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Dissociation between adipose tissue fluxes and lipogenic gene expression in *ob/ob* mice

S. M. Turner^{1,3}, S. Roy¹, H. S. Sul¹, R. A. Neese¹, E. J. Murphy², W. Samandi¹, D. J. Roohk¹, and M. K. Hellerstein^{1,2}

¹ Department of Nutritional Sciences and Toxicology, University of California, Berkeley

² Division of Endocrinology and Metabolism, Department of Medicine, San Francisco General Hospital, University of California, San Francisco

³ KineMed, Emeryville, California

Abstract

Recent evidence has been presented that expression of lipogenic genes is downregulated in adipose tissue of *ob/ob* mice as well as in human obesity, suggesting a functionally lipoatrophic state. Using ²H₂O labeling, we measured three adipose tissue biosynthetic processes concurrently: triglyceride (TG) synthesis, palmitate de novo lipogenesis (DNL), and cell proliferation (adipogenesis). To determine the effect of the *ob/ob* mutation (leptin deficiency) on these parameters, adipose dynamics were compared in *ob/ob*, leptin-treated *ob/ob*, food-restricted *ob/ob*, and lean control mice. Adipose tissue fluxes for TG synthesis, de novo lipogenesis (DNL), and adipogenesis were dramatically increased in *ob/ob* mice compared with lean controls. Low-dose leptin treatment (2 µg/day) via miniosmotic pump suppressed all fluxes to control levels or below. Food restriction in *ob/ob* mice only modestly reduced DNL, with no change in TG synthesis or adipogenesis. Measurement of mRNA levels in age-matched *ob/ob* mice showed generally normal expression levels for most of the selected lipid anabolic genes, and leptin treatment had, with few exceptions, only modest effects on their expression. We conclude that leptin deficiency per se results in marked elevations in flux through diverse lipid anabolic pathways in adipose tissue (DNL, TG synthesis, and cell proliferation), independent of food intake, but that gene expression fails to reflect these changes in flux.

Keywords

deuterated water; lipogenesis; isotope; gene expression

The *ob/ob* mouse exhibits obesity, insulin resistance, hyperphagia, sterility, and other metabolic and hormonal disturbances due to a mutation in the leptin gene (23). Histological (1) and biochemical evidence (22,25,26,28,46) suggests that these disturbances include abnormal dynamics of both the lipid and cellular components of adipose tissue.

De novo lipogenesis (DNL) has been well studied in the *ob/ob* mouse. In vivo ³H₂O labeling studies have shown increased hepatic (14,22,29) and adipose tissue (6,14,22,29) DNL. Inhibitory effects of leptin on DNL have also been demonstrated (2).

Triglyceride (TG) synthesis and degradation (lipolysis) can also now be measured in adipose tissue by administration of $^2\text{H}_2\text{O}$ (5,39,44). Measurement of TG synthesis contributes another metric of adipose tissue metabolism in vivo and also allows for more accurate assessment of DNL rates (39). Generally, DNL is measured as a fraction of total lipid or palmitate present in a lipid sample. By measuring the fraction of new TG from all sources concurrently with the fraction of TG from DNL, the true contribution of DNL to newly synthesized TG can be calculated (39). We have shown elsewhere (39) that cell proliferation, DNL, and TG synthesis can be measured concurrently using $^2\text{H}_2\text{O}$ labeling.

Leptin has also been shown to inhibit differentiation of preadipocytes (2,42) and to reverse the differentiation of mature adipocytes (47) in vitro. These effects on adipocyte differentiation have been proposed to have a role in the weight-reducing effects of leptin treatment (2,42, 47). Changes in the rate of preadipocyte differentiation may or may not alter the proliferation rates of mature adipocytes, depending upon whether there is a change in the fate of new preadipocytes in vivo. The effect of leptin on proliferation of mature adipose cells has not been determined in vivo. The effects of leptin deficiency or caloric restriction on adipocyte proliferation have also not been directly measured.

Although many biochemical studies in *ob/ob* mice have reported increased lipogenesis, gene expression microarray data have painted a different picture of adipose tissue dynamics in *ob/ob* mice (32,37) and obese humans (8). Expression of mRNAs for lipogenic enzymes [e.g., fatty acid (FA) synthase (FAS), ATP citrate lyase, phosphoenolpyruvate carboxykinase (PEPCK)] and sterol response element-binding protein-1c (SREBP-1c) were reported to be reduced, not elevated, in adipose tissue of *ob/ob* mice (32). Paradoxically, treatment of *ob/ob* and lean mice with exogenous leptin further reduced the expression of similar sets of such lipogenic genes (32). On the basis of these gene expression results, Nadler and Attie (31) reasonably suggested that adipose tissue in *ob/ob* mice operates as if in a functionally replete state, with reduced capacity for lipid anabolism and storage. According to this model, adipose tissue function in *ob/ob* mice is analogous to a lipotrophic state rather than an avidly lipid anabolic milieu. It is important to resolve these apparently discordant models of obesity suggested by gene expression vs. flux measurements.

Reduced expression of lipogenic genes has also been reported in human obesity (8). Increased hepatic DNL fluxes were measured with stable isotopes, yet there was reduced adipose lipogenic gene expression, perhaps secondary to increased circulating leptin levels (8), providing further evidence of a functionally lipotrophic state in obesity.

Here, we compare lipogenic fluxes in vivo and gene expression in the same tissue (adipose). One of our objectives was to establish the independent effects of leptin and calorie intake. Accordingly, a low dose of leptin (2 $\mu\text{g}/\text{day}$) was administered, since this dose has previously been shown (17) to reduce food intake but not plasma insulin levels. Also, caloric restriction of *ob/ob* mice results in gorging or a “meal” feeding pattern, followed by a prolonged fast, which may by itself affect DNL compared with ad libitum feeding. We therefore controlled the pace of food intake in the food-restricted *ob/ob* mice by using an automatic pellet dispenser to administer food at a fixed rate over each 24-h period.

We show here that, in the C57BL/6J^{lep/lep} (*ob/ob*) genetic model of obesity, fluxes through lipogenic and adipogenic pathways in adipose tissue are markedly elevated and that gene expression is not necessarily reflective of flux through these lipid anabolic pathways.

METHODS

Animals

Stable isotope studies—All animal experiments were performed in compliance with the University of California Berkeley animal care and use committee. Six- to seven-week-old female, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were studied: C57BL/6J^{+/+} controls (*ctrl*), ad libitum fed C57BL/6J^{lep/lep} (*ob/ob*), leptin treated *ob/ob* (*ob-lep*), and food restricted *ob/ob* (*ob-r*). The rationale for studying *ob-lep* and *ob-r* was to determine whether changes in adipose tissue dynamics in *ob/ob* are driven by excess food intake or leptin deficiency per se. Mice were housed individually in hanging wire cages and fed American Institute of Nutrition 93 purified diets (Bio Serv, Frenchtown, NJ). Mice were given 3 days to acclimate to the environment, after which they grew normally. All treatments and interventions began 5 days prior to the start of labeling with ²H₂O. Five animals were studied in each group.

Food for the *ob-r* group was administered in a continuous manner with automatic pellet dispensers (Coulbourn Instruments, Allentown, PA). Food intake was set at 2.8 g/day, which represents the lower range of intake for lean mice, partially adjusting for the reduced total energy expenditure in *ob/ob* (4,27,36). This ensured that sufficient caloric restriction was achieved for comparison to the effects of leptin administration [which also reduces food intake (see below)].

Ob-lep mice received murine leptin (a gift from Amgen, Thousand Oaks, CA) subcutaneously at a dose of 2 µg/day via a 28-day Alzet miniosmotic pump (Alza, Palo Alto, CA). This dose has previously been shown (17) to achieve near-physiological leptin concentrations in *ob/ob* mice.

Mice received ²H₂O (deuterated water) as a priming dose at 0.012 ml/g body wt via intraperitoneal injection. The normal drinking water was then replaced with water enriched to 4% ²H₂O, which was continued for a total of 21 days. ²H₂O treatment had no impact on food intake or body weight. Prior to death, mice were fasted for 4 h, anesthetized with isoflurane, and exsanguinated via cardiac puncture.

Gene expression studies—To measure the expression of lipogenic genes, 8-wk-old female *ob/ob* (*n* = 5), *ob-lep* (*n* = 5), and control (*n* = 5) mice were studied. Leptin was administered for 7 days as described above. Mice were killed at the end of the dark cycle (7 AM) without a prior fast (to reflect maximally stimulated lipid anabolism in the circadian cycle). Inguinal adipose tissue was immediately dissected and frozen in liquid nitrogen.

Blood Measurements

At the end of the 3-wk study period, blood was collected by cardiac puncture and placed on ice until plasma was separated and frozen. Plasma glucose concentrations were measured on a YSI (Yellow Springs, OH) auto analyzer. Plasma insulin and leptin assays were performed by assay services at Linco Research (St. Charles, MO).

Changes in Fat Pad Weight

The gain in weight of each fat pad was compared with the net synthesis of TG. The weight of each fat pad at the beginning of the ²H₂O labeling period was estimated by sacrificing three separate *ob/ob* (30 ± 5 g) and 5 control mice (18 ± 0.6 g) at the same age as the study mice at the start of ²H₂O labeling period (6 wk old). The relative percentage of body weight from each pad was calculated and averaged for each group. This percentage was then applied to the starting weight of the study animals to estimate the initial weight of each depot. The average

starting weight was then subtracted from the final measured weight of the isolated pads to estimate the absolute fat gain in each depot.

Adipose Tissue Preparation and Isolation of Adipocytes

Immediately following dissection, inguinal, perimetrial, retroperitoneal, and mesenteric fat pads were placed in HBSS with calcium in preweighed tubes for isolation of mature adipocytes according to the method of Rodbell (35). Inguinal and retroperitoneal pads from the left and right sides were pooled for analysis. Minced tissue was placed in HBSS with 0.1% type II collagenase (Worthington Biochemical, Lakewood, NJ). Tissue was incubated at 37°C for ≤90 min with shaking. Samples were spun at 800 rpm for 10 min. The adipocyte enriched, stromal-vascular cell-depleted fraction was then carefully removed and frozen.

Bone marrow cells were isolated from the hind limb femur as described previously (33).

DNA Isolation and Derivatization from Adipose Cells

The frozen slurry enriched in adipocytes was lyophilized, and the dry weight of the sample was determined. The samples were digested, and DNA was isolated as described elsewhere (33) using Qiagen DNeasy tissue kits. The yield of DNA from each sample was determined with a Pharmacia Biotech Genequant II spectrophotometer.

Ten to twenty-five micrograms of DNA were hydrolyzed to free deoxyribonucleic acids, as described in detail elsewhere (33). Isolated deoxyadenosine was reduced and the deoxyribose (dR) moiety acetylated, as described previously (33). The resulting pentosetetraacetate (PTA) derivative of dR was injected in ethyl acetate into the gas chromatograph-mass spectrometer (GC-MS) for measurement of isotope enrichments.

Derivatization and Analysis of TG, FA, and Body Water

Samples of adipose TG were taken following the incubation with collagenase and transesterified by incubation with 3 N methanolic HCl (Sigma-Aldrich). FA methyl esters were separated from glycerol by Folch extraction. The phase containing free glycerol was then lyophilized, and the glycerol was converted to glycerol triacetate by incubation with acetic anhydride-pyridine, 2:1, as described elsewhere (21). The phase containing FA methyl esters was concentrated under nitrogen and injected directly into the GC-MS.

$^2\text{H}_2\text{O}$ enrichments in body water were measured in tetrabromoethylene derivatized from plasma samples, as described in detail elsewhere (7).

GC-MS Analyses

Model 5970, 5971, or 5973 GC-MS instruments (Agilent, Palo Alto, CA) were used for measuring isotopic enrichments of glycerol triacetate, FA methyl esters, and tetrabromoethylene (H_2O).

Glycerol triacetate was analyzed using a DB-225 fused silica column, monitoring mass-to-charge ratios (m/z) 159 (parent ion, M_0) and 160 (M_1) or m/z 159, 160, and 161 (M_0 , M_1 , and M_2 , respectively). Methane chemical ionization (CI) was used with selected ion monitoring.

FA methyl ester composition was analyzed by flame ionization detection and for ^2H enrichment by GC-MS, as described elsewhere (21,24).

Tetrabromoethylene (H_2O) was analyzed using a DB-225 fused silica column, monitoring m/z 265 and 266 (M_0 and M_1 ions). Standard curves of known $^2\text{H}_2\text{O}$ enrichment were run before and after each group of samples to calculate isotope enrichment.

PTA samples (dR) were analyzed for incorporation of deuterium using methane CI with a 30-m DB-225 column under selected ion monitoring of m/z 245–246 (M_0 and M_1 ions). Natural abundance (unenriched) dA samples were measured concurrently, and the excess M_1 (EM_1) abundance in the adipose PTA samples were calculated by subtracting the M_1 abundance measured in the unenriched standard from the M_1 abundance in the sample. Bone marrow DNA samples were run simultaneously. Bone marrow DNA represents a completely, or nearly completely, turned over tissue and is used as a reference value for calculating the fraction of newly divided cells in a tissue of interest, as described previously (33).

RNA Isolation and Reverse Transcription

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy (Qiagen, Valencia, CA). During purification, an on-column DNase I digestion step was carried out as described in the RNeasy Mini Protocol. Five-hundred nanograms of total RNA from each sample and 2 μ g of total RNA pooled from each sample from the control group were reverse transcribed with random hexamers using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA).

Real-time PCR

Real-time PCR was performed in 96-well format using the ABI Prism 7900 HT sequence detection system and analyzed using SDS 2.0 software (Applied Biosystems). For each gene transcript, 10 ng of cDNA from each sample was analyzed as an unknown against a standard curve derived from a fivefold dilution series of cDNA reverse transcribed from RNA pooled from the control group. Each 25- μ l PCR reaction was carried out using TaqMan Universal PCR Master Mix (Applied Biosystems) and primer and probe sets from Applied Biosystems Assays on demand. Relative mRNA levels for each gene were measured in arbitrary units and normalized to GAPDH levels.

Calculations

TG synthesis—The fraction (f) of TG newly synthesized during the labeling period in an adipose depot was calculated on the basis of the precursor-product relationship, as described previously (44,20), using the measured body water enrichment to estimate the maximal or asymptotic enrichment in TG-glycerol. The absolute rate of retained TG synthesis was calculated by multiplying f times the TG mass present at the time of measurement.

Estimate of net lipolysis—Net lipolysis was calculated as described previously (44): net lipolysis ($\text{mg} \cdot \text{kg}^{-1} \cdot 21 \text{ days}^{-1}$) = absolute TG synthesized – absolute TG accumulated.

In the case of the leptin treated mice, in which TG retained was a negative value (i.e., body fat was lost), the absolute TG synthesis rate was added to the quantity of TG lost to yield the net lipolysis rate (i.e., the negative number was subtracted).

DNL—Fractional DNL was calculated from the incorporation of ^2H into TG-palmitate, using mass isotopomer distribution analysis as described in detail elsewhere (39). Fractional DNL represents the fraction of FA synthesized via the DNL pathway during the labeling period, relative to all other sources of TG-FAs. However, this underestimates the contribution of DNL to newly synthesized TG to the extent that preexisting fat is present, since “non-DNL” TG could represent preexisting TG or newly synthesized TG from non-DNL pathways. This problem can be resolved by correcting DNL for the proportion of TG that was newly synthesized during the labeling period. The ratio of fractional DNL to fractional TG synthesis reveals the true contribution of DNL to new TG-palmitate (39). Data presented here are expressed as the corrected value, representing the fractional contribution from DNL to newly synthesized TG.

The absolute rate of palmitate DNL was calculated by multiplying measured corrected fractional DNL times 0.8 (the estimated fraction by weight of TG in adipose tissue) times the weight of the fat pad times the percentage of FA in the depot that is palmitate. This value represents the absolute amount (g) of palmitate synthesized during the labeling period.

Adipose cell proliferation (adipogenesis)—Fractional adipose cell proliferation (f) was calculated as the EM_1 in adipose DNA divided by EM_1 in bone marrow DNA, as described in detail elsewhere (33). Bone marrow DNA enrichment is used to approximate the maximum DNA enrichment achievable within each animal at the body water enrichment present (33). The absolute adipose cell proliferation rate was estimated by multiplying f times total DNA yield from the tissue and converting to cell numbers (assuming $\sim 10 \mu\text{g DNA}/106 \text{ cells}$).

Statistical Analysis

Data are presented as means $\pm$ SD unless otherwise stated. Significance was determined by one-way ANOVA followed by Tukey's pairwise comparisons. $P < 0.05$ was considered significant.

RESULTS

Food Intake

Food intake in the control mice was $3.1 \pm 0.4 \text{ g/day}$. Food intake of the food-restricted *ob/ob* mice (*ob-r*) was fixed at 2.8 g/day . *ob/ob* Mice consumed $5.1 \pm 1.1 \text{ g/day}$, and the leptin-treated *ob/ob* mice (*ob-lep*) ate $3.8 \pm 0.9 \text{ g/day}$. Significant differences were present between *ob/ob* and controls, *ob-lep*, and *ob-r* ($P < 0.05$; Table 1).

Body Weight

The effects of leptin treatment and food restriction on body weight during the $^2\text{H}_2\text{O}$ labeling period are shown (Fig. 1). Leptin treatment reduced body weight significantly more than food restriction despite higher food intake in the leptin-treated group.

Plasma Insulin, Glucose, and Leptin Concentrations

Plasma insulin, glucose, and leptin levels were measured from the terminal blood collection after a 4-h fast in all groups at the end of the study. Insulin concentrations were significantly higher in all *ob/ob* groups, including the leptin-treated group, compared with control mice, as has been previously reported (15) (Table 1). Plasma glucose concentrations were significantly higher in the *ob/ob* group compared with controls (Table 1). Both food restriction and leptin treatment tended to reduce plasma glucose concentration in *ob/ob* mice, but only the leptin-treated group showed significant reductions compared with *ob/ob*. Plasma leptin levels in the leptin-treated *ob/ob* and control mice were similar (5.7 ± 2.9 and $4.1 \pm 1.9 \text{ ng/ml}$, respectively; Table 1). In the *ob/ob* and food-restricted *ob/ob* mice, plasma leptin concentrations were undetectable.

Body Fat Distribution

The untreated *ob/ob* animals had larger fat pads (Table 2) that represented a higher percentage of total body weight compared with controls. Food restriction significantly reduced the weight of all fat pads, and leptin treatment resulted in a significantly greater weight reduction in inguinal and mesenteric pads.

Absolute Rates of TG Synthesis (All-Source Lipogenesis)

The absolute TG synthesis rate was markedly elevated in the *ob/ob* and food-restricted *ob/ob* mice compared with control and leptin-treated mice (Fig. 2). Leptin treatment also significantly reduced the f of newly synthesized TGs (Table 3), compared with *ob/ob*, for each fat pad.

DNL Contribution to Adipose TG-Palmitate

The contribution from DNL to newly synthesized TG-palmitate is shown in Table 4. In the *ob/ob* and control mice, the DNL fractional contribution to newly deposited adipose TG was close to 100%. The leptin-treated group exhibited a substantial reduction in the contribution from DNL to new TG. Food restriction also lowered the DNL contribution to newly synthesized TG compared with *ob/ob* and controls.

Absolute Rate of DNL

The absolute rate of DNL (g palmitate synthesized de novo over the 21-day labeling period) was significantly elevated in the *ob/ob* group compared with controls (Fig. 3). Food restriction had a small (nonsignificant) impact on absolute DNL. Leptin treatment, in contrast, reduced absolute DNL to levels equal to or below those seen in the control mice in all depots.

Net Lipolysis

ob/ob Mice had higher rates of net lipolysis (calculated as TG synthesis minus fat accumulated over 21 days) than the controls (Fig. 4). Food restriction (tended toward increased lipolysis) tended to increase lipolysis, and leptin treatment further increased (tended to increase) lipolytic rates (further; significant in inguinal fat pad only). The absolute magnitude of the increase in lipolysis induced by leptin treatment (range 0–0.6 g/21 days) was approximately one-half the magnitude of the reduction in TG synthesis due to leptin treatment (0.4–1.2 g/21 days; compare with Fig. 2).

Adipogenesis

The absolute rate of adipose cell proliferation (adipogenesis) was markedly increased in the perimetrial and retroperitoneal fat pads of *ob/ob* mice compared with controls (Fig. 5). Leptin treatment, but not food restriction, markedly reduced adipose tissue cell proliferation in all fat pads compared with *ob/ob* mice.

Gene Expression

Expression levels of mitochondrial glycerol-3-phosphate acyltransferase (GPAT), SREBP1, diacylglycerol acyltransferase (DGAT)1, glucose transporter 4 (GLUT4), FA-binding protein (FABP)4, PEPCK, acetyl-CoA acetyltransferase 1 (ACAT1), and peroxisome proliferator-activated receptor- γ (PPAR γ) were not elevated in *ob/ob* compared with controls (Fig. 6), confirming previously reported microarray results (32,37). Notably, the expression of PEPCK, ACAT1, and FABP4 were reduced, whereas only DGAT2 and FAS expression were significantly elevated in *ob/ob* compared with controls. Interestingly, leptin treatment increased PEPCK and GLUT4 expression, and FAS expression also trended upward with leptin treatment, whereas DGAT2 expression was no longer significantly elevated after leptin treatment. Most mRNA levels studied were not significantly altered by leptin administration.

DISCUSSION

Leptin provides a signal from the adipose tissue to the central nervous system and other organs that regulates energy intake, energy expenditure, and nutrient partitioning (11,15–17). Here, we show in vivo that this endocrine signal completes a full circle back to the adipose tissue, inducing dramatic effects on adipose lipid dynamics and cell proliferation, independent of food

intake or insulin concentrations. Of perhaps equal interest, the measured changes in flux through lipid anabolic pathways in *ob/ob* vs. lean control mice, or in response to leptin administration, were not generally reflected by adipose tissue mRNA levels.

Several areas of adipose metabolism were studied simultaneously, using heavy water labeling techniques recently developed (33,39,44). Previous studies (6,14,22) have shown increases in DNL in adipose tissue of *ob/ob* mice, with labeled water ($^2\text{H}_2\text{O}$ or $^3\text{H}_2\text{O}$) used to measure adipose tissue TG synthesis as well as DNL. However, a comprehensive evaluation of the integrated dynamics of different adipose tissue components in *ob/ob* mice in response to leptin administration or food restriction had not previously been carried out.

We found that leptin administration to *ob/ob* mice reduced rates of total TG synthesis (Fig. 2) and stimulated lipolysis (Fig. 4) in vivo, consistent with previous studies (12,30,45). Our data clearly show that leptin deficiency, independent of hyperphagia or hyperinsulinemia, results in markedly increased lipogenic fluxes in *ob/ob* mice, consistent with reported in vitro (30) and in vivo (47) effects.

The long-term contribution from DNL to adipose tissue TG stores could be measured accurately because $^2\text{H}_2\text{O}$ can be administered easily for an extended period of time. This method cannot distinguish between adipose DNL and hepatic DNL as the initial site of biosynthesis but measures the accumulation in adipose tissue via either pathway. The measured DNL contribution to newly synthesized adipose TG-palmitate in lean controls as well as *ob/ob* mice was 70–100% (Table 1), a higher value than we previously estimated based on short-term labeling studies in rodents, which measured primarily hepatic DNL (24). DNL, therefore, makes a quantitatively substantial contribution to the accrual of adipose fat in rodents on chow diets, and this pathway is stimulated in the *ob/ob* mouse. In humans, by contrast, DNL contributes about 20% of newly synthesized adipose TG-palmitate (39).

Of note, leptin suppressed adipose TG synthesis and DNL without significantly reducing plasma insulin concentrations. The dose of leptin (2 $\mu\text{g}/\text{day}$) was selected to alter body weight, but not plasma insulin levels, and was based on the work of Harris et al. (17). The reduction in food intake with leptin treatment was also less than was achieved by food restriction alone, but leptin administration reduced lipogenic fluxes to a much greater extent than was observed with food restriction. Thus, leptin deficiency, independent of hyperphagia or hyperinsulinemia, results in major alterations in flux through lipid anabolic pathways in adipose tissue.

In addition to the inhibition of adipose cell proliferation rates reported here, leptin has been shown to have inhibitory or stimulatory effects on the proliferation of numerous cell types. Proliferation and differentiation of hematopoietic cells (3,13), promotion of angiogenesis and epithelial wound repair (38), and proliferation and suppression of apoptosis in pancreatic β -cells (34,41) have been reported. Leptin inhibits the differentiation of marrow stromal cells into adipocytes, consistent with our observations here (42).

The mature adipocyte-enriched fraction on which we measured cell proliferation may contain residual vascular-stromal cells. The values reported for adipogenesis should therefore be interpreted as reflecting adipose tissue as an organ rather than as exclusively mature adipocytes. Previous work (9) has demonstrated, however, that the stromal-vascular compartment of adipose tissue also expands in settings of adipose tissue lipid and cell accumulation.

A result of general interest is the comparison between directly measured metabolic flux rates through complex pathways and expression levels of related genes. Three previous reports have examined the expression of lipogenic genes in adipose tissue from obese humans (8) or *ob/ob* mice (32,37). These studies reported a reduction in the expression of the genes controlling expression of lipogenic enzymes, namely SREBP-1c, and genes coding lipogenic enzymes

(FAS, PEPCK, ATP citrate-lyase, pyruvate carboxylase). We surveyed genes involved in several aspects of lipid storage in adipose tissue. GPAT, DGAT1, DGAT2, and PEPCK are involved in the synthesis of glycerol phosphate and triacyl-glycerols; GLUT4 and FAS are essential in the uptake of glucose and conversion to TG; SREBP1 and PPAR γ act as signals regulating several aspects of lipid biosynthesis and the differentiation of preadipocytes. We confirm that low or normal levels of mRNAs were present for most of these enzymes in *ob/ob* adipose tissue, and few effects were observed with leptin treatment (Fig. 6). The mRNA levels of FAS were significantly elevated in *ob/ob* mice but, paradoxically, were not suppressed by leptin treatment; in fact, message levels were further increased by leptin. Only expression of DGAT2 was elevated in *ob/ob* mice and reduced by leptin treatment, consistent with its recently described role in obesity and adipose metabolism (40). Thus, with some exceptions, the use of gene expression results by themselves to understand adipose tissue fluxes or to identify therapeutic targets would not have been reliable. Indeed, mRNA expression could be quite misleading if considered independent of flux analysis. The effects of leptin treatment, for example, might appear to be absent or even prolipogenic, whereas, in fact, leptin potently reduced measured flux rates through all the lipid anabolic pathways studied.

Straightforward interpretation of previous gene expression results led to the suggestion (31) that lipogenic and lipid storage capacities are reduced in *ob/ob* mice. Our direct measurements of fluxes clearly show that this is not the case. Essentially identical results were obtained from four different depots, and the metabolic alterations were normalized by administration of leptin but not by caloric restriction, as expected from previous literature concerning the effects of leptin on other metabolic alterations (27).

This disparity between mRNA levels and the flow of molecules in vivo through complex pathways is instructive (18,19,43). Considerable work from the field of metabolic engineering has led to the conclusion that, even in systems as simple as bacteria, manipulating gene expression does not typically alter metabolic flux distributions in a predictable manner within complex pathways and metabolic networks. Fischer and Sauer (10) recently reported, for example, that the vast majority of gene deletions have no effect on flux through central metabolic pathways in *Bacillus subtilis*, whereas those genes that altered one flux tended to alter many fluxes. Thus, a simple relationship between gene expression and metabolic flux should not be assumed in evolved metabolic networks, and caution must be used in extrapolating from gene expression to fluxes through the pathways for which they code.

The specific explanation for dissociation between mRNA levels and metabolic fluxes in the *ob/ob* mouse is not clear. For example, although mRNA levels are reduced, enzyme concentration or activity may not be decreased because of altered translation controls or changes in the catabolic rates of the enzymes. Alternatively, lipogenic enzymes may not be rate limiting but may be present in excess in vivo. Cofactors or the availability of substrates could be responsible for higher rates of synthesis through these pathways, independent of enzyme levels, or enzyme activities could be regulated allosterically. In a general sense, the multiple levels of potential flux control that exist beyond gene transcription give mRNA levels low predictive power for lipogenic and adipogenic fluxes in this model of obesity.

In summary, these results emphasize the general rule, often ignored, that gene expression data should be interpreted cautiously with regard to functional outputs of complex pathways (10, 18,19,43).

References

1. Abraham RR. Some cellular characteristics of the epididymal adipose tissue in lean and obese-hyperglycaemic mice. *Diabetologia* 1973;9:303–306. [PubMed: 4767370]

2. Bai Y, Zhang S, Kim KS, Lee JK, Kim KH. Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. *J Biol Chem* 1996;271:13939–13942. [PubMed: 8663251]
3. Bennett BD, Solar GP, Yuan JQ, Mathias J, Thomas GR, Matthews W. A role for leptin and its cognate receptor in hematopoiesis. *Curr Biol* 1996;6:1170–1180. [PubMed: 8805376]
4. Breslow MJ, Min-Lee K, Brown DR, Chacko VP, Palmer D, Berkowitz DE. Effect of leptin deficiency on metabolic rate in *ob/ob* mice. *Am J Physiol Endocrinol Metab* 1999;276:E443–E449.
5. Brunengraber DZ, McCabe BJ, Kasumov T, Alexander JC, Chandramouli V, Previs SF. Influence of diet on the modeling of adipose tissue triglycerides during growth. *Am J Physiol Endocrinol Metab* 2003;285:E917–E925. [PubMed: 12799315]
6. Cawthorne MA, Cornish S. Lipogenesis in vivo in lean and genetically obese (*ob/ob*) mice fed on diets with a high fat content. *Int J Obes* 1979;3:83–90. [PubMed: 528121]
7. Collins ML, Eng S, Hoh R, Hellerstein MK. Measurement of mitochondrial DNA synthesis in vivo using a stable isotope-mass spectrometric technique. *J Appl Physiol* 2003;94:2203–2211. [PubMed: 12562673]
8. Diraison F, Dusserre E, Vidal H, Sothier M, Beylot M. Increased hepatic lipogenesis but decreased expression of lipogenic gene in adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2002;282:E46–E51. [PubMed: 11739082]
9. Faust IM, Miller WH Jr, Sclafani A, Aravich PF, Triscari J, Sullivan AC. Diet-dependent hyperplastic growth of adipose tissue in hypothalamic obese rats. *Am J Physiol Regul Integr Comp Physiol* 1984;247:R1038–R1046.
10. Fischer E, Sauer U. Large-scale in vivo flux analysis shows rigidity and suboptimal performance of *Bacillus subtilis* metabolism. *Nat Genet* 2005;37:636–640. [PubMed: 15880104]
11. Friedman JM. Leptin, leptin receptors, and the control of body weight. *Nutr Rev* 1998;56:s38–s46. discussion s54–s75. [PubMed: 9564176]
12. Fruhbeck G, Aguado M, Martinez JA. In vitro lipolytic effect of leptin on mouse adipocytes: evidence for a possible autocrine/paracrine role of leptin. *Biochem Biophys Res Commun* 1997;240:590–594. [PubMed: 9398609]
13. Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, Ng A, Nicola NA, Alexander WS, Hilton DJ. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc Natl Acad Sci USA* 1996;93:14564–14568. [PubMed: 8962092]
14. Godbole VY, Grundler ML, Thenen SW. Early development of lipogenesis in genetically obese (*ob/ob*) mice. *Am J Physiol Endocrinol Metab* 1980;239:E265–E268.
15. Halaas JL, Boozer C, Blair-West J, Fidathusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci USA* 1997;94:8878–8883. [PubMed: 9238071]
16. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995;269:543–546. [PubMed: 7624777]
17. Harris RB, Zhou J, Redmann SM Jr, Smagin GN, Smith SR, Rodgers E, Zachwieja JJ. A leptin dose-response study in obese (*ob/ob*) and lean (+/?) mice. *Endocrinology* 1998;139:8–19. [PubMed: 9421392]
18. Hellerstein MK. In vivo measurement of fluxes through metabolic pathways: the missing link in functional genomics and pharmaceutical research. *Annu Rev Nutr* 2003;23:379–402. [PubMed: 12704218]
19. Hellerstein MK, Murphy E. Stable isotope-mass spectrometric measurements of molecular fluxes in vivo: emerging applications in drug development. *Curr Opin Mol Ther* 2004;6:249–264. [PubMed: 15264427]
20. Hellerstein MK, Neese RA. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am J Physiol Endocrinol Metab* 1999;276:E1146–E1170.
21. Hellerstein MK, Neese RA, Schwarz JM. Model for measuring absolute rates of hepatic de novo lipogenesis and reesterification of free fatty acids. *Am J Physiol Endocrinol Metab* 1993;265:E814–E820.

22. Hems DA, Rath EA, Verrinder TR. Fatty acid synthesis in liver and adipose tissue of normal and genetically obese (ob/ob) mice during the 24-hour cycle. *Biochem J* 1975;150:167–173. [PubMed: 1237298]
23. Houseknecht KL, Baile CA, Matteri RL, Spurlock ME. The biology of leptin: a review. *J Anim Sci* 1998;76:1405–1420. [PubMed: 9621947]
24. Jung HR, Turner SM, Neese RA, Young SG, Hellerstein MK. Metabolic adaptations to dietary fat malabsorption in chylomicron-deficient mice. *Biochem J* 1999;343:473–478. [PubMed: 10510316]
25. Kaplan ML, Fried GH. Adaptive enzyme responses in adipose tissue of obese hyperglycemic mice. *Arch Biochem Biophys* 1973;158:711–719. [PubMed: 4782531]
26. Kaplan ML, Leveille GA. Development of lipogenesis and insulin sensitivity in tissues of the *ob/ob* mouse. *Am J Physiol Endocrinol Metab* 1981;240:E101–E107.
27. Levin N, Nelson C, Gurney A, Vandlen R, de Sauvage F. Decreased food intake does not completely account for adiposity reduction after ob protein infusion. *Proc Natl Acad Sci USA* 1996;93:1726–1730. [PubMed: 8643697]
28. Loten EG, Rabinovitch A, Jeanrenaud B. In vivo studies on lipogenesis in obese hyperglycaemic (ob-ob) mice: possible role of hyperinsulinaemia. *Diabetologia* 1974;10:45–52. [PubMed: 4275919]
29. Memon RA, Grunfeld C, Moser AH, Feingold KR. Fatty acid synthesis in obese insulin resistant diabetic mice. *Horm Metab Res* 1994;26:85–87. [PubMed: 8200619]
30. Muller G, Ertl J, Gerl M, Preibisch G. Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *J Biol Chem* 1997;272:10585–10593. [PubMed: 9099705]
31. Nadler ST, Attie AD. Please pass the chips: genomic insights into obesity and diabetes. *J Nutr* 2001;131:2078–2081. [PubMed: 11481397]
32. Nadler ST, Stoehr JP, Schueler KL, Tanimoto G, Yandell BS, Attie AD. The expression of adipogenic genes is decreased in obesity and diabetes mellitus. *Proc Natl Acad Sci USA* 2000;97:11371–11376. [PubMed: 11027337]
33. Neese RA, Siler SQ, Cesar D, Antelo F, Lee D, Misell L, Patel K, Tehrani S, Shah P, Hellerstein MK. Advances in the stable isotope-mass spectrometric measurement of DNA synthesis and cell proliferation. *Anal Biochem* 2001;298:189–195. [PubMed: 11700973]
34. Okuya S, Tanabe K, Tanizawa Y, Oka Y. Leptin increases the viability of isolated rat pancreatic islets by suppressing apoptosis. *Endocrinology* 2001;142:4827–4830. [PubMed: 11606450]
35. Rodbell M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* 1964;239:375–380. [PubMed: 14169133]
36. Scarpace PJ, Matheny M, Pollock BH, Tumer N. Leptin increases uncoupling protein expression and energy expenditure. *Am J Physiol Endocrinol Metab* 1997;273:E226–E230.
37. Soukas A, Cohen P, Socci ND, Friedman JM. Leptin-specific patterns of gene expression in white adipose tissue. *Genes Dev* 2000;14:963–980. [PubMed: 10783168]
38. Stallmeyer B, Kampfer H, Podda M, Kaufmann R, Pfeilschifter J, Frank S. A novel keratinocyte mitogen: regulation of leptin and its functional receptor in skin repair. *J Invest Dermatol* 2001;117:98–105. [PubMed: 11442755]
39. Strawford A, Antelo F, Christiansen M, Hellerstein MK. Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with $^2\text{H}_2\text{O}$. *Am J Physiol Endocrinol Metab* 2004;286:E577–E588. [PubMed: 14600072]
40. Suzuki R, Tobe K, Aoyama M, Sakamoto K, Ohsugi M, Kamei N, Nemoto S, Inoue A, Ito Y, Uchida S, Hara K, Yamauchi T, Kubota N, Terauchi Y, Kadowaki T. Expression of DGAT2 in white adipose tissue is regulated by central leptin action. *J Biol Chem* 2005;280:3331–3337. [PubMed: 15550388]
41. Tanabe K, Okuya S, Tanizawa Y, Matsutani A, Oka Y. Leptin induces proliferation of pancreatic beta cell line MIN6 through activation of mitogen-activated protein kinase. *Biochem Biophys Res Commun* 1997;241:765–768. [PubMed: 9434783]
42. Thomas T, Gori F, Khosla S, Jensen MD, Burguera B, Riggs BL. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Endocrinology* 1999;140:1630–1638. [PubMed: 10098497]
43. Turner SM, Hellerstein MK. Emerging applications of kinetic biomarkers in preclinical and clinical drug development. *Curr Opin Drug Discov Devel* 2005;8:115–126.

44. Turner SM, Murphy EJ, Neese RA, Antelo F, Thomas T, Agarwal A, Go C, Hellerstein MK. Measurement of TG synthesis and turnover in vivo by $^2\text{H}_2\text{O}$ incorporation into the glycerol moiety and application of MIDA. *Am J Physiol Endocrinol Metab* 2003;285:E790–E803. [PubMed: 12824084]
45. Wang MY, Lee Y, Unger RH. Novel form of lipolysis induced by leptin. *J Biol Chem* 1999;274:17541–17544. [PubMed: 10364187]
46. Yen TT, Allan JA, Yu PL, Acton MA, Pearson DV. Triacylglycerol contents and in vivo lipogenesis of ob/ob, db/db and Avy/a mice. *Biochim Biophys Acta* 1976;441:213–220. [PubMed: 952988]
47. Zhou YT, Wang ZW, Higa M, Newgard CB, Unger RH. Reversing adipocyte differentiation: implications for treatment of obesity. *Proc Natl Acad Sci USA* 1999;96:2391–2395. [PubMed: 10051652]

Fig. 1.

Change in body weight of *ob/ob*, *ob-r*, *ob-lep*, and control mice. Food restriction and leptin treatment began on day -5, and $^2\text{H}_2\text{O}$ was administered on day 0 and continued until the end of the study. All mice were 37–44 days old at the start of the study. $\square$, *ob/ob*; Δ , *ob/ob* restricted; $\circ$ *ob/ob* leptin; $\blacksquare$ control mean $\pm$ SD.

Fig. 2.

Absolute rates of triglyceride (TG) synthesis over 21 days (g). Absolute amount of TG synthesized over the labeling period was calculated from fractional TG synthesis measured from $^2\text{H}_2\text{O}$ incorporated into TG-glycerol, as described in the text. Data are means $\pm$ SD; values not sharing a superscripted letter are significantly different within each column (40).

Fig. 3.

Absolute rates of palmitate de novo lipo genesis (DNL; g palmitate synthesized over 21-day labeling period). Absolute amount of palmitate synthesized over the labeling period, calculated from fractional palmitate synthesis as described in the text. Data are means $\pm$ SD; values not sharing a superscripted letter are significantly different within each column.

Fig. 4.

Net adipose tissue lipolysis (g TG/21 days). Net lipolysis was calculated from absolute TG synthesis (Fig. 2) minus estimated increase in fat pad mass. Data are means $\pm$ SD; values not sharing a superscripted letter are significantly different within each column.

Fig. 5.

Absolute rates of adipose cell proliferation (cells/21 days). Adipose cell proliferation was calculated from fractional cell proliferation times number of cells/fat pad. Data are means $\pm$ SD; values not sharing a superscripted letter are significantly different within each column.

Fig. 6.

Quantitative RT-PCR of lipogenic genes in C57BL/6 mice (control, $n = 5$), *ob/ob* mice ($n = 5$), and *ob/ob* mice administered leptin ($n = 5$). Total RNA was isolated from inguinal adipose tissue and reverse transcribed. Ten nanograms of cDNA were used in each PCR reaction.

Relative mRNA levels for each gene were normalized to an internal standard, GAPDH, and are expressed as this ratio. Data are expressed as means $\pm$ SE. For each gene, 1-way repeated-measures ANOVA was used to test for significant differences between the 3 groups. If groups differed significantly, pairwise comparisons were performed and P values calculated using the Holm-Sidak method. Statistical analysis was done using SigmaStat 3.0. * $P < 0.05$ compared with control; † $P < 0.01$ compared with control; ‡ $P < 0.05$ compared with *ob/ob*. GPAT, mitochondrial glycerol-3-phosphate acyltransferase; SREBP1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase; DGAT1, diacylglycerol *O*-acyltransferase 1; DGAT2, diacylglycerol *O*-acyltransferase 2; GLUT4, solute carrier family 2 (facilitated glucose transporter), member 4; FABP4, adipocyte fatty acid-binding protein-4; PEPCK, cytosolic phosphoenolpyruvate carboxykinase 1; ACAT1, acetyl-CoA acetyltransferase 1; PPAR γ , peroxisome proliferator-activated receptor- γ .

Table 1

Plasma concentrations of insulin, glucose, and leptin

	Insulin, ng/ml	Glucose, mg/ml	Leptin, ng/ml	Food intake, g/day
Con	0.36±0.15 ^a	2.2±0.3 ^b	5.7±2.9 ^a	3.1±0.4 ^a
<i>ob/ob</i>	1.9±1.1 ^b	3.7±0.6 ^a	bdl	5.1±1.1 ^b
<i>ob-r</i>	3.0±3.7 ^b	3.0±1.2 ^{a,b}	bdl	2.8 ^a
<i>ob-lep</i>	1.8±1.9 ^b	2.0±0.5 ^b	4.05±1.9 ^a	3.8±0.9 ^{a,b}

Values are means ± SD; values not sharing a superscripted letter are significantly different within each column. Con, control; bdl, below detection level.

Table 2

Individual fat pad weights

	Perimetrial	Inguinal	Mesenteric	Retroperitoneal
Con	0.36±0.20.6 ^{b,c}	0.46±0.2 ^a	0.85±0.03 ^a	0.16±0.1 ^a
<i>ob/ob</i>	2.0±0.3 ^b	2.8±0.2 ^b	0.85±0.2 ^b	1.1±0.1 ^b
<i>ob-r</i>	1.4±0.4 ^c	2.2±0.3 ^c	0.63±0.2 ^c	0.84±0.1 ^c
<i>ob-lep</i>	1.6±0.6 ^{b,c}	1.5±0.5 ^d	0.34±0.1 ^a	0.66±0.3 ^c

Values are means ±SD and in g; values not sharing a superscripted letter are significantly different within each column.

Table 3

Fractional TG synthesis (%) in adipose tissue

	Perimetrial	Inguinal	Mesenteric	Retroperitoneal
Con	66±5 ^a	62±4 ^{a,d}	70±7 ^a	63±5 ^a
<i>ob/ob</i>	43±6 ^b	56±5 ^{b,d}	51±7 ^b	47±8 ^b
<i>ob-r</i>	66±2 ^a	65±3 ^a	69±5 ^a	61±6 ^a
<i>ob-lep</i>	25±3 ^c	25±3 ^c	26±5 ^c	23±2 ^c

Values are means ± SD; values not sharing a superscripted letter are significantly different within each column. TG, triglyceride.

Table 4Fractional DNL^{*} synthesis (%) in adipose tissue

	Perimetrial	Inguinal	Mesenteric	Retroperitoneal
Con	93±20 ^a	92±11 ^a	100±9 ^a	87±13 ^a
<i>ob/ob</i>	114±10 ^a	65±3 ^b	103±10 ^a	112±17 ^b
<i>ob-r</i>	60±7 ^b	37±6 ^c	59±6 ^b	57±6 ^c
<i>ob-lep</i>	16±4 ^c	14±2 ^d	38±14 ^c	24±6 ^d

Values are means ± SD; values not sharing a superscript are significantly different within each column. DNL, de novo lipogenesis.

* Calculated as measured fractional DNL ÷ fractional TG synthesis.