

Changes in Gene Expression in Skeletal Muscle in Response to Fat Overfeeding in Lean Men

Emmanuelle Meugnier,*†‡|| Cécile Bossu,§|| Myriam Oliel,§ Sakina Jeanne,¶ Angélique Michaut,*†‡§
Monique Sothier,§ John Brozek,¶ Sophie Rome,*†‡ Martine Laville,*†‡§ and Hubert Vidal*†‡§

Abstract

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Objective: The adaptive mechanisms in response to excess energy supply are still poorly known in humans. Our aims were to define metabolic responses and changes in gene expression in skeletal muscle of healthy volunteers during fat overfeeding.

Research Methods and Procedures: Eight lean young healthy men were given a diet rich in saturated fat with an excess of ~550 kcal/d for 4 weeks. Using oligonucleotide microarrays, gene expression changes in skeletal muscle were analyzed at Day 0, Day 14, and Day 28.

Results: Fat overfeeding led to an increase in body weight (1.0 ± 0.3 kg) and waist circumference (2.2 ± 0.5 cm, $p = 0.005$) and a significant decrease in fasting non-esterified fatty acid plasma levels ($-29 \pm 5\%$, $p = 0.028$). Respiratory quotient was significantly increased (0.84 ± 0.01 to 0.88 ± 0.02 , $p = 0.034$) and lipid oxidation rate tended to decrease. The expression of 55 genes was modified in skeletal muscle. The main pathways indicated a coordinated

stimulation of triacylglycerol synthesis, inhibition of lipolysis, reduction of fatty acid oxidation, and development of adipocytes. Promoter analysis of the regulated genes suggests that sterol regulatory element binding proteins might be important players of the short-term adaptation to fat overfeeding in human skeletal muscle.

Discussion: This combined metabolic and genomic investigation shows that fat overfeeding for 28 days promotes the storage of the excess energy in lean men and demonstrates the usefulness of a transcriptomic approach to a better understanding of the metabolic adaptation to changes in nutritional behavior in human.

Key words: nutrition, lipid metabolism, gene expression

Introduction

Lifestyle changes over the past decades, associated with a consumption of high energy foods and low energy expenditure, are major determinants of the current obesity epidemic. However, there is considerable interindividual variation in the susceptibility to weight gain in response to overeating (1–4), suggesting differences in the adaptive mechanisms to a positive energy balance. These mechanisms are not yet clearly defined. They could involve an increase in energy expenditure and changes in the metabolic fate of the nutrients and fuel substrates (4,5). Defects in these adaptive processes could explain why an individual becomes obese or not when exposed to an excessive intake of highly palatable food for a long period of time (6).

A large number of overfeeding studies have been performed during the past 50 years, and it was consistently demonstrated that most of the excess energy is stored, primarily as triacylglycerols in the adipose tissue (7,8). It has been argued that this very efficient energy storage is an evolutionary benefit as it allowed for a longer survival during famines. The observed individual differences in energy storage have been attributed to thermogenic adaptations, such as non-exercise activity thermogenesis (4). However, the contribution of adaptive changes in energy

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*Centre de Documentation en Éthique de l'Inserm (INSERM), U870, Lyon, France; †Institut Scientifique de Recherche Agronomique (INRA), Unité Mixte de Recherche (UMR) 1235, Lyon, France; ‡Université Lyon 1, IFR62, Laennec Faculty of Medicine, Lyon, France; §Human Nutrition Research Center of Lyon, Laennec Faculty of Medicine, Lyon, France; ¶Genfit, Loos, France.

||These authors contributed equally to this study.

Address correspondence to Hubert Vidal, UMR INSERM U-449/INRA-1235, Faculté de Médecine R Laennec, Rue G. Paradin, F-69372 Lyon Cedex 08, France or Martine Laville, CRNH-Rhône-Alpes, Faculté de Médecine R Laennec, Rue G. Paradin, F-69372 Lyon Cedex 08, France.

E-mail: vidal@sante.univ-lyon1.fr or martine.laville@chu-lyon.fr

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Table 1. Changes in body weight and body composition during fat overfeeding

	Baseline	Overfeeding 14 days	Overfeeding 28 days
Body weight (kg)	64.6 ± 2.3	65.2 ± 2.4†	65.5 ± 2.5†
Fat body mass (kg)	8.5 ± 0.5		8.9 ± 0.5
Lean body mass (kg)	56.7 ± 1.9		57.5 ± 2.1
Body fat (%)	13.3 ± 0.4		13.3 ± 0.4
Waist-to-hip ratio	0.84 ± 0.01	0.86 ± 0.01*	0.85 ± 0.01
Waist circumference (cm)	73.6 ± 1.9	75.6 ± 1.8†	75.4 ± 1.9*
Abdominal fat area (cm ³)	9.5 ± 0.6		10.09 ± 1.1
Visceral fat (%)	47.5 ± 5.3		43.9 ± 4.3
Subcutaneous fat (%)	52.5 ± 5.3		56.1 ± 4.3

Data are mean ± standard error.

* $p < 0.05$ vs. baseline.

† $p < 0.02$ vs. baseline.

expenditure is not completely understood as they were not observed in all overfeeding studies (3,9). Moreover, diet composition strongly influences the balance between energy expenditure and storage. Horton et al. (8) demonstrated that carbohydrate overfeeding increases total energy expenditure and carbohydrate oxidation, which results in 80% of stored excess energy. However, fat overfeeding has only minimal effect on fat oxidation and energy expenditure (10,11), leading to storage of >90% of excess energy (8).

The molecular mechanisms triggered by overfeeding in skeletal muscle, one of the major tissues involved in the regulation of energy expenditure, have not been clearly defined yet in humans. Nutrigenomics is a developing field in science aiming at understanding how a diet interacts with gene expression to influence the balance between health and disease (12–14). The application of high-throughput genomic methods to nutrition studies could allow identification of coordinated groups of genes that contribute to the adaptation of the metabolic pathways (15). Using bioinformatic tools, such as the analysis of promoter sequences of the regulated genes, could help in identifying master regulators. It is conceivable that a defective activity or expression of these regulators can play a role in the maintenance in body weight in response to nutritional modifications, as recently proposed with the nuclear receptor peroxisome proliferator-activated receptor- γ (16).

The aim of this study was to retrieve the common clusters of genes that contribute to the adaptation to fat overfeeding for 4 weeks in the skeletal muscle in a small group of healthy young lean men with no personal or family history of obesity, eating disorders, or diabetes. Affymetrix (Santa Clara, CA) DNA chips (~38,500 genes) were used to study the changes in gene expression in muscle biopsies. These

biopsies were taken before overfeeding, after 2 and 4 weeks of a fat diet that was designed to provide isoenergetic amounts of lipids 30% above energy requirements.

Research Methods and Procedures

Subjects

Eight lean, young men (age = 23 ± 1 year; BMI = 20.7 ± 0.7 kg/m²) participated in the study. Their characteristics are shown in Tables 1 and 2. Medical questionnaire, physical examination, and routine laboratory analyses were performed before inclusion and all subjects were healthy, with normal fasting glycemia, lipid profile, blood pressure, blood coagulation, and no allergy to lidocaine. All subjects were white and had a stable body weight for at least 6 months at the start of the study. None had personal or familial history of obesity, diabetes, dyslipidemia, or eating disorder. The study was approved by the Ethics Committee of Hospices Civils de Lyon, and all of the volunteers gave a written informed consent.

Study Design

The subjects were overfed during 28 days (4 weeks) with a diet supplying 30% energy excess than the baseline energy requirements. Two weeks before the study, they were invited for a first examination to determine basal metabolic rate by indirect calorimetry and to receive instructions from the dietitian. A baseline dietary record was established by self-reporting over a period of 5 days. During the 4 weeks of overfeeding, subjects maintained their normal lifestyle and were asked to have a stable physical activity. They were studied at Day 0 for the baseline condition, at Day 14 for an intermediary assessment and at Day 28 after completing the

Table 2. Changes in metabolic parameters during fat overfeeding

	Baseline	Overfeeding 14 days	Overfeeding 28 days
Fasting glucose (mM)	4.8 ± 0.1	4.9 ± 0.1	5.1 ± 0.1
Fasting insulin (mU/L)	7.4 ± 1.5	7.0 ± 1.1	7.4 ± 1.0
Total cholesterol (mM)	4.0 ± 0.2	4.1 ± 0.2	4.0 ± 0.1
Low-density lipoprotein-cholesterol (mM)	2.3 ± 0.2	2.2 ± 0.1	2.3 ± 0.2
Fasting triacylglycerols (mM)	0.71 ± 0.08	0.76 ± 0.13	0.55 ± 0.07
Fasting free fatty acids (μM)	401 ± 28	290 ± 21†	307 ± 34†
Fasting glycerol (μM)	41 ± 4	40 ± 2	34 ± 4
Leptin (μg/L)	1.9 ± 0.4	2.3 ± 0.5	2.0 ± 0.4
Ghrelin (nM)	2.6 ± 0.3	2.7 ± 0.3	2.8 ± 0.3
Adiponectin (mg/L)	9.5 ± 1.6	11.1 ± 2.0*	10.5 ± 2.0
Respiratory quotient	0.84 ± 0.01	0.85 ± 0.01	0.88 ± 0.02*
Lipid oxidation (mg/kg per min)	0.80 ± 0.09	0.76 ± 0.07	0.64 ± 0.15
Carbohydrate oxidation (mg/kg per min)	1.79 ± 0.18	1.84 ± 0.15	2.15 ± 0.25
Protein oxidation (mg/kg per min)	0.88 ± 0.14	0.96 ± 0.11	1.03 ± 0.11
Basal metabolic rate (kcal/d)	1663 ± 72	1700 ± 55	1731 ± 75

Data are mean ± standard error.

* $p < 0.05$ vs. baseline.

† $p < 0.02$ vs. baseline.

overfeeding period. Dietary records were established between Day 9 and Day 14 (intermediary report) and between Day 23 and Day 28 (final report). Skeletal muscle biopsies were taken at Day 0, Day 14, and Day 28 for analysis of the changes in gene expression. We also tried to take biopsies of subcutaneous abdominal fat; but due to the leanness of the subjects, the size and the quality of the adipose tissue biopsies did not allow the genomic analysis in this tissue.

Dietary Intervention

The objective of the overfeeding protocol was to provide an excess of energy of 760 kcal/d by adding 70 g of lipids to the usual daily diet. This was reached with 20 g of butter, 100 g of cheese (Emmental), and 40 g of almonds. These food items were provided to the subjects as commercially available portions in daily packages for a 2-week period at Day 0 and at Day 14. The lipid composition of the supplementary foods was mainly saturated fatty acids (44%, with predominantly palmitic acid) and monounsaturated (42%, with mostly oleic acid) fatty acids, whereas polyunsaturated fatty acids represented 8% (with predominantly linoleic acid). Up to 120 mg of cholesterol was also provided through the supplementary diet. The fat overfeeding diet was explained by a dietitian with verifications of the compliance using the 5-day dietary records at Day 14 and Day 28. Nutritional advice was proposed to the subjects after the study until they recovered their initial weight.

Measurements

All measurements were carried out in the morning, after an overnight fast. Body composition was determined by DXA (QR 4500; Hologic, Inc., Bedford, MA). The abdominal fat repartition (intra-abdominal vs. subcutaneous) was assessed by magnetic resonance imaging (Magnetom Symphony 1.5 Tesla; Siemens AG, Munich, Germany) before the beginning and at the end of the overfeeding period. Basal metabolic rate was measured in the fasting state by indirect calorimetry using a ventilated-hood system (Deltatrac; Datex, Helsinki, Finland), as previously reported (17). Glucose was measured using a glucose oxidase method (glucose analyser II; Beckman Coulter, Paris, France), triacylglycerol and free fatty acid plasma concentrations were determined by enzymatic colorimetric methods using commercial kits (TG PAP kit, Biomerieux, Lyon, France; and Wako-NEFA-C kit, Oxoid, Dardilly, France, respectively). Plasma total cholesterol and high-density lipoprotein cholesterol were assayed using Modular Analytics P800-module (Roche Diagnostics, Meylan, France). Plasma low-density lipoprotein cholesterol was calculated using the Friedwald-Frederickson's equation. Plasma insulin was measured by radioimmunoassay (Ins Irma, Kip 1251; MDS, Inc., Nordion, France), and the serum concentrations of leptin, adiponectin, and ghrelin were determined by commercial radioimmunoassays (Quantikine leptin, Oxford,

U.K.; adiponectin assay kit, BioCat, Heidelberg, Germany; and ghrelin assay, Linco Research, Inc., St. Charles, MO).

Statistical Analysis of the Metabolic Parameters

Differences between baseline (Day 0), Day 14, and the end (Day 28) of the fat overfeeding period were analyzed using the non-parametric Wilcoxon rank sums test. Results were considered as significant if $p < 0.05$. All results in tables and text are reported as means \pm standard error.

Skeletal Muscle Biopsies. Biopsies were taken under local anesthesia (2% lidocaine) at Day 0, Day 14, and Day 28 of the overfeeding period, by percutaneous biopsies of the vastus lateralis muscle using Weil Blakesley pliers as previously described (18). The size of the biopsies averaged 60 mg. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

RNA Extraction and Preparation for Affymetrix Microarray. Total RNA from skeletal muscle biopsies was prepared as previously reported (19). RNA concentration and quality were verified using the Agilent 2100 BioAnalyser (Agilent Technologies, Massy, France). Total RNA (1 μg) was used to synthesize double-stranded cDNA using Superscript II and DNA Polymerase I (Invitrogen, Eragny, France), and the template for an in vitro transcription reaction was used to synthesize biotin-labeled antisense cRNA (GeneChip Expression 3'-Amplification Reagents for IVT Labeling; Affymetrix, Inc.); 15 μg of labeled cRNA was fragmented (Tris-acetate, KOAc and MgOAc buffer; 94°C for 35 minutes), hybridized to the Affymetrix HG U133 Plus 2.0 microarray and scanned (Affymetrix GeneChip Scanner GCS3000), according to the Affymetrix GeneChip protocol. Very stringent quality verifications at the different steps of the protocol, including total RNA integrity, reverse transcription rate, amplification and cRNA labeling efficiency, hybridization on the chips and quality of the probe sets using the intensity signals of housekeeping reference genes, led to the exclusion of a number of samples. We finally performed the statistical analyzes using the microarray data from 5 different subjects. These 5 subjects did not differ from the whole group regarding their responses to fat overfeeding (weight gain of 1.3 ± 0.3 kg after 28 days of overfeeding).

Microarray Data Analysis. Background correction and quantile normalization were performed for the raw microarray data using Bioconductor tools resulting in probe intensities for each GeneChip. Subsequently, only the Perfect Match probes from each of the 54,000 probe sets were fit with a linear model for identification of differential gene expression. Because the level of expression could be slightly different between probes corresponding for one transcript, we add a factor called "probe" to control for probe-specific biases in the statistical analysis. The p values were determined using a t-distribution and they were not adjusted for multiple testing. Fold-changes were calculated

from the regression coefficient. Statistical models addressing time effect (Day 0 as reference) were fit to the data under patient hypothesis (identification of differentially expressed genes for each patient separately) and under population hypothesis (identification of differentially expressed genes for all patients).

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)¹. The mRNA concentrations of target genes were determined by RT-qPCR using a Light-Cycler (Roche Diagnostics, Meylan, France) (20). The list of the primers and real-time PCR assay conditions is available on request (vidal@sante.univ-lyon1.fr). The results were normalized using cyclophilin A mRNA concentration, measured as a reference gene by RT-qPCR as previously shown (20).

Analysis of Gene Promoter Sequences. To identify transcription factors that may have contributed to the regulation of gene expression during fat overfeeding, we analyzed promoter sequences of the genes with significant changes in mRNA levels during the diet. The gene promoter sequences (1000 base pairs) were retrieved from TRASER (TRAnscript SEquence Retriever; <http://genome-www6.stanford.edu/cgi-bin/Traser/traser>) with EZRetrieve (<http://siriusb.umdj.edu:18,080/EZRetrieve/index.jsp>), and analyzed using either MatInspector from the Genomatix software package (Genomatix Suite release 3.0; München, Germany) or the TRANSFAC system (21). In this latter approach, a statistical method based on a z-test (comparison of two proportions) was used to calculate the enrichment of each transcription factor binding sites in the promoter dataset of the genes of interest by comparison to their occurrence in the whole human promoter dataset.

Results

Dietary Intake

Dietary intakes were estimated from self-reported dietary records performed during 5 successive days before Day 0, at Day 14, and at Day 28 of the overfeeding period, respectively. Table 3 shows that the compliance to fat overfeeding was good with significant increase in energy intake closed to the expected value of 30% (actually 25% of excess energy, corresponding to ~ 500 to 600 kcal/d). Most excess calorie intake came from lipids, resulting in a strong modification of the relative percentages of macronutrients in the diet, with $\sim 48\%$ of the energy provided by lipids during the overfeeding period (Table 3). Regarding the type of ingested fatty acids, the proportion of saturated fat significantly increased, as expected from the proposed supplement-

¹ Nonstandard abbreviations: RT-qPCR, real-time quantitative polymerase chain reaction; FABP, fatty acid binding protein; ACS, acyl-CoA synthetase; PDK4, pyruvate dehydrogenase kinase 4; EBOX, E-box motif; SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element.

Table 3. Dietary records

	Baseline	Overfeeding 14 days	Overfeeding 28 days
Energy intake (kcal/d)	2471 ± 109	3104 ± 102†	2885 ± 149*
Lipids (kcal/d)	925 ± 72	1462 ± 101†	1336 ± 81†
Carbohydrates (kcal/d)	1149 ± 51	1145 ± 33	1015 ± 52
Proteins (kcal/d)	397 ± 21	497 ± 25†	475 ± 24†
Lipids (%)	37.0 ± 1.9	46.7 ± 2.0†	47.7 ± 1.4†
Carbohydrates (%)	46.7 ± 1.2	37.2 ± 1.6†	35.6 ± 1.0†
Proteins (%)	16.0 ± 1.1	16.1 ± 1.0	16.7 ± 0.6
Saturated fatty acids (%)	35.6 ± 1.5	38.6 ± 1.4	39.5 ± 1.7*
Monounsaturated fatty acids (%)	54.9 ± 1.4	50.5 ± 1.0†	50.8 ± 1.4†
Polyunsaturated fatty acids (%)	9.5 ± 0.6	10.9 ± 0.8	9.7 ± 0.8

Data are mean ± standard error.

* $p < 0.05$ vs. baseline.

† $p < 0.02$ vs. baseline.

tation (butter and fat-rich cheese). Globally, the subjects did not report major difficulties with the diet and there were no indications of malabsorption.

Effects of Fat Overfeeding on Body Weight, Body Composition, and Metabolic Parameters

Four weeks of fat overfeeding induced an average weight gain of 1.0 ± 0.3 kg in the studied group of thin healthy young men (Table 1). Body composition did not reveal significant changes, although the fat mass tended to increase during the diet ($p = 0.091$). Interestingly, the measured waist circumference was significantly higher (2.2 ± 0.5 cm, $p = 0.005$) at the end of the overfeeding period and this effect was already significant after 2 weeks (1.9 ± 0.6 cm, $p = 0.011$), suggesting abdominal deposition of fat tissue. This was further supported by magnetic resonance imaging, which revealed a trend for an increase in abdominal fat area ($p = 0.115$). The deposition of fat tissue seemed more important in the subcutaneous region ($p = 0.075$) than in the visceral area ($p = 0.345$) (Table 1).

As shown in Table 2, 4 weeks of fat overfeeding did not alter fasting glycemia or insulinemia. Total cholesterol and low-density lipoprotein-cholesterol levels were also not modified by the diet. In contrast, the plasma concentrations of triacylglycerols seemed decreased after 28 days ($p = 0.128$), and, more strikingly, the levels of non-esterified fatty acid were significantly reduced ($-29 \pm 5\%$, $p = 0.028$). This reduction was already significant after 14 days (Table 2). There was no change in the plasma levels of leptin and ghrelin, but the circulating concentrations of adiponectin significantly rose at Day 14, during fat overfeeding (Table 2).

The substrate oxidation rates were estimated by indirect calorimetry in the fasting state. Although the results did not reach significance, Table 2 clearly shows that there was a tendency for a reduction in lipid oxidation ($p = 0.063$ at Day 28) and for a concomitant increase in the rate of glucose oxidation ($p = 0.063$ at Day 28). This was also supported by an increase in the respiratory quotient from 0.84 ± 0.01 to 0.88 ± 0.02 at Day 28 ($p = 0.034$).

Changes in Skeletal Muscle Gene Expression During Fat Overfeeding

Using Affymetrix oligonucleotide chips to determine changes in gene expression in biopsies taken at baseline, Day 14, and Day 28, we identified 55 genes with significant changes in expression level in skeletal muscle during fat overfeeding (Table 4). More than 85% of the genes were up-regulated and only 7 were down-regulated. Using Gene Ontology and manual assignment based on SOURCE, OMIM, and PUBMED, the genes were classified into several functional groups. Most of them code for proteins directly involved in lipid metabolism (Table 4). Using RT-qPCR, we confirmed the changes observed with the Affymetrix chips for fatty acid binding protein (FABP)-3 (fold change = 2.0 ± 0.5), FABP4 (fold change = 1.9 ± 0.3), acyl-CoA synthetases (ACS) (fold change = 1.8 ± 0.4), and pyruvate dehydrogenase kinase 4 (PDK4) (fold change = -2.4 ± 0.7). It should be indicated, however, that due to very low level of expression, we were not able to detect reproducible adiponectin mRNA using RT-qPCR in the muscle samples. Finally, because there was a tendency for an inhibition of fatty acid oxidation, we investigated the expression of carnitine palmitoyl-transferase-1, the rate-

Table 4. Significantly regulated genes during fat overfeeding in the skeletal muscle of thin men

UG Cluster	Symbol	Genbank	Name	FC	
				Day 14	Day 28
Regulators of metabolism					
Hs.80485	ADIPOQ	NM_004797	Adiponectin	3.20	2.52
Adipocyte development					
Hs.477128	URB	AW303375	Steroid sensitive gene 1		3.93
Hs.504187	ASAM	BG112263	Adipocyte-specific adhesion molecule		2.68
Hs.507798	LHFP	AA569225	Lipoma HMGIC fusion partner		1.61
Transport and uptake of lipids					
Hs.439312	PLTP	NM_006227	Phospholipid transfer protein		3.27
Hs.50223	RBP4	NM_006744	Retinol binding protein 4	2.10	2.75
Hs.554739	FABP3	NM_004102	Fatty acid binding protein 3, muscle and heart	1.60	2.52
Hs.150122	OSBPL10	NM_017784	Oxysterol binding protein-like 10		2.12
Hs.446291	MSR1	AI299239	Macrophage scavenger receptor 1		1.65
Hs.127445	LIPA	NM_000235	Lipase A, lysosomal acid, cholesterol esterase		1.60
Hs.391561	FABP4	NM_001442	Fatty acid binding protein 4, adipocyte	1.43	1.53
Hs.162757	LRP1	NM_002332	Low density lipoprotein-related protein 1		1.53
Fatty acid and triacylglycerol synthesis					
Hs.558396	SCD	AB032261	Stearoyl-CoA desaturase (delta-9-desaturase)	4.03	3.62
Hs.520189	ELOVL5	AL136939	ELOVL family member 5		1.80
Hs.103253	PLIN	NM_002666	Perilipin	2.15	1.68
Hs.379191	SCD5	AL571375	Stearoyl-CoA desaturase 5	1.48	1.52
Hs.11638	ACSL5	AW173691	Acyl-CoA synthetase long-chain family member 5		1.51
Protection against lipid peroxidation and oxidative stress					
Hs.154654	CYP1B1	NM_000104	Cytochrome P450, subfamily B, polypeptide 1		4.09
Hs.389700	MGST1	D16947	Microsomal glutathione S-transferase 1	1.6	2.74
Hs.459538	ALDH1A3	NM_000693	Aldehyde dehydrogenase 1 family, member A3		2.18
Hs.500645	ALDH18A1	U76542	Aldehyde dehydrogenase 18 family, member A1		1.61
Eicosanoids					
Hs.302085	PTGIS	NM_000961	Prostaglandin I2 (prostacyclin) synthase		2.27
Hs.78183	AKR1C3	AB018580	Aldo-keto reductase family 1, member C3		1.98
Hs.558319	AKR1C2	AA594609	Aldo-keto reductase family 1, member C2	1.50	1.88
Hs.445000	PTGER3	AW242315	Prostaglandin E receptor 3 (subtype EP3)	1.43	1.67
Phospholipids					
Hs.477869	PLSCR4	NM_020353	Phospholipid scramblase 4	1.55	1.74
Hs.130759	PLSCR1	AI825926	Phospholipid scramblase 1		1.69
Carbohydrate metabolism					
Hs.305971	SLC2A10	NM_030777	Facilitated glucose transporter, member 10		2.20
Hs.23363	PGM2	BF512139	Phosphoglucomutase 2	1.32	2.10

Table 4. Continued

UG Cluster	Symbol	Genbank	Name	FC	
				Day 14	Day 28
Hs.89643	TKT	BF696840	Transketolase (Wernicke-Korsakoff syndrome)		1.55
Hs.148266	GPD2	AA613031	Glycerol-3-phosphate dehydrogenase 2	-1.53	-1.23
Hs.530003	SLC2A5	NM_003039	Facilitated glucose/fructose transporter, member 5	-1.52	-1.31
Hs.8364	PDK4	NM_002612	Pyruvate dehydrogenase kinase, isoenzyme 4	-2.08	-2.83
Signaling pathways					
cyclic AMP					
Hs.513578	ADCY7	NM_001114	Adenylate cyclase 7		1.93
Hs.191046	PDE1A	AU146305	Phosphodiesterase 1A, calmodulin-dependent	1.49	1.89
Hs.467898	ADCY3	AF033861	Adenylate cyclase 3		1.78
Hs.370581	CAP1	NM_006367	CAP, adenylate cyclase-associated protein 1		1.72
Hs.9333	PDE8A	W73272	Phosphodiesterase 8A	1.44	1.67
Adrenergic receptors					
Hs.249159	ADRA2A	AF284095	Adrenergic, alpha-2A-, receptor		2.18
Hs.517493	ADRBK2	AI651212	Adrenergic, beta, receptor kinase 2	1.37	1.70
Other signaling pathways					
Hs.494312	NTRK2	AI346341	Neurotrophic tyrosine kinase, receptor, type 2	1.89	2.17
Hs.477375	MYLK	NM_005965	Myosin, light polypeptide kinase	1.60	2.03
Hs.310456	PIK3AP1	AW575754	Phosphoinositide-3-kinase adaptor protein 1		1.75
Hs.512973	HSPC121	AJ271091	Butyrate-induced transcript 1	1.53	1.59
Hs.256067	PRKAA2	AV693202	Protein kinase, AMP-activated, alpha 2 catalytic	-1.50	-1.40
Hs.485572	SOCS2	NM_003877	Suppressor of cytokine signaling 2	-2.31	-1.54
Hs.237028	NPR3	AI628360	Natriuretic peptide receptor C	-1.45	-2.29
Transcription factors					
Hs.326035	EGR1	AI459194	Early growth response 1	1.51	2.34
Hs.555906	CREB5	AI819043	cAMP responsive element binding protein 5		1.95
Hs.457853	EBF2	NM_022659	Early B-cell factor 2	1.58	1.74
Hs.511626	RORA	L14611	RAR-related orphan receptor A	-1.45	-1.54
Unknown function					
Hs.482730	EDIL3	AA053711	EGF-like repeats and discoidin I-like domains 3	2.36	4.44
Hs.335079	MAP1B	AA554833	Microtubule-associated protein 1B	1.51	1.67
Hs.106650	FLJ20533	NM_017866	Hypothetical protein FLJ20533	-1.97	-1.82

UG, UniGene; FC, fold change.

limiting step of long-chain fatty acid oxidation in the mitochondria (22). Using RT-qPCR, a decrease in the mRNA levels of carnitine palmitoyl-transferase-1 (muscle isoform) was found in the skeletal muscle of the subjects during fat overfeeding (fold change = -1.9 ± 0.3 , $p = 0.043$).

For more insight into the transcription factors that coordinate the changes in gene expression, we analyzed the promoter sequences of the regulated genes. Analysis with MatInspector from the Genomatix software (Table 5) revealed that 20 cis-acting sequences are statistically over-

Table 5. Search for potential transcription binding sites in the promoter region (1000 pb upstream of the start site) of the 55 genes that are regulated during fat overfeeding.

Promoter analysis using Genomatrix			
Family matrix	% sequences in overfeeding gene set	% sequences in vertebrate gene set	<i>p</i>
V\$EGRF	83.6	59.3	9.98 e-05
V\$EBOX	81.8	57.9	1.55 e-04
V\$SP1F	81.8	64.8	4.63 e-03
V\$MZF1	80.0	55.6	1.40 e-04
V\$E2FF	78.1	53.8	1.63 e-04
V\$EKLF	78.1	55.0	3.15 e-04
V\$MAZF	76.3	48.7	2.68 e-05
V\$ZBPF	74.5	52.9	8.42 e-04
V\$NFKB	72.7	49.0	4.20 e-04
V\$AHRR	56.3	37.2	3.05 e-03
V\$HIFF	56.3	39.4	8.17 e-03
V\$INSM	50.9	34.4	8.74 e-03
V\$ZF5F	50.9	24.4	2.12 e-05
V\$PLAG	49.1	31.8	5.71 e-03
V\$AP2F	45.4	27.7	3.76 e-03
V\$NRF1	43.6	22.0	2.91 e-04
V\$NOLF	41.8	23.5	2.04 e-03
V\$GABF	41.8	17.8	3.01 e-05
V\$CDEF	40.0	19.0	2.60 e-04
V\$MTF1	32.7	14.2	4.19 e-04
Promoter analysis using TRANSFAC			
Transcription factor motifs	% in overfeeding gene set	% in human promoter gene set	<i>p</i>
M00221_SRE	69.2	47.3	<0.005
M00143_BSAP	7.6	0.8	<0.005
M00050_E2F	71.1	55.7	<0.01
M00215_SRF	13.4	6.5	<0.01
M00055_N-Myc	73.0	59.5	<0.05

represented in the set of regulated genes during overfeeding when compared with the genomes of vertebrates ($p < 0.01$). Among them, the cis-acting elements identified as epidermal growth factor receptor, E-box motif (EBOX), and transcription factor Sp1 motif had the highest enrichment. The EBOXs could bind proteins of the basic-helix-loop-helix-leucine zipper family, including the sterol regulatory element-binding proteins (SREBPs). Interestingly, the promoter analysis executed with TRANSFAC (Table 5) showed that sterol regulatory element (SRE), another cis-acting sequence that binds the SREBPs, was significantly over-represented in the set of regulated genes compared with the total human promoter dataset (69.2% vs. 47.3%).

Discussion

The adaptive mechanisms in response to excess energy supply are still poorly known in humans. In the present study, we used a transcriptomic approach in skeletal muscle to define the mechanisms that underline the metabolic responses to a high-fat diet in healthy lean volunteers. We show that fat overfeeding for 28 days with an energy excess of 25% can induce metabolic adaptations favoring energy storage. This is in agreement with previous reports that have demonstrated that >80% of excess energy is stored during fat overfeeding (8). Fat overfeeding induced a reduction in fasting plasma levels of non-esterified fatty acids, which

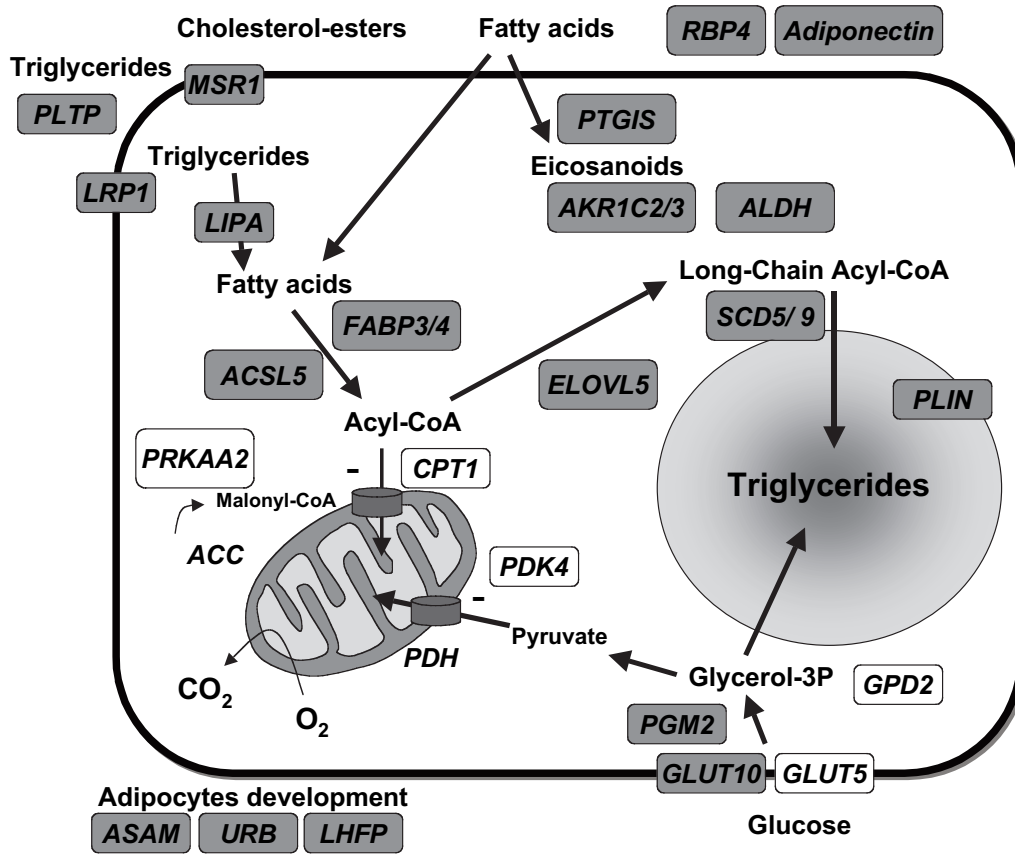


Figure 1: Schematic representation of the main lipid metabolic pathways affected in skeletal muscle during 4 weeks of fat overfeeding. Genes indicated in white boxes were down-regulated during the dietary study, whereas genes indicated in gray boxes were up-regulated, as indicated in Table 4. The names of the genes are indicated in Table 4.

was already observed after 14 days of dieting. This is in favor of an inhibition of lipolysis in adipose tissue and consistent with a stimulation of triacylglycerol deposition. There was probably also an induction of adipocyte differentiation and/or of fat cell size in the subcutaneous area, as strongly suggested by the observed increase in waist circumference and by the data of fat tissue distribution using magnetic resonance imaging. Unfortunately, due to the leanness of the subjects, the quality of the adipose tissue biopsies was not sufficient to allow molecular analysis in this tissue.

Using oligonucleotide DNA chips, we investigated the global regulation of gene expression in skeletal muscle. Most of the regulated genes are linked to lipid metabolism pathways that contribute to lipid transport and uptake and to intracellular synthesis of fatty acids and triacylglycerols. These pathways were confirmed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Although the fold changes in mRNA levels were globally rather small, it is likely that they were translated at the biological level because the affected pathways were very consistent with the metabolic modifications observed during

fat overfeeding. As shown in Figure 1, genes encoding the proteins and enzymes of triacylglycerols and cholesterol-ester transport and metabolism were induced by fat overfeeding, including macrophage scavenger receptor 1, low density lipoprotein-related receptor 1, or the lysosomal lipase A, which catalyzes the hydrolysis of triacylglycerols and cholesterol-esters (23). In the cells, the free fatty acids generally bind to fatty acid binding proteins, two isoforms of which are induced during the study (FABP3 and FABP4) and are further activated into acyl-CoA owing to the action of ACS. The expression of one isoform of ACS, ACSL5, was increased after 28 days of overfeeding. The metabolic fate of acyl-CoA is either oxidation in the mitochondria or elongation/desaturation to form more complex fatty acids and to be stored as triacylglycerols. Fat overfeeding clearly induced the expression of several genes involved in long-chain fatty acid metabolism and triacylglycerol synthesis, such as *ELOVL5*, a member of the elongase family, and the stearoyl-CoA desaturases-9 and stearoyl-CoA desaturases-5 (24). Triacylglycerol synthesis requires glycerol-3 phosphate for fatty acid esterification. In agreement, the gene expression of the glycolytic enzyme phosphoglucomutase

was induced, whereas glycerol-3-P dehydrogenase was repressed during the dietary intervention. Perilipin is a protein that coats lipid storage droplets in adipocytes and that also plays a role in the control of lipolysis (25). Perilipin knock-out mice display a decrease in triacylglycerol synthesis and an increase in fatty acid oxidation (26). Induction of the expression of perilipin is, thus, consistent with lipid storage, and inhibition of lipolysis and fatty acid oxidation during fat overfeeding.

The gene expression data also revealed an inhibition of key genes related to the fatty acid oxidative pathway (PDK4, carnitine palmitoyl transferase-1, and protein kinase, adenosine monophosphate-activated, alpha 2 catalytic subunit; PRKAA2). Pyruvate dehydrogenase kinase 4, PDK4, through phosphorylation of pyruvate dehydrogenase inhibits glucose oxidation and favors fatty acids as energy fuels (27). Reduction of its expression during fat overfeeding was in agreement with the observed trend for lower oxidation of lipids and increased oxidation of glucose. Carnitine palmitoyl transferase-1 codes for the rate-limiting step of long-chain acyl-CoA entry in the mitochondria and, thus, of β -oxidation (22). In addition, carnitine palmitoyl transferase-1 activity is inhibited by malonyl-CoA. The reduction of the expression of PRKAA2, one of the catalytic subunits of adenosine monophosphate-activated protein kinase, could contribute to higher malonyl-CoA levels (28). These observations are globally in agreement with a recent study showing the down-regulation of several genes involved in mitochondrial oxidative phosphorylation in the skeletal muscle of healthy subjects submitted to an isoenergetic high-fat diet for 3 days (29).

Surprisingly, we also observed the induction of genes that are normally expressed at high levels in adipose tissue, such as adiponectin, FABP4, or perilipin. A very recent report showed the presence of adiponectin in rat skeletal muscle during a high fat–high sucrose diet (30). Although we were not able to confirm adiponectin mRNA expression in human muscle using RT-qPCR, the observed coordinated regulation of these adipocyte-related genes using Affymetrix chips, together with the up-regulation of some genes related to fat cell development (steroid sensitive gene 1, adipocyte-specific adhesion molecule, and lipoma HMGIC fusion partner) (31), suggest that adipose cells were induced in the muscle of the subjects during fat overfeeding. It is indeed important to indicate that muscle biopsies were not dissected to take out connective tissue and potential fat before gene expression analysis and, thus, that changes in the amount of contaminating tissues, such as adipose tissue, could have contributed to the results. A histological analysis of the muscle would be of interest to confirm this hypothesis.

To identify master regulators orchestrating the changes in genes expression during fat overfeeding, we analyzed the promoter sequences of the regulated genes. With this approach, we were able to define cis-acting sequences poten-

tially recognized by transcription factors and to statistically analyze their relative abundance in the set of genes of interest with regard to the gene promoters of the whole human genome. Using 2 different methods for promoter sequences analysis, we found that cis-acting sequences corresponding to the E-box motifs (EBOX) and to the SREs were significantly enriched in the set of the 55 genes regulated by fat overfeeding in skeletal muscle. The sterol regulatory element-binding proteins (SREBP1a, SREBP1c, and SREBP2), which belong to the basic-loop-helix family of transcription factors, are well known to act via SRE and EBOX cis-acting elements (32). They were first identified as implied in cholesterol metabolism (33) and in adipocyte differentiation (34). A significant amount of data demonstrate that the SREBPs are major regulators of lipid metabolism, particularly of lipogenesis and triacylglycerol synthesis (35,36). The role of SREBP in coordinating gene expression in response to changes in food intake is clearly documented in rodent in the fasting–re-feeding transition and during high carbohydrate diet (37,38), but there are yet little data reporting a direct role in the response to high fat feeding. In vitro experiments have shown that polyunsaturated fatty acids and cholesterol promote a down-regulation of SREBP expression and transcriptional activity (39). However, SREBP-1c was recently involved in the renal accumulation of lipids during a diet enriched in saturated lipids in mice (40). Furthermore, Mingrone et al. found that intramyocytic triacylglycerol accumulation is associated with increased expression of SREBP-1c in the skeletal muscle of severely obese subjects and that both parameters were reduced after massive lipid malabsorption due to bariatric surgery (41). Therefore, our present data strongly suggest that the SREBPs might be important players of the short-term adaptation to fat overfeeding in human skeletal muscle, promoting lipid storage through the induction of the key genes of the triacylglycerol synthesis pathway.

In summary, this combined metabolic and genomic investigation study clearly demonstrates that fat overfeeding for 28 days efficiently promotes the storage of an excess energy in thin men. This metabolic orientation toward lipid storage is performed by the coordinated stimulation of triacylglycerol synthesis, inhibition of lipolysis, reduction of fatty acid oxidation, and development of adipocytes. These effects result in lower circulating levels of fatty acids, and less triacylglycerols, during the fasting state. Using microarrays, key lipid metabolic pathways were identified and analysis of the promoter sequences of the regulated genes strongly suggests involvement of the SREBP transcription factors in skeletal muscle metabolic adaptations to fat overfeeding. Together, these results demonstrate the validity and the usefulness of the nutrigenomic approach to a better understanding of the metabolic adaptations to changes in nutritional behavior in human. Lipid accumulation in skeletal muscle has been associated with insulin resistance (42).

Insulin sensitivity was not measured in the present study and will be an important parameter to investigate in future experiments. Another important step will be the search for potential alterations in these adaptive processes in subjects prone to develop obesity or metabolic complications.

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