, could also eventually affect gene expression in peripheral tissues.

In summary, the present study demonstrates that 3 hours 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 in 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 post-prandial 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.

ACKNOWLEDGEMENTS

The authors acknowledge Genopole Rhône-Alpes and CeCIL for access to their equipment. The technical help of Annie Tradif, Emmanuelle Loizon, Sandra Pesenti (technicians) and Cindy Naud (nurse), as well as patient contribution, are highly appreciated. This work was supported in part by research grants from INSERM (ATC-nutrition n°4NU10G), INRA and the Inter-EPST Bioinformatic Program. This study was funded by a grant from Canadian Institute for Health Research (grant # OHP-65393) and start-up fund from Diabète-Québec to RRL. MF is supported by post-doctorate fellowship from CIHR and RRL by a fellowship from the Fonds de recherche en santé du Québec (FRSQ).

REFERENCES

- 1- Yki-Järvinen H: Glucose toxicity. *Endocrine Reviews* 13:415-431, 1992
- 2- Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54:1615-25, 2005
- 3- Robertson RP: Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J Biol Chem* 279:42351-4, 2004
- 4- Monnier L, Mas E, Ginet C, Michel F, Villon L, Cristol JP, Colette C: Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *JAMA* 295:1681-7, 2006
- 5- Brindisi MC, Rabasa-Lhoret R, Chiasson JL: Postprandial hyperglycaemia: to treat or not to treat? *Diabetes Metab* 32:105-11, 2006
- 6- Girard J, Ferre P, Foufelle F: Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 17:325-52, 1997
- 7- Yamashita H, Takenoshita M, Sakurai M, Bruick RK, Henzel WJ, Shillinglaw W, Arnot D, Uyeda K: A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc Natl Acad Sci U S A* 98:9116-21, 2001
- 8- Ma L, Tsatsos NG, Towle HC: Direct role of ChREBP.Mlx in regulating hepatic glucose responsive genes. *J Biol Chem* 280:12019-27, 2005
- 9- Yki-Järvinen H: Acute and chronic effects of hyperglycemia on glucose metabolism. *Diabetologia* 33: 579-585, 1990
- 10- Del Prato S, Matsuda M, Simonson DC, Groop LC, Sheehan P, Leonetti F, Bonadonna RC, DeFronzo RA: Studies on the mass action effect of glucose in NIDDM and IDDM: evidence for glucose resistance. *Diabetologia* 40:687-97, 1997
- 11- Meneilly GS, Ryan EA, Radziuk J, Lau DC, Yale JF, Morais J, Chiasson JL, Rabasa-Lhoret R, Maheux P, Tessier D, Wolever T, Josse RG, Elahi D: Effect of acarbose on insulin sensitivity in elderly patients with diabetes. *Diabetes Care* 23:1162-7, 2000
- 12- Rome S, Clement K, Rabasa-Lhoret R, Loizon E, Poitou C, Barsh GS, Riou JP, Laville M, Vidal H: Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. *J Biol Chem* 278:18063-8, 2003
- 13- Ducluzeau PH, Perretti N, Laville M, Andreelli F, Vega N, Riou JP, Vidal H: Regulation by insulin of gene expression in human skeletal muscle and adipose tissue. Evidence for specific defects in type 2 diabetes. *Diabetes* 50:1134-42, 2001
- 14- Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM: High-fidelity mRNA amplification for gene profiling. *Nat Biotechnol* 16: 457-459, 2000
- 15- Jenson SD, Robetorye RS, Bohling SD, Schumacher JA, Morgan JW, Lim MS, Elenitoba-Johnson KS: Validation of cDNA microarray gene expression data obtained from linearly amplified RNA. *Mol Pathol* 56:307-12, 2003

- 16- Clement K, Viguier N, Poitou C, Carette C, Pelloux V, Curat CA, Sicard A, Rome S, Benis A, Zucker JD, Vidal H, Laville M, Barsh GS, Basdevant A, Stich V, Cancellato R, Langin D: Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J* 18:1657-69, 2004
- 17- Tusher G V, Tibshirani R, Chu G: *Proc Natl Acad Sci U S A* 98:5116-5121, 2001
- 18- Debard C, Laville M, Berbe V, Loizon E, Guillet C, Morio-Liondore B, Boirie Y, Vidal H: Expression of key genes of fatty acid oxidation, including adiponectin receptors, in skeletal muscle of Type 2 diabetic patients. *Diabetologia* 47:917-25, 2004
- 19- Brindisi MC, Hahn J, Chiasson JL, Rabasa-Lhoret R: Under-utilization of capillary glucose monitoring by type 2 diabetic patients. *Diabetes Res Clin Pract* 2006 Jun [Epub ahead of print]
<http://www.jbc.org/cgi/ijlink?linkType=ABST&journalCode=pnas&resid=98/9/5116>
- 20- Raben A, Holst JJ, Madsen J, Astrup A: Diurnal metabolic profiles after 14 d of an ad libitum high-starch, high-sucrose, or high-fat diet in normal-weight never-obese and postobese women. *Am J Clin Nutr* 73:177-89, 2001
- 21- Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787-90, 2000
- 22- Marfella R, Quagliari L, Nappo F, Ceriello A, Giugliano D: Acute hyperglycemia induces an oxidative stress in healthy subjects. *J Clin Invest* 108:635-6, 2001
- 23- Sato M, Bremner I: Oxygen free radicals and metallothionein. *Free Radic Biol Med* 14:325-37, 1993
- 24- Thornalley PJ, Vasak M: Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim Biophys Acta* 827:36-44, 1985
- 25- Gunes C, Heuchel R, Georgiev O, Muller KH, Lichtlen P, Bluthmann H, Marino S, Aguzzi A, Schaffner W: Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. *EMBO J* 17:2846-54, 1998
- 26- Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruyter ND, Bos JL, Burgering BM: FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 23:4802-12, 2004
- 27- Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollma B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol Chem* 280:4617-26, 2005

- 28- Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222-6, 2000
- 29- Du X, Stocklauser-Farber K, Rosen P: Generation of reactive oxygen intermediates, activation of NF-kappaB, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? *Free Radic Biol Med* 27:752-63, 1999
- 30- Evans JL, Goldfine ID, Maddux BA, Grodsky GM: Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev* 23:599-622, 2002
- 31- Newbury SF: Control of mRNA stability in eukaryotes. *Biochem Soc Trans* 34:30-4, 2006
- 32- Bonora E, Muggeo M: Postprandial blood glucose as a risk factor for cardiovascular disease in Type II diabetes: the epidemiological evidence. *Diabetologia* 44:2107-14, 2001
- 33- Wright E Jr, Scism-Bacon JL, Glass LC: Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *Int J Clin Pract* 60:308-14, 2006
- 34- Piconi L, Quagliaro L, Da Ros R, Assaloni R, Giugliano D, Esposito K, Szabo C, Ceriello A: Intermittent high glucose enhances ICAM-1, VCAM-1, E-selectin and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase. *J Thromb Haemost* 2:1453-9, 2004
- 35- Risso A, Mercuri F, Quagliaro L, Damante G, Ceriello A: Intermittent high glucose enhances apoptosis in human umbilical vein endothelial cells in culture. *Am J Physiol Endocrinol Metab* 281:E924-30, 2001

Table 1: Baseline characteristics of the lean healthy men (n = 7)

Characteristics	Mean ± SD
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
Non esterified 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

Table 2: List of the top 15 up- and down-regulated genes in skeletal muscle and adipose tissue during the hyperglycemic-euinsulinemic clamp.

Skeletal muscle

UGCluster	Name	Symbol	Fold Change	Cytoband
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) superfamily, 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 superfamily domain containing 7	MFSDF7	-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

UGCluster	Name	Symbol	Fold Change	Cytoband
Hs.293274	Adenomatosis polyposis coli down-regulated 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.

The fold changes in mRNA levels during the hyperglycemic clamp were measured on tissue samples from 7 subjects for skeletal muscle and 6 subjects for adipose tissue. RT-qPCR was performed as indicated under “research design and methods”. Values were corrected by the mRNA level of hypoxanthine phosphoribosyl transferase (HPRT) used as a house-keeping 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 n.r. (not regulate). ND : not detectable in a reliable manner using RT-qPCR.

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

Table 4: Gene promoter analysis

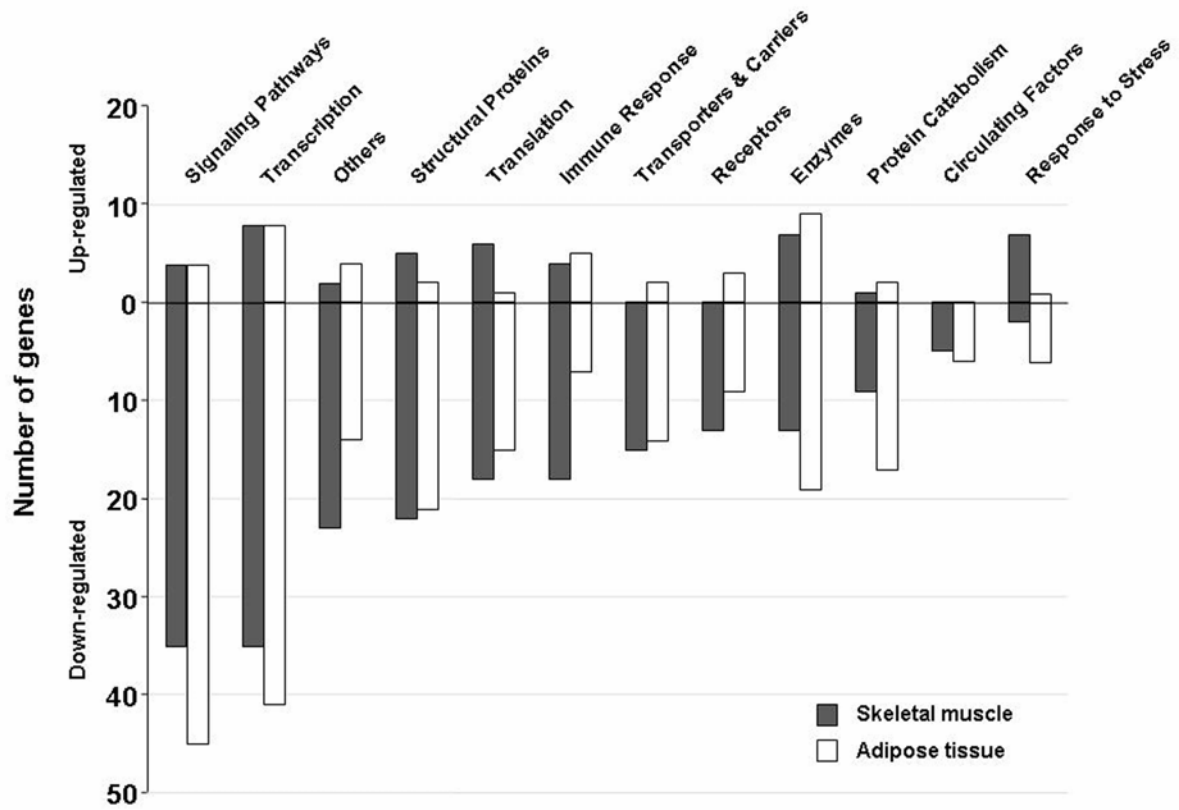
The promoter sequences (1,000 base pairs upstream the transcription start site) of the genes up- (n = 84) and down-regulated (n = 472) during the clamp were analyzed for the presence of transcription factor matrices using MatInspector from Genomatix. The table shows the matrices displaying a significantly different frequency in the sets of regulated genes when compared to same size sets of randomly drawn genes using a z-test. A significant difference (alpha error lower than 5%) corresponds to z-score > 1.98. The score of 3 transcription factor matrices that did not reach significance and that are discussed in the manuscript (CHRE, NFkB and SP1) are also presented.

Transcription factor matrix	Up-regulated 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
NFKB	66.7	73.8	1.012
SP1F	92.8	90.1	0.806

	Down-regulated genes (n = 472)	Randomly drawn genes (n = 472)	
	%	%	
SF1F	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
CHRE	14.2	16.7	1.080
NFKB	70.1	66.7	1.120
SP1F	84.4	82.2	1.325

FIGURE LEGENDS

Figure 1: Functional group distribution of the up- and down-regulated genes during the hyperglycemic clamp in skeletal muscle and in adipose tissue.



SUPPLEMENTARY DATA (Available at <http://dx.doi.org/10.2337/db06-1242>)

Supplementary Table 1: List of the 316 genes regulated during the hyperglycemic euinsulinemic clamp in skeletal muscle.

Supplementary Table 2: List of the 336 genes regulated during the hyperglycemic euinsulinemic clamp in adipose tissue.

Supplementary Table 3: Changes in genes expression measured by RT-quantitative PCR in skeletal muscle and adipose tissue during the somatostatin control study.

Supplementary Table 4: Classification of the 35 up-regulated genes containing a recognition motif for the transcription factor MTF1 in their promoter sequences.