Title

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Journal

Obesity, 12(8)

ISSN

1930-7381

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Publication Date

2004-08-01

DOI

10.1038/oby.2004.158

Peer reviewed

The Yellow Agouti Mutation Alters Some But Not All Responses to Diet and Exercise

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Abstract

CHIU, SALLY, JANIS S. FISLER, GLENDA M. ESPINAL, PETER J. HAVEL, JUDITH S. STERN, AND CRAIG H. WARDEN. The yellow agouti mutation alters some but not all responses to diet and exercise. *Obes Res.* 2004;12:1243–1255.

Objective: Effects of ectopic expression of the agouti signaling protein were studied on responses to diet restriction and exercise in C57BL/6J (B6) mice and obese B6 mice congenic for the yellow agouti mutation [B6.Cg-A^y (A^y)].

Research Methods and Procedures: Adult male A^y mice were either kept sedentary or exercised on a running wheel and fed ad libitum or diet restricted until weight matched to ad libitum-fed B6 control mice. Body composition, plasma lipids, leptin, and adiponectin were measured. mRNA levels for leptin, adiponectin, lipoprotein lipase, and pyruvate dehydrogenase kinase 4 were measured in a visceral (epididymal) and a subcutaneous (femoral) fat depot by real-time polymerase chain reaction.

Results: Correlations among traits exhibited one of three patterns: similar lines for B6 and A^y mice, different slopes for B6 and A^y mice, and/or different intercepts for B6 and A^y mice. Correlations involving plasma leptin, mesenteric and epididymal adipose weights, or low-density lipoprotein-cholesterol were most likely to have different slopes and/or intercepts in B6 and A^y mice. mRNA levels for leptin, Acrp30, pyruvate dehydrogenase kinase 4, and lipoprotein lipase in epididymal adipose tissue were not correlated with corresponding levels in femoral adipose tissue.

Discussion: The agouti protein interferes with leptin signaling at melanocortin receptors in the hypothalamus of A^y mice. Our results are consistent with the hypothesis that the melanocortin portion of the leptin-signaling pathway mediates effects primarily on certain fat depots and on some, but not all, components of cholesterol homeostasis.

Key words: agouti, epididymal adipose tissue, subcutaneous adipose tissue, quantitative PCR, cholesterol

Introduction

Diet (energy) restriction and exercise are two key environmental factors that promote weight loss in obese individuals. Although much is known about biochemical responses to diet restriction and exercise, it is apparent that all individuals do not benefit equally from the same treatments (1). Interactions between alleles of certain genes and the environment are one possible explanation for dissimilar responses to dieting and exercise. Although at least some mechanisms by which genes influence gain of fat mass are known, very little is known about mechanisms by which genes influence loss of fat mass.

Fat depots are metabolically and genetically heterogeneous (2–6). However, previous studies have examined only a limited number of genes, compared models that are genetically diverse (e.g., different mouse strains), or surveyed limited environmental conditions. In the present study, we tested the hypothesis that weights and gene expression of each fat depot are independently regulated by exercise and diet restriction. Many studies show that different fat depots are differentially related to plasma cholesterol levels in humans (7–12) and mice (2). Thus, we also examined correlations of fat depot weights with cholesterol homeostasis in mice after diet restriction and/or exercise.

Mechanistic studies of the effects of diet restriction and exercise on obesity in mice have concentrated on mice with mutations in the leptin pathway (13–19). Leptin, a hormone produced in adipose tissue, acts on the hypothalamus and is a putative indicator of fat and, therefore, energy stores (20).

Received for review June 23, 2003.
Accepted in final form June 14, 2004.

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Mice with mutations in the leptin gene (*Lep*^{ob}) and the leptin receptor gene (*Lepr*^{db}) have virtually identical phenotypes when the mutation is on the same background, including severe spontaneous obesity, hyperphagia, and type 2 diabetes-like phenotypes (21,22). Diet restriction, even to levels below lean controls, does not normalize body fat content in these mice. Despite diet restriction and/or weight matching to lean controls, Lep^{ob} and Lepr^{db} mice still retain an obese phenotype (13–15). Likewise, exercise, either alone or in addition to pair feeding, does not normalize their body fat content (17–19).

The B6.Cg-A^{y} (A^{y})[1] mouse arose from a spontaneous dominant mutation and was first studied by Cuenot in 1905 (23). It is characterized by moderate, late-onset obesity, insulin resistance, and a yellow coat color. Obesity results from both hyperphagia and reduced energy expenditure (24). Molecular studies revealed a dominant mutation in the agouti (*a*) gene that resulted in ectopic expression of agouti (25). In the hypothalamus, agouti antagonizes binding of α-MSH to the melanocortin 4 receptor (MC4R) and the melanocortin 3 receptor, both constituents of a pathway involved in food intake regulation (26). Early observations suggested that A^{y} mice have a lower food requirement for fat deposition compared with lean mice (27,28). Like the Lep^{ob} mouse, pair-fed A^{y} mice do not normalize their weight gain and body composition (28), indicating metabolic efficiency or a preferred energy partitioning to adipose tissue. Exercise has been shown to slow weight gain in A^{y} mice (29,30). No studies have looked at effects of combined exercise and diet restriction on the phenotype of this milder form of obesity. Therefore, we chose to study the response to diet restriction and exercise in the lethal yellow mouse (A^{y}).

The purpose of the present study was to use an integrated approach to examine the whole-body phenotypic response to diet restriction and exercise in A^{y} and C57BL/6J (B6) mice. We determined the effects of dietary energy restriction, exercise, and agouti genotype on food intake, running wheel activity, body composition, plasma leptin and adiponectin (Acrp30), plasma cholesterol, and the expression of several genes related to obesity and lipid metabolism including leptin, Acrp30, lipoprotein lipase (*Lpl*), pyruvate dehydrogenase kinase 4 (*Pdk4*), 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase, low-density lipoprotein (LDL) receptor, cholesterol 7α-hydroxylase (*Cyp7a1*), and uncoupling protein 3 (*Ucp3*) in two adipose depots, femoral and epididymal, and/or in liver and gastrocnemius muscle. All genes in A^{y} mice originate from the background B6 strain, except for the A^{y} mutation and a small surrounding congenic region on mouse chromosome 2. None of the genes analyzed is located on mouse chromosome 2; therefore, none of the genotype effects is due to allele variations among the mice studied. This genetic homogeneity allows us to examine integrated responses of molecular, metabolic, and whole-body phenotypes as weight and body fat change in response to dietary energy restriction and/or exercise without the potential confounding allele effects that would likely be observed in mice with different genetic backgrounds. These mouse models also allow us to examine the impact this agouti mutation has on various phenotypes.

We report that diet restriction and exercise had differential effects on adipose tissue distribution. Several strong correlations among traits reveal potentially important relationships among the expression of specific genes, hormones, and adipose tissue depots in the regulation of fat mass and lipid metabolism as well as differences due to the agouti genotype in the nature of some of these relationships.

Research Methods and Procedures

Animals

B6 and A^{y} male mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and fed a standard rodent chow diet (Deans Animal Feed, San Carlos, CA). At 8 weeks of age, mice were fed a low-fat, defined diet (AIN-76A; Research Diets, New Brunswick, NJ). Mice were singly housed on a 14-/10-h light/dark cycle at 70 °F. All mice were given free access to water, and ad libitum (ad lib) mice were given free access to food. All animals were housed and cared for under conditions meeting the NIH standards as stated in the Guide for the Care and Use of Laboratory Animals, American Association for Accreditation of Laboratory Animal Care accreditation standards, and the Animal Welfare Act PL85-544. The study design and measurements taken are presented in Figure 1.

Exercise and Diet Restriction

To examine phenotypes of exercised (ex) mice, six A^{y} and four B6 mice were placed in polycarbonate cages equipped with electronically monitored running wheels (Mini Mitter Co., Bend, OR) at 15 weeks of age and acclimated for 1 week. AIN-76A pellets were crushed and placed in a food cup. Food intake was measured in 2-day intervals for 2 weeks, and daily values were averaged within each genotype. Three A^{y} mice were diet restricted (rest'd) to 83% average A^{y} intake for 1 month and switched to 73% for the remainder of the experiment. Food was given once daily, and spill was measured. Remaining mice were fed ad lib. We did not include rest'd B6 mice in the exercise group because we studied all animals simultaneously and could accommodate only 10 mice in our running wheel facilities.

[1] Nonstandard abbreviations: A^{y}, B6.Cg-A^{y}; MC4R, melanocortin 4 receptor; B6, C57BL/6J; Acrp30, adiponectin; Lpl, lipoprotein lipase; Pdk4, pyruvate dehydrogenase kinase 4; HMG, 3-hydroxy-3-methylglutaryl; CoA, coenzyme A; LDL, low-density lipoprotein; Cyp7a1, cholesterol 7α-hydroxylase; Ucp3, uncoupling protein 3; ad lib, ad libitum; ex, exercised; rest'd, diet restricted; BW, body weight; sed, sedentary; HDL, high-density lipoprotein; PCR, polymerase chain reaction; CT, threshold cycle.

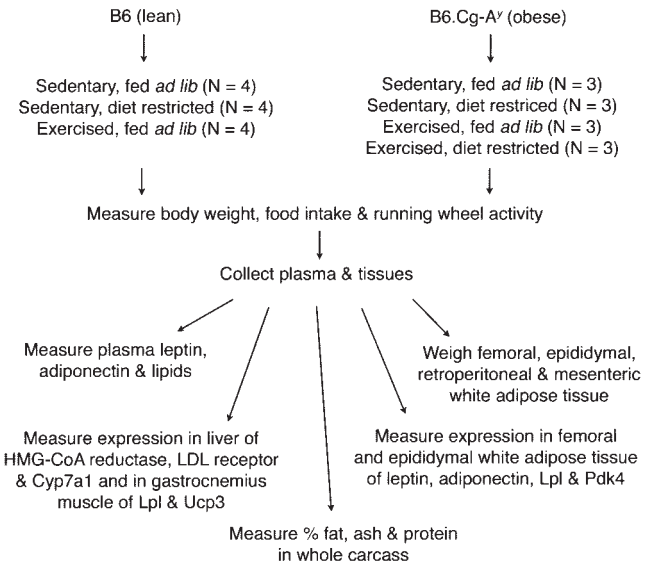

Figure 1: Experimental design and measurements.

Body weights (BWs) were taken weekly, and mice were sacrificed when the weight of rest'd A^y mice equaled that of B6 controls. Running wheel data, expressed as total revolutions per day, were collected at 15-minute intervals using the VitalView Data Acquisition System (Mini Mitter Co.) beginning 1 week after mice were placed on wheels.

To examine phenotypes of sedentary (sed) mice, six A^y and eight B6 mice were singly housed in polycarbonate cages. As with ex mice, food intake was measured and averaged for each strain. Three A^y and four B6 mice were rest'd to 83% average food intake for 1 month and switched to 73% for the remainder of the experiment. BWs were taken weekly, and mice were sacrificed when rest'd A^y mice were weight-matched to B6 ad lib mice.

Body Composition

Mice were fasted overnight, anesthetized with inhaled isoflurane, and bled through the retroorbital sinus. Anal-nasal length was measured in anesthetized mice. Animals were weighed and killed by cervical dislocation, and dissected tissues were frozen immediately in liquid nitrogen and stored at −80 °C. Epididymal, femoral, retroperitoneal, and mesenteric white adipose fat depots were dissected, weighed, flash-frozen, and stored. The weights of the four fat depots were corrected for BW (fat depot percentage) and expressed as fat depot (grams)/BW (grams) multiplied by 100. For correlation analyses, the absolute weights of the fat pads were used. The animal carcass was used to measure precise body composition by the method of Bell and Stern (31). Body fat percentage was calculated as [carcass fat mass + 0.8 (total fat depot weight)] × 100/BW.

Plasma Analyses

Blood was collected into separator tubes (Microtainer; BD Biosciences, San Jose, CA), and centrifuged for 10 minutes, and plasma was separated. Plasma was analyzed in triplicate for fasting total cholesterol, high-density lipoprotein (HDL), free fatty acids, and triglycerides enzymatically as described (32,33). LDL levels were calculated by subtraction of HDL from total cholesterol. Fasting plasma leptin was measured by enzyme-linked immunosorbent assay using a mouse leptin kit (R&D Systems, Minneapolis, MN). Fasting plasma adiponectin was measured by radioimmunoassay (Linco Research, Inc., St. Charles, MO) using mouse adiponectin standards and I^{125}-iodinated mouse adiponectin tracer. All together, these assays consumed all available plasma from most animals.

Quantitative Polymerase Chain Reaction (PCR)

Femoral and epididymal white adipose tissue, gastrocnemius, and liver RNA were isolated using TRIzol reagent (Gibco/BRL, Grand Island, NY). RNA was quantified, and integrity was confirmed by 1% denaturing gel electrophoresis. cDNA was generated from 1 μg of total RNA as instructed by the manufacturer (Applied Biosystems, Foster City, CA). Quantitative real-time PCR reactions were carried out with 30 ng of cDNA using SYBR Green I dye detection of the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Melting curve analysis incorporated into the 7900HT was used to demonstrate that each primer produced only a single product from each sample. The results ensure quantitation of one specific gene for each reaction. The reagents and amplification conditions were used as suggested by the manufacturer. The following primer pairs were designed with ABI Primer Express software and used to validate the genes of interest: leptin, F5′-CAGCCTGCCTTCCCAAAA-3′ and R5′-AGATGG-AGGAGGTCT CGGAGAT-3′; Acrp30, F5′-CAGTGGA-TCTGACGACACCAA-3′ and R5′-TGGGCA GGATTAA-GAGGAACA-3′; Pdk4, F5′-GGGAG GCTGAAGGGT-AAGGAT-3′ and R5′-CCCTCGCTCCTCGTTTTAATT-3′; Lpl, F5′-CCAATGGAGGCACTTTCCA-3′ and R5′-CA-CGTCTCCGAGTCCTCTCTCT-3′; Ucp3, F5′-GGAG-TCTCACCTGTTTACTGACAACT-3′ and R5′-GCACA-GAAGCCAGCTCCAA-3′; calmodulin, F5′-AGCAGAGCT TCGCCAT GTG-3′ and R5′-ATGTCTGCTTCCCTGAT-CATCTC-3′; LDL receptor, F5′- CTGTGGGCTCCA TA-GGCTATCT-3′ and R5′-GCGGTCCAGGGTCATCTTC-3′; HMG-CoA reductase, F5′-GGGAACTATTGCACCGA-CAAG-3′ and R5′-CGGCTTCACAAACCACAGTCT-3′; Cyp7a1, F5′GACCTCCGGGCCTTCCTA-3′ and R5′-ATCACT CGGTAGCAGAAGGCATA-3′; and acidic ribosomal phosphoprotein PO (36B4), F5′-GGACC CGAGAA-GACCTCCTCCTT-3′ and R5′-TCAATGGTGCCTCTG-GAGATT-3′. Samples were run in triplicate reactions to confirm consistency in the amount of PCR products. Calmodulin was used as the control gene for adipose and gastrocnemius, and 36B4 was used as the control gene for liver. These genes corrected for the amount of input mRNA and were selected

based on the least variation in threshold cycle (Ct) values. This indicates that the control chosen had the least variation in expression across experimental groups. Levels of mRNA are expressed as fold difference over the lowest value for a given trait. First, fold difference over the control gene is calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct(gene) - Ct(control\ gene)$. Ct is the PCR cycle where fluorescent signal associated with the exponential growth exceeds the threshold (10 × noise level) and is the output result of SYBR Green I dye detection assay. All values for a trait are then divided by the lowest $2^{-\Delta Ct}$ value (fold difference over lowest value) and termed as relative expression. To validate controls, the calmodulin Ct values from one sample of each treatment and genotype group were compared with 36B4 Ct value for adipose and liver. The results showed a highly significant correlation ($R^2 > 0.93$) between calmodulin and 36B4 expression, suggesting no differential regulation of either gene among groups.

Statistical Analyses

All data were analyzed using JMP (SAS Institute Inc., Cary, NC) and expressed as means ± SE. Three-way ANOVA was used to determine effects of genotype, diet, and exercise, with $p \leq 0.01$ considered significant for main and interactions effects. Nonsignificant effects were omitted from the model. Differences among group means were analyzed by one-way ANOVA with Tukey HSD post hoc test for multiple comparisons, with $p < 0.05$ considered significant. Simple regression analysis was used to examine the relationship between phenotypes. Differences in the slopes of the regression lines among strains were determined as the strain × phenotype interaction effect and differences in intercept as the strain effect in the regression analysis. The false discovery rate controlling procedure was used to control for multiple comparisons in regression analysis (34) for each group analyzed, with $p < 0.05$ considered significant.

Results

Food Intake and BW

Food intake was measured to determine both the level of diet restriction and the effects of the A^y mutation and exercise on food intake. At 18 weeks old, ex A^y mice ate 4.8 ± 0.1 g/d, ex B6 mice ate 4.5 ± 0.1 g/d, sed A^y mice ate 4.1 ± 0.1 g/d, and sed B6 mice ate 3.7 ± 0.1 g/d. Ex mice ate significantly more than their sed counterparts ($p < 0.05$). Sed A^y mice ate significantly more than sed B6 mice ($p < 0.05$). However, ex A^y mice did not eat significantly more than ex B6 mice.

Weekly BWs for ex and sed mice are shown beginning 3 weeks before diet restriction (Figure 2, A and B). Ad lib mice continued to slowly gain weight throughout the course of the experiment. At the end of the experiment, rest'd, sed A^y mice were 81% of ad lib sed A^y mice, whereas rest'd sed B6 mice were 72% of ad lib sed B6 mice. Rest'd ex A^y mice were 79% of ad lib ex A^y BW.

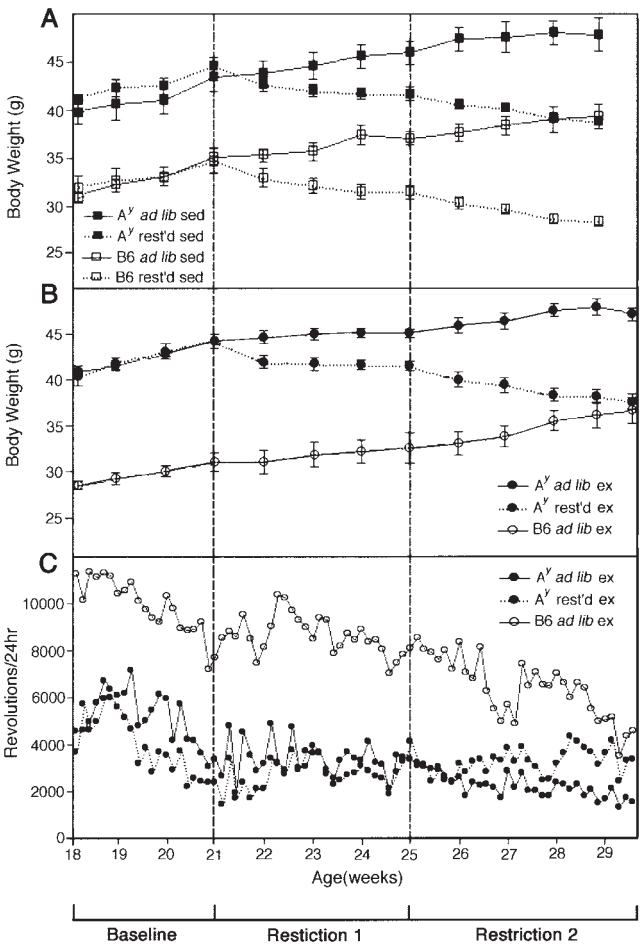

Figure 2: BWs and running wheel patterns of lean (B6) and obese (A^y) mice. (A) BWs of sed lean (B6) and obese (A^y) mice. One-half of the sed B6 and one-half of the sed A^y mice were rest'd to 83% at 21 weeks and then to 73% at 25 weeks of age. Mice were sacrificed when weight-matched to ad lib sed mice (29 weeks of age). (B) BWs of ex lean (B6) and obese (A^y) mice. One-half of the ex A^y mice were rest'd to 83% ad lib at 21 weeks and to 73% at 25 weeks of age. Mice were sacrificed when weight-matched to ad lib B6 mice (29.5 weeks). (C) Total running wheel revolutions for each 24-hour period for ad lib B6, ad lib A^y, and rest'd A^y mice.

Running Wheel Activity

Revolutions were continuously monitored for ex animals. Twenty-four-hour total running wheel revolutions are shown in Figure 2C. Over the course of the entire experiment, B6 mice ran an average of 656 ± 123 × 10^3 revolutions, ad lib A^y mice ran 277 ± 84 × 10^3 revolutions, and rest'd A^y mice ran 269 ± 56 × 10^3 revolutions. The total number of revolutions run did not differ among groups. Patterns shown suggest that rest'd A^y mice increased their running as their weight decreased, and B6 mice decreased

Table 1. Body fat composition

Group	Body fat (%)	Total body fat (g)	Femoral fat (%)	Epididymal fat (%)	Mesenteric fat (%)	Retroperitoneal fat (%)
A^y ad lib sed	36.8 ± 0.6^{a}	15.4 ± 0.5^{a}	3.1 ± 0.2^{a}	3.2 ± 0.3^{b}	3.1 ± 0.2^{a}	1.4 ± 0.1^{a}
A^y ad lib ex	35.4 ± 0.6^{a}	$16.2 \pm 0.5^{a,b}$	$2.3 \pm 0.2^{a,b,c}$	2.8 ± 0.2^{b}	$2.7 \pm 0.1^{a,b}$	1.5 ± 0.1^{a}
A^y rest'd sed	34.4 ± 0.4^{a}	$13.0 \pm 0.2^{a,b,c}$	$2.9 \pm 0.1^{a,b}$	2.9 ± 0.1^{b}	$2.2 \pm 0.0^{b,c}$	1.1 ± 0.1^{a}
A^y rest'd ex	32.8 ± 1.3^{a}	$11.9 \pm 0.4^{b,c}$	$2.1 \pm 0.3^{b,c,d}$	2.6 ± 0.1^{b}	$2.3 \pm 0.1^{b,c}$	$1.0 \pm 0.1^{a,b}$
B6 ad lib sed	29.6 ± 0.9^{a}	10.6 ± 0.7^{c}	$2.0 \pm 0.1^{c,d}$	4.8 ± 0.3^{a}	$1.7 \pm 0.1^{c,d}$	1.3 ± 0.1^{a}
B6 ad lib ex	21.7 ± 2.9^{b}	7.2 ± 1.2^{d}	1.4 ± 0.2^{d}	3.2 ± 0.5^{b}	$1.3 \pm 0.2^{d,e}$	$1.1 \pm 0.2^{a,b}$
B6 rest'd sed	18.1 ± 2.1^{b}	4.9 ± 0.6^{d}	1.4 ± 0.2^{d}	2.7 ± 0.3^{b}	0.8 ± 0.2^{e}	0.7 ± 0.1^{b}
Significance of ANOVA						
Genotype	<0.0001	<0.0001	<0.0001	NS	<0.0001	0.0031
Diet	0.0027	<0.0001	NS	0.0044	<0.0001	0.0003
Exercise	NS	NS	0.0025	NS	NS	NS

Values are means ± SE. Values for the same trait with different superscripts are significantly different by Tukey's HSD test ($p < 0.05$). p Values are from three-way ANOVA. There were no significant two- or three-way interactions.

their running with time. Previous investigators have observed that distance run decreases with age (35). Diet restriction has also been shown to increase spontaneous running in rodents (36), which is consistent with our results for the rest'd A^y mice.

Effects of Treatments on Body Composition

Both genotype (A^y vs. B6) and diet (rest'd vs. ad lib) had significant effects on percentage body fat and total body fat (Table 1). Untreated B6 and A^y mice tended to have different distributions of body fat. Sed ad lib A^y mice had larger percentage fat/BW in the femoral and mesenteric depots than sed ad lib B6 mice, whereas the opposite was observed for percentage epididymal fat/BW. There were significant effects of genotype and diet on all fat pads with two marked exceptions (Table 1). There was no overall effect of genotype on epididymal fat mass. Interestingly, dietary energy restriction reduced the size of all visceral fat depots (epididymal, mesenteric, and retroperitoneal) but not the subcutaneous (femoral) depot. In contrast, exercise reduced only the amount of femoral, subcutaneous fat but not visceral adiposity or other body composition traits.

Strain, diet, and exercise all affected anal-nasal length. A^y mice were longer than B6 mice ($p < 0.0001$), ad lib fed mice were longer than rest'd mice ($p = 0.0015$), and ex mice were longer than sed mice ($p < 0.0001$). Carcass protein and water (both expressed as grams) were reduced in rest'd animals ($p = 0.0004$) but were not affected by either strain or exercise group. Body ash was unaffected by genotype, diet, or exercise.

Plasma Analysis

Genotype, diet restriction, and exercise influenced cholesterol homeostasis (Table 2). A^y mice had higher cholesterol levels than B6 mice. Diet restriction in A^y mice lowered total plasma, HDL-, and LDL-cholesterol, whereas exercise alone lowered only HDL cholesterol. Exercise or diet restriction in B6 mice lowered total and HDL-cholesterol only. Plasma triglyceride and free fatty acid levels were not different between A^y and B6 mice and were unaffected by diet and/or exercise. Plasma leptin was lower in B6 than in A^y mice. Diet restriction decreased plasma leptin concentrations in both A^y and B6 mice, but exercise had no effect. Plasma adiponectin concentrations were lower in A^y mice than in B6 mice and were increased by dietary energy restriction in both genotypes, but not by exercise. The ex and rest'd A^y group was omitted from plasma analyses due to lack of sufficient sample.

Adipose Depot-Specific Regulation of mRNA Levels

We examined levels of mRNA expression for genes known to be associated with obesity, cholesterol homeostasis, and diabetes in four separate tissues: femoral and epididymal adipose and liver and skeletal (gastrocnemius) muscle. Leptin, Acrp30, Lpl, and Pdk4 mRNA expression levels were measured in both femoral and epididymal adipose depots (Table 3). A^y mice have over 6 times greater expression of leptin mRNA in femoral tissue than B6 mice. Diet restriction with exercise tended to decrease leptin mRNA in femoral adipose of A^y mice, although this decrease did not reach statistical significance. Only genotype had a significant effect on mRNA expression of leptin in femoral adipose tissue. In epididymal adipose, there was no effect of diet, exercise, or genotype. This indicates that the regulation of leptin mRNA depends on the particular adipose depot studied and on the model examined. In contrast to leptin, B6 mice had significantly higher Acrp30 gene

Table 2. Plasma lipids and hormones

Group	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Leptin (ng/ml)	Adiponectin (μg/ml)
A^y ad lib sed	296 ± 7^a	221 ± 4^a	76 ± 7^a	73 ± 3^a	12.2 ± 0.8^c
A^y ad lib ex	259 ± 17^a	196 ± 6^b	63 ± 11^a	80 ± 6^a	13.0 ± 1.7^c
A^y rest'd ex	184 ± 3^b	157 ± 1^c	27 ± 2^b	52 ± 2^b	$19.6 \pm 0.9^{a,b}$
B6 ad lib sed	180 ± 2^b	166 ± 2^c	14 ± 3^b	23 ± 3^c	$15.4 \pm 0.5^{b,c}$
B6 ad lib ex	136 ± 6^c	122 ± 3^d	15 ± 4^b	$13 \pm 3^{c,d}$	$17.9 \pm 0.4^{a,b,c}$
B6 rest'd sed	109 ± 7^c	100 ± 6^e	9 ± 2^b	7 ± 1^d	21.2 ± 2.2^a
Significance of ANOVA					
Genotype	<0.0001	<0.0001	<0.0001	<0.0001	0.0031
Diet	<0.0001	<0.0001	0.0032	0.0005	<0.0001
Exercise	0.0001	<0.0001	NS	NS	NS

Values are means ± SE. Values for the same trait with different superscripts are significantly different by Tukey's HSD test ($p < 0.05$). *p* Values are from three-way ANOVA. Values were not determined for the A^y rest'd sed group. There were no significant two- or three-way interactions. NS, not significant.

expression than their A^y counterparts in epididymal but not femoral tissue. As a group, B6 mice had 4-fold greater expression of Acrp30 in epididymal fat than A^y mice; however, neither exercise nor diet restriction influenced its expression. There was no effect of diet, exercise, or genotype on Acrp30 expression in femoral adipose tissue. In epididymal tissue, B6 mice had 2-fold higher Lpl expression than A^y mice, yet there was no effect of diet or exercise. B6 mice had 5-fold higher mRNA expression of Pdk4 in femoral fat and approximately 3-fold higher in epididymal fat than A^y mice. Exercise increased epididymal Pdk4 with a particularly marked ($p = 0.0002$) increase in B6 mice.

We also examined genes involved in regulation of plasma cholesterol levels including HMG-CoA reductase, LDL receptor, and Cyp7a1 mRNA levels in liver and Lpl and Ucp3 in the gastrocnemius muscle (data not shown). Diet restriction increased HMG-CoA reductase mRNA levels ($p = 0.0003$). There was no effect of exercise, diet restriction, or genotype on expression of LDL receptor or Cyp7a1 mRNA levels in the liver. Likewise, there was no significant effect of genotype, exercise, or diet restriction on expression of either Lpl or Ucp3 in muscle.

Correlation of Physical and Plasma Phenotypes: Similarities and Differences between Strains

Correlations of body fat measures and plasma phenotypes are shown in Figure 3 and Table 4. Because strain had a large effect on many of the phenotypes, we investigated similarities and differences in the relationships of various phenotypes between the two strains (B6 and A^y). In some instances, the slopes of the regression lines were significantly different. In addition, there were a few relationships that had similar slopes in both A^y and B6 mice, but the heights of the line, or intercepts, were markedly different. Where there were no significant differences in slope or intercept, both strains were analyzed together by simple regression.

Figure 3 shows the relationship of percentage body fat with several physical, hormone, and lipid phenotypes. We calculated Adiposity Index (total fat pad weight/BW × 100), an often-used surrogate for percentage body fat. Both strains had similar slopes for Adiposity Index vs. percentage body fat but significantly different intercepts. A^y mice have a lower Adiposity Index for a given percentage body fat. This has implications when using Adiposity Index as a body fat indicator because different strains may have dissimilar relationships. This same pattern is also seen when comparing epididymal adipose weights with percentage body fat, suggesting that the difference in height of the Adiposity Index line is due to the disparity in epididymal adipose size between strains. With the exception of mesenteric adipose tissue, where an increase in percentage body fat resulted in a much steeper increase in mesenteric fat in A^y mice, all phenotypes vs. percentage body fat had nonsignificant differences in slope.

The relationships of the epididymal adipose depot with other BW/fat variables, leptin, and cholesterol also had different intercepts or slopes in the two strains (Table 4). For a given fatness, A^y mice have less epididymal adipose than B6 mice. Also, A^y mice have higher cholesterol for any

Table 3. Adipose tissue gene expression

	Leptin		Acrp30		Lpl		Pdk4	
	fem	**epi**	**fem**	**epi**	**fem**	**epi**	**fem**	**epi**
A^y ad lib sed	80.9 ± 15.2[a]	12.3 ± 1.5	13.0 ± 1.0	2.4 ± 0.2[a]	1.2 ± 0.1	1.7 ± 0.2[a]	1.1 ± 0.1	4.9 ± 0.6[a]
A^y ad lib ex	64.5 ± 21.6[a,b,c]	5.4 ± 1.5	14.4 ± 4.5	1.8 ± 0.5[a]	1.5 ± 0.1	1.6 ± 0.4[a]	3.0 ± 1.1	8.2 ± 0.6[a]
A^y rest'd sed	67.9 ± 14.1[a,b]	12.1 ± 5.7	18.8 ± 2.6	4.0 ± 1.7[a,b]	1.8 ± 0.1	2.6 ± 0.8[a]	0.5 ± 0.1	1.6 ± 0.3[a]
A^y rest'd ex	30.0 ± 15.9[a,b,c,d]	4.3 ± 1.7	11.3 ± 5.2	2.3 ± 0.4[a]	1.6 ± 0.1	2.0 ± 0.4[a]	0.5 ± 0.1	1.6 ± 0.1[a]
B6 ad lib sed	8.6 ± 4.3[d]	10.8 ± 4.0	7.1 ± 2.7	10.0 ± 1.5[b,c]	1.8 ± 0.1	3.8 ± 0.5[a,b]	4.6 ± 1.0	8.3 ± 1.5[a]
B6 ad lib ex	10.3 ± 3.1[b,c,d]	7.3 ± 2.8	13.2 ± 4.1	10.3 ± 2.7[b,c]	1.5 ± 0.3	4.2 ± 0.7[a,b]	8.1 ± 3.2	29.3 ± 5.6[b]
B6 rest'd sed	9.9 ± 6.1[c,d]	10.1 ± 4.	17.8 ± 4.1	13.1 ± 0.3[c]	2.5 ± 0.2	5.6 ± 0.7[b]	4.0 ± 1.7	4.3 ± 0.4[a]
Significance of ANOVA								
Genotype	0.0003	NS	NS	<0.0001	NS	<0.0001	0.0004	<0.0001
Diet	NS	NS	NS	NS	NS	NS	NS	NS
Exercise	NS	NS	NS	NS	NS	NS	NS	<0.0001

fem, femoral adipose tissue depot; epi, epididymal adipose tissue depot. Femoral leptin mRNA values were log-transformed prior to analyses. Values are fold difference from lowest value within each individual gene and fat pad (relative expression) and expressed as mean ± SE. Values for the same trait with different superscripts are significantly different by Tukey's HSD test ($p < 0.05$). p Values are from three-way ANOVA. There was a significant ($p = 0.0002$) interaction between genotype and exercise on Pdk4 expression in epididymal adipose. Otherwise, no two- or three-way interactions were found. NS, not significant.

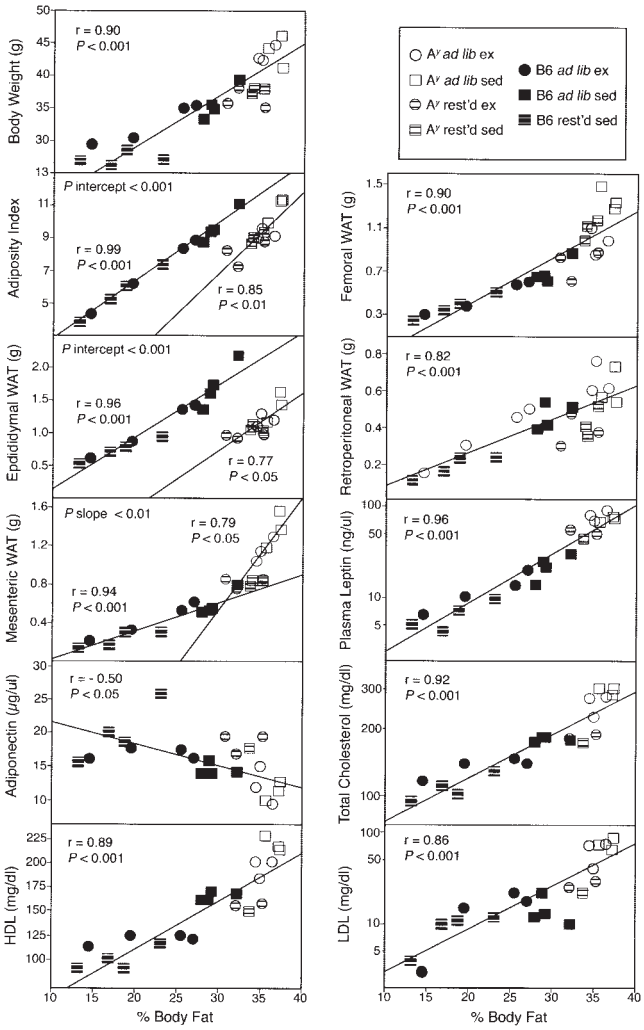

Figure 3: Relationships of percentage body fat with other phenotypes. When the slope or intercept of the regression lines differs between strains, data are separated by strain. WAT, white adipose tissue.

given epididymal weight. Epididymal adipose differs from the other adipose depots in these relationships.

Although plasma leptin had a single, strong correlation with percentage body fat in both A^y and B6 mice, its relationships with the various individual fat pads yielded both different slope and intercept, with the exception of the epididymal depot, where the lines were only of different height (Table 4). A^y mice had smaller variations in leptin for a given range of adipose depot weight. Adiponectin is reduced with increasing BW, percentage body fat, fat pad weights with the exception of epididymal fat, and leptin and cholesterol levels (Figure 3; Table 4).

Cholesterol is strongly related to BW, percentage body fat (Figure 3), femoral and mesenteric adipose, adiponectin, and leptin, but not to epididymal or retroperitoneal adipose tissue (Table 4). The association of fatness is stronger with total and HDL- than with LDL-cholesterol in both strains. The relationship between cholesterol and the epididymal fat pad is similar to that between percentage body fat and the epididymal fat pad, but in this case, there was a significant relationship only in B6 mice. In A^y mice only, there is a strong inverse association between cholesterol and adiponectin.

Correlations with mRNA Expression Levels in Femoral and Epididymal Fat Depots

We tested whether gene expression is differentially regulated in epididymal and femoral adipose tissue by examining correlations with other phenotypes (Table 5). There were no significant differences in slope between regression lines of A^y and B6 mice. Leptin expression in femoral (subcutaneous) adipose was well correlated with percentage body fat and plasma lipids. In contrast, leptin gene expression in epididymal (visceral) adipose tissue was not correlated with any parameters. The opposite is true with Acrp30 expression, with significant inverse correlations with body fat in the epididymal but not femoral fat depots. Expression of Lpl was well correlated with body fat and plasma lipid measures in both epididymal and femoral adipose tissue, with stronger correlations with epididymal expression. Pdk4 expression was correlated with percentage body fat in femoral but not epididymal adipose. Several correlations had significant differences in intercept between B6 and A^y mice, with Acrp30 expression in epididymal having the most intercept differences between strains.

Discussion

It was previously shown that, compared with normal mice, some obese mouse models exhibit an impairment in the reduction of body fat in response to diet and exercise (13–19,27). We show here that given the interventions of diet restriction, voluntary exercise, or the combination, only diet restriction had a significant effect on body fat percentage in A^y mice, whereas either diet restriction or exercise decreased body fat percentage in lean mice. Each treatment caused differential responses in the four adipose depots and in the measured plasma and molecular phenotypes. Diet and exercise, alone or in combination, had different effects on fat depot weights of B6 and A^y mice. The results emphasize that there is no real surrogate for direct measurement of actual fat mass when examining the effects of treatment or genotype. The complementary effects of diet and exercise also emphasize the importance of treatments that use both approaches because the combination produced the greatest effects. This is consistent with a recommendation that both together are necessary for a healthy lifestyle.

Our results demonstrate that the ectopic expression of the agouti protein in A^y mice specifically alters some, but not all, responses to diet restriction and exercise that are medi-

Table 4. Correlation matrix of obesity and plasma phenotypes

Phenotype 1	Phenotype 2	*p* slope	*p* intercept	*r* all	*r* A^y	*r* B6
BW	fem WAT	NS	NS	0.87‡		
	epi WAT	<0.05	<0.001		0.75*	0.93‡
	retro WAT	NS	NS	0.91‡		
	mes WAT	NS	<0.05		0.87†	0.96‡
	Leptin	<0.05	<0.001		0.88*	0.94‡
	Adiponectin	NS	NS	0.76‡		
	TC	NS	NS	0.94‡		
	HDL	NS	NS	0.93‡		
	LDL	NS	NS	0.85‡		
fem WAT	epi WAT	<0.01	<0.001		0.59	0.96‡
	retro WAT	NS	NS	0.71‡		
	mes WAT	NS	NS	0.90‡		
	Leptin	<0.01	<0.001		0.40	0.92‡
	Adiponectin	NS	NS	−0.61†		
	TC	NS	NS	0.93‡		
	HDL	NS	NS	0.92‡		
	LDL	NS	NS	0.86‡		
epi WAT	retro WAT	NS	<0.001		0.71*	0.91‡
	mes WAT	<0.01	<0.001		0.91†	0.96‡
	Leptin	NS	<0.001		0.60	0.95‡
	Adiponectin	NS	NS	−0.43		
	TC	NS	<0.001		0.68	0.89‡
	HDL	NS	<0.001		0.67	0.88†
	LDL	NS	<0.001		0.63	0.55
retro WAT	mes WAT	NS	<0.001		0.74*	0.94‡
	Leptin	<0.01	<0.001		0.76	0.95‡
	Adiponectin	NS	NS	0.64‡		
	TC	NS	<0.01		0.60	0.86†
	HDL	NS	<0.05		0.62	0.77*
	LDL	NS	<0.01		0.56	0.75*
mes WAT	Leptin	<0.01	<0.01		0.80	0.95‡
	Adiponectin	NS	NS	−0.65†		
	TC	NS	NS	0.94‡		
	HDL	NS	NS	0.92‡		
	LDL	NS	NS	0.89‡		
leptin	Adiponectin	NS	<0.05		−0.88*	−0.44
	TC	NS	<0.05		0.87*	0.90‡
	HDL	NS	NS	0.90‡		
	LDL	NS	NS	0.87‡		
adiponectin	TC	NS	<0.01		−0.91†	−0.44
	HDL	NS	<0.01		−0.91†	−0.52
	LDL	NS	<0.001		−0.90†	0.08
TC	HDL	NS	NS	0.99‡		
	LDL	NS	NS	0.90‡		
HDL	LDL	NS	NS	0.84‡		

fem WAT, femoral white adipose tissue; epi WAT, epididymal white adipose tissue; retro WAT, retroperitoneal white adipose tissue; mes WAT, mesenteric white adipose tissue; TC, total cholesterol; NS, not significant. Plasma leptin, TC, and LDL were log transformed prior to analyses. *p* slope indicates whether the slopes between A^y and B6 correlations are significantly different. *p* intercept indicates whether the intercept between A^y and B6 correlations are significantly different. For significant *p* slope or *p* intercept values, correlation coefficients are given for A^y & B6 separately.

* $p < 0.05$.

† $p < 0.01$.

‡ $p < 0.001$.

Table 5. Correlations between mRNA expression levels in femoral and epididymal adipose depots and other phenotypes

	Leptin		Acrp30		Lpl		Pdk4	
	fem	**epi**	**fem**	**epi**	**fem**	**epi**	**fem**	**epi**
Body fat (%)								
r all	0.66†	0.12	−0.01	−0.76‡	−0.52*	0.67†	−0.46*	−0.38
p intercept	NS	NS	NS	NS	NS	NS	NS	NS
r A^y								
r B6								
BW								
r all	0.69‡	−0.01	−0.05		−0.56†	−0.74‡	−0.33	
p intercept	NS	NS	NS	<0.01	NS	NS	NS	<0.05
r A^y				−0.30				0.50
r B6				−0.43				0.24
fem WAT								
r all	0.80‡	0.14	0.14		−0.46*	−0.68†	−0.45*	−0.39
p intercept	NS	NS	NS	<0.01	NS	NS	NS	NS
r A^y				−0.11				
r B6				−0.31				
epi WAT								
r all		0.18	−0.18		−0.36			0.05
p intercept	<0.001	NS	NS	<0.001	NS	<0.001	<0.05	NS
r A^y	0.34			−0.14		0.50	0.10	
r B6	0.27			−0.37		−0.02	0.00	
retro WAT								
r all		0.08	−0.11		−0.53†			
p intercept	0.01	NS	NS	<0.001	NS	<0.01	<0.05	<0.05
r A^y	0.04			−0.26		−0.35	0.40	0.74*
r B6	0.36			−0.24		−0.16	0.05	0.30
mes WAT								
r all		0.04	0.00		−0.57*	−0.75	−0.41	−0.29
p intercept	<0.05	NS	NS	<0.05	NS	NS	NS	NS
r A^y	0.39			−0.36				
r B6	0.35			−0.37				
Leptin								
r all	0.74‡	0.07	−0.08	−0.81‡	−0.62†	−0.76‡	−0.44	−0.30
p intercept	NS	NS	NS	NS	NS	NS	NS	NS
r A^y								
r B6								
Adiponectin								
r all		0.14	0.26		0.35			0.02
p intercept	<0.01	NS	NS	<0.001	NS	<0.01	<0.05	NS
r A^y	−0.56			0.37		0.51	−0.61	
r B6	0.22			0.36		0.67	−0.07	

Table 5. Continued

	Leptin		Acrp30		Lpl		Pdk4	
	fem	**epi**	**fem**	**epi**	**fem**	**epi**	**fem**	**epi**
TC								
r all	0.85‡	0.04	−0.08		−0.61†	−0.76‡	−0.31	−0.30
p intercept	NS	NS	NS	<0.05	NS	NS	NS	NS
r A^y				−0.61				
r B6				−0.26				
HDL								
r all	0.65†	0.06	−0.18		−0.60†	−0.76‡	−0.33	−0.36
p intercept	NS	NS	NS	<0.05	NS	NS	NS	NS
r A^y				−0.55				
r B6				−0.29				
LDL								
r all	0.93‡	0.02	0.07		−0.49*	0.59†	−0.26	−0.30
p intercept	NS	NS	NS	<0.05	NS	NS	NS	NS
r A^y				−0.66				
r B6				0.09				

* $p < 0.05$.
† $p < 0.01$.
‡ $p < 0.001$.
fem, femoral adipose tissue depot; epi, epididymal adipose tissue depot; WAT, white adipose tissue. Plasma leptin, total cholesterol, LDL cholesterol, and femoral leptin mRNA were log-transformed prior to analyses. There were no significant differences in slope between A^y and B6 mice. *p* intercept indicates whether the intercept between A^y and B6 correlations are significantly different. For significant *p* intercept values, correlation coefficients are given for A^y and B6 separately.

ated by physiological levels of plasma leptin. This is demonstrated for traits whose correlations exhibit different slopes or intercepts for B6 and A^y mice and suggests that the melanocortin signaling pathway influences only some of the traits regulated by plasma hormones such as leptin. Because other peripheral hormones, such as ghrelin and polypeptide-$YY_{3–36}$, also activate the same pathway, differences between B6 and A^y mice may reflect properties specific to melanocortin signaling, which is only partially controlled by leptin. Two studies measuring the responses of A^y mice to pharmacological doses of injected leptin reached different conclusions. A study using relatively low doses (5 μg/h) injected peripherally and centrally concluded that A^y mice are resistant to leptin (37). In contrast, other investigators, administering 2 mg/kg leptin subcutaneously twice daily, concluded that leptin and A^y have separate pathways of action (38). Leptin or polypeptide-$YY_{3–36}$ administration studies in *POMC*$^{-/-}$ mice also suggest melanocortin-independent pathways of action (39). In the present study, we examined the effect of the melanocortin pathway on the responsiveness of leptin and other pathways at physiological hormone levels. Our data suggest that A^y mice are responsive to leptin but that the dose-response curve is altered because B6 and A^y mice have different slopes for most correlations involving plasma leptin. This suggests that treatment of people with mutations in the *MC4R* gene by diet restriction and exercise alone will produce different results than similar treatments of people with wild-type *MC4R* alleles. Indeed, it has been reported that presence of *MC4R* mutations influences weight loss response to gastric bypass surgery (40,41).

Adipose depot heterogeneity was observed for nearly every trait examined. Leptin, adiponectin/Acrp30, and Lpl gene expression exhibited significant genotype effects in only one of the two adipose depots examined. Control of individual fat depot size is complex, with genes on several chromosomes influencing depot size (2,42). Other investigators have reported depot specific responses of mRNA levels of adipose-expressed genes (33–37). In humans, measures of adiposity are correlated to subcutaneous leptin expression, whereas expression in more central depots, such as omental and visceral, are weakly correlated or not correlated at all (43,44). In rodents, epididymal adipose tends to contain greater amounts of leptin mRNA than subcuta-

neous depots (45,46), with serum leptin correlating strongly with inguinal depot expression and, to a weaker extent, epididymal expression (46). Adiponectin/Acrp30 mRNA is lower in visceral adipose tissue of obese rats compared with their lean litter mates, but there is no difference in subcutaneous tissue between the lean and obese rats (47). Visceral adipose tissue mass is well-known to be more closely related to features of the metabolic syndrome (e.g., insulin resistance, hypertension, and dyslipidemia) than total body adiposity. Cultured adipose tissue from intraabdominal stores produces more adiponectin than subcutaneous adipose tissue from humans (48) and rat (P.J. Havel, unpublished data). Accordingly, visceral fat distribution is more predictive of lower adiponectin levels in humans than subcutaneous fat (20,49). Our findings are consistent with increasing evidence that individual fat depot weights are controlled by distinctly different genes and exhibit markedly different underlying biochemical and metabolic responses.

Our results demonstrate that relationships exist that are both general to all groups and specific to a single group. Because effects seen in these animals represent long-term adaptation to exercise and diet restriction, the correlations give insight into the relationships among whole body, intermediate, and molecular phenotypes resulting from these adaptations. The fact that many correlations are strong for many comparisons with both strains and all treatments combined indicates that these relationships are driven by the degree of obesity and are not dependent on the specific strain or treatment within the general model. Although we have examined only a small subset of the total collection of genes involved in the regulation of energy homeostasis and cholesterol metabolism, the results of this study demonstrate several effects and interactions in the regulation of lipid storage in different adipose depots with hormonal and gene expression responses during dietary energy restriction and exercise.

Acknowledgments

We thank Kimber Stanhope and James Graham for expert technical assistance in performing the plasma leptin and adiponectin assays, Larry Castellani for performing the plasma lipid assays, and Sue Bennett and Noreene Shibata for maintaining the mouse facility. This work was supported by NIH Grant R01 DK52581, by NIH Training Grants PHS DK07355 and T32 GM007377, by the University of California Davis Clinical Nutrition Research Unit Grant NIH DK35747, and a grant from the Nora Eccles Treadwell Foundation.

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