The hypothesis that particular lipoprotein patterns are associated with susceptibility to obesity and diabetic phenotypes, we fed the diabeticogenic diet to C57BL/6 mice carrying mutations at loci for the low-density lipoprotein (LDL) receptor or apolipoprotein E (apoE). Mutations at these genes result in dyslipidemic states because of impaired lipoprotein production and metabolism (3, 20, 32, 47). The low-density lipoprotein receptor (LDLR) is involved with clearing LDL and lipoprotein remnants containing apoE (4, 17) and may also play a role in regulating hepatic lipoprotein production (45). ApoE is a structural component of all lipoprotein particles except LDL, and it serves as a high-affinity ligand for both the apoB and remnant receptor (11, 19). This allows for the specific uptake of apoE-containing lipoproteins by the liver (7, 11). In addition, apoE is involved with promoting hepatic very-low-density lipoprotein (VLDL) secretion (27). Thus both of these proteins play an integral role in the efficient production and clearance of lipoproteins.

Compared with wild-type C57BL/6 mice fed the diabeticogenic diet, LDLR-deficient (LDLR−/−) mice showed the greatest gains in adiposity and hypertriglyceridemia. This strain also demonstrated increased glucose levels compared with the other strains, demonstrating that dyslipidemia induced by the LDLR mutation is associated with impaired glucose metabolism. Remarkably, apoE-deficient (apoE−/−) mice were resistant to diet-induced hypertriglyceridemia or hyperglycemia despite significant weight gain. Thus diet in conjunction with distinct lipoprotein profiles, as induced by the mutations in LDLR and apoE genes, contribute to determining susceptibility to diabetes and dyslipidemia. Our results demonstrate that alleles of the LDLR and apoE should be considered as candidates for identifying diabetes susceptibility and severity among individuals.

METHODS

Mice and diets. Male C57BL/6 wild-type, LDLR−/−, and apoE−/− mice, 6 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). LDLR−/− and apoE−/− mice are maintained on the C57BL/6 background strain. Mice...
were fed either rodent chow (Wayne Rodent BLOX 8604, Teklad, Madison, WI) or a diabetogenic diet (no. F1850, Bioserve, Frenchtown, NJ), with 20 mice per group. Rodent chow contains 4% fat (wt/wt) and 72% carbohydrate. The diabetogenic diet contains 35.5% fat (primarily lard) and 36.6% carbohydrate (primarily sucrose) and contains no cholesterol. The diabetogenic diet has been previously described and demonstrated to induce obesity and type 2 diabetes in C57BL/6 mice (36, 37, 40, 41). Mice were maintained in a temperature-controlled (25°C) facility with a strict 12:12-h light-dark cycle and given free access to food and water. Blood was collected after a 4-h fast from the retroorbital sinus into tubes containing 1 mM EDTA, and plasma was stored at −70°C before analysis. The Animal Care and Use Committee of the University of Washington approved this project.

Adiposity measurements using magnetic resonance spectroscopy. To determine murine whole body fat mass, magnetic resonance spectroscopy (MRS) was performed as described by Mystkowski et al. (30). This technique exploits the differential water and lipid proton resonances and involves the placement of the mouse within the coil and to place the coil at the center of the mouse within the coil and to place the coil at the center of the magnetic field and affects the shape of the field. The resonant proton signal at 200.1 MHz, and scanned using a 4.7 T research magnet (Bruker/GE Omega CSI-II, Fremont, CA) at the University of Washington Department of Radiology. Shimming (which corrects for magnetic field inhomogeneities) was performed before each spectral acquisition, and two acquisitions were obtained per animal. Care was taken to center the mouse within the coil and to place the coil at exactly the same longitudinal and rotational position for each reading. Areas under the curve (AUC) for individual spectral peaks were obtained using line integrals (GE Omega version 6.0.2 Fremont, CA), with the AUC for the water peak (AUCW) demarcated by the region from 8.0 ppm to the relative minimum transformed signal between the water and lipid peaks. The AUC for the lipid peak (AUClipid) was demarcated by the same relative minimum value between peaks and −1.5 ppm. Body fat percentage by MRS was defined as %FATMRS = AUClipid/0.83 × AUCW + AUCF, and the value for each animal was determined by averaging the %FATMRS calculated per spectrum.

Analytical procedures. Plasma insulin and leptin levels were measured using radioimmunoassay kits (nos. RI-13K and ML-82K, Linco, St. Louis, MO), with rat insulin and mouse leptin as the respective standards (36). Plasma glucose levels were determined colorimetrically (cat. no. 315-100, Sigma, St. Louis, MO). Plasma triglyceride concentrations were assessed colorimetrically after the removal of free glycerol (diagnostic kit no. 450032, Boehringer Mannheim, Indianapolis, IN). Plasma free fatty acids (FFA) were determined colorimetrically (diagnostic kit no. 99075401, Wako, Dallas, TX) with oleic acid as the standard.

Fat absorption. Fat absorption was measured in mice by modifying the procedure described by Ishibashi et al. (21). Mice were fed the diets for 12 wk, fasted overnight, and then gavaged with 1 μCi [3H]retinol (cat. no. NET 927, NEN Life Science Products, Boston, MA) in 200 μl corn oil. Plasma samples were obtained at 1, 2, 5, 8, 12, and 24 h after gavage, and plasma radioactivity was quantified. Total plasma radioactivity was determined by multiplying the disintegrations per minute per microliter by total plasma volume, which was estimated at 5.77% of body weight (2).

Statistics. Values are reported as means ± SE. To compare the longitudinal relationship between body weight and leptin levels across strains, a generalized estimating equation (GEE) approach was used (24). The GEE approach corrects for the dependence of multiple measurements taken within a single mouse across successive time points. ANOVA analyses were used to determine interactions, and Bonferroni post hoc tests were applied to determine differences between means. In some cases, the Student’s t-test was used to compare independent means. P < 0.05 was accepted as statistically significant.

RESULTS

Diabetogenic diet-fed LDLR−/− mice are heavier than wild-type or apoE−/− mice. Mice at the age of 6 wk were fed either the chow or diabetogenic diet for a total of 16 wk. At the beginning of the diet study, wild-type C57BL/6 mice were heavier (24.0 ± 0.2 g) than LDLR−/− (21.0 ± 0.3 g; P < 0.001) or apoE−/− (21.0 ± 0.3 g; P < 0.001) mice. Mice fed rodent chow for 16 wk had final body weights of 27–30 g. After 16 wk of the diabetogenic diet, body weights for wild-type, LDLR−/−, and apoE−/− mice were 44.9 ± 2.1, 49.3 ± 1.3, and 35.2 ± 2.6 g (P < 0.001 vs. wild type), respectively. Weights for wild-type and apoE−/− mice were 1.8-fold higher than initial values, whereas LDLR−/− mice had weight increases of 2.4-fold after 16 wk (P < 0.001 vs. wild-type, Fig. 1A). Thus diabetogenic diet-fed LDLR−/− mice gained significantly more weight than either wild-type or apoE−/− mice.

Leptin is a long-term satiety signal that often reflects changes in body weight (10, 25). Marked elevations in leptin are thought to reflect leptin resistance and a diminution of satiety responses (1). LDLR−/− mice were singular in the early and marked increase in plasma leptin levels when fed the diabetogenic diet (Fig. 1B). By 4 wk, leptin levels were 2.3-fold higher in LDLR−/− mice than in the other strains. Between 8 and 16 wk, leptin levels were 20-fold higher than for rodent chow-fed LDLR−/− mice. In contrast, wild-type mice experienced a 10-fold increase, and diabetogenic diet-fed apoE−/− mice had only a 3.7-fold increase in leptin over their chow-fed counterparts. To determine whether leptin expression is disproportional to body weight in LDLR−/− mice, the body weight vs. leptin values for mice fed the diabetogenic diet for 4–16 wk were examined using GEEs (Fig. 1C; see METHODS). For all mice, body weights correlated significantly with leptin values (P < 0.0001). The relationship between leptin and mouse weight for C57BL/6 was not significantly different from that observed for apoE−/− mice (P = 0.56), with a regression line described by leptin = 2.04 × (body weight) − 56. However, this relationship was significantly different in LDLR−/− mice (P < 0.0001 compared with either C57BL/6 or apoE−/− mice), with a regression line described by leptin = 2.83 × (body weight) − 68. Thus leptin levels were consistently higher in the LDLR−/− mice as reflected by the 38% increase in the slope of the regression line for this strain.

Because body weight and leptin levels were highest for LDLR−/− mice fed the diabetogenic diet, we expected food intake to be greatest for this strain. How-

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ever, as shown in Fig. 2A, diabetogenic diet-fed wild-type mice and LDLR−/− mice had similar food consumption profiles throughout the study. When averaged throughout the study, apoE−/− mice showed greater food intake (19.3 ± 0.7 calories per mouse per day) than wild-type or LDLR−/− mice (17.7 ± 0.6 calories per mouse per day; \( P = 0.05 \)). Therefore, increased food intake does not account for increased weight gain in the LDLR−/− mice. To determine whether fat absorption was altered in either the LDLR−/− or the apoE−/− mice, radiolabeled retinol was gavaged into mice fed the diabetogenic diet for 12 wk, and the plasma radioactivity was followed for the subsequent 24 h (Fig. 2B). Results demonstrate that retinol was readily absorbed by all strains, with maximum plasma values obtained between 5 and 8 h after gavage treatment. Therefore, fat absorption was not different between strains and cannot account for the increased weight gain in diabetogenic diet-fed LDLR−/− mice. We conclude that caloric intake or fat absorption does not account for variation in body weights between the strains.

To evaluate weight gain in terms of adiposity, body fat percentage was determined in a subset of mice using proton MRS, a recently validated noninvasive method for the precise and accurate assessment of body composition (30). MRS capitalizes on the differential behavior of protons associated with lipid vs. water when placed in a magnetic field, when the determination of aqueous vs. lipid composition of each mouse is allowed (Fig. 3A). The left peak (8 ppm cutoff) represents the aqueous percentage, and the right peak (−1.5 ppm cutoff) represents the lipid percentage. The areas under such peaks were used to calculate the percent adiposity for each mouse (Fig. 3B).

ANOVA evaluation of adiposity measurements showed that both diet \( (P < 0.0001) \) and genotype \( (P < 0.0001) \) significantly contributed to adiposity levels. Chow-fed LDLR−/− and apoE−/− mice were 30% leaner than wild-type mice at 16 wk \( (P < 0.05) \). All mice had significant increases in adiposity when fed the diabetogenic diet \( (P < 0.001, \) Fig. 3B). Both wild-type and apoE−/− mice showed a twofold increase in adiposity (17.2 ± 1.3 to 32.5 ± 1.5% for wild-type; 17.2 ± 1.3 to 32.5 ± 1.5% for apoE−/−).
12.4 ± 0.9 to 23.5 ± 3.1% for apoE−/−), demonstrating that loss of apoE does not alter the extent of obesity compared with diabetogenic diet-fed wild-type mice. In contrast, adiposity increased 3.6-fold for LDLR−/− mice (11.1 ± 0.9% to 39.5 ± 1.5%), showing that LDL receptor deficiency results in increased diet-induced obesity. For diabetogenic diet-fed but not chow-fed mice, adiposity positively correlated with body weight (r = 0.79–0.95, P < 0.007). These results also suggest that the increased leptin levels in the LDLR−/− mice (Fig. 1C) are at least partially resulting from the increased adiposity observed in this strain.

To examine how adiposity was distributed, liver, inguinal, and epididymal adipose fat pads were removed from chow or diabetogenic diet-fed mice and weighed (Fig. 4). For chow-fed mice, the percent body weight of these two fat depots (%adipose weights) were significantly lower in apoE−/− mice compared with wild-type mice (P < 0.03). This result is consistent with the MRS analysis showing decreased adiposity in this strain. Inguinal adipose was moderately higher in chow-fed LDLR−/− mice compared with wild-type mice (P < 0.05). Adipose weights increased significantly for all strains fed the diabetogenic diet (P = 0.004–0.00001). Compared with diabetogenic diet-fed wild-type mice, LDLR−/− mice showed significantly increased inguinal but not epididymal %adipose weights (Fig. 4B). Thus loss of the LDLR results in increased adiposity that was due, in part, to increased subcutaneous adipose accumulation.

%Adipose depot weights were significantly lower in apoE−/− mice compared with wild-type mice, whereas %liver weights were increased (Fig. 4). Liver lipid content was measured to determine whether hepatic lipid storage was increased in apoE−/− mice. For mice fed the chow diet, apoE−/− mice had moderately higher hepatic triglyceride (TG) content (1.6 ± 0.4 mg TG/g liver) compared with wild-type mice (1.1 ± 0.3 mg TG/g liver), although differences did not achieve statistical significance. When wild-type or LDLR−/− mice were fed the diabetogenic diet, hepatic TG levels increased twofold to 2.3 ± 0.4 mg TG/g liver (P < 0.01 vs. chow-fed mice). However, apoE−/− mice were resistant to any changes in hepatic TG content in response to this diet, with a final hepatic lipid content of 1.7 ± 0.2 mg TG/g liver.

Hyperlipidemia occurs in LDLR−/− mice fed the diabetogenic diet. The increase in adiposity in LDLR−/− mice could result from increased TG availability from circulating lipoproteins. To test this possibility, plasma TG levels were measured in mice fed the chow or diabetogenic diet. Chow-fed LDLR−/− and apoE−/− mice had consistently higher TG levels than wild-type mice (Fig. 5A). During the course of the diabetogenic diet feeding study, LDLR−/− mice showed a marked and rapid elevation in plasma TG levels.
levels (Fig. 5B). In contrast, TG levels were reduced by 30% in wild-type and apoE<sup>−/−</sup> mice in response to dietary treatment (P < 0.01). Plasma FFA were also highest for LDLR<sup>−/−</sup> mice fed the diabetogenic diet (0.41 ± 0.04 meq/l for wild-type; 0.99 ± 0.14 meq/l for LDLR<sup>−/−</sup>, but not apoE<sup>−/−</sup> mice, develop hyperglycemia and hyperinsulinemia in response to the diabetogenic diet. The association between obesity, hypertriglyceridemia, and type 2 diabetes is well documented (6, 12, 33, 34). To test for the development of insulin resistance and diabetes, plasma levels of glucose and insulin, and insulin-to-glucose ratios, were determined in chow- and diabetogenic diet-fed mice (Fig. 6).

All strains fed rodent chow had glucose levels of ~130–150 mg/dl. For wild-type mice fed the diabetogenic diet, glucose levels fluctuated, showing increases up to 209 mg/dl (Fig. 6A). After 16 wk of treatment, final values were 172 ± 14 mg/dl. Glucose levels for LDLR<sup>−/−</sup> mice increased consistently with time, and final values were 247 ± 19 mg/dl, a level corresponding to frank diabetes (40). During the first 12 wk of feeding the apoE<sup>−/−</sup> mice the diabetogenic diet, glucose levels
remained consistently lower than in the other strains. However, after 16 wk of feeding the diabetogenic diet, glucose levels for this strain were not different from those observed for wild-type mice.

Insulin levels for mice fed rodent chow remained constant at ~0.8 ng/ml. Plasma insulin levels increased significantly for diabetogenic diet-fed wild-type and LDLR−/− mice, but not for apoE−/− mice (Fig. 6B). Final insulin values for diabetogenic diet-fed mice were 4.2-fold higher in wild-type and 5.3-fold higher in LDLR−/− mice compared with chow-fed counterparts (P < 0.02). Overall, insulin levels were highest for diabetogenic diet-fed LDLR−/− mice, but differences were only statistically significant at the 8-wk time point (P < 0.003). A measure of insulin resistance is the ratio of insulin to glucose (I/G). This ratio increases as insulin resistance develops, indicating that more insulin is needed to maintain normal glucose levels. All chow-fed mice showed similar I/G ratios (Fig. 6C). Evidence of insulin resistance was seen for wild-type and LDLR−/− mice, because I/G ratios increased two-to threefold compared with chow-fed mice (P < 0.03). Despite the greater obesity and hypertriglyceridemia observed in the LDLR−/− mice, I/G ratios were similar to those of diabetogenic diet-fed wild-type mice. ApoE−/− mice showed no diet-induced changes in I/G, demonstrating maintenance of low glucose and insulin levels despite the dietary challenge.

**DISCUSSION**

This report explores the role of genes involved with lipid metabolism in the susceptibility to obesity and complications associated with obesity. Loss of the LDLR increases susceptibility to diet-induced obesity, hyperleptinemia, and hypertriglyceridemia and influences the sites of lipid deposition, demonstrating an important role of LDLR function in regulating adipose lipid storage and leptin secretion. However, despite the profound hypertriglyceridemia observed in the LDLR−/− mice, glucose and insulin levels were only modestly increased compared with wild-type mice, and I/G ratios were comparable to those of wild-type mice. Therefore, severe hypertriglyceridemia did not exacerbate insulin resistance beyond what induced by the diabetogenic diet. ApoE deficiency did not alter susceptibility to diet-induced obesity from wild-type values but resulted in resistance to diet-induced hyperglycemia and hyperinsulinemia. Taken together, these findings help link the factors that regulate lipid metabolism with the development of obesity and diabetes.

The direct role of the LDLR on obesity has not been well studied. In humans, LDLR polymorphisms have been associated with obesity (26), suggesting a link between LDLR function and peripheral lipid deposition. In our study, loss of the LDL receptor increased adipose deposition, especially in subcutaneous adipose depots. This suggests either that the loss of adipose LDLR expression directly contributes to increased TG uptake in adipose or, more likely, that loss of hepatic LDLR expression increases circulating lipoproteins, providing increased TG substrate for uptake by adipocytes. Alternatively, loss of the LDLR may have indirect effects on reducing lipolysis in fat cells or altering thermogenesis, which would also increase lipid storage in adipose tissue. LDLR deficiency in mice and humans results in increased levels of circulating apoB-100 particles due to both increased hepatic lipoprotein production and impaired clearance (21, 39, 42, 45). Furthermore, LDLR−/− mice are highly susceptible to high-fat diet-induced hypertriglyceridemia (28, 43). The TG-enriched lipoprotein particles may then be preferentially cleared by nonhepatic tissues, such as adipose, resulting in increased obesity. Interestingly, male LDLR−/− mice appear to be more susceptible than female mice to obesity associated with hypertriglyceridemia (23). We have also observed that male C57BL/6 mice are more susceptible to diabetogenic diet-induced obesity [(36) and unpublished observations]. Therefore, increased diet-induced obesity may be dependent on both LDLR activity and gender-dependent factors.

Increased lipid accumulation in adipose would be expected to increase leptin production. Circulating leptin levels are known to reflect whole body adiposity in humans and rodents (10, 25). The temporal change and magnitude of leptin increases were distinct for LDLR−/− mice. The LDLR−/− mice as a group were the heaviest and showed the highest leptin levels of all three strains, increasing 3.8-fold over wild-type and apoE−/− by 8 wk. Interestingly, subcutaneous adipose was preferentially increased in diabetogenic diet-fed LDLR−/− mice. Subcutaneous adipose is more responsive in producing leptin than visceral adipose (13, 29). It is likely that the increased subcutaneous adipose in the LDLR−/− mice is contributing to the increased leptin levels observed in this strain. However, regression analysis demonstrated that, even between weight-matched mice of the three strains, leptin levels were significantly higher for the LDLR−/− mice. Therefore, it is also possible that LDL receptor deficiency results in overproduction of leptin by adipose tissue. Whether this results from LDLR deficiency within the adipocyte itself or from indirect effects through hepatic LDL receptor deficiency remains to be determined.

The overproduction of leptin in LDLR−/− mice was not reflected in the food intake by this strain. Leptin is produced only in adipose tissue and elicits satiety responses by binding to leptin receptors in the brain (9, 22, 48). On the basis of increased leptin levels that we observed in the LDLR−/− mice, we hypothesized that food intake would be decreased. However, results from our food intake and fat absorption studies clearly showed that this was not the case. Therefore, these mice are likely becoming leptin resistant, a disorder observed in human obesity (18, 49). Studies evaluating leptin production and signaling responses in the obese LDLR−/− mice should help us understand the relationship between leptin production and responsiveness, which may open new avenues of leptin therapy treatment in obese individuals.

The response of apoE−/− mice to the diabetogenic diet was quite different from that observed for the
LDLR−/− mice. Several possibilities for this observation exist. First, we considered whether loss of apoE−/− impaired fat absorption. Our fat absorption studies showed that there were no apparent defects in gut fat absorption compared with wild-type mice. Also, apoE−/− mice fed the diabetogenic diet gained weight, demonstrating a positive energy balance. Second, a study demonstrated that apoE deficiency reduced hepatic VLDL secretion by >60% and increased hepatic TG content threefold (27). No increases in hepatic TG levels were observed in diabetogenic diet- vs. chow-fed apoE−/− mice, suggesting that hepatic TG were being shunted away from lipid storage or VLDL production. We suggest that fatty acid oxidation is accelerated in these mice to remove hepatic TG stores. Triscari et al. (44) showed that in high fat-fed rodents, β-oxidation is increased compared with chow diet-fed controls. Furthermore, several studies have demonstrated that, in livers from obese mice, uncoupling protein 2 (UCP2) mRNA and protein levels are increased (14, 46). UCP2 uncouples respiration from oxidative phosphorylation, resulting in ATP depletion and energy release in the form of heat. Our data suggest that apoE may be an important toggle that directs hepatic fatty acids away from fatty acid oxidation and into TG production for lipoprotein secretion. In this scenario, apoE deficiency would promote β-oxidation, possibly providing more substrate for UCP while preventing fatty liver formation. This concept is currently under investigation.

In addition to effects on hepatic tissue, we must consider whether dietary lipids are being preferentially stored in adipose or muscle from apoE−/− mice. This seems unlikely, because the apoE−/− mice did not exhibit extraordinary increases in adiposity. However, the ability of these tissues to activate UCPs in response to this diet has not been studied in apoE−/− mice, and increased hepatic TG content threefold (27). No increases in hepatic TG levels were observed in diabetogenic diet- vs. chow-fed apoE−/− mice, suggesting that hepatic TG were being shunted away from lipid storage or VLDL production. We suggest that fatty acid oxidation is accelerated in these mice to remove hepatic TG stores. Triscari et al. (44) showed that in high fat-fed rodents, β-oxidation is increased compared with chow diet-fed controls. Furthermore, several studies have demonstrated that, in livers from obese mice, uncoupling protein 2 (UCP2) mRNA and protein levels are increased (14, 46). UCP2 uncouples respiration from oxidative phosphorylation, resulting in ATP depletion and energy release in the form of heat. Our data suggest that apoE may be an important toggle that directs hepatic fatty acids away from fatty acid oxidation and into TG production for lipoprotein secretion. In this scenario, apoE deficiency would promote β-oxidation, possibly providing more substrate for UCP while preventing fatty liver formation. This concept is currently under investigation.

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In conclusion, apoE−/− mice provided in a diabetogenic diet-fed model of obesity exhibit extraordinary increases in adiposity. How-


