Increased Serum Leptin Protects From Adiposity Despite the Increased Glucose Uptake in White Adipose Tissue in Mice Lacking p85α Phosphoinositide 3-Kinase

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Mice lacking the p85α regulatory subunit of phosphoinositide (PI) 3-kinase (Pik3r1$^{-/-}$) showed increased glucose uptake in white adipose tissue (WAT) and skeletal muscle due to increased phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P3] production and on a normal diet had a body weight and fat mass similar to wild-type mice. After 3 months on a high-fat diet, Pik3r1$^{-/-}$ mice still had increased insulin sensitivity and better glucose tolerance than wild-type mice, but showed markedly greater increases in body weight and WAT mass than wild-type mice. On the normal diet, serum leptin levels of Pik3r1$^{-/-}$ mice were significantly higher than in wild-type mice as a result of increased leptin secretion from adipocytes, presumably due to the increased PtdIns(3,4,5)P3 production in adipocytes. Leptin (5 μg/g body wt per day) caused a reduction in food intake and decrease in body weight by the wild-type mice as well as Pik3r1$^{-/-}$ mice, suggesting Pik3r1$^{-/-}$ mice having leptin sensitivity similar to wild-type mice. The slightly increased serum leptin compensated for the increased glucose uptake by adipocytes in Pik3r1$^{-/-}$ mice, thereby preventing adiposity on the normal diet. On the high-fat diet, leptin (5 μg/g body wt per day) failed to decrease food intake or body weight in either genotype, indicating that both genotypes had indeed become severely leptin resistant. Consequently, leptin secretion was unable to sufficiently compensate for the severe leptin resistance caused by the high-fat diet, thereby failing to prevent obesity in Pik3r1$^{-/-}$ mice. Our findings suggest that primary increase in serum leptin on the normal diet play a role in the protection from adiposity in Pik3r1$^{-/-}$ mice. *Diabetes* 53:2261–2270, 2004

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GFAT, glutamine:fructose-6-phosphate amidotransferase; GTT, glucose tolerance test; HPLC, high-performance liquid chromatography; IRS, insulin receptor substrate; KRBH, Krebs Ringer bicarbonate buffer; PI, phosphoinositide; PMSF, phenylmethylsulfonyl fluoride; PtdIns(3,4,5)P3, phosphatidylinositol (3,4,5)-triphosphate; WAT, white adipose tissue.

Obesity is defined as a state of pathophysiologically increased adipose mass and is closely linked to major diseases, such as diabetes, hyperlipidemia, hypertension, and cardiovascular disease (1). The prevalence of obesity, which often develops in adulthood, is increasing sharply in both Western countries and Japan, and the increase can be explained by drastic changes in lifestyle, such as a high-fat diet and sedentary lifestyle. Although adipocytes were previously viewed as passive participants in the pathogenesis of obesity, they have recently been recognized as playing a more active role in the regulation of energy homeostasis and body composition (1), and rapid advances are now being made in our knowledge of the mechanisms whereby many biological molecules secreted by adipocytes play a physiological role in the regulation of body weight and glucose homeostasis.

Leptin is a protein encoded by the obese gene (*ob*) and is synthesized and released in response to increased energy storage in adipose tissue (2). Leptin is an adiposity signal that acts on the hypothalamus, where it stimulates catabolic effector pathways and inhibits anabolic effector pathways, leading to decreased body weight and increased insulin sensitivity (3). The insulin signaling pathway and phosphoinositide (PI) 3-kinase (PI 3-kinase) have been suggested to be involved in the regulation of leptin production and secretion (4–6). Glucose utilization by adipocytes (7) and the hexosamine biosynthetic pathway (8,9) have been suggested to be key factors linking leptin production and secretion to body weight mass and energy availability. In fact, mice overexpressing the rate-limiting enzyme for hexosamine synthesis, glutamine:fructose-6-phosphate amidotransferase (GFAT), in muscle and fat have been shown to be hyperleptinemic (10,11). Moreover, in acute glucosamine and glucose infusion experiments (9), plasma leptin levels were found to be increased 2.5- to 3.0-fold compared with the control animals, whereas adipose tissue leptin levels were increased by only 50%. These results suggest that insulin, glucose metabolism, and hexosamine flux in fat regulate leptin synthesis and secretion, although it remains to be determined whether they are physiological regulators in vivo.

Adiponectin is another biological molecule secreted by adipocytes and was identified independently by four groups using different approaches (12–15). Mouse cDNAs

for adiponectin have also been termed Acrp30 (13) and AdipoQ (14). Adiponectin expression and plasma level have been reported to be significantly reduced in obese/diabetic mice and humans (14,16,17). It was reported that a proteolytic cleavage product of Acrp30 increases fatty acid oxidation in muscle and causes weight loss in mice (18), and we (19) and others (20) have recently reported that adiponectin is a potent insulin-sensitizing hormone linking adipose tissue and whole-body glucose metabolism. Thus, adiponectin may contribute to the suppression of obesity and insulin resistance. Importantly, adiponectin is reportedly produced and secreted in a PI 3-kinase–dependent manner (21).

P85α is a regulatory subunit of PI 3-kinase that dimerizes the p110 catalytic subunit. Insulin activates PI 3-kinase by tyrosine phosphorylation of insulin receptor substrates (IRSs), such as IRS-1, and subsequent binding of p85α associated with p110 (22,23). We generated mice lacking only the p85α isoform of the Pik3r1 gene (24–26) (Pik3r1$^{-/-}$) and found that they exhibited increased insulin sensitivity and hypoglycemia due to increased glucose transport in skeletal muscle and adipocytes (27). This phenotype can be explained by the fact that insulin-dependent generation of phosphatidylinositol (3,4,5)-triphospahate [PtdIns(3,4,5)P3] is increased in Pik3r1$^{-/-}$ mice in vivo (27). Mice lacking all three isoforms of Pik3r1 also were shown to display hypoglycemia, lower insulin levels, and increased glucose tolerance (28), confirming the findings in our previous study (27). Importantly, insulin-stimulated PtdIns(3,4,5)P3 production was prolonged in the knockout animals (29).

Selective overexpression of GLUT4 in the adipose tissue in mice has been shown to lead to increased glucose uptake by adipose tissue and to adiposity (30), demonstrating that adiposity can be promoted by increased glucose uptake and lipogenesis by adipocytes. On a normal diet, however, Pik3r1$^{-/-}$ mice have normal body weight and adipose tissue mass despite the increased glucose uptake by adipose tissue (27). Interestingly, on a high-fat diet, the knockout animals became markedly obese compared with wild-type mice. We therefore attempted to identify the mechanisms by which Pik3r1$^{-/-}$ mice maintain a normal body weight on a normal diet and became obese on the high-fat diet, as compared with wild-type mice.

RESEARCH DESIGN AND METHODS

Pik3r1$^{+/-}$ mice (C57BL/6J and CBA background) were generated as described previously (27). They had been backcrossed with C57BL/6J mice more than three times, and because their genetic background was not completely homogeneous, female offspring obtained from intercrosses of Pik3r1$^{+/-}$ mice were analyzed in this study. The animals were given free access to water and ordinary laboratory or high-fat diet and received standard animal care according to our institutional guidelines.

Measurement of serum parameters and in vivo glucose homeostasis. Glucose, insulin, and leptin levels were determined with a Glucose Test Sensor (Sanwa Kagaku Kenkyujo, Nagoya, Japan), an insulin kit (BIOTRAK; Amersham Life Science), and the Quantikine M Mouse Leptin Immunoassay kit (R&D Systems), respectively, according to the manufacturer's instructions. The glucose tolerance test (GTT) and insulin tolerance test were carried out according to previously described methods (27,31). The insulin resistance index was calculated by multiplying the fasting blood glucose level (mg/dl) by the fasting insulin level (ng/ml).

Histological and immunohistochemical analysis and determination of adipocyte size. Adipose tissue was fixed with formaldehyde, and after cutting 10-μm sections and mounting them on glass slides, they were stained with hematoxylin and eosin. White adipocyte areas were measured in 300 or more cells per mouse in each group, as described elsewhere (32).

Assessment of the effects of intraperitoneal leptin administration. Leptin (Pepro Tech EC) was administered as a daily intraperitoneal injection of 5 μg/g body wt per day to wild-type and Pik3r1$^{-/-}$ mice on each of the diets, and isotonic PBS was administered to controls. Changes in food intake and body weight were measured to assess the effects of leptin (32).

RNA preparation, Northern blot analysis, and RNase protection assay. Total RNA was prepared from white adipose tissue (WAT) by using TRIzol Reagent Total RNA isolation reagent (Gibco/BRL). Northern blot analysis was performed according to a standard protocol. RNase protection assay to measure leptin and GFAT mRNAs was performed by using HybspeedTM RPA (Ambion), as described previously (32). The leptin cRNA transcript is 615-bp long, whereas the RNase-protected product is 517-bp long (corresponding to nucleotides 50–566 of the mouse sequence) (Genebank U18812). The GFAT cRNA transcript is 615-bp long, whereas the RNase-protected product is 546-bp long (corresponding to nucleotides 1,469–2,014 of the mouse sequence) (Genebank U00932). Cyclophilin expression was used to normalize leptin or GFAT expression in each sample.

Determination of leptin content and leptin secretion. To determine the leptin contents of perimetric fat pads, tissue fragments were immediately placed in extraction buffer A (0.3 mol/l NaCl, 1 mmol/l EDTA, 0.05 mmol/l Tris-HCl, pH 7.4, 1% Triton X-100, and 1 mmol/l phenylmethylsulfonyl fluoride [PMSF]) and sonicated. After removing cell debris by repeated centrifugation at 15,000*g* at 4°C, the protein concentration and leptin concentration in the buffer were determined with a protein assay kit (BioRad) and the Quantikine M Mouse Leptin Immunoassay kit, respectively. Leptin content was calculated as nanogram of leptin per milligram of protein.

To obtain tissue samples for the leptin secretion experiment, perimetric fat pads were dissected into proximal and distal segments during removal from the animal and then further sectioned into several pieces in Krebs Ringer bicarbonate buffer (KRBH) (120 mmol/l NaCl, 4 mmol/l KH_2PO_4, 1 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 10 mmol/l $NaHCO_3$, and 30 mmol/l HEPES, pH 7.4) containing 0.5% BSA (Fraction V) (5). Each sample (~100 mg) consisted of two proximal pieces and two distal pieces, thereby ensuring that each sample contained a mixture of large and small cells, since leptin levels appear to correlate with adipose cell size. Samples were incubated with or without 100 nmol/l insulin for 1 h. To assess the inhibition of insulin-induced leptin secretion by wortmannin, WAT was incubated with 100 nmol/l wortmannin for 20 min before insulin stimulation and during insulin stimulation. The amount of leptin in the medium was measured in the presence or absence of 100 nmol/l insulin or 100 nmol/l wortmannin using the Quantikine M Mouse Leptin Immunoassay kit.

Measurement of glucosamine metabolites. Uridinediphospho-glucose (UDP-Glc), uridinediphospho-galactose (UDP-Gal), uridinediphospho-*N*-acetyl-glucosamine (UDP-GlcNAc), and uridinediphospho-*N*-acetyl-galactosamine (UDP-GalNAc) concentrations were determined by two sequential chromatographic separations and UV detection (33,34). Briefly, the first purification was performed on a solid-phase, strong anion-exchanging cartridge by using phosphate buffers of increasing molarity. This first purification allows partial separation of the two compounds of interest from nonpolar cellular and most polar intracellular substrates with optimal recoveries. UDP-GlcNAc and UDP-GalNAc coelute with UDP-Glc and UDP-Gal during the solid-phase extraction. The second step is a reverse-phase ion-pairing high-performance liquid chromatography (HPLC isocratic method on a C18 column. All HPLC analyses were performed on an HPLC system (TOSOH) by a reverse-phase ion-pairing isocratic method on a C18 column, namely, an Inertosil ODS-2 reverse-phase column (0.46 × 15 cm). The mobile phase was 0.55 mol/l boric acid, 2 mol/l glycerol, 0.3 mmol/l hexadecyltrimethyl ammonium bromide, and 40% methanol (vol/vol) (35). Under our experimental conditions, the retention times for UDP-Glc, UDP-Gal, UDP-GlcNAc, and UDP-GalNAc were 38.0, 43.9, 35.5, and 42.5 min, respectively.

High-fat diet study. The composition of the normal diet (CLEA Rodent Diet CE-2; CLEA, Japan) was 50.7% (wt/wt) carbohydrate, 4.6% fat, 25.2% protein, 4.4% dietary fiber, 6.5% crude ash, 3.6% mineral mixture, 1% vitamin mixture, and 4% moisture. The composition of the high-fat diet was 32% safflower oil, 33.1% casein, 17.6% sucrose, 5.6% cellulose, 9.8% mineral mixture, 1.4% vitamin mixture, 0.5% DL-methionine (32,36).

Determination of adiponectin content, serum adiponectin, and adiponectin secretion. To determine the adiponectin contents of fat pads, tissue fragments were immediately placed in extraction buffer A and sonicated, and after removing cell debris by repeated centrifugation at 15,000*g* at 4°C, the protein concentration in the buffer was determined using a protein assay kit (BioRad). The same amounts of proteins were subjected to SDS-PAGE, followed by immunoblotting with the polyclonal antibody to GBP28 (human adiponectin) (15), as described elsewhere (27,37). The serum adi-

ponectin level was determined by immunoblotting with the antibody to GBP28. To measure the amounts of adiponectin secreted by adipose tissue, 100 mg of tissue fragments was incubated in KRBH buffer containing 0.5% BSA with or without insulin stimulation (100 nmol/l) for up to 60 min. To assess the inhibition of insulin-induced adiponectin secretion by wortmannin, adipose tissue was incubated with 100 nmol/l wortmannin for 20 min before insulin stimulation and during insulin stimulation. The amounts of adiponectin in the medium were measured by Western blotting.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using the Statview software system (Abacus Concepts, Berkeley, CA). Statistical differences were analyzed by using Student's *t* test for unpaired comparisons. A P value <0.05 was considered statistically significant.

RESULTS

Increased insulin sensitivity and normal body weight of Pik3r1$^{-/-}$ mice on a normal diet. At 5 months of age on a normal diet, under fed conditions the blood glucose levels of Pik3r1$^{-/-}$ mice were lower than those in wild-type mice (115 ± 2 vs. 129 ± 2 mg/dl, $P < 0.01$). A GTT showed that blood glucose levels of the Pik3r1$^{-/-}$ mice were significantly lower than those of the wild-type mice, and the Pik3r1$^{-/-}$ mice had significantly lower fasting insulin levels than those of the wild-type mice (data not shown). These findings were consistent with the results of our previous study that male Pik3r1$^{-/-}$ mice showed increased insulin sensitivity (27). On the normal diet, the body weight (Fig. 1*A*), fat mass (Fig. 1*B*), and size of the adipocytes (Fig. 1*C*) of the Pik3r1$^{-/-}$ mice were similar to those of the wild-type mice.

Increased serum leptin levels of Pik3r1$^{-/-}$ mice on a normal diet. On a normal diet, the serum leptin levels of the Pik3r1$^{-/-}$ mice were significantly higher than those of the wild-type mice (5.0 ± 0.6 vs. 2.9 ± 0.4 ng/ml, $P < 0.01$) (Fig. 2*A*), despite having similar body weight and fat mass. To determine whether the higher serum leptin levels in the Pik3r1$^{-/-}$ mice were due to increased production, we investigated leptin expression in WAT. Northern blot analysis revealed indistinguishable levels of leptin expression in the Pik3r1$^{-/-}$ and wild-type mice on a normal diet (Fig. 2*B*), and similar results were obtained by RNase protection assay (data not shown). Because the leptin content of the WAT was somewhat decreased in the Pik3r1$^{-/-}$ mice compared with the wild-type mice (Fig. 2*C*), it is likely that leptin secretion was increased in the Pik3r1$^{-/-}$ mice. We next assessed leptin secretion by isolated adipose tissue over a 60-min period. Although insulin did not increase leptin secretion by wild-type adipose tissue, it caused a robust increase in leptin secretion by Pik3r1$^{-/-}$ adipose tissue (Fig. 2*D*). Wortmannin, a PI 3-kinase inhibitor, suppressed this effect in Pik3r1$^{-/-}$ adipose tissue, suggesting that the increased leptin secretion was caused by increased PtdIns(3,4,5)P3 production in the Pik3r1$^{-/-}$ adipocytes in response to insulin as compared with wild-type adipocytes, as we previously reported (27). When fractional leptin secretion was calculated by normalizing the amount of secreted leptin by the leptin content of WAT in the presence of insulin stimulation, Pik3r1$^{-/-}$ adipose tissue was found to secrete more leptin than wild-type tissue (Fig. 2*E*).

UDP-GlcNAc level in Pik3r1$^{-/-}$ adipose tissue was not increased. To determine the potential involvement of hexosamine pathway in the increased leptin secretion, we studied the hexosamine biosynthesis pathway (Fig. 3*A*). We noted a significantly (by 42%) lower level of GFAT expression in the WAT of the Pik3r1$^{-/-}$ mice on the normal diet than in wild-type mice (Fig. 3*B*). We next measured UDP-Glc and UDP-GlcNAc, the end product of the hexosamine biosynthesis pathway, in WAT. The UDP-GlcNAc level was increased by only 18% in Pik3r1$^{-/-}$ mice compared with wild-type mice, whereas the UDP-Glc level was increased by 56%, albeit not significantly (Fig. 3*C*). It seems likely that a combination of potentially increased

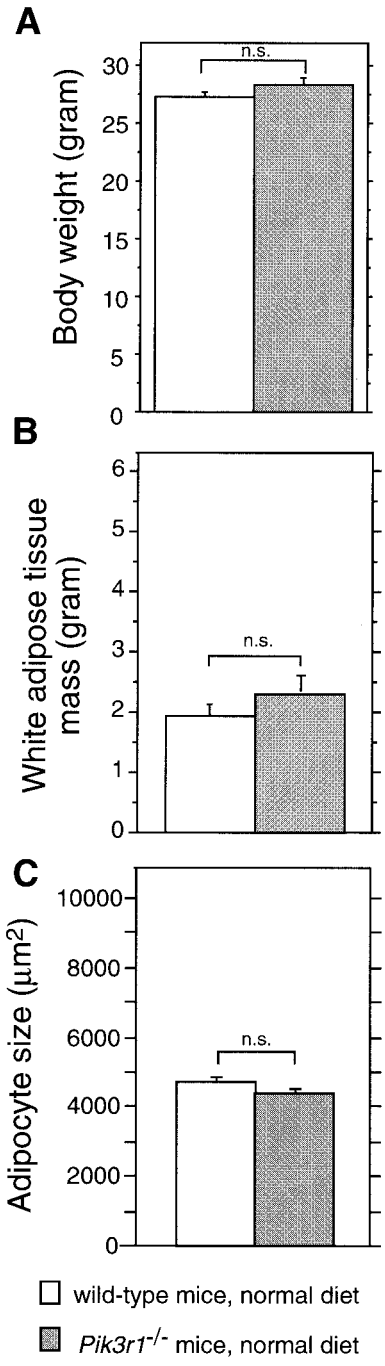

FIG. 1. Normal body weight and fat mass in Pik3r1$^{-/-}$ on a normal diet. *A*: Body weight of 5-month-old wild-type and Pik3r1$^{-/-}$ mice on a normal diet. Values are expressed as means ± SE (wild-type, $n = 45$; Pik3r1$^{-/-}$ mice, $n = 38$). *B*: Total weight of the WAT (perimetric, retroperitoneal, and perirenal fat pads) of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Values are expressed as means ± SE ($n = 8$). *C*: Adipocyte size of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Values are expressed as means ± SE ($n = 4$).

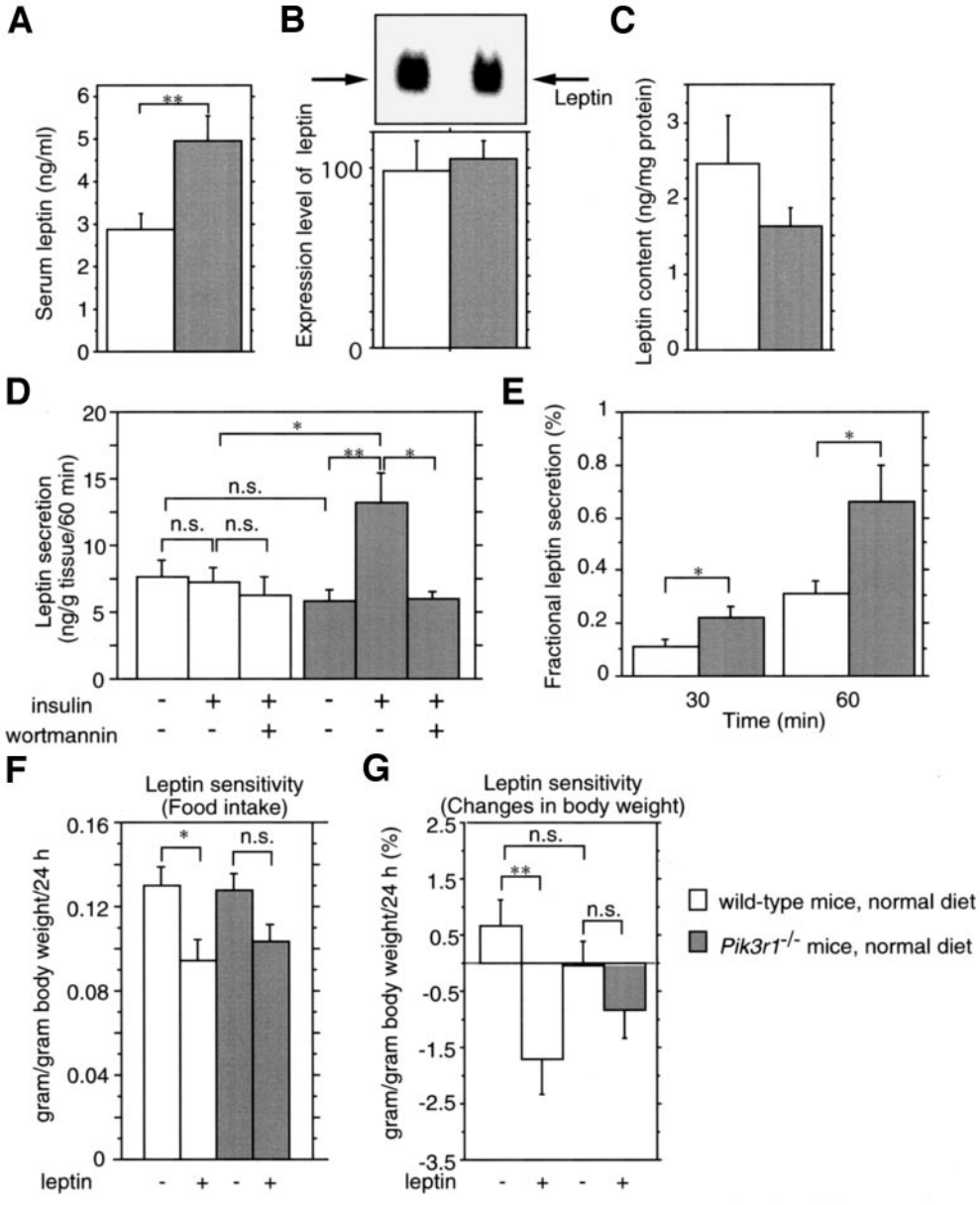

FIG. 2. Increased leptin secretion by Pik3r1$^{-/-}$ adipose tissues associated with increased PI 3-kinase activity on the normal diet. *A*: Serum leptin levels of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Values are expressed as means ± SE of data obtained from wild-type mice (n = 42) and Pik3r1$^{-/-}$ mice (n = 36). *B*: Leptin expression levels of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Northern blot analysis of total RNA from the WAT of each genotype. Representative images are shown in upper panel. Data have been normalized to 36B4 and calculated as fold intensity. Bars represent means ± SE (wild-type, n = 7; Pik3r1$^{-/-}$ mice, n = 8). *C*: Leptin content of perimetric WAT in wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Values are expressed as means ± SE (n = 5). *D*: Leptin secretion by the adipose tissue of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. The amount of leptin in the medium was measured after incubation for 60 min in the presence or absence of 100 nmol/l insulin or 100 nmol/l wortmannin. Experiments were carried out three times, and similar results were obtained. Therefore, the results were collected, and values are expressed as means ± SE (n = 9–12) (ng of leptin secreted by 1 g of adipose tissue). *E*: Fractional leptin secretion by adipose tissue from wild-type and Pik3r1$^{-/-}$ mice on the normal diet. The amount of leptin in the medium was measured in the presence of 100 nmol/l insulin at the times indicated. Experiments were carried out twice, and similar results were obtained. Therefore, the results were collected, and values are shown as fractional leptin secretion (ng of leptin secreted in the medium/ng of leptin content of adipose tissue; %) (means ± SE, n = 12). *F* and *G*: Effect of intraperitoneal leptin administration on the food intake and body weight of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. Mice received a daily intraperitoneal injection of either leptin (5 μg · g^{-1} · day^{-1}) (lep +) or isotonic sodium chloride solution (lep −). After administration for 5 days, changes in food intake (*F*) and body weight per day (gram) (*G*) were measured. Values are expressed as means ± SE of the values obtained in each group (n = 9).

glucose influx, as evidenced by the increased UDP-Glc levels, and downregulation of GFAT expression caused only a minimal increase in UDP-GlcNAc levels in WAT and that the increased leptin secretion by Pik3r1$^{-/-}$ WAT cannot be satisfactorily explained by the hexosamine flux.

Increased serum leptin compensated for the increased glucose uptake by adipocytes, thereby preventing adiposity in Pik3r1$^{-/-}$ mice on the normal diet. On the normal diet, Pik3r1$^{-/-}$ mice had a normal fat mass and body weight despite having higher serum leptin levels than the wild-type mice. These results can be interpreted as meaning that the Pik3r1$^{-/-}$ mice were leptin resistant on the normal diet, because it took more leptin to maintain a fat mass similar to that of the wild-type mice. To compare the sensitivity of the mouse groups on the normal diet to endogenous and exogenous leptin, we measured changes in the food intake and body weight of mice with or without leptin administration. On the normal diet the Pik3r1$^{-/-}$ and wild-type mice had similar food intake (Fig. 2*F*). Leptin (5 μg/g body wt per day by intraperitoneal injection), however, caused a 34% reduction ($P < 0.05$) in food intake in the wild-type mice and a 22% reduction (NS) in the *Pik3r1*$^{-/-}$ mice. Leptin caused a 2.41% decrease in the body weight ($P < 0.01$) of the wild-type mice, but only a 0.78% decrease (NS) in the Pik3r1$^{-/-}$ mice (Fig. 2*G*). Thus, Pik3r1$^{-/-}$ mice had mild leptin resistance compared with wild-type mice, but the difference in leptin sensitivity between the two groups was

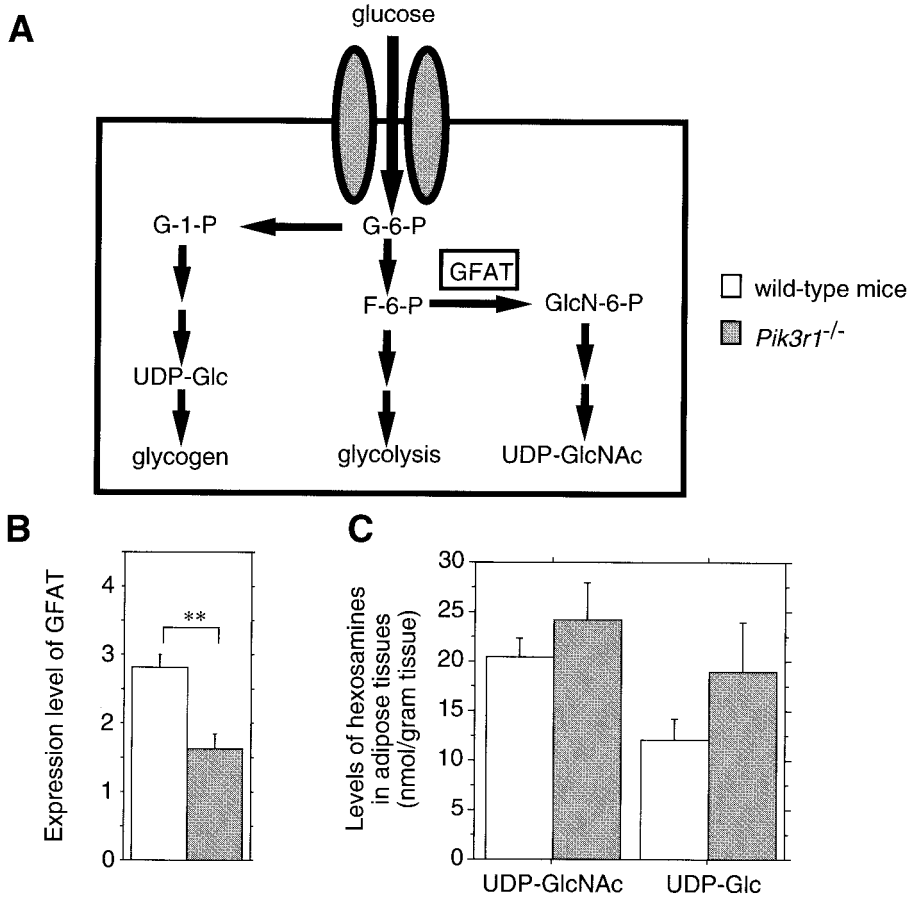

FIG. 3. Minimally increased UDP-GlcNAc in Pik3r1$^{-/-}$ adipose tissue. *A*: Schematic representation of the hexosamine biosynthesis pathway. After glucose entry into adipose cells via the glucose transport system and rapid phosphorylation to glucose-6-phosphate (G-6-P), it is primarily utilized by the glycogen synthesis and glycolysis pathways. The hexosamine pathway receives ~1–3% of incoming glucose via conversion of fructose-6-phosphate (F-6-P) to glucosamine-6-phosphate (GlcN-6-P) by the rate-limiting enzyme GFAT. The principal end product of this pathway, UDP-GlcNAc, is the substrate for virtually all glycosylation pathways. *B*: Level of expression of GFAT mRNA in perimetric WAT from wild-type and Pik3r1$^{-/-}$ mice on a normal diet. Data have been normalized to 36B4 and calculated as fold intensity. The bars represent means ± SE (n = 3). *C*: Levels of UDP-GlcNAc and UDP-Glc in the adipose tissue of mice on the normal diet. The body weight of wild-type and Pik3r1$^{-/-}$ mice was 25.3 ± 1.0 g (n = 8) and 27.1 ± 1.7 g (n = 6) (NS), respectively, and their serum leptin levels were 1.3 ± 0.7 and 4.4 ± 1.1 ng/ml, respectively (P < 0.05). The bars represent means ± SE. *P < 0.05 vs. wild-type mice.

quite small. It therefore seems likely that primary increase in serum leptin compensated for the increased glucose uptake by adipocytes in Pik3r1$^{-/-}$ mice, thereby maintaining normal body weight and food intake.

High-fat diet–induced obesity in Pik3r1$^{-/-}$ mice. Both mouse groups were fed a normal diet until 5 months of age and were then fed either a normal or high-fat diet. We performed GTTs in wild-type and Pik3r1$^{-/-}$ mice after 3 months on a normal or high-fat diet. The blood glucose levels during GTTs were higher in the wild-type mice on the high-fat diet than on the normal diet. This development of glucose intolerance was also observed in the Pik3r1$^{-/-}$ mice on the high-fat diet, but their glucose tolerance was still much better than that of wild-type mice on the high-fat diet (Fig. 4*A*), and the Pik3r1$^{-/-}$ mice had lower fasting insulin levels on the high-fat diet than the wild-type mice (Fig. 4*B*). On the high-fat diet, the insulin resistance index (calculated by multiplying the fasting blood glucose level by the fasting insulin level) of the Pik3r1$^{-/-}$ mice was decreased by 63% compared with the wild-type mice (Fig. 4*C*). These results may be due to the increased hepatic insulin sensitivity in Pik3r1$^{-/-}$ mice even on the high-fat diet compared with wild-type mice on the high-fat diet. Blood glucose levels 90 min after insulin injection in Pik3r1$^{-/-}$ mice were significantly lower than in the wild-type mice (data not shown), suggesting Pik3r1$^{-/-}$ mice to have better peripheral insulin sensitivity than wild-type mice. Thus, after 3 months on the high-fat diet, Pik3r1$^{-/-}$ mice still showed increased insulin sensitivity and better glucose tolerance than the wild-type mice.

Next we studied the effect of the high-fat diet on body weight, fat mass, and adipocyte size. After 3 months on the normal diet (at 8 months of age), the body weight of the *Pik3r1*$^{-/-}$ mice was similar to that of the wild-type mice (36.5 ± 1.7 vs. 35.0 ± 1.4 g, NS) (Fig. 4*D*). By contrast, after 3 months on the high-fat diet, Pik3r1$^{-/-}$ mice showed markedly heavier body weight (49.1 ± 2.8 vs. 39.4 ± 1.5 g, $P < 0.01$) (Fig. 4*C*) and WAT mass (4.7 ± 0.5 vs. 3.4 ± 0.2 g, $P < 0.05$) (Fig. 4*E*) than wild-type mice, even though Pik3r1$^{-/-}$ mice had body weight similar to that of wild-type mice on the normal diet. Although adipocyte size in both types of mice was significantly larger on the high-fat diet than on the normal diet, adipocyte size in the high-fat Pik3r1$^{-/-}$ group was significantly larger than in the high-fat wild-type group (Fig. 4*F*). The number of adipocytes in both types of mice was similar whether on the normal or the high-fat diet (data not shown). Thus, the Pik3r1$^{-/-}$ mice developed greater adiposity than the wild-type mice on the high-fat diet.

Leptin action in Pik3r1$^{-/-}$ mice on the normal diet was abrogated on the high-fat diet. On a normal diet, the serum leptin levels of the Pik3r1$^{-/-}$ mice were significantly higher than in the wild-type mice, despite similar body weight and fat mass. After 3 months on the high-fat diet, the leptin levels had risen to 45.0 ± 6.6 ng/ml in the Pik3r1$^{-/-}$ mice and 26.7 ± 4.0 ng/ml in the wild-type mice (P = 0.02) (Fig. 5*A*), suggesting that both genotypes became leptin resistant on the high-fat diet as compared with the normal-diet groups and that the Pik3r1$^{-/-}$ mice were more leptin resistant than wild-type mice on the

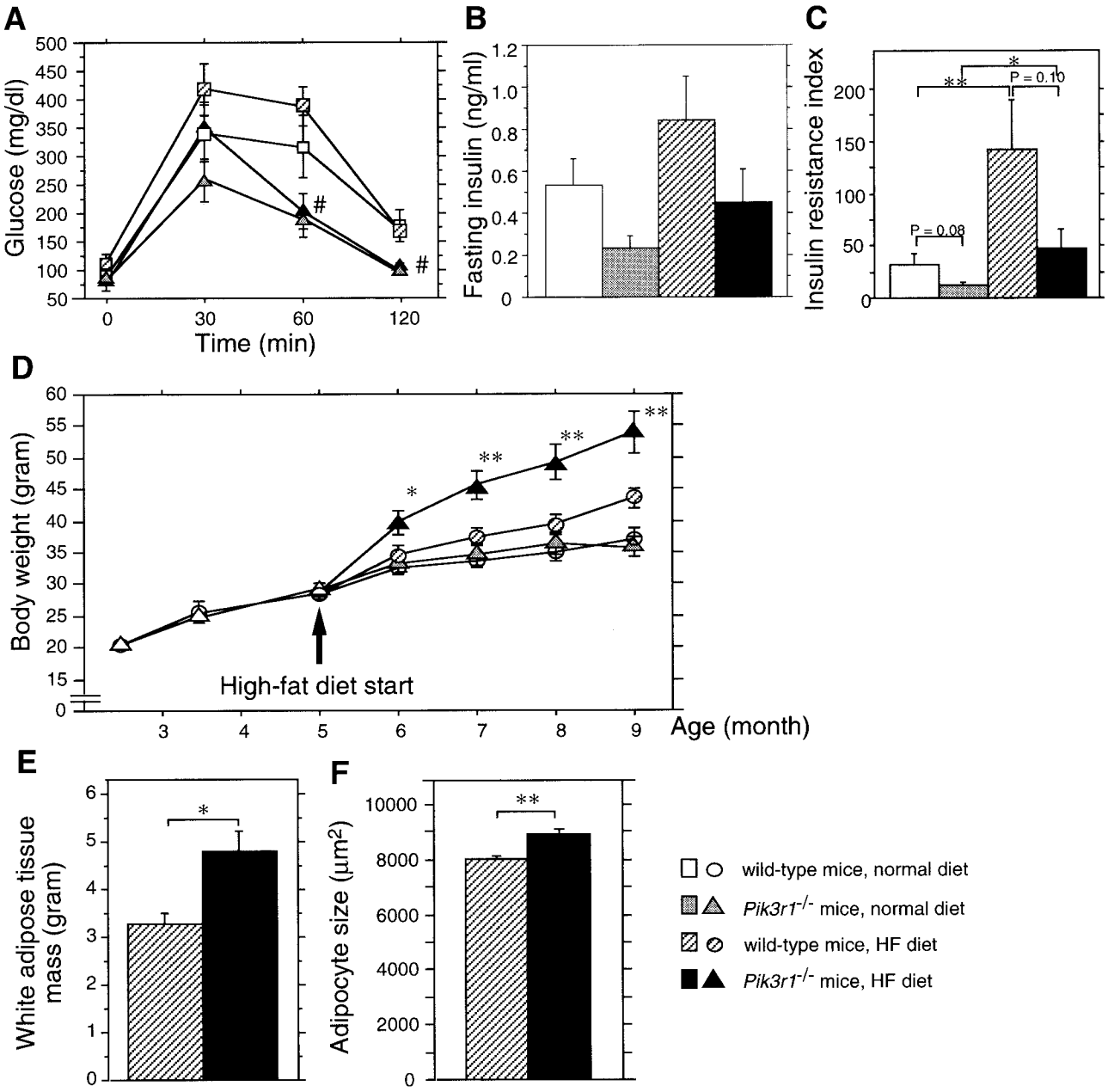

FIG. 4. High-fat (HF) diet–induced obesity in *Pik3r1*$^{-/-}$ mice. *A* and *B*: Glucose tolerance in wild-type and Pik3r1$^{-/-}$ mice after 3 months on the normal or high-fat diet. Plasma glucose levels (*A*) were measured at the times indicated. Fasting insulin levels (*B*) were also measured. Values are expressed as means ± SE of the data obtained from wild-type mice on the normal diet (squares, $n = 11$), wild-type mice on the high-fat diet (circles, $n = 9$), Pik3r1$^{-/-}$ mice on the normal diet (diamonds, $n = 10$), and Pik3r1$^{-/-}$ mice on the high-fat diet (triangles, $n = 5$). #$P < 0.05$ vs. wild-type mice on the high-fat diet. *C*: Insulin sensitivity index, calculated by multiplying the fasting glucose level by the fasting insulin level, in wild-type and Pik3r1$^{-/-}$ mice after 3 months on the normal or high-fat diet. *D*: Changes in body weight of wild-type and Pik3r1$^{-/-}$ mice. Both mouse groups were fed a normal diet until 5 months of age and then fed either a normal or high-fat diet. Values are expressed as means ± SE ($n = 10$–14). *E*: Total weight of the WAT (perimetric, retroperitoneal, and perirenal fat pads) of wild-type and Pik3r1$^{-/-}$ mice after 3 months on the high-fat diet. Values are expressed as means ± SE ($n = 8$). *F*: Adipocyte size of wild-type and Pik3r1$^{-/-}$ mice after 3 months on the high-fat diet. Values are expressed as means ± SE ($n = 1{,}200$). *$P < 0.05$; **$P < 0.01$.

high-fat diet. To compare leptin sensitivity and action in the high-fat diet groups, we measured changes in food intake and body weight in mice injected and not injected with leptin. After 12 weeks on the high-fat diet, Pik3r1$^{-/-}$ mice showed greater food intake than the wild-type mice (Fig. 5*B*) and a body weight gain similar to that of the wild-type mice (Fig. 5*C*). Although leptin administration (5 μg/g body wt per day) decreased food intake in both genotypes on the normal diet (Fig. 2*F*), it did not decrease food intake or body weight in either genotype on the high-fat diet, indicating that both genotypes had indeed become severely leptin resistant on the high-fat diet. Thus, the balanced leptin action seen in Pik3r1$^{-/-}$ mice on the normal diet appears to be abrogated by the severe leptin resistance on the high-fat diet.

Differences in body weight and fat mass may be important confounding factors in the high-fat studies. We therefore compared weight-matched groups to avoid these factors. To this end, we compared wild-type mice on the high-fat diet (33.8 ± 1.8 g, $n = 8$) with Pik3r1$^{-/-}$ mice on

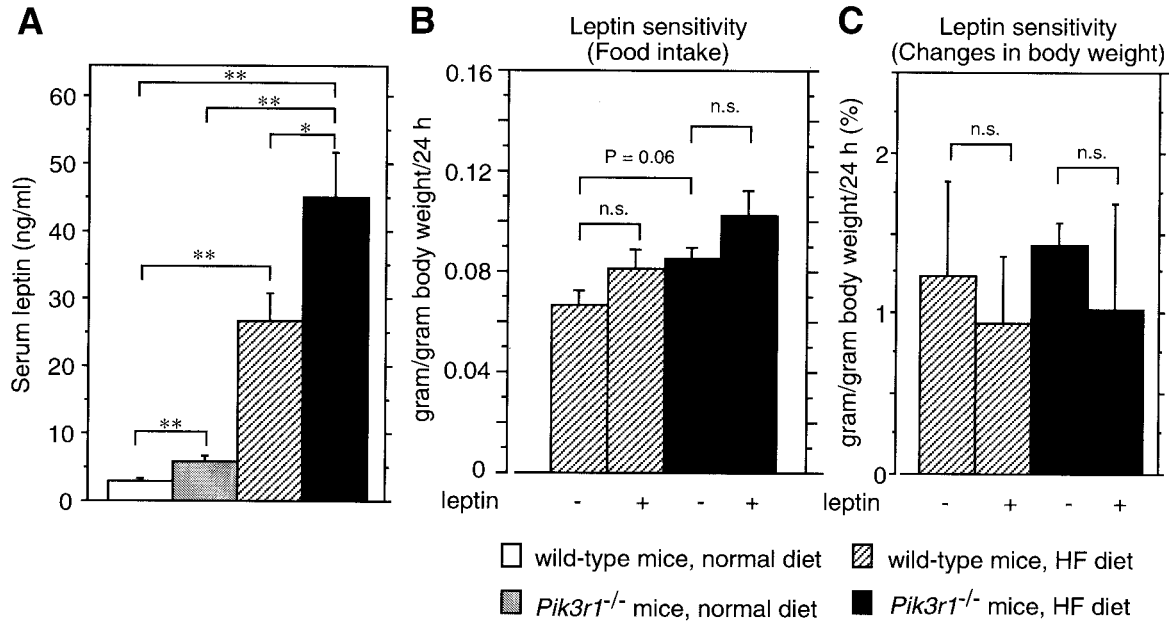

FIG. 5. Balanced leptin action in Pik3r1$^{-/-}$ mice on the normal diet abrogated on the high-fat (HF) diet by severe leptin resistance. *A*: Serum leptin levels of wild-type and Pik3r1$^{-/-}$ mice after 3 months on the normal and high-fat diet. Values are expressed as means ± SE of the data obtained from the wild-type mice on the normal diet (n = 42), Pik3r1$^{-/-}$ mice on the normal diet (n = 36), wild-type mice on the high-fat diet (n = 15), and Pik3r1$^{-/-}$ mice on the high-fat diet (n = 10). *B* and *C*: Effect of intraperitoneal leptin administration on the food intake and body weight of wild-type and Pik3r1$^{-/-}$ mice after 3 months on the high-fat diet. Mice received either a daily intraperitoneal injection of either leptin (5 μg · g^{-1} · day^{-1}) (lep +) or isotonic sodium chloride solution (lep −). After administration for 5 days, changes in food intake (*B*) and body weight (*C*) were measured. Values are expressed as means ± SE of the data obtained from the respective mouse groups (n = 4–7). **P < 0.01.

the high-fat diet (34.7 ± 1.4 g, n = 9) (P = 0.71). Serum leptin was 32.8 ± 4.0 vs. 49.2 ± 5.7 ng/ml (P < 0.05), respectively. Food intake was 0.066 ± 0.004 vs. 0.075 ± 0.006 g · g body wt^{-1} · 24 h^{-1} (P = 0.21), respectively, and change in body weight was 0.92 ± 0.45 vs. 0.83 ± 0.28 g · g body wt^{-1} · 24 h^{-1} (%) (P = 0.87), respectively. Thus, even weight-matched Pik3r1$^{-/-}$ mice on the high-fat diet had significantly higher serum leptin levels than wild-type mice on the high-fat diet but showed food intake and body weight gain similar to those of wild-type mice on the high-fat diet. These results suggest that increased serum leptin in Pik3r1$^{-/-}$ mice on the high-fat diet compared with wild-type mice on the high-fat diet is independent of body weight.

Increased serum adiponectin in Pik3r1$^{-/-}$ mice as compared with wild-type mice. On a normal diet, expression of adiponectin was significantly increased by 90% in Pik3r1$^{-/-}$ WAT compared with wild-type WAT (Fig. 6*A*), despite the two genotypes having similar body weight, WAT mass, and adipocyte size (Fig. 1*C–E*). The level of expression was decreased in both groups on the high-fat diet, consistent with previous reports (14,19). We next determined serum adiponectin levels by Western blotting. Serum adiponectin was detected as a 35-kDa protein (Fig. 6*B*, *upper panel*), and on the normal diet its level was approximately fourfold higher in the Pik3r1$^{-/-}$ mice than in the wild-type mice (Fig. 6*B*, *lower panel*). WAT adiponectin content on the normal diet was increased by 60% in Pik3r1$^{-/-}$ mice compared with wild-type mice (P = 0.06) (Fig. 6*C*). We then investigated adiponectin secretion by WAT in the presence and absence of insulin. Insulin-dependent adiponectin secretion after incubation for 60 min was significantly greater in Pik3r1$^{-/-}$ tissue than wild-type tissue, and wortmannin suppressed this effect (Fig. 6*D*). Taken together, these findings indicate that adiponectin secretion and adiponectin production are increased in Pik3r1$^{-/-}$ WAT as a result of increased PtdIns(3,4,5)P3 production in the Pik3r1$^{-/-}$ adipocytes, as compared with wild-type adipocytes, in response to insulin, as we previously reported (27). Thus, these findings raise the possibility that insulin-stimulated PI 3-kinase activation regulates serum adiponectin levels.

DISCUSSION

Selective overexpression of GLUT4 in the adipose tissue demonstrated that adiposity can be promoted by increased glucose uptake and lipogenesis by adipocytes (30). On a normal diet, Pik3r1$^{-/-}$ mice have normal body weight and adipose tissue mass despite the increased glucose uptake by adipose tissue. In the present study, we attempted to identify the mechanisms by which Pik3r1$^{-/-}$ mice maintained normal body weight on the normal diet. After 3 months on a high-fat diet, Pik3r1$^{-/-}$ mice showed markedly greater increases in body weight and WAT mass than wild-type mice. On the normal diet, serum leptin levels of Pik3r1$^{-/-}$ mice were significantly higher than in wild-type mice as a result of increased leptin secretion from adipocytes. Pik3r1$^{-/-}$ mice had mild leptin resistance compared with wild-type mice, but the difference in leptin sensitivity between the two groups was quite small. It therefore seems likely that primary increase in serum leptin compensated for the increased glucose uptake by adipocytes in Pik3r1$^{-/-}$ mice, thereby maintaining normal body weight and food intake. On the high-fat diet, the increased leptin secretion was insufficient to compensate for the leptin resistance caused by high-fat diet and thus failed to prevent obesity.

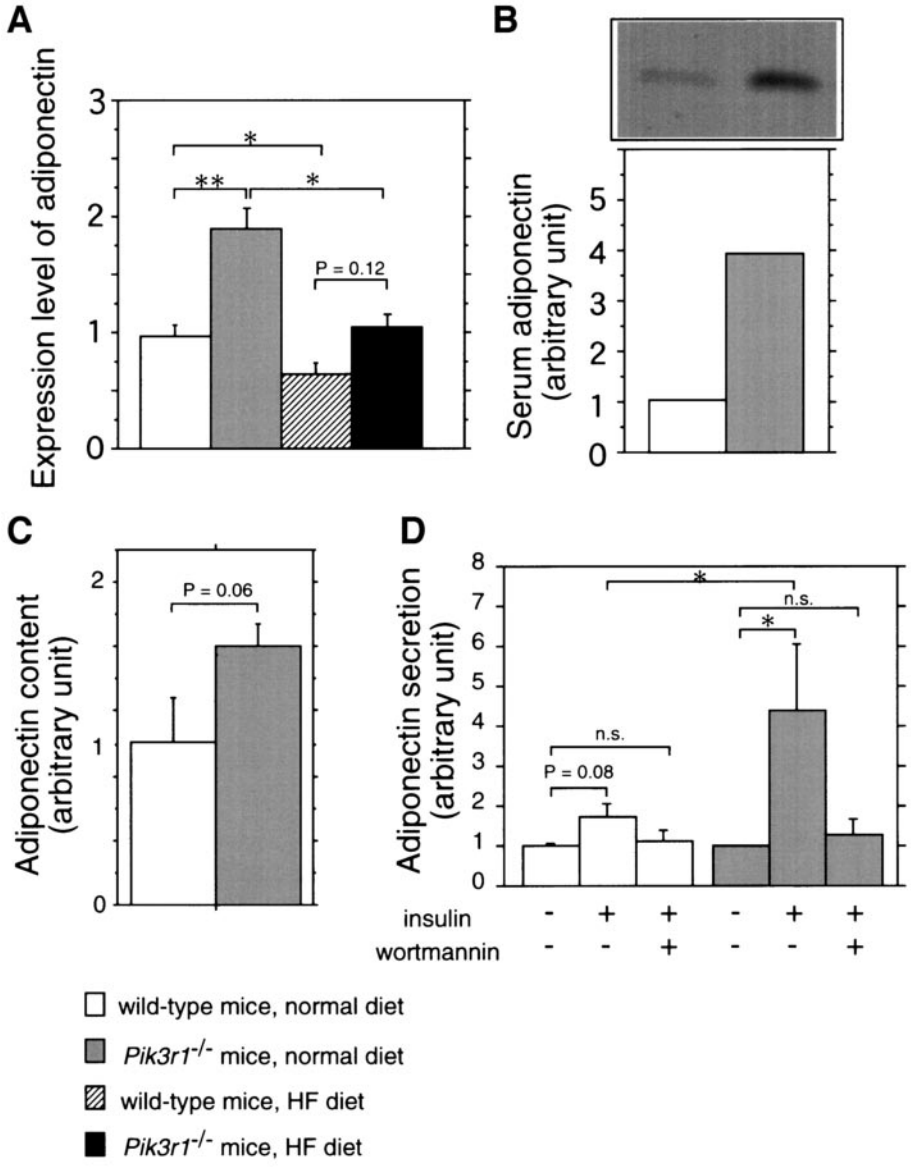

FIG. 6. Increased adiponectin production and secretion in Pik3r1$^{-/-}$ WAT. *A*: Adiponectin expression levels in the perimetric WAT of wild-type and Pik3r1$^{-/-}$ mice on the normal and high-fat (HF) diets. Data have been normalized to 36B4 and calculated as fold intensity. The bars represent means ± SE (n = 3). *B*: Serum adiponectin levels of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. The serum adiponectin level was determined by Western blotting. Serum samples from five animals were collected for each genotype. Adiponectin was detected as a 35-kDa protein with anti-adiponectin antibody (*upper panel*). *C*: Adiponectin content of wild-type and Pik3r1$^{-/-}$ mice on the normal diet. The same amounts of proteins were subjected to SDS-PAGE. Values are expressed as means ± SE (n = 10). *D*: Adiponectin secretion by adipose tissues from wild-type and Pik3r1$^{-/-}$ mice on the normal diet. The same amounts of adipose tissue fragments were preincubated in KRBH buffer containing 0.5% BSA for 20 min and then incubated for 60 min with or without insulin stimulation (100 nmol/l) or preincubated in KRBH buffer containing 0.5% BSA and 100 nmol/l wortmannin for 20 min and then incubated for 60 min with 100 nmol/l insulin. Values are expressed as fold stimulation compared with the respective groups without insulin stimulation. Bars represent means ± SE (n = 6–11). *P < 0.05; **P < 0.01.

On the normal diet, the serum leptin levels of the Pik3r1$^{-/-}$ mice were increased as compared with wild-type mice (Fig. 2*A*), despite having similar body weight and fat mass (Fig. 1*C* and *D*). Assuming normal leptin sensitivity and normal glucose uptake by adipocytes on the normal diet, Pik3r1$^{-/-}$ mice would be lean due to the increased leptin action, as reported in leptin transgenic mice (38). In marked contrast to their body weight being similar to that of wild-type mice on the normal diet, on the high-fat diet the Pik3r1$^{-/-}$ mice showed markedly greater increases in body weight and WAT mass than the wild-type mice (Fig. 5*C* and *D*), a phenotype similar to that of IRS-2 knockout (IRS-2$^{-/-}$) mice (39,40) and neuron-specific insulin receptor knockout mice (41). How does the leptin resistance in these mice develop? Both insulin and leptin receptors are present in hypothalamic regions that control energy homeostasis, and insulin, like leptin, has been reported to hyperpolarize hypothalamic glucose-responsive neurons in lean rats by opening ATP-dependent potassium (K_{ATP}) channels (42,43). Hypothalamic K_{ATP} channel function has been suggested to be crucial to physiological regulation of food intake and body weight, and involvement of PI 3-kinase in insulin signaling as well as leptin signaling pathways in hypothalamic neurons has been suggested (42,44).

Pik3r1$^{-/-}$ mice exhibited increased glucose transport in adipocytes due to increased insulin-dependent generation of PtdIns(3,4,5)P3 in vivo (27). Insulin-stimulated PI 3-kinase activity associated with IRSs was mediated via p85α in wild-type mice, whereas it was mediated via the p50α isoform in Pik3r1$^{-/-}$ mice, and this isoform switch was associated with an increase in insulin-induced generation of PtdIns(3,4,5)P3 in Pik3r1$^{-/-}$ adipocytes. On the other hand, Pik3r1$^{-/-}$ mice showed selective decrease in the number of mature B-cells due to markedly decreased PI 3-kinase activation (45). We observed abundant expression of the p50α in T-cells of both Pik3r1$^{-/-}$ mice and normal mice, whereas B-cells expressed only a low amount of this isoform. Thus, it seems likely that in vivo PI 3-kinase activity in respective tissue of Pik3r1$^{-/-}$ mice is dependent on the relative existence of p50α and p85α. Western blot

analysis revealed that, while expression of p85α was not altered among hypothalamus, WAT, and liver of wild-type mice, p50α expression was by far lower in the hypothalamus than in the other two tissues (data not shown). It is therefore possible that disruption of p85α PI 3-kinase results in decreased activation of PI 3-kinase in hypothalamic neurons, leading to leptin resistance in Pik3r1$^{-/-}$ mice. In the future, hypothalamus-specific Pik3r1 gene knockout should provide a genetic explanation for the role of PI 3-kinase in the hypothalamus.

After 3 months on the high-fat diet, both wild-type and Pik3r1$^{-/-}$ mice became severely leptin resistant. In marked contrast to having a similar body weight to wild-type mice on the normal diet, on the high-fat diet Pik3r1$^{-/-}$ mice showed greater increases in body weight and WAT mass than the wild-type mice (Fig. 4*D* and *E*). High-fat diet–induced leptin resistance abrogated the balanced leptin action in Pik3r1$^{-/-}$ mice, leading to the obese phenotype. Thus, against a background of excess glucose transport into adipocytes caused by increased production of PtdIns(3,4,5)P3, a concomitant increase in leptin plays a physiological role in the suppression of adiposity due to excess glucose influx into adipocytes, and this should explain the difference between Pik3r1$^{-/-}$ mice and adipose tissue–specific GLUT4 transgenic mice. While involvement of the insulin signaling pathway and PI 3-kinase has been suggested in the regulation of leptin production and secretion (4–6), under our experimental conditions, insulin increased leptin secretion by Pik3r1$^{-/-}$ WAT via PI 3-kinase–dependent pathway (Fig. 2*D*). Studies on insulin exposure of isolated human adipose cells have generally not shown acute stimulation of leptin secretion either (46). The difference may be explained by the balance between PI 3-kinase activation in response to insulin and other antagonizing effects (47).

Because many biological molecules regulate food intake and energy expenditure in vivo, molecules other than leptin may contribute to the phenotype in Pik3r1$^{-/-}$ mice on a normal diet, and we focused on adiponectin (19,20). We previously showed that adiponectin expression is negatively correlated with adipocyte size (19,48). Interestingly, however, on the normal diet adiponectin expression in WAT was increased in the Pik3r1$^{-/-}$ mice as compared with wild-type mice (Fig. 6*A*), despite having similar body weight, WAT mass, and adipocyte size (Fig. 1*C–E*). Even more unexpectedly, adiponectin expression in WAT on the high-fat diet was higher in Pik3r1$^{-/-}$ mice than in the wild-type mice (Fig. 6*A*), despite larger adipocyte size (Fig. 4*E*). Serum adiponectin was elevated in Pik3r1$^{-/-}$ mice compared with wild-type mice (Fig. 6*B*), and insulin-dependent adiponectin secretion by Pik3r1$^{-/-}$ WAT was significantly increased compared with secretion by wild-type WAT (Fig. 6*D*). Because adiponectin secretion has been demonstrated to be increased by insulin in a PI 3-kinase–dependent fashion (21), we interpreted these findings as indicating that the kinetics of adiponectin secretion are upregulated by the increased PtdIns(3,4,5)P3 production in Pik3r1$^{-/-}$ adipocytes. Thus, insulin-stimulated PI 3-kinase activation not only increases adiponectin expression but also stimulates its secretion by adipocytes, leading to an elevation of serum adiponectin. It is possible that the increased serum adiponectin in Pik3r1$^{-/-}$ mice contributes to increased insulin sensitivity in peripheral tissues during both the normal and the high-fat diet in addition to a primary increase in PtdIns(3,4,5)P3 in adipocytes and presumably skeletal muscles in Pik3r1$^{-/-}$ mice. To study the role of adiponectin in Pik3r1$^{-/-}$ mice, we are now generating mice lacking adiponectin in addition to p85α.

In summary, serum leptin was increased in Pik3r1$^{-/-}$ mice due to increased secretion by WAT. Serum adiponectin was also increased in Pik3r1$^{-/-}$ mice as a result of increased production and secretion. The slightly increased serum leptin compensated for the increased glucose uptake by adipocytes in Pik3r1$^{-/-}$ mice, thereby preventing adiposity on the normal diet. The results of this study provide important biochemical links among the increased availability of nutrients, production of PIs in vivo, and serum leptin and adiponectin levels, all of which contribute to the regulation of fat storage and insulin sensitivity.

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