Transcriptional Profiling Reveals Global Defects in Energy Metabolism, Lipoprotein, and Bile Acid Synthesis and Transport with Reversal by Leptin Treatment in Ob/ob Mouse Liver*

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Leptin, a hormone secreted by adipose tissue, has been shown to have a major influence on hepatic lipid and lipoprotein metabolism. To characterize changes in lipid and lipoprotein gene expression in mouse liver, suppression subtractive hybridization and cDNA microarray analysis were used to identify mRNAs differentially expressed after leptin treatment of ob/ob mice. Ob/ob mice showed a profound decrease in mRNAs encoding genes controlling bile acid synthesis and transport as well as a variety of apolipoprotein genes and hepatic lipase with reversal upon leptin administration, suggesting that leptin coordinately regulates high density lipoprotein and bile salt metabolism. Leptin administration also resulted in decreased expression of genes involved in fatty acid and cholesterol synthesis, glycolysis, gluconeogenesis, and urea synthesis, and increased expression of genes mediating fatty acid oxidation, ATP synthesis, and oxidant defenses. The changes in mRNA expression are consistent with a switch in energy metabolism from glucose utilization and fatty acid synthesis to fatty acid oxidation and increased respiration. The latter changes may produce oxidant stress, explaining the unexpected finding that leptin induces a battery of genes involved in antioxidant defenses. Expression cluster analysis revealed responses of several sets of genes that were kinetically linked. Thus, the mRNA levels of genes involved in fatty acid and cholesterol synthesis are rapidly (<1 h) repressed by leptin administration, in association with an acute decrease in plasma insulin levels and decreased sterol regulator element-binding protein-1 expression. In contrast, genes participating in fatty acid oxidation and ketogenesis were induced more slowly (24 h), following an increase in expression of their common regulatory factor, peroxisome proliferator-activated receptor α. However, the regulation of genes involved in high density lipoprotein and bile salt metabolism shows complex kinetics and is likely to be mediated by novel transcription factors.

Forms of the leptin receptor are widely expressed, leptin actions on appetite and energy metabolism are thought to be mediated mainly through the receptor with a long intracellular domain present in the central nervous system (1). The binding of leptin to its receptor in the brain leads to the activation of Janus kinases and signal transducers and activators of transcription, thereby altering the expression and release of a number of neuropeptide effectors known to be involved in the control of feeding behavior and energy balance (1, 2).

Mice with genetic defects in the leptin signaling pathway (e.g. ob/ob and db/db mice) are obese and infertile, and show reduced activity and body temperature (3). They also exhibit hyperlipidemia, hyperinsulinemia, hyperglycemia, and resistance to insulin. Daily administration of recombinant leptin can partially or fully reverse these abnormal phenotypes (4–10). Gene expression profiling in adipose tissue (11, 12), skeletal muscle (13), hypothalamus (14), and pituitary (15) has been used to characterize differences in ob/ob and wild-type (wt) lean mice, and the response to leptin at the molecular level. Interestingly, hypercholesterolemia in ob/ob and db/db mice is due to elevated high-density lipoprotein (HDL)† levels (16). Our laboratory has shown that elevated HDL results from a defect in uptake of HDL by hepatocytes (9, 10). The mRNA for apoA-I, the major apolipoprotein of HDL, is also reduced in ob/ob mice, indicating sluggish HDL turnover. Defects in both synthesis and catabolism of HDL are reversed after about 2 days of low-dose leptin treatment. To gain fuller understanding of hepatic metabolic defects in ob/ob mice, we have used suppression subtractive hybridization (SSH) in combination with microarray analysis to characterize the metabolic response to leptin in mouse liver. These studies have revealed global defects in HDL metabolism and reverse cholesterol transport, including marked decreases in mRNAs encoding hepatic lipase, several apolipoproteins, cholesterol 7α-hydroxylase (the rate-limiting step in bile acid synthesis), and hepatic bile salt transporters. Our transcriptional profiling also shows altered mRNA expression for many genes regulating hepatic energy and other metabolic pathways.

MATERIALS AND METHODS

Animals—Female C57BL/6J obese ob/ob and lean +/+ mice (8–10 weeks of age) were purchased from The Jackson Laboratory. They were fed a chow diet and allowed to acclimate for at least 1 week before starting the experiments. For leptin treatments, the mice were injected intraperitoneally once per day either with recombinant mouse leptin

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† The abbreviations used are: HDL, high density lipoprotein; SSH, suppression subtractive hybridization; wt, wild-type; SREBP-1, sterol regulatory element-binding protein-1; FFAα, peroxisome proliferator-activated receptor α; Cyp7A, cholesterol 7α-hydroxylase; C/EBPα, CCAAT/enhancer-binding protein α; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; HMG-CoA, hydroxymethylglutaryl-CoA.
Coordinate Regulation of Hepatic Leptin-target Genes

(R&D Systems, Inc.) at a dose of 1 μg/g body weight or with saline as a control. Leptin- and saline-injected mice were pair-fed on the same diet. At the indicated times after injection, the mice were anesthetized and sacrificed, and liver was removed and snap frozen in liquid nitrogen. The tissues were stored at −70 °C for future use.

SSH—Liver RNA (total 15 μg) (mRNA (total 15 μg) was purified from total RNA with a mini-oligo(dT) cellulose spin column kit (5 Prime — 3 Prime, Inc.). For Northern analysis, liver total RNA from three mice in each treatment group was pooled, separated on 1% agarose-formaldehyde gels, blotted onto a nitrocellulose membrane, and hybridized with the radiolabeled probes. Hybridization and washing conditions were according to the manufacturer’s recommendations. The membranes were exposed to x-ray film to detect the hybridized probe. 

RESULTS

The primary goal of this study was to identify genes regulated at the transcriptional level by leptin signaling in mouse liver. SSH (17) was carried out using liver mRNA from pair-fed ob/ob mice daily injected with either leptin (1 μg/g body weight) or saline for 2 days. The subtracted cDNA fragments were then subcloned and propagated in bacteria as a cDNA library. To screen the target genes in an efficient way, we adopted microarray technology for pilot expression analysis of cDNA clones isolated from the library. Genes either induced or repressed by leptin signaling after 2 days of treatment, as confirmed by microarray hybridization (performed in triplicate), were further characterized by cDNA sequencing and Northern analysis. We then used microarray analysis to characterize the kinetics of changes in mRNA expression after leptin treatment (0.5-48 h), and to assess differential expression in wt versus ob/ob mouse liver.

Identification of Subtracted cDNA Clones from ob/ob Mouse Liver—Our screening yielded a total of 273 differentially expressed unique genes matched to nucleotide sequences found in the GenBank™ data base by BLAST searches, and about 40 clones were found without significant similarity to any sequences in the data base. The known genes can be divided into several large categories (Tables I and II). Microarray hybridization indicated that 173 of the identified genes (63%) are up-regulated by leptin, while the rest of the genes are down-regulated (Tables I and II). A large majority of the genes repressed by leptin were found to have higher expression in ob/ob versus wt liver, and, conversely, genes induced by leptin had lower expression in ob/ob versus wt liver (Tables I and II). Although the changes in mRNA levels detected by microarray hybridization were generally moderate (1.2–2.5-fold), in each case where the changes were confirmed by Northern analysis, they were shown to be much more dramatic than corresponding microarray results. For example, the mRNA levels of hepatic lipase and dicarboxylate transport protein are induced 5.5- and 1.6-fold by Northern analysis versus 1.7 (±0.2)- and 1.2 (±0.1)-fold by microarray analysis, respectively, by leptin. Northern analysis showed a 6.7- and 2.3-fold reduction in expression levels of glucose transporter-2 and stearyl-CoA desaturase after leptin treatment, compared with 2.4 (±0.2)- and 1.4 (±0.1)-fold decreases, respectively, indicated by microarray analysis. A similar underestimate of the degree of changes of mRNA expression by microarray analysis has been reported in other studies (11). About 40% of the genes were also evaluated by Northern analysis and in each case changes detected by microarray analysis were confirmed (Tables I and II). For each of the isolated genes with 1.2- or 1.3-fold change detected by microarray analysis, the responses to leptin were found to be significant (p ≤ 0.10 and 0.02, respectively) by Northern analysis.

Leptin Modulates Expression of Genes Involved in Cholesterol and Bile Salt Synthesis and Transport—Using SSH, we have identified a number of differentially expressed genes involved in the cholesterol and bile acid metabolic pathways (Table I). Leptin was found to repress the expression of an SREBP-1 target gene encoding cytosolic HMG CoA synthase, and also the gene for 4-sterol methyl oxidase involved in cholesterol synthesis (1.9- and 2.9-fold by Northern analysis). In contrast, the transcription of several genes responsible for bile acid metabolism is induced under the same conditions. For example, the mRNAs of the key regulatory enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (Cyp7a1), and of two bile acid transporters, sister of P-glycoprotein (localized on the basolateral membrane; Ref. 24) and the mRNAs of the key regulatory enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (Cyp7a1), and of two bile acid transporters, sister of P-glycoprotein (localized on the basolateral membrane; Ref. 24), increase upon leptin injection into ob/ob mice (6.1, 2.2-, and 2.5-fold, respectively). The nuclear receptor HNF4α, oxysterol-binding nuclear receptor
liver X receptor α, and the orphan receptor liver receptor homolog-1 have been shown to control the expression of cpy7α gene (26, 27). However, results from Northern analysis and microarray hybridization showed no significant differences in the expression of these three transcription factors after leptin treatment in ob/ob liver (data not shown), suggesting alternative mechanisms responsible for the induction of the cpy7α gene.

**Leptin Induces mRNAs Encoding Hepatic Lipase and Several Apolipoproteins**—The expression of several mRNAs encoding proteins involved in lipoprotein metabolism is decreased in ob/ob liver, and up-regulated after leptin treatment (Table I). Many of these genes are involved in reverse cholesterol transport (28). For example, the mRNA of hepatic lipase, an enzyme involved in the hydrolysis of triglycerides and phospholipids in HDL, dramatically increases after leptin injection into obese mice (5.5-fold by Northern analysis). Leptin also induces mRNAs of several HDL apolipoproteins, e.g. apoA-I (2.1-fold), apoA-II (2.4-fold), apoH (2.2-fold), and apoM (2.4-fold). However, mRNA of some other HDL apolipoproteins such as apoC-I and apoE are not affected by leptin (data not shown). The expression of another lipase, lysosomal acid lipase, which is required for the hydrolysis of low-density lipoprotein-derived cholesteryl esters and triglycerides in lysosomes, increases after leptin treatments (2.5-fold; Table I), while the mRNA level of hepatic monoglyceride lipase, an enzyme involved in the hydrolysis of stored triglycerides, does not change under the same condition (data not shown). We also found by Northern analysis that the mRNAs of two transcription factors, apolipoprotein regulatory protein-1 and early growth response factor-1, known to be important for the regulation of apoA-I transcription (29, 30) were not altered by leptin (data not shown). The expression of another lipase, lysosomal acid lipase, which is required for the hydrolysis of low-density lipoprotein-derived cholesteryl esters and triglycerides in lysosomes, increases after leptin treatments (2.5-fold; Table I), while the mRNA level of hepatic monoglyceride lipase, an enzyme involved in the hydrolysis of stored triglycerides, does not change under the same condition (data not shown). We also found by Northern analysis that the mRNAs of two transcription factors, apolipoprotein regulatory protein-1 and early growth response factor-1, known to be important for the regulation of apoA-I transcription (29, 30) were not altered by leptin (data not shown). The expression of another lipase, lysosomal acid lipase, which is required for the hydrolysis of low-density lipoprotein-derived cholesteryl esters and triglycerides in lysosomes, increases after leptin treatments (2.5-fold; Table I), while the mRNA level of hepatic monoglyceride lipase, an enzyme involved in the hydrolysis of stored triglycerides, does not change under the same condition (data not shown).
| Pathway                                           | Gene                                                                 | Fold change  | Northern | ob/wt |
|--------------------------------------------------|----------------------------------------------------------------------|-------------|----------|-------|
| **Carbohydrate metabolism**                      | Glucose transporter (GLUT-2; liver-specific)                        | 2.4 ↓       | (6.7 ↓)  | 1.6   |
|                                                  | Aldolase B                                                          | 1.6 ↓       | (2.3 ↓)  | 2     |
|                                                  | Glucose 6-phosphatase                                                | 1.5 ↓       | (2.1 ↓)  | 1.5   |
|                                                  | Glyceraldehyde-3-phosphate dehydrogenase                           | 1.3 ↓       |          | 1.7   |
|                                                  | Fructose bisphosphatase 2                                           | 1.4 ↓       |          | 1.1   |
|                                                  | UDP-glucose pyrophosphorylase 2                                     | 1.6 ↓       | (2.5 ↓)  | 1.2   |
|                                                  | Sorbitol dehydrogenase                                              | 1.4 ↓       |          | 0.6   |
| **Respiratory chain and ATP synthesis**          | Electron-transferring-flavoprotein dehydrogenase                    | 1.4 ↑       |          | 0.7   |
|                                                  | NADH dehydrogenase subunit 1, 2, 4, and 5                          | 1.4 ↑       |          | 0.9   |
|                                                  | Cytochrome c oxidase subunit I and III                             | 1.3 ↑       |          | 1.3   |
|                                                  | Mitochondrial cytochrome b                                          | 1.4 ↑       |          | 0.7   |
|                                                  | ATP synthase F1 subunit α                                           | 1.3 ↑       |          | 0.8   |
|                                                  | ATP synthase F1 subunits 6 and 8                                    | 1.4 ↑       | (2.1)    | 0.7   |
|                                                  | Adenine nucleotide translocase-2                                    | 1.4 ↑       |          | 0.6   |
|                                                  | Dicarboxylate transport protein                                     | 1.2 ↑       | (1.6)    | 0.8   |
|                                                  | Tricarboxylate transport protein                                    | 1.5 ↑       |          | 0.6   |
| **Urea metabolism**                              | Carbamyl phosphate synthetase I                                    | 1.6 ↓       |          | 1.3   |
|                                                  | Argininosuccinate synthetase                                        | 1.4 ↓       |          | 1.4   |
|                                                  | Argininosuccinate lyase                                             | 1.5         |          | 1.1   |
|                                                  | Arginase                                                           | 1.4 ↓       | (2.2 ↓)  | 1.3   |
|                                                  | Homogentisate 1,2-dioxygenase                                       | 1.3 ↓       |          | 1.2   |
| **Redox pathway**                                | Glutathione S-transferase                                           | 1.3 ↑       |          | 0.8   |
|                                                  | Glutathione S-transferase α3                                         | 2.1 ↑       |          | 0.6   |
|                                                  | Glutathione S-transferase II                                        | 1.2 ↑       |          | 0.7   |
|                                                  | Glutathione transferase GT9.3                                       | 1.8 ↑       |          | 1.5   |
|                                                  | Glutathione S-transferase class μ                                   | 1.3 ↓       |          | 0.9   |
|                                                  | Glutathione peroxidase 1                                             | 1.5 ↑       |          | 0.9   |
|                                                  | Epoxide hydrolase 2 (Epox2)                                         | 1.4 ↑       | (2.3)    | 0.8   |
|                                                  | Cu-Zn superoxide dismutase                                          | 1.4 ↑       | (1.9)    | 0.8   |
|                                                  | Leukotriene C4 synthase                                             | 1.7 ↑       |          | 0.5   |
|                                                  | NADP-dependent isocitrate dehydrogenase                            | 1.3 ↑       |          | 0.7   |
|                                                  | γ-Glutamyl cysteine synthetase light subunit                        | 1.3 ↓       |          | 1.5   |
|                                                  | Heme-binding protein HBP29                                           | 1.3 ↑       |          | 0.7   |
|                                                  | Plasma selenoprotein P                                              | 1.4 ↑       | (1.8)    | 0.6   |
|                                                  | α-Tocopherol (vitamin E) transfer protein                           | 1.3 ↑       |          | 0.8   |
|                                                  | 5-Oxo-l-prolinase                                                   | 1.4 ↑       |          | 0.8   |
|                                                  | Flavin-containing monooxygenase 5                                   | 1.4 ↓       |          | 0.6   |
| **Cytochrome P450 family**                       | Cytochrome P450 MUT-2                                              | 1.4 ↑       |          | 0.8   |
|                                                  | Cytochrome P450 cb                                                 | 1.1 ↓       |          | 0.8   |
|                                                  | Cytochrome P450 2d10                                               | 1.2 ↓       |          | 0.7   |
|                                                  | Cytochrome P450 2f15                                               | 1.4 ↓       |          | 0.8   |
|                                                  | Cytochrome P450 3A                                                 | 1.4 ↑       | (1.9)    | 0.6   |
|                                                  | Cytochrome P450 4A10                                               | 1.3 ↑       |          | 0.7   |
|                                                  | Cytochrome P450 naphthalene hydroxylase                            | 1.5 ↑       | (2.6)    | 0.8   |
|                                                  | Cytochrome P450 P-450Md                                            | 1.3 ↑       |          | 0.6   |
|                                                  | Cytochrome P450 17 α hydroxylase                                    | 1.3 ↑       |          | 1.1   |
|                                                  | Steroid cytochrome P450 7 α hydroxylase                            | 1.5 ↑       | (2.2)    | 0.7   |
|                                                  | Testosterone 16α-hydroxylase type b                                 | 1.4 ↑       |          | 0.6   |
| **Extracellular matrix and plasma proteins**     | EGF-containing fibulin-like extracellular matrix protein 1          | 1.7 ↑       |          | 0.6   |
|                                                  | Vascular cell adhesion molecule-1 (VCAM1)                           | 1.4 ↓       |          | 1.1   |
|                                                  | Syndecan-1                                                         | 1.2 ↓       |          | 0.6   |
|                                                  | Fibronectin                                                        | 1.4 ↑       |          | 0.5   |
|                                                  | Fibrinogen α-, β-, and γ-chain                                     | 1.6 ↓       | (3.1 ↓)  | 0.7   |
|                                                  | P-selectin                                                         | 2.0         |          | 0.5   |
|                                                  | Vitronectin                                                        | 1.2 ↓       |          | 0.8   |
|                                                  | Albumin/α-fetoprotein                                              | 1.4 ↑       |          | 0.7   |
|                                                  | Amyloid A protein                                                  | 2.3 ↑       | (8.1)    | 0.5   |
|                                                  | α2-HS-glycoprotein/fetuin homolog                                  | 1.3 ↑       |          | 0.4   |
|                                                  | Heptoglobin                                                        | 1.6 ↑       |          | 0.7   |
|                                                  | Complement factor B (MHC class III antigen)                        | 1.5 ↑       |          | 0.8   |
|                                                  | Complement component C3 α chain                                    | 1.4 ↑       |          | 0.6   |
|                                                  | Complement component C3 β chain                                    | 1.6 ↑       | (2.8)    | 0.8   |
|                                                  | Complement component C4                                            | 1.5 ↑       |          | 0.6   |
|                                                  | α2-Macroglobulin                                                   | 1.4 ↑       |          | 0.5   |
|                                                  | α1-Microglobulin                                                   | 1.3 ↑       |          | 0.6   |
|                                                  | β2 Microglobulin                                                   | 1.2 ↑       |          | 0.7   |
| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|----------------|----------------|----------|
| Zn <br> α2-glycoprotein | 1.2 ↓ | 0.8 |  |
| Ciliary neurotropic factor | 1.6 ↑ | 0.7 |  |
| Prekiniogen | 1.6 ↑ | 0.8 |  |
| Heparin cofactor II | 1.3 ↑ | 0.7 |  |
| Coagulation factor X | 1.2 ↑ | 0.3 |  |
| Coagulation factor XIII subunit B | 1.3 ↓ | 0.8 |  |
| Antithrombin III | 1.4 ↑ | 0.7 |  |
| Carboxypeptidase U | 1.2 ↑ | 0.7 |  |
| Carbonic anhydrase inhibitor | 1.2 ↑ | 0.7 |  |
| Regeneration-associated serpin-1 | 1.2 ↑ | 0.7 |  |
| SDF3 (human pigment epithelium-derived factor homolog) | 1.5 ↑ | 0.5 |  |
| Plasminogen | 1.3 ↑ | 0.8 |  |
| Cardiotrophin-1 | 1.3 ↑ | 0.7 |  |
| α-Catenin | 1.4 ↑ | 1.7 |  |
| α-Amylase | 1.2 ↑ | 0.7 |  |
| Amino acid, polyamine, and nucleotide metabolism |  |  |  |
| Phenylalanine hydroxylase | 1.2 ↓ | 1.5 |  |
| Branched chain α-ketoacid decarboxylase E1a subunit | 1.6 ↓ | 1.9 |  |
| Glycine N-methyltransferase | 1.2 ↓ | 1.8 |  |
| Betaine homocystein methyltransferase | 1.3 ↓ | 1.2 |  |
| Tryptophan-2,3-dioxygenase | 1.2 ↑ | 1.1 |  |
| Pyrroline-5-carboxylate dehydrogenase | 1.4 ↓ | 0.9 |  |
| Prolyl 4-hydroxylase | 1.4 ↓ | 1.1 |  |
| Tyrosine aminotransferase | 1.4 ↓ | 0.6 |  |
| Aspartate aminotransferase | 1.2 ↑ | 0.7 |  |
| Aspartate aminotransferase 2 | 1.3 ↑ | 0.8 |  |
| Kynurenine 3-hydroxylase | 1.5 ↓ | 1.2 |  |
| Serine dehydratase | 1.4 ↓ | 0.6 |  |
| S-Adenosylhomocysteine hydrolase | 1.3 ↓ | 0.8 |  |
| 10-Formyltetrahydrofolate dehydrogenase | 1.3 ↑ | 0.8 |  |
| S-Adenosylmethionine synthetase | 1.4 ↑ | 1.4 |  |
| Adenosine kinase | 1.4 ↑ | 0.7 |  |
| Protein synthesis, transport and folding |  |  |  |
| Aspartyl-tRNA synthetase a2 protein | 1.5 ↑ | 0.6 |  |
| Ribosomal protein L10a | 1.3 ↑ | 0.7 |  |
| Ribosomal protein L41 | 1.4 ↓ | 0.7 |  |
| Ribosomal protein S8 | 1.3 ↓ | 0.6 |  |
| Translational initiation factor 4AII | 1.3 ↑ | 1.1 |  |
| Translational elongation factor 2 | 1.4 ↑ | 0.7 |  |
| Ribosome-associated membrane protein RAMP4 | 1.3 ↑ | 0.6 |  |
| Ribosome-binding protein p34 | 1.4 ↓ | 0.9 |  |
| Sec24 | ND<sup>d</sup> | ND<sup>d</sup> |  |
| Sec61 | 1.2 ↓ | 0.3 |  |
| Sec62 | 1.4 ↓ | 0.7 |  |
| Human docking protein SRP receptor homolog | 1.5 ↑ | 0.7 |  |
| Transitional endoplasmic reticulum ATPase | 1.4 ↑ | 0.7 |  |
| Valosin-containing protein | 1.3 ↑ | 0.6 |  |
| Peroxisome PTS1 receptor | 1.3 ↑ | 0.8 |  |
| Peroxisome PTS2 receptor | 1.4 ↑ | 0.7 |  |
| Chaperonin subunit 4 | 1.3 ↑ | 0.7 |  |
| Syntaxin 7 | 1.4 ↓ | 1.7 |  |
| Synexin (annexin VII) | ND<sup>d</sup> | ND<sup>d</sup> |  |
| Cytoskeleton |  |  |  |
| β-Tubulin isotype 2 | 1.7 ↑ | 1.6 |  |
| Destrin (actin-depolymerizing factor) | 1.2 ↓ | 0.6 |  |
| Keratin-associated protein 8—1 | 1.3 ↓ | 0.7 |  |
| Hormone and vitamin metabolism |  |  |  |
| 11-β-hydroxysteroid dehydrogenase | 1.3 ↑ | 0.6 |  |
| 10-Formyltetrahydrofolate dehydrogenase | 1.8 ↓ | 0.8 |  |
| Plasma vitamin D-binding protein | 1.4 ↑ | 0.6 |  |
| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| Hydroxysteroid sulfotransferase | 1.2 ↑ | | 0.6 |
| Sulfortransferase M-STRP | 1.4 ↑ | | 0.7 |
| Sulfortransferase STα1 | 1.5 ↓ | | 2.8 |
| Cis-retinol/3α-hydroxysterol short-chain dehydrogenase | 1.5 ↑ | | 0.4 |

**Iron and heme metabolism**

| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| Protoporphyrinogen oxidase | 1.3 ↑ | | 0.5 |
| Ferrochelatase (protoheme ferrolyase) | 1.3 ↑ | | 0.6 |
| Hemopexin | 1.7 ↑ | | 0.6 |
| Transferrin | 1.5 ↑ | | 0.6 |

**Signal Transduction**

| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| TGF-β receptor-binding protein | 1.4 ↑ | | 0.4 |
| Insulin-like growth factor I | 1.6 ↑ | | 0.5 |
| Fibroblast growth factor receptor 4 | 1.4 ↑ | | 0.6 |
| Protein phosphatase type 1 catalytic subunit | 1.3 ↑ | | 0.6 |
| Protein Ser/Thr phosphatase 2A catalytic subunit | 1.4 ↑ | | 1.1 |
| Protein tyrosin kinase 9 homolog | 1.5 ↑ | | 0.7 |
| Human protein tyrosine kinase 9 homolog | 1.5 ↓ | | 1.4 |
| Adenylate cyclase activating polypeptide | 1.4 ↑ | | 0.6 |
| Cyclin G-associated kinase | 1.3 ↑ | | 0.4 |
| α protein | 1.2 ↑ | | 0.8 |
| Developmentally regulated GTP-binding protein | 1.4 ↑ | | 0.8 |
| Rho GTPase (CDC42) | 1.5 ↓ | | 0.5 |
| ADP-ribosylation-like 4 | 1.4 ↓ | | 0.5 |
| Human guanine nucleotide-exchange protein 2 homolog | 1.5 ↑ | | 0.6 |
| Ras-related RAB7 | 1.3 ↓ | | 1.8 |
| Ras-related GTP-binding protein | 1.4 ↑ | | 1.8 |
| 14–3-3 protein (γ subtype) I | 1.6 ↓ | | 1.4 |
| Calmodulin | 1.3 ↓ | | 0.5 |

**Apothesis**

| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| Apoptosis-related protein | 1.3 ↑ | | 0.5 |
| Silencer of death domain SODD | 1.4 ↓ | | 2.8 |
| Death-associated kinase 3 | 1.3 ↓ | | 1.7 |

**Nuclear or nucleic acid-binding proteins**

| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| Poly(A)-binding protein | 1.2 ↑ | | 0.6 |
| Vigilin | 1.3 ↑ | | 1.5 |
| Human HPBRII-7 homolog | 1.2 ↓ | | 0.7 |
| Nonsense-mediated mRNA decay trans-acting factor | 1.5 ↑ | | 1.3 |
| Fragile-X-related protein | 1.4 ↑ | | 0.6 |
| Centrosomin | 1.3 ↓ | | 0.7 |
| Homeo box B6 | 1.4 ↑ | | 0.6 |
| Cut-like homeoprotein | 1.4 ↑ | | 1.7 |
| Nuclear receptor binding factor 2 | 1.6 ↓ | | 2.0 |
| p100 co-activator | 1.4 ↑ | | 0.6 |
| Transcriptional coactivator ALY | ND<sup>d</sup> | ND<sup>d</sup> | |
| C-terminal binding protein 2 | 1.4 ↑ | | 0.6 |
| Telomerase protein-1 | 1.4 ↓ | | 1.5 |

**Others**

| Gene | Fold change<sup>a</sup> | Northern<sup>b</sup> | ob/wt<sup>c</sup> |
|------|-------------------------|-------------------|----------------|
| Putative N-acetyltransferase Camello 2 | 1.6 ↑ | | 0.3 |
| Cytosolic malate dehydrogenase | 1.3 ↑ | | 0.8 |
| GTP-specific succinyl-CoA synthetase | 1.4 ↑ | | 0.8 |
| Succinate dehydrogenase | 1.3 ↑ | | 1.4 |
| Alcohol dehydrogenase ADH-AA | 1.6 ↓ | | 1.5 |
| Alcohol dehydrogenase 1 | 1.3 ↓ | | 0.7 |
| Aldehyde dehydrogenase (Ahd-2-like) | 1.3 ↓ | | 0.7 |
| Human esterase D homolog | 1.3 ↓ | | 0.8 |
| Voltage-gated sodium channel (type I) β | 1.5 ↑ | | 0.5 |
| GM2 activator protein | 1.4 ↑ | | 1.3 |
| Galectin | 1.3 ↓ | | 1.8 |
| Heparin-binding protein-44 | 1.2 ↓ | | 0.6 |
| Vimentin | 1.5 ↓ | | 1.7 |
| TGF-β regulated gene 1 | 1.5 ↑ | | 0.6 |
| GPI-anchored protein homolog p137 | 1.3 ↑ | | 1.1 |
| Selenium-binding protein 1 | 1.6 ↑ | | ND<sup>d</sup> |
| Prothymosin a | 1.2 ↓ | | 0.6 |
| Chondromodulin II | 1.5 ↑ | | 0.9 |
| Neurodegeneration associated protein 1 | 1.4 ↓ | | 1.0 |
| Autocrine motility factor receptor | 1.3 ↑ | | 0.6 |
| Testis enhanced gene transcript | 1.3 ↑ | | 0.8 |
| Mouse p19 mRNA factor | 1.5 ↓ | | 0.6 |
| Mouse SA mRNA | 1.3 ↑ | | 1.5 |
| Mouse I54 mRNA | 1.3 ↑ | | 1.3 |
apoM mRNAs are up-regulated at 24 h, while cholesterol 7α-hydroxylase and MRP3 are significantly induced only after 48 h, indicating differential temporal regulation by leptin (data not shown).

Coordinate Control of Genes Regulating Fatty Acid Synthesis by Insulin and SREBP-1—Our cDNA subtraction approach has allowed us to isolate a large group of differentially expressed genes involved in hepatic lipid metabolism in ob/ob liver (Table I). The mRNAs of genes encoding fatty acid synthase, malic enzyme, and steroyl-CoA desaturase were shown to be increased in ob/ob liver and be dramatically decreased upon leptin treatment (5.1-, 3.7-, and 2.3-fold, respectively; Table I). Cluster analysis showed that the mRNA levels of these genes involved in fatty acid synthesis pathway are reduced and mapped to a single cluster with a similar kinetic response to leptin treatment (5.1-, 3.7-, and 2.3-fold, respectively; Table I). The mRNAs of genes encoding fatty acid synthase, malic enzyme, and stearoyl-CoA desaturase were shown to be increased by leptin (2-fold), and several enzymes involved in fatty acid synthesis (see above). Northern analysis confirmed that leptin increases the mRNAs encoding intracellular fatty acid transport proteins, including carnitine octanoyltransferase (2.6-fold) and peroxisomal membrane protein PMP70 (2-fold), and several enzymes involved in β-oxidation, such as medium-chain acyl-CoA dehydrogenase (2.8-fold) (Table I). Accompanying these changes, the mRNAs of two peroxisomal protein-targeting receptors, PTS1 and PTS2, are also induced by leptin (Table II), presumably reflecting the response to the increased loading of newly synthesized peroxisomal cargo. In addition, two cytochrome P450 enzymes, CYP2E1 and CYP4A14, known to be involved in fatty acid ω-oxidation in microsomes (34) are up-regulated by leptin (2–3-fold by Northern analysis, Table I).

Since many of the genes in these pathways are modulated by the transcription factor PPARα (35), we reasoned that leptin may increase the expression level of and the transcriptional activity of PPARα, thereby leading to the induction of genes involved in β- and ω-oxidation. To test this hypothesis, we performed Northern analysis using labeled PPARα cDNA probe. As shown in Fig. 2B, 2 days of leptin treatment up-regulated PPARα mRNA expression (2.3-fold) in ob/ob liver. The induction of PPARα expression by leptin occurs within 12 h, prior to the increase in mRNA of its target genes (data not shown). Thus these results strongly support the notion that PPARα may directly mediate the coordinate expression of genes involved in hepatic fatty acid oxidation following leptin treatment. It is known that the stimulation in β-oxidation can lead to an increase in mitochondrial ketogenesis. We found the expression of a regulatory enzyme mitochondrial HMG-CoA synthase (isolated by SSH) in the latter pathway, which is
controlled by PPARα (36), is also induced by leptin (2.5-fold by Northern analysis; Table I).

Leptin Reduces mRNAs of Genes Mediating Glycolysis and Gluconeogenesis, and Induces Mitochondrial Respiratory Genes—The finding that leptin treatment restores blood glucose levels and insulin sensitivity in ob/ob mice indicates the importance of its regulatory roles in carbohydrate metabolism (37). We have identified several differentially expressed genes in the sugar metabolic pathways (Table II). As revealed by Northern analysis, the mRNAs of two enzymes aldolase B (2.3-fold) and glucose-6-phosphatase (2.1-fold) involved in glycolysis and gluconeogenesis, respectively, were found to be elevated in ob/ob liver and were repressed by leptin (Table II; Ref. 37). In addition, the liver-specific glucose transporter (GLUT-2) gene is strikingly down-regulated after leptin injection (6.7-fold). These results suggest a reduction of hepatic glucose oxidation and production following 2 days of leptin injection, as determined by microarray hybridization experiments. D, leptin significantly decreases plasma insulin levels in 1 h after treatment in ob/ob mice.

Four of five enzymes in this pathway were found to be down-regulated at the mRNA level by leptin in ob/ob liver. This is consistent with the decreases in mRNAs of enzymes of gluconeogenesis, with which the urea cycle shares argininosuccinate synthetase and argininosuccinate lyase reaction steps. Expression cluster analysis indicated that several of the genes in these two pathways are grouped into one cluster with a similar kinetic pattern of repression starting at 24 h (data not shown). Previously it was shown that a transcription factor CCAAT/enhancer-binding protein α (C/EBPα) might coordinate the expression of urea cycle and gluconeogenic enzymes (39). Temporal expression analysis by microarray hybridization showed that, the hepatic mRNA level of C/EBPα is reduced 2-fold after 1 day leptin treatment in ob/ob mice (data not shown), thereby suggesting its potential role in the leptin-mediated regulation of both groups of genes.

Leptin Induces Genes Involved in Antioxidant Defenses—Another interesting group of genes is involved in the redox pathway (Table II). These genes had lower expression in ob/ob versus wt liver, and were induced by leptin. Northern analysis confirmed that genes encoding the antioxidant defensive enzymes, CuZn-superoxide dismutase, epoxide hydrolase, and plasma selenoprotein P, are induced by leptin (1.9-, 2.3-, and 1.8-fold, respectively). Considering that leptin can lead to the accumulation of detrimental reactive oxygen species inside cells (40) and that fatty acid oxidation and oxidative phosphorylation can produce these reactive radicals (41), one may hypothesize that the synthesis of the redox genes allows the ob/ob liver to eliminate the reactive molecules and to restore intra-
borne cytokines, such as IL-1, IL-6, and TNF-α, hepatic synthesis of acute-phase proteins is regulated by blood-borne cytokines. Since similar HDL particles accumulate in hepatic lipase hepatectomies (10). In particular the deficiency of hepatic lipase responsible for the defective transport of circulating HDL into HDL and hepatic lipase. The significant reduction in expression of genes encoding the major protein components of HDL (28). Previously ob/ob mice were shown to have elevated HDL cholesterol in plasma as a result of defects in HDL holoparticle uptake by liver (9, 10). This decrease of HDL catalolism can be reversed in vivo by leptin treatment, suggesting the involvement of leptin signaling in HDL metabolism and reverse cholesterol transport. In this study, we have identified several hepatic genes encoding key proteins participating in reverse cholesterol transport (Table 1). Leptin induces the transcription of genes encoding the major protein components of HDL and hepatic lipase. The significant reduction in expression of these proteins in ob/ob mice (Table 1) is probably partly responsible for the defective transport of circulating HDL into hepatocytes (10). In particular the deficiency of hepatic lipase probably makes a major contribution to the accumulation of large, apoE and cholesterol-rich HDL particles in ob/ob mice, since similar HDL particles accumulate in hepatic lipase knockout mice (47).

The biosynthesis of bile acid, a cholesterol catalytic product, is an important step in reverse cholesterol transport. We discovered that several genes involved in bile acid metabolism are markedly repressed in ob/ob versus wt liver and are induced in ob/ob liver by leptin (Table 1). This included a dramatic induction of Cyp7A, the rate-limiting enzyme in bile salt biosynthesis, as well as induction of the hepatocyte basal and canalicular

**FIG. 2.** Transcriptional regulation of genes involved in fatty acid oxidation in ob/ob liver by leptin signaling. A, an expression cluster of genes including those involved in fatty acid oxidation. Microarray analysis was carried out as described in the legend to Fig. 1A. B, the hepatic mRNA expression of expression factor PPARα is up-regulated by leptin. Northern analysis was performed as described in the legend to Fig. 1B.

cellular redox homeostasis. The induction of mRNA encoding inhibitory TGF-β receptor-binding protein (Table II) may also contribute to the relief of mitochondrial oxidative stress (42).

Cytochrome P450α and Pregnane X Receptor—We have identified a battery of leptin-responsive genes of the cytochrome P450 family. Leptin stimulates the expression of the mRNA encoding CYP3A (1.9-fold), a steroid hydroxylase, cytochrome P450 naphthalene hydroxylase (2.6-fold), and steroid cytochrome P450 7α hydroxylase (2.2-fold), whereas it attenuates the mRNA level of cytochrome P450 17α hydroxylase (Table II). Previously it was shown that a transcription factor, pregnane X receptor, is responsible for the induction of the cyp3A gene in liver (43). To examine whether leptin can modulate the mRNA levels of this factor, we performed Northern analysis to detect the response of its expression to leptin in ob/ob liver. Our results indicated that leptin up-regulates hepatic pregnane X receptor mRNA expression after 2 days of leptin injection (2.1-fold), suggesting a regulatory role of pregnane X receptor in the transcriptional activation of cyp3A and possibly other P450 family members.

Acute-phase Proteins Regulated by leptin—Our results indicate that leptin affects transcription of many genes encoding plasma acute-phase proteins (Table II). For example, the mRNA levels for amyloid A and complement component C3 are dramatically increased by 8.1- and 2.8-fold, respectively, while the expression of the fibrinogen gene is repressed 3.1-fold after leptin injection into ob/ob mice. It is well known that the hepatic synthesis of acute-phase proteins is regulated by blood-borne cytokines, such as IL-1, IL-6, and TNF-α. Leptin modulates the production of several pro- and anti-inflammatory cytokines after lipopolysaccharide treatment (44). To test whether the differential expression of the acute-phase proteins genes is caused by changes of cytokine levels in sera, we had measured serum IL-6 and TNF-α in leptin-injected ob/ob animals without endotoxin lipopolysaccharide challenge. However, we found no significant changes in level of these cytokines following leptin treatment (data not shown).

**DISCUSSION**

The present study has taken a global approach to the analysis of alterations in hepatic metabolism in ob/ob mice treated with leptin, compared with pair-fed controls. Gene expression profiling and cluster analysis provided insights into the transcriptional mechanisms underlying some of the altered patterns of gene expression in response to leptin. Several new findings have emerged from these studies. The mRNAs encoding genes of bile salt synthesis and transport were profoundly reduced in ob/ob liver and induced following leptin injection. Along with the increased synthesis and catabolism (9, 10) of several HDL apolipoproteins, as well as a marked induction of hepatic lipase, these findings indicate increased HDL turnover and bile salt formation and transport, *i.e.* leptin stimulates sequential steps of reverse cholesterol transport and bile salt synthesis and transport. A very rapid fall in plasma insulin following leptin treatment is probably responsible for the coordinate repression of a cluster of SREBP-1 target genes, resulting in decreased fatty acid and cholesterol biosynthesis (Fig. 1). In contrast, much slower changes in expression of PPARα, perhaps in response to alterations in fatty acid flux (45), may account for the slower induction of genes encoding enzymes of fatty acid β- and ω-oxidation and ketogenesis (Fig. 2). An intriguing finding was the discovery that a battery of antioxidant defenses is repressed in ob/ob liver and induced after leptin treatment. This could be a response to oxidant stress generated by increased fatty acid oxidation and mitochondrial respiration following leptin treatment. The reduction in expression of genes participating in both gluconeogenesis and the urea cycle correlates well with the decrease in the level of transcription factor C/EBPα, another insulin-sensitive transcription factor (46).
bile salt export pumps. From these results, it appears that ob/ob mice have impaired hepatic bile synthesis and transport, which is reversed by leptin treatment. Interestingly, there is substantial evidence that HDL-derived cholesterol is a preferential source for the cholesterol that appears in bile (28). Following binding of HDL to scavenger receptor BI on the basal side of hepatocytes, there is rapid uptake of free cholesterol and cholesteryl ester by hepatocytes, followed by their appearance in bile (48). Recent evidence suggests that different steps in the reverse cholesterol transport pathway may be coordinately regulated by the transcription factor, liver X receptor, which is activated by specific oxysterols (49). The present findings indicate that leptin signaling may be an important factor regulating reverse cholesterol transport at sequential steps, i.e. synthesis of HDL proteins, uptake of HDL cholesterol and protein by hepatocytes, conversion of HDL-derived cholesterol into bile salts (by Cyp7A), as well as bile salt transport. However, the mechanisms underlying these different effects of leptin remain unknown. Recently the expression of hepatic lipase and bile acid transporters was found to be markedly reduced in HNF-1α knockout mice (50), suggesting a potential role of this transcription factor in the regulation of bile acid and HDL metabolism by leptin.

The stimulation of lipid oxidation and oxidative phosphorylation in ob/ob liver is probably associated with enhancement of reactive oxygen formation within cells (40, 41). This would in turn trigger various defense responses, including elevated expression of antioxidant enzymes, reversing the deficiency in ob/ob liver (Table II; Ref. 51). Previously it was shown that the oxidative stress generated in endothelial cells by leptin treatment leads to activation of the redox-sensitive transcription factors activator protein 1 and, to a lesser extent, nuclear factor κB (NF-κB) (40). Microarray expression analysis indicated that hepatic NF-κB mRNA levels were not altered by leptin (data not shown). Thus it is possible that leptin-mediated enhancement of fatty acid oxidation and oxidative phosphorylation in ob/ob liver can elevate redox gene expression by stimulating the activities and/or transcription of redox-sensitive transcription factors, such as activator protein 1, NF-κB, specificity protein 1 (Sp1), or Nrf-1 and -2 basic-leucine zipper proteins (52). For example, CuZn-superoxide dismutase, which is induced by leptin (Table II), is a known target gene of Sp1 (53). Our data provide the first in vivo evidence suggesting that leptin treatment produces oxidative stress, followed by induction of antioxidant defenses.

Leptin was originally defined as a satiety signal, providing negative feedback signaling to the central nervous system to increase sympathetic outputs, inhibit food intake, reduce body weight, and stimulate energy expenditure (1, 2). Evidence has accumulated for complex effects of leptin on carbohydrate and fat metabolism (37, 45). Our study suggests that changes in the liver may be mediated at least in part by alterations in humoral factors, which act on a small number of key transcription factors. Leptin reduced the mRNA encoding hepatic lipogenic and cholesterol synthetic enzymes by inhibiting the transcription of their common regulatory transcription factor SREBP-1. These changes in hepatic SREBP-1 mRNA are likely to be paralleled by changes in SREBP-1 protein processing into the active form (11). Similar findings have been reported in rodent adipocytes where leptin was shown to regulate both SREBP-1 mRNA levels as well as cleavage of SREBP-1 protein into the active form (11). The increased expression and activity of SREBP-1 in ob/ob liver is associated with an elevation of fatty acid biosynthesis and a fatty liver in this strain of mice (54; Table I). In ob/ob adipose tissue, however, the mRNA levels of SREBP-1 and its target genes are much lower than in wild-type lean mice (11, 12), whereas the converse is true in the liver (Table I). These data suggest that the liver has become the major site for fatty acid synthesis in ob/ob mice. Recent evidence has revealed a role of insulin in the aberrant production of hepatic SREBP-1 in ob/ob mice (33). The cluster analysis and time course study that we performed indicated a coordinate repression of SREBP-1 target genes after leptin administration. A striking feature is the rapidity of these coordinate changes, which parallel decreases in plasma insulin levels. The decrease of insulin concentration is probably due to the stimulation of hepatic insulin disposal (55) or to the reduction in insulin synthesis and secretion by leptin (37). These results thus support the hypothesis that leptin-mediated reduction in the expression of SREBP-1 and its target genes may be secondary to the effects of leptin on plasma insulin.

Chronic leptin treatment induce the expression of genes involved in hepatic fatty acid oxidation and was accompanied by the decreased expression of genes involved in glucose metabolism, including glycolysis. This suggests a switch of the energy substrate from glucose to fatty acids, consistent with the findings obtained by biochemical approaches (45). The upregulation by leptin of gene expression in fatty acid oxidation...
and ketogenic pathways is kinetically clustered and appears to be directly mediated through the transcription factor PPARα. PPARα is known to be responsible for the transcriptional activation of these genes in liver (35), and the hepatic expression of PPARα is increased upon leptin treatment (Fig. 2b). Previously leptin was found to induce the transcription of PPARα gene in pancreatic β cells (56) but not in adipose tissue (11). The mechanisms underlying the induction of PPARα transcription by leptin is unclear, but it could perhaps be related to changes in fatty acid flux mediated by leptin signaling (45).

Fig. 3 proposes a model to illustrate some of the leptin-mediated transcriptional responses regulating hepatic metabolic pathways in ob/ob mice. Leptin treatment causes the transcriptional induction of genes involved in fatty acid oxidation and ketogenesis through the enhancement of hepatic PPARα expression and activity. This may in turn lead to an increase in oxidative stress, thereby activating redox-sensitive transcription factors and modulating the expression of their target genes. In contrast, leptin rapidly decreases the levels of plasma insulin, and causes reduced expression of SREBP-1 and its target genes participating in fatty acid and cholesterol synthesis. In addition, leptin attenuates the expression of a transcription factor C/EBPα that results in a coordinated repression of its target genes in the metabolic pathways of gluconeogenesis and the urea cycle. Finally, a battery of genes encoding key proteins required for reverse cholesterol transport (including HDL and bile acid metabolism) are up-regulated by leptin signaling via an unknown mechanism.

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