Transcript Profiling Suggests That Differential Metabolic Adaptation of Mice to a High Fat Diet Is Associated with Changes in Liver to Muscle Lipid Fluxes

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Genetically homogenous C57Bl/6 mice display differential metabolic adaptation when fed a high fat diet for 9 months. Most become obese and diabetic, but a significant fraction remains lean and diabetic or lean and non-diabetic. Here, we performed microarray analysis of “metabolic” transcripts expressed in liver and hind-limb muscles to evaluate: (i) whether expressed transcript patterns could indicate changes in metabolic pathways associated with the different phenotypes, (ii) how these changes differed from the early metabolic adaptation to short term high fat feeding, and (iii) whether gene classifiers could be established that were characteristic of each metabolic phenotype. Our data indicate that obesity/diabetes was associated with preserved hepatic lipogenic gene expression and increased plasma levels of very low density lipoprotein, and, in muscle, with an increase in lipoprotein lipase gene expression. This suggests increased muscle fatty acid uptake, which may favor insulin resistance. In contrast, the lean mice showed a strong reduction in the expression of hepatic lipogenic genes, in particular of Scd-1, a gene linked to sensitivity to diet-induced obesity; the lean and non-diabetic mice presented an additional increased expression of eNos in liver. After 1 week of high fat feeding the liver gene expression pattern was distinct from that seen at 9 months in any of the three mouse groups, thus indicating progressive establishment of the different phenotypes. Strikingly, development of the obese phenotype involved re-expression of Scd-1 and other lipogenic genes. Finally, gene classifiers could be established that were characteristic of each metabolic phenotype. Together, these data suggest that epigenetic mechanisms influence gene expression patterns and metabolic fates.

The current epidemic of obesity leads to an increased occurrence of type 2 diabetes (1). The change in nutrition, in particular the consumption of diets rich in calories, coming in great part from lipids, appears as a major contributor to obesity development (2). Genetic predispositions, however, also cooperate with changes in nutritional behavior in the development of the common forms of obesity and diabetes. In rare instances, mutations in single genes, for instance those for leptin, leptin receptor, melanocortin 4 receptor, proopiomelanocortin, or pre-convergase genes (3–6), lead to Mendelian forms of obesity. But even in these cases, the impact of a given mutation may be strongly modified by the genetic background. This is exemplified by the various phenotypes caused by the ob/ob mutation when present in different mouse strains (7) and by the widely different degree of glucose intolerance caused by targeted mutations of the insulin receptor and insulin receptor substrate genes in mice with different genetic background (8). In the absence of targeted mutations, the susceptibility of rodents to develop obesity and/or diabetes in response to high fat diet feeding is also strongly dependent on the genetic background, with some strains being very sensitive and others completely resistant (9–12). These observations, as well as genetic linkage studies in humans, suggest that complex gene-gene interactions define the degree of susceptibility to these metabolic diseases and that most of the genes involved contribute individually only to a small extent to the overall pathogenic risk. Besides the genetic basis for variability in gene expression and function, there is now strong evidence that epigenetic mechanisms may participate in the development of several diseases. This has been well established in cancer biology (13, 14) where the impact of epigenetic variations, in particular DNA or histone methylation, may cause oncogenic transformation of somatic cells. The impact of epigenetic variations on metabolic diseases is less well characterized, but there is increasing interest in evaluating how such mechanisms may contribute to these pathologies (15, 16). A striking recent report has demonstrated that variable degrees of methylation of the viable agouti (A<sup>vy</sup>) allele can be obtained upon changes in food composition that lead to corresponding variations in this allele expression and changes in coat color (17).

High fat diet-induced obesity/diabetes in the mouse, in the absence of mutation of selected genes, is considered a good model for the pathogenesis of the human conditions. Recently, we reported that genetically homogenous C57Bl/6 mice fed a high fat diet (HFD)<sup>1</sup> developed differential metabolic adapta-

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<sup>1 The abbreviations used are: HFD, high fat diet; ObD, obese and diabetic mice; LD, lean and diabetic mice; LnD, lean and non-diabetic mice; NC, normal chow; AUC, area under the curve; RT, reverse transcription; qRT, quantitative real time; MDS, multidimensional scaling; VLDL, very low density lipoprotein; Scd-1, stearoyl-CoA desaturase;
tion (18). Approximately 50% of the HFD mice became obese and diabetic (ObD mice), whereas ~12% remained lean and diabetic (LD mice), and ~12% lean and non-diabetic (LnD mice); the rest of the mice displayed intermediary phenotypes. The differential metabolic adaptation was first observed as an increase in glucose intolerance after 3 months of HFD feeding followed by a progressive and differential increase in body weight so that the three distinct groups were observed at 6 months and well established at 9 months. Euglycemic clamp analysis revealed that the different groups of mice were all insulin-resistant. This was associated, in fasted ObD mice, with a decreased glucose clearance rate and hyperinsulinemia; in LD mice with normal glucose clearance in the presence of hyperglycemia and relative insulinopenia; in LnD mice, with increased glucose clearance in the presence of mild insulinopenia. Furthermore, insulin-stimulated glucose uptake by muscles isolated from each type of mice was indistinguishable from that of normal chow fed mice thus indicating that insulin resistance was mostly induced by the diabetic milieu rather than caused by muscle gene expression defects.

Thus, evaluation of the molecular events underlying the differential metabolic adaptation of C57Bl/6 mice to HFD feeding should provide interesting and important information about the pathogenesis of obesity/diabetes but also about the changes in gene expression associated with resistance to obesity and/or diabetes development. In addition, because the mice studied were genetically homogenous, these data may lead to an appreciation that changes in gene expression may be controlled by non purely genetic factors.

Therefore, in the present study, we evaluated the expression pattern of a set of “metabolic” genes in liver and muscles of the HFD mice. We showed that there were marked changes in the expression of genes that together suggested that obesity/diabetes was associated with an increase in lipid flux from the liver to the periphery and that an increase in fatty acid uptake by muscle could explain the various levels of insulin resistance in the different subgroups. The lean phenotype was associated with a reduced liver expression of Scd-1 and of other lipogenic genes, and the mice resisting diabetes development were characterized by increased expression of eNOS in liver. Finally, the expression profiles showed group-specific characteristics, which were sufficiently consistent so that automated gene selection and classification methods showed good discrimination in a cross-validation test. In particular, genes whose RNA expression levels are good indicators of a diabetic condition could be identified.

MATERIALS AND METHODS

Animals and RNA Preparation—Four- to five-week-old C57Bl/6J male mice (IFFACREDO, L’Arbresle, France) were fed a normal chow (NC) diet (energy content: 12% fat, 28% protein, and 60% carbohydrate) or a high fed diet (HFD) (energy content: 72% fat (corn oil and lard), 28% protein, and <1% carbohydrate) for 9 months. Their body weight and tolerance to intraperitoneal glucose injections (AUC; area under the curve) were determined as described previously (18). These two criteria were used to classify the mice in three subgroups, lean non-diabetic (LnD), lean diabetic (LD), and obese diabetic (ObD), which represented 11.8%, 12.4%, and 47.2%, respectively, of the total population of mice. The rest of the mice displayed intermediate phenotypes (18).

Total RNA was extracted from the liver and muscle of the HFD and NC mice according to Chomczynski and Sacchi (19). Polyadenylated (poly(A)) RNA was isolated by one round of selection with oligo(dT) cellulose (Sigma), and its quality was analyzed by Northern blot detection of GLUT-2 mRNA for liver and β-actin mRNA for muscle. Samples presenting no sign of degradation were used for microarray analysis.

Probes for cDNA Microarray Preparation—Using gene-specific primers, PCR products with an average length of 300–400 bp were obtained by RT-PCR using RNA from liver, skeletal muscle, adipose tissue, hypothalamus, and β-Tet-tet cells. After purification by agarose gel electrophoresis, PCR products were cloned into pcR2.1Topo (Invitrogen), sequenced-verified, and amplified by PCR using the gene-specific primers (GATC Biotech AG, Konstanz, Germany). The PCR products were purified either using the MultiScreen-PCR, 96-Well Filtration System for PCR products (Millipore, Eschborn, Germany), the PeqGold AmpLiPure HTI 96 Kit/96 Well Filtration System for PCR products (Peqlab, Erlangen, Germany) or the Qiagen-96 columns (Qiagen). An aliquot of each purified PCR product was analyzed by agarose gel electrophoresis for evaluation of quality and quantity. After liphylization, the amplified inserts were resuspended in 10 μl of spotting solution (1.5 M betaine, 3 × SSC: 0.45 M NaCl, 0.045 M sodium citrate—12% lean and non-diabetic (LnD mice) and 47.2% obese and diabetic (ObD mice) of the total population of mice). The final concentration was adjusted to 100–500 ng/μl. Control genes for data normalization included ribosomal genes mRNAs.

Printing of cDNA Microarrays—Microarray printing was done according to published methods (20–23). Briefly, PCR products were arrayed onto reactive aldehyde-coated microscope slides (Peqlab QM1220, Peqlab, Erlangen, Germany) and SuperAldehyde slides (Zeiss International) using a high precision gridding robot (GeneMachines, San Carlos, CA) fitted with four printing tips (Telechem International). Post-printing slide treatments were performed as recommended by the supplier, and slides were stored at room temperature.

RNA Labeling and Hybridization—RNA samples from the HFD animals were always treated individually, whereas RNAs from control animals were pooled after RNase digestion and quality control. We synthesized biotinylated cRNAs starting from 10 μg genomic DNA and then dried by centrifugation. An aliquot of the dried cRNAs was resuspended in 25 μl hybridization solution and mixed with 38 pmol of Cy3-conjugated SuperScriptII transcripts (Invitrogen) and 190 ng/g mouse Cot-1 DNA (Invitrogen). Immediately before hybridization, the probes were denatured for 1 min 30 s at 95 °C and then rapidly applied onto the microarray under a coverslip. The hybridization reaction was performed for 14–16 h at 64 °C in a hybridization cassette (Telechem International). Slides were washed with buffer of decreasing ionic strength to remove non-specifically hybridized DNA and then dried by centrifugation.

Microarrays Scanning and Normalization—Microarrays were screened using a scanning laser microscope (ScanArray 4000, GSI Lunomics, Watertown, MA). Separate images were acquired for each fluorophore at a resolution of 10 μm per pixel. For scaling of the two channels with respect to signal intensity, photomultiplier and laser power settings were adjusted to achieve a signal ratio of control genes as close to 1.0 as possible. The spot average fluorescence intensity for each gene and for each fluorophore at a resolution of 10 μm was measured with the ImaGene software (Biodiscory Inc., Los Angeles, CA). Data were transformed into log2 intensities ratio (Cy5/Cy3) and normalized with a locally weighted linear regression (Lowess) method. The expression data were filtered based on flag and background intensity criteria (24). All these previous steps were performed with the R software for statistical computing available at the Comprehensive R Archive Network (cran.r-project.org).

cDNA Microarrays Validation and Definition of the Confidence Interval—Spotted microarrays were prepared as described above with cDNAs encoding proteins controlling several metabolic pathways (Table 1). We first validated these microarrays using mRNA prepared from the liver (self versus self) and from the liver of fasted and fed mice. Correct normalization of data was verified by visualizing data on M versus A plots and quantile-quantile plots (Supplemental Fig. S1, a and c). The boundaries of a 98% confidence interval were determined to be −0.47 and +0.47, based on log2 gene expression ratios for self versus self experiments as shown on the frequency histograms (Supplemental Fig. S1b). As an additional validation of the microarrays, hybridization experiments were performed with mRNA extracted from fed and fasted
A letter code was defined to represent the different pathways to which genes present on the cDNA microarray belong. The number of genes present in each pathway is indicated on the list.

A) Glucose metabolism: 69
   A1) Glycolysis: 21 genes
   A2) Krebs cycle: 17
   A3) Pentose phosphate shunt: 6
   A4) Gluconeogenesis: 4
   A5) Glycogen biosynthesis/degradation: 11
   A6) Transporters, Carriers: 9
   A7) Others: 1

B) Lipid metabolism: 85
   B1) Activation and β-oxidation of fatty acids (even/odd chain, polyunsaturated): 18
   B2) Biosynthesis of Triglycerides and Phospholipids: 16
   B3) Cholesterol metabolism: 29
   B4) Lipid transport, Lipoproteins, Lipases: 22

C) Mitochondrial metabolism: 16

D) Proteins involved in insulin secretion and β-cell function: 100
   D1) Insulin and insulin secretion: 11
   D2) Glucose signaling: 3
   D3) Insulin biosynthesis: 27
   D4) Transcription factors: 15
   D5) Receptors/Ligands: 8
   D6) Cytokines, Signaling: 36

E) Proteins involved in insulin/glucagon action and insulin resistance: 73

F) Adipose tissue: 61
   F1) Adipocyte differentiation factors, transcription factors: 34
   F2) Adipocyte cytokines/hormones, intracellular signaling: 27

G) Hypothalamus: 52
   Peptides and peptide receptors

H) Pancreatic development: 106

I) Ribosomal genes: 7

K) Miscellaneous: 85

mouse livers. Based on the established confidence interval, 131 genes were differentially expressed (Supplemental Fig. S2). Where previous data existed, the regulated expression of these genes was found to correctly reflect their known regulation during the fed to fast transition.

**Analysis of Gene Expression in the Liver of LnD, LD, and ObD Mice and Statistics**—The median value of six hybridizations was determined for each gene. After 10,000 bootstraps of the median values, the distributions of the 1st and of the 99th percentiles were determined. The mean of each percentile distribution was used as limits of the 98% confidence interval. The whole procedure was done with the R software.

The BRB-Array Tools software (Biometric Research Branch of the NCI, National Institutes of Health) was used for supervised analysis. A leave-one-out cross-validation procedure was performed with 297 genes and (21-1) mice for liver or 203 genes and (18-1) mice for muscle to generate prediction models in defining classifiers containing genes that best discriminate between the ObD, LD, and LnD phenotypes, between the diabetic and non-diabetic phenotypes or between the obese and lean phenotypes. For each iteration of the leave-one-out cross-validation, one mouse was left out, and genes were selected using an univariate t- (for two groups comparison) or ANOVA F-test (for more than two groups comparison) with a specific p value (<0.005 or <0.01 depending on the group comparison). The permutation distribution of the t- or F statistic, based on 2000 random permutations, was used to confirm statistical significance. The discrimination power of the classifier was evaluated using the supervised k-nearest neighbor method (with k = 1), which consists in predicting the group label of a new mouse based on the label of its nearest neighbors positioned in a space of n dimensions (n corresponding to the number of genes present in the classifier). The metric used was the Euclidian distance. The whole procedure was repeated until all mice had been left out once. The overall performance of the classifier was measured by calculating the classification rate following the entire cross-validation procedure. The probability of obtaining a high classification rate by chance was determined by repeating the entire cross-validation procedure using 2000 random permutations of the group labels of mice. The proposed final classifiers (Table S4) are lists of genes selected with a t- or ANOVA F-tests, using p value cutoff used for the leave-one-out cross-validation, applied on all liver (21 mice) or muscle tissues (18 mice).

The correlation between the expression values of a given gene and the AUC index was assessed by univariate least square linear regression, using the lm function of the “base package” of the R language. The t statistic is a measure of the hypothesis that the regression coefficient is equal to zero and its p value were computed for every gene. Genes having a p value < 0.0075 were selected for the classification procedure. The class discrimination power of this gene selection method was tested by a leave-one-out cross-validation procedure using the k-nearest neighbor method as previously described. p values for the significance of the deviation from independence between true and predicted labels were obtained with the Fisher exact test (test package for R) and with the alternative hypothesis set to “greater.”

The multidimensional scaling (MDS) method projects the data points from a high dimensional space to a low dimensional space (two or three dimensions), in a way that best preserves their relative distance. MDS was performed with the expression values of genes present in classifiers generated by the F- or t-tests or the linear regression method.

**Real-time Quantitative PCR—**Quantitative RT-PCR was performed to confirm the expression levels of some genes obtained by cDNA microarrays. First-strand cDNA was synthesized from 2.5 μg of total RNA using Superscript II RT (Invitrogen) primed with 50 pmol of random hexamers. Reactions without reverse transcriptase served as a control for amplification of genomic DNA. The reactions were carried out in a 20-μl volume in Lithos qPCR Mastermix containing 1× SYBR Green (Eurogentec) with cDNA template from 250 ng of total RNA and 10 pmol of specific primers. The list of the primers is available in Table S5 of the Supplemental Materials. Quantitative PCR was carried out using the LightCycler System (Roche Diagnostics). For each sample, duplicate amplification measurements are presented as average.

**Measurements of Plasma Lipid Concentrations—**Total cholesterol and high density lipoprotein and triglycerides plasma concentrations were determined using enzymatic colorimetric assays produced by Biomerieux (France) on a Cobas Mira Plus analyzer from Roche Applied Science. VLDL levels were calculated by subtracting the high density lipoprotein value from the total cholesterol value.

**RESULTS**

**Gene Expression in Liver of High Fat Fed Mice—**To evaluate the pattern of “metabolic” gene expression in tissues from mice displaying differential metabolic adaptation at 9 months of high fat feeding, we isolated mRNA from the experimental groups and used mRNA from mice fed a normal chow (NC) for 9 months. In a first set of experiments, microarray analysis were performed with independent liver mRNA preparations obtained from each group (7 for ObD, 6 for LD, and 8 for LnD) and compared with a pool of mRNA isolated from the liver of 10 NC mice. The characteristics of the mice used are presented in Table II. The complete list of genes whose expression was significantly regulated in the different groups of mice is presented in Supplemental Table S1. A summary of these data is presented in Table III. These data show that a set of genes was similarly regulated in all three groups but that each subgroup presented additional specific gene expression patterns.
Genes Similarly Regulated in All Groups—These genes and their encoded proteins belonged to different metabolic pathways where they play important regulatory roles. Glucose-6-phosphate translocase (glucoseoneogenesis) was reduced; CPT-1 (β-oxidation) was increased as were UCP-2 (mitochondrial uncoupling) and lactate dehydrogenase (production of lactate from pyruvate).

Genes Specifically Regulated in Each Group—In the ObD mice, there was a coordinated up-regulation of eight genes involved in fatty acid oxidation, including two involved in peroxisomal fatty acid oxidation and an increase in the fatty acid oxidation enzymes, and reduced levels of mRNA for fatty acid synthase. There was also a reduction in cytokine-inducible SH2-containing protein (CIS-1, an inhibitor of growth hormone-induced signaling pathway) gene expression, which was stronger in both diabetic as compared with the non-diabetic groups. Lipoprotein lipase increases the hydrolysis of chylomicron and VLDL thereby enhancing the availability of fatty acids for muscles, which may increase insulin resistance. There was also a reduction in cytokine-inducible SH2-containing protein (CIS-1, an inhibitor of growth hormone-induced signaling pathway) gene expression, which was stronger in both diabetic as compared with the non-diabetic group.

Gene Expression in Muscle of High Fat Fed Mice—For these experiments, hindlimb muscles were prepared from mice of the different HFD groups and from NC mice. As for the liver analysis, hybridizations were performed with mRNA prepared from mice from each group (6 for ObD, 7 for LD, and 5 for LnD) and compared with mRNA pooled from 13 NC mice. As in liver, there was a set of genes that were similarly regulated in each group and additional group-specific variations (Table IV; the complete list of regulated genes is presented in Table S2).

Genes Similarly Regulated in All Groups—These genes encode proteins belonging to several metabolic pathways and play critical regulatory roles. The mRNA for pyruvate dehydrogenase kinase 4 (involved in phosphorylation and inhibition of the pyruvate dehydrogenase complex) was increased; mRNA for mitochondrial and peroxysomal fatty acid oxidation (CPT-1, fatty acyl-CoA dehydrogenase, and peroxisomal enoyl-CoA hydratase) were increased as were the mRNA for the uncoupling proteins UCP-2 and UCP-3. There was a reduction in the mRNA for CIS-1, which is further discussed below.

Genes Specifically Regulated in Each Group—In the ObD mice there was a further reduction in glycolytic gene expression; four genes for Krebs’ cycle enzymes were up-regulated, and there was also a further increase in the β-oxidation genes for medium and very long chain-specific fatty acyl-CoA dehydrogenase. In the LD mice a number of genes were significantly and specifically regulated (Table S2); there were reduced levels of mRNA for glycolytic enzymes and for fatty acid synthesis and increased levels of the mRNAs for β-oxidation.

In the LnD mice there were further reductions in the expression of mRNAs for glycolytic enzymes, increased levels of mRNA for Krebs’ cycle and β-oxidation enzymes, and reduced levels of mRNA for fatty acid synthesis. There was also a reduction in one gene for oxidative phosphorylation (complex III, cytochrome c₁).

When gene expression was compared between the diabetic groups (ObD and LD) and the non-diabetic one (LnD), there was a significant increase in lipoprotein lipase mRNA expression only in the diabetic groups. Lipoprotein lipase increases insulin resistance. There was also a reduction in cytokine-inducible SH2-containing protein (CIS-1, an inhibitor of growth hormone-induced signaling pathway) gene expression, which was stronger in both diabetic as compared with the non-diabetic group. Interestingly, the phosphatidylinositol 3-kinase subunit p110 was increased in the LnD mice, which might contribute to the improved insulin signaling in comparison to the diabetic mice. The lean (LD and LnD), as compared with the obese (ObD), mice have an increase in another gene for β-oxidation (dieneoyl-CoA reductase) and a marked reduction in fatty acid synthase.

### Table III

| Groups          | ObD         | LD          | LnD          | HFD 1 week |
|-----------------|-------------|-------------|--------------|------------|
| Glucose-6-phosphate translocase (A6) | -0.48       | -0.75       | -0.89        | -0.73      |
| Lactate dehydrogenase (A7) | 0.50        | 1.21        | 1.03         | 0.38       |
| Carnitine palmitoyltransferase I (B1) | 0.99        | 0.66        | 0.61         | 0.89       |
| UCP2 (F1) | 1.49        | 1.48        | 1.23         | 3.61       |

| Pathways        | Groups       |
|-----------------|--------------|
| Glycolysis (A1) | ↑ 6          |
| Kreb’s cycle (A2) | ↑ 4         |
| β-Oxidation (B1) | ↑ 8          |
| Fatty acid synthesis (B2) | ↓ 6          |
| Cholesterol biosynthesis (B3) | ↓ 1         |
| Oxidative phosphorylation (C1) | ↓ 1         |
| Insulin signaling (E) | ↓ 5         |

| Genes differentially regulated in each subgroup | Groups |
|-----------------------------------------------|--------|
| Scd-1 (B2) | -0.31 |
| eNOS (E) | 0.01 |
| PPARα (E) | 0.51 |

*The symbol “*” means that the gene was not detected by the cDNA microarray.*
Validation of Microarray Data and Plasma VLDL Measurements—Validation of microarray gene expression data by quantitative RT-PCR was sought for some genes. Fig. 1a presents the expression, relative to NC control, of liver (Sed-1, UCP-2, carnitine O-acyltransferase, malic enzyme, squalene monooxygenase, eNOS) or muscle (lipoprotein lipase) mRNAs. Data were compared with the values obtained by the microarray analysis of the same mRNA preparations. There was a very good correlation between microarray and qRT-PCR analysis but with some differences in the absolute values of the ratio. In particular the increase in the expression level of lipoprotein lipase was much higher when assessed by qRT-PCR.

Because the gene expression data suggested a relative increase in fatty acid synthesis and VLDL production in the ObD as compared with the LD and LnD groups, we measured plasma VLDL and total cholesterol levels in mice from each group. There was a significant increase in VLDL and total cholesterol levels in mice from each group. To obtain such classifiers, supervised analyses were performed as described under “Material and Methods.” Final classifiers were obtained based on the liver or the muscle gene expression data following either a three-way ObD versus LD versus LnD comparison (Table S4) or comparisons of diabetic (ObD and LD) versus non-diabetic (LnD) (Table V) or obese (ObD) versus lean (LD and LnD) (Table S4a) mice using, respectively, an ANOVA F-test (three-group comparison) or a t test (two-group comparison) as selection methods. The performance of each classifier to discriminate between the metabolic phenotypes was estimated during a leave-one-out cross-validation procedure. Table VI shows that in all cases discrimination was significantly better than would be expected by chance.

The obese and lean phenotypes could be recognized with accuracy, despite heterogeneity in both groups, using a t statistic for gene selection and the nearest neighbor classification principle. The gene selection was applied in turn to each learning set, selecting different genes. The results indicated that sixteen genes (Table S4a) defined characteristic differences in the pattern of expression between the lean and obese individuals. A summary of the dissimilarities for the expression of these sixteen genes between the single mice was visualized with an MDS plot (Fig. 2a). It shows that the expression in the LD group is intermediate between well separated LnD and

### Table IV

**Summary of differentially regulated genes and pathways in the hindlimb muscle of 9-month HFD mice as compared to NC mice**

Each individual value corresponds to the median of log2 ratios of gene expression. Upward and downward arrows represent up- or down-regulated pathways, respectively. The numbers represent the number of key genes regulating the indicated pathways. In parenthesis is the letter code of the pathway comprising the stated gene (see Table I). Values in bold correspond to the expression level of genes considered to be differentially expressed based on the 98% confidence interval.

| Genes similarly regulated in all subgroups | ObD | LD | LnD |
|------------------------------------------|-----|----|-----|
| Pyruvate DH kinase 4 (A1)                | 1.21| 1.85| 2.06|
| Carnitine palmitoyltransferase 1 (B1)    | 0.78| 0.39| 1.09|
| Fatty acid-CoA DH, long chain specific (B1) | 0.68| 0.92| 1.09|
| Enoyl-CoA hydratase (peroxisomal) (B1)  | 1.23| 0.69| 0.68|
| UCP2 (F1)                                | 1.29| 0.78| 1.23|
| UCP3 (F1)                                | 0.61| 0.81| 0.51|
| Cytokine-inducible SH2-containing protein (CIS-1) (D6) | −2.12| −1.39| −0.55|

| Pathways                                  | ObD | LD | LnD |
|-------------------------------------------|-----|----|-----|
| Glycolysis (A1)                           | ↓ 3 | ↓ 2| ↓ 3 |
| Kreb’s cycle (A2)                         | ↑ 4 |    | ↑ 3 |
| β-Oxidation (B1)                          | ↑ 8 | ↑ 5| ↑ 5 |
| Fatty acid synthesis (B2)                 | ↓ 1 |    | 1   |

| Genes differentially regulated in each subgroup |
|-----------------------------------------------|
| Phosphofructokinase 1 C isozyme (A1)         | −0.11| −0.64| −0.91|
| 2,4-Dienol-CoA reductase (mitochondrial) (B1)| 0.28| 0.57| 0.73|
| Fatty acid synthase (B2)                     | −0.53| −1.44| −1.68|
| Lipoprotein lipase (B4)                     | 0.46| 0.65| 0.28|
| Complex III, cytochrome c5 (heme protein) (C)| −0.44| 0.28| −0.94|

**Changes in Gene Expression after a Short Period of High Fat Feeding**—To evaluate whether the changes in gene expression induced by a short period of high fat feeding was related to the patterns seen after 9 months, we performed analysis of liver gene expression after 1 week of HFD or NC feeding. Table III shows a summary of the changes in pathway-specific gene expression (complete data are given in Table S3). The number of genes that were significantly regulated in these conditions was much greater than in the 9-month HFD mice. There was an increase in the mRNA for glycolytic enzymes, in particular an increase in glucokinase and pyruvate dehydrogenase expression and a strong up-regulation in four Krebs’ cycle enzyme mRNAs. There was a coordinated up-regulation of the β-oxidation genes and of peroxisome proliferator-activated receptor α, similar to that of the ObD group. The genes for fatty acid synthesis (fatty acid synthase, malic enzyme, and Sed-1) were decreased. Many of the genes for cholesterol biosynthesis were markedly regulated but not in a coordinated manner, with some being up-regulated and some down-regulated. The genes of oxidative phosphorylation were markedly down-regulated, similarly to the LnD group. Finally, several genes encoding insulin signaling molecules (IRS-1, phosphatidylinositol 3-kinase p85, Grb14, Grb2, and MEK1) were down-regulated, but this was not observed in any of the HFD groups at 9 months.

Thus, a short period of high fat feeding induced an overall pattern of gene regulation that was quite distinct from any of the three groups of HFD mice. This suggests that, following an initial adaptive response to high fat diet, there was a subsequent transition toward the different patterns of gene expression characteristic of the distinct metabolic states.

**Phenotypic Classifiers and Multidimensional Scaling (MDS)**—One potentially important use of microarray data is to assess whether differences in gene expression can be used to derive expression profiles (classifiers or phenotypic predictors) that allow for automatic identification of the phenotypic groups. To obtain such classifiers, supervised analyses were performed as described under “Material and Methods.” Final classifiers were obtained based on the liver or the muscle gene expression data following either a three-way ObD versus LD versus LnD comparison (Table S4) or comparisons of diabetic (ObD and LD) versus non-diabetic (LnD) (Table V) or obese (ObD) versus lean (LD and LnD) (Table S4a) mice using, respectively, an ANOVA F-test (three-group comparison) or a t test (two-group comparison) as selection methods. The performance of each classifier to discriminate between the metabolic phenotypes was estimated during a leave-one-out cross-validation procedure. Table VI shows that in all cases discrimination was significantly better than would be expected by chance.

The obese and lean phenotypes could be recognized with accuracy, despite heterogeneity in both groups, using a t statistic for gene selection and the nearest neighbor classification principle. The gene selection was applied in turn to each learning set, selecting different genes. The results indicated that sixteen genes (Table S4a) defined characteristic differences in the pattern of expression between the lean and obese individuals. A summary of the dissimilarities for the expression of these sixteen genes between the single mice was visualized with an MDS plot (Fig. 2a). It shows that the expression in the LD group is intermediate between well separated LnD and
ObD groups, analogous to their physiological phenotypes. This indicates that the expression of the genes selected as indicators for obesity were also related to diabetic status.

The discrimination between the diabetic and non-diabetic mice was also reliable (Table VI). Gene selection was based on the significance of the linear regression of AUC glucose against gene expression. The nine most significant genes are shown in Table V, and the corresponding MDS plot (Fig. 2b) visualizes the dissimilarity relationships. Interestingly, because this classifier's genes were selected by regression analysis, they are good indicators of the diabetes phenotype. The linear regression for three of these genes (long-chain fatty acid-CoA ligase,
eNOS, and phosphofructokinase-1) is presented in Fig. 3. These data show a positive correlation between glucose intolerance and expression of long-chain fatty acid-CoA ligase (a gene for $\beta$-oxidation) and an inverse correlation with phosphofructokinase (a key glycolytic gene) and eNOS expression.

A three-way classifier was tested with the gene expression data from muscle tissue. It was based on the ANOVA F-test for the MDS plot in this case shows, however, a good distinction of the three phenotypes (Fig. 2c). Together, the above results demonstrated that there were identifiable and consistent bona fide differences of gene expression between the mouse subgroups and that these could be used as a diagnostic of the metabolic state of a single animal and to reveal interesting positive or negative correlations between a continuous variable and genes participating in key metabolic pathways.

**DISCUSSION**

This study was aimed at evaluating whether the differential metabolic adaptation of C57Bl/6 mice to a high fat diet could be correlated to specific patterns of gene expression and whether genetic classifiers could be used to identify the different metabolic phenotypes.

**Gene Expression in Liver and Muscle of High Fat Fed Mice**—As compared with their expression in the liver of NC mice, the genes for $\beta$-oxidation were increased in the liver of ObD, similar in LD, and reduced in the LnD groups. In contrast, fatty acid synthesis genes were present at a similar level in ObD and NC mice but were markedly decreased in the lean groups (LnD and LD mice), as revealed by reductions in Scd-1, fatty acid synthase, and malic enzyme mRNA levels. Genes for oxidative phosphorylation were similarly expressed in the ObD and NC mice and markedly decreased in the LnD and to a lesser extent in LD mice. These data therefore suggest that the pathways for production of metabolic energy through $\beta$-oxidation and oxidative phosphorylation are more activated in the liver of ObD mice than in the LnD mice. It is nevertheless well known that the activity of CPT-1 is under strong allosteric regulation by malonyl-CoA, and the actual activity of this pathway can be modulated independently of gene expression levels.

In muscle, changes in gene expression indicated, in the three HFD groups, a similar reduction in the expression of genes for glucose oxidation and an increase in those for fatty acid oxidation, suggesting corresponding changes in the activity of these pathways. Decreased glucose oxidation and increase $\beta$-oxidation are also established metabolic defects in human diabetic muscles (28). However, the marked overexpression of both UCP2 and UCP3 suggests decoupling of oxidative phosphorylation.

**Liver and Muscle Metabolic Cross-talk in the Establishment of the Different Phenotypes**—There are a number of salient findings that can potentially explain the metabolic adaptation of the different groups of HFD mice and that are summarized in Fig. 4. First, when comparing the lean groups (LD and LnD) with the ObD group, there was a striking reduction in Sed-1 in the liver of the lean mice. Stearoyl-CoA desaturase 1 catalyzes the formation of oleate from stearate, a reaction that is required for the synthesis of triglycerides and VLDL formation, thus for the channeling of lipids to peripheral tissues (29). Sed-1 is a target of leptin action in the liver (30), and inactivation of this gene protected mice against the development of diet-induced obesity and fatty liver (31). It is therefore striking that resistance to weight gain in this model of high fat feeding is correlated with a strong reduction in the expression of the mRNA of this enzyme. An additional feature of the liver from the lean mice was a marked reduction in the expression of four and eight lipogenic genes in LD and LnD mice, respectively. Together, these results suggest that the liver tissue of the lean mice has a reduced ability to synthesize and transfer lipids to the peripheral tissues as compared with the ObD mice. This is also in agreement with the measured plasma levels of VLDL and cholesterol that were lower in the lean as compared with the ObD mice.

At the level of the hindlimb muscles, there was an increase in lipoprotein lipase expression in the two diabetic groups (ObD and LD, see microarray and qRT-PCR data), suggesting an increased capability to take up free fatty acids hydrolyzed from triglycerides present in chylomicrons or VLDLs. Studies with transgenic mice overexpressing lipoprotein lipase in muscle or liver have demonstrated that a local increase in this gene expression led to tissue-selective insulin resistance, probably caused by increased fatty acid uptake (32). Thus, the two diabetic groups may have increased insulin resistance in part because of an increase in muscle LPL gene expression.

It is interesting to mention that the capacity for fatty acid uptake by muscle has been demonstrated to correlate with development of insulin resistance. For instance, increasing fatty acid uptake in muscles by CD36 transgenic expression or reducing this uptake by cd36 or fatty acid transport protein-1

### Table V

*Subset of genes expressed in the liver constituting a classifier obtained using the linear regression as gene selection method*

| Gene name                                      | $t$ value | $p$ value |
|-----------------------------------------------|-----------|-----------|
| eNOS                                          | -5.12     | 0.00905   |
| Caspase-7                                     | -3.47     | 0.0025    |
| Long-chain fatty acid-CoA ligase 2             | 3.44      | 0.0027    |
| Glycerol-3 phosphate dehydrogenase cytoplasmic| 3.40      | 0.003     |
| Phosphofructokinase 1 C isozyme               | -3.38     | 0.0031    |
| Golgi SNARE GS27                              | 3.19      | 0.0047    |
| Enolase, $\alpha$-isozyme (most tissues)      | 3.15      | 0.0053    |
| JNK2                                          | 3.05      | 0.0067    |
| Fatty acyl-CoA dehydrogenase, long chain-specific | 3.03     | 0.0069    |

### Table VI

*Performance of classifiers determined using the k-nearest neighbour method included in a leave-one-out cross-validation procedure*

Lists of genes present in classifiers are visualized in the Table V.

| Organ     | Classifier category | Groups | Number of mice | Number of mice misclassified | Percent correctly classified | Classifier $p$ value | $p$ value for gene selection | Method for gene selection |
|-----------|---------------------|--------|----------------|-------------------------------|----------------------------|-----------------------|-----------------------------|--------------------------|
| Liver     | Obese versus lean   | ObD    | 7              | 1                             | 90                         | 0.003                 | 0.0005                      | t test                   |
|           |                     | LD and LnD | 14         |                               |                            |                       |                             |                          |
| Diabetic  | versus non-diabetic | ObD and LD | 13         | 0                             | 86                         | 0.003                 | 0.0075                      | Regression               |
|           |                     | LnD    | 8              | 3                             |                            |                       |                             |                          |
| Muscle    | ObD versus LD versus LnD | ObD    | 6              | 3                             | 72                         | 0.008                 | 0.01                        | F-test                   |
|           |                     | LD    | 7              | 0                             |                            |                       |                             |                          |
|           |                     | LnD   | 5              | 2                             |                            |                       |                             |                          |
gene knockout increases or, respectively, protects against high fat diet-induced insulin resistance (33–35).

An unexpected finding was the decreased expression of CIS-1. This protein is a negative regulator of STAT5, which is the initial intracellular transducer of several growth factor receptors, including that for growth hormone (36). Because growth hormone is known to cause insulin resistance, the strong reduction of this negative regulator may increase insulin resistance.

The above data therefore suggest that two mechanisms, increased fatty acid uptake and reduced inhibition of growth hormone signaling, may increase insulin resistance independently of a change in the expression of genes encoding proteins directly involved in the insulin signaling cascade. Although confirmation of this hypothesis would require specific studies on the role of CIS-1 and on measurement of LPL enzymatic activity, it would be consistent with our previous demonstration that insulin resistance of glucose uptake in muscle was observed in vivo but could not be seen in vitro with isolated muscles. This suggested that the insulin resistance in vivo was due to the diabetic milieu rather than to cell autonomous defects.

Finally, a striking observation that could explain the resistance to diabetes development in the LnD mice was the increase in eNOS liver expression. Indeed, preceding studies have demonstrated that mice with homozygous inactivation of the enos gene display several features of the metabolic syndromes, including insulin resistance, hyperlipidemia, and hypertension (37, 38). Heterozygous inactivation of the enos gene in the mouse was associated to a normal phenotype, but enos\textsuperscript{+/-} mice fed a high fat diet developed insulin resistance and hypertension (39). There is thus a graded response to the level of eNOS

**FIG. 2.** Multidimensional scaling (MDS) representation of expression of selected genes in the ObD, LD, and LnD groups. MDS was applied to represent the distances between mice based on the expression profiles of the genes constituting the final classifiers. Each symbol corresponds to one mouse in a three-dimensional space representation (black triangles, ObD; gray squares, LD; and black circles, LnD). For the obese versus lean comparison the classifier was derived from liver genes using a t test (a), and for the diabetic versus non-diabetic comparison the classifier was derived using a linear regression method (b). For the ObD versus LD versus LnD comparison the classifier was derived from muscle genes using an ANOVA F-test (c).
gene expression, and it can be proposed that the increase in eNOS expression in the liver of the LnD mice participate in the protection against diabetes development. This is further supported by the striking inverse correlation between glucose intolerance and eNOS expression detected in the regression analysis of the data (Fig. 3).

**Development of the Three Phenotypes**—Comparison of the gene expression patterns in the liver of mice fed for 1 week or 9 months with a HFD diet indicated that the initial changes in gene expression were much more numerous and usually of higher magnitude than those seen after 9 months. In addition, these changes were distinct from those of any of the three HFD groups. For instance, the strong reduction in the expression of mRNAs encoding proteins involved in insulin signaling after 1 week of HFD feeding was no longer seen after 9 months. This suggest that the mechanisms causing insulin resistance may vary over time.

Of particular interest is the regulation of Scd-1, fatty acid synthase, and malic enzyme mRNA expression. These lipogenic genes were strongly reduced in all the mice after a short period of high fat diet feeding. Their expression remained low in the lean but returned to the level of the control mice in the 9-month obese HFD mice. Thus, these data indicate that one critical step in the development of obesity upon HFD feeding may be the re-expression of Scd-1 and the other lipogenic liver genes to their normal level. These observations therefore suggest that a better understanding of the molecular events controlling Scd-1 and other lipogenic gene expression over the time course of the HFD feeding experiment may provide important information regarding the initial mechanisms that may eventually lead to the differential metabolic adaptation. Scd-1 expression is down-regulated by leptin (30), and the leptin levels in the ObD mice were much higher than in the other groups (44.8 ± 3.0 ng/ml in the ObD versus 14.4 ± 2.6 ng/ml and 18.3 ± 1.2 ng/ml in the LnD and LD mice, respectively). Thus, leptin resistance could potentially explain the restoration of normal Scd-1 expression levels in the liver of ObD mice. However, leptin has also been reported to increase β-oxidation (26), and ObD mice

![Graph](image-url)

**FIG. 3.** Linear regression between gene expression and glucose intolerance. Three examples of linear regression between glucose intolerance (AUC glucose) and expression level of genes belonging to the classifier that discriminates the diabetic versus the non-diabetic phenotypes. A positive correlation was observed between the glucose intolerance and the expression of long-chain fatty acyl-CoA ligase (or synthetase), which catalyzes the activation of free fatty acids for entry into the mitochondria and β-oxidation. A negative correlation was observed between glucose intolerance and phosphofructokinase (a key enzyme in the control of glycolysis) or eNOS mRNA levels (see "Discussion"). The gene expression values are expressed as log₂ ratios (black triangles, ObD; gray squares; LD; and black circles, LnD).
display up-regulation of eight genes involved in this pathway suggesting that the liver of these mice is still, at least partly, sensitive to leptin action.

Supervised Analysis—Analysis of differentially expressed genes allowed identification of group-specific characteristics, which were sufficiently consistent so that automated gene selection and classification methods showed good discrimination in a cross-validation procedure despite the small number of individuals.

One particularly interesting classifier was that derived from the linear regression method, which tried to find genes, whose expression best correlated with the AUC glucose, a physiological parameter. Together, the data indicated that glucose intolerance was inversely correlated with phosphofructokinase 1, a gene increasing the initial step in glycolysis, and with eNOS. Interestingly, eNOS was the gene selected with the smallest p value further supporting an important role of its expression level in preventing the diabetic phenotype as discussed above.

Glucose intolerance was positively correlated with genes increasing β-oxidation (long-chain fatty acid-CoA ligase and fatty acyl-CoA dehydrogenase) and with JNK2. Previous data have demonstrated that JNK1, but not JNK2 knockout mice, presented a protection against development of insulin resistance during diet-induced obesity (27). In our experimental conditions, however, one can not exclude that JNK2 could also play a role in protection against glucose intolerance.

The specific expression profiles obtained here provide the interesting information that disease classifiers can be established on the basis of liver or muscle gene expression. The latter observation is particularly interesting, because it suggests that comparison could be made with human patients from which small muscle biopsies could be prepared for microarray analysis.

Summary—The differential metabolic adaptation of mice to a high fat diet that we previously described was now shown to be associated with striking differences in gene expression patterns. The mechanisms triggering this differential adaptation are not presently known. Purely genetic causes appear unlikely, because C57Bl/6 mice are highly inbred. Our present data would rather suggest that epigenetic modifications might contribute to the observed phenotypic diversity. Whatever the exact mechanism, these data indicated that the differential metabolic adaptations are associated with higher lipogenic gene expression in the liver of ObD as compared with the lean (LD and LnD) mice. This was correlated with increased plasma VLDL and, at the muscle level, an augmented expression of LPL gene expression. This gene expression pattern is compatible with the hypothesis, which need to be further evaluated, that there is a higher flux of fatty acids from the liver to peripheral tissues in the obese as compared with the lean mice, which, together with a decrease in CIS-1 expression, may contribute to the higher insulin resistance of obese mice. In lean mice, resistance to obesity development was associated with a striking reduction in liver Scd-1 expression. The resistance to

**FIG. 4. Liver and muscle metabolic cross-talk in the establishment of the different phenotypes.** The scheme describes the changes in gene expression and plasma VLDL levels that suggest a higher flux of lipids from liver to muscle in the ObD mice relative to the two groups of lean mice. Top, in ObD mouse liver, lipogenic genes are expressed at the same level as in NC mouse liver but are increased as compared with their expression in the liver of LD and LnD mice and in the liver of mice fed a high fat diet for only 1 week. There is a significant increase in plasma VLDL concentration and, at the muscle level, a higher expression of LPL. This suggests an increased capacity for fatty acid uptake, which can cause insulin resistance. A reduction in CIS-1 expression may further aggravate the insulin resistance by increasing growth hormone signaling. Middle, in the LD mice, there is a reduction in hepatic lipogenic gene expression, including Scd-1. Plasma levels of VLDL are elevated, although not significantly, and there is a marked increase in LPL expression at the muscle level. The resulting increase in fatty acid uptake together with the reduction in CIS-1 expression may cause the observed insulin resistance. Bottom, in the LnD group, there is also a reduction in liver lipogenic gene expression, which is more marked than in LD mice (nine versus five lipogenic genes are down-regulated, respectively). At the muscle level, LPL expression in not increased as compared with the normal chow fed mice and CIS-1 is only moderately decreased. These observations are compatible with the lower degree of insulin resistance observed in these mice. The up-regulation of eNOS in the liver of these mice may contribute to the protection against diabetes development.
diabetes development in LnD mice may be associated with an additional increase in liver eNOS gene expression.

The different transcriptional programs appear after an initial stage where high fat feeding induces coherent changes in gene expression in liver in all mice tested. These are followed by a subsequent modification of gene expression toward patterns characteristic of each phenotype. How the transition toward the different steady states occurs is not yet elucidated at the molecular level and will require further detailed studies.

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