The farnesoid X receptor (FXR) is a bile acid (BA)-activated nuclear receptor that plays a major role in the regulation of BA and lipid metabolism. Recently, several studies have suggested a potential role of FXR in the control of hepatic carbohydrate metabolism, but its contribution to the maintenance of peripheral glucose homeostasis remains to be established. FXR-deficient mice display decreased adipose tissue mass, lower serum leptin concentrations, and elevated plasma free fatty acid levels. Glucose and insulin tolerance tests revealed that FXR deficiency is associated with impaired glucose tolerance and insulin resistance. Moreover, whole-body glucose disposal during a hyperinsulinemic-euglycemic clamp is decreased in FXR-deficient mice. In parallel, FXR deficiency alters distal insulin signaling, as reflected by decreased insulin-dependent Akt phosphorylation in both white adipose tissue and skeletal muscle. Whereas FXR is not expressed in skeletal muscle, it was detected at a low level in white adipose tissue in vivo and induced during adipocyte differentiation in vitro. Moreover, mouse embryonic fibroblasts derived from FXR-deficient mice displayed impaired adipocyte differentiation, identifying a direct role for FXR in adipocyte function. Treatment of differentiated 3T3-L1 adipocytes with the FXR-specific synthetic agonist GW4064 enhanced insulin signaling and insulin-stimulated glucose uptake. Finally, treatment with GW4064 improved insulin resistance in genetically obese ob/ob mice in vivo. Although the underlying molecular mechanisms remain to be unraveled, these results clearly identify a novel role of FXR in the regulation of peripheral insulin sensitivity and adipocyte function. This unexpected function of FXR opens new perspectives for the treatment of type 2 diabetes.

The farnesoid X receptor (FXR) (NR1H4) is a nuclear receptor that is activated by bile acids (BAs) (1). A major physiological role of FXR is to protect liver cells from the deleterious effect of BA overload by decreasing their endogenous production and by accelerating BA biotransformation and excretion (1). In addition, the generation and characterization of FXR-deficient (FXR−/−) mice has also established a critical role of FXR in lipid metabolism, since these mice display elevated serum levels of triglycerides and high density lipoprotein cholesterol (2). Recently, several studies have suggested that FXR might also regulate hepatic carbohydrate metabolism (3). The first indication came from the observation that hepatic FXR expression is reduced in several rodent models of diabetes (4). FXR expression also varies in mouse liver during nutritional changes, being increased during fasting and decreased upon refeeding (5, 6). Moreover, FXR activation by BAs or the synthetic non-steroidal specific agonist GW4064 (7) modulates the expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (3). However, conflicting data report either a positive (8) or a negative effect (9, 10) of BA and/or GW4064 on phosphoenolpyruvate carboxykinase gene expression. A recent study, using FXR−/− mice, highlighted also a role of FXR in regulating the kinetics of hepatic carbohydrate metabolism during the fasting–refeeding transition phase. Indeed, FXR appears to modulate glycolytic and lipogenic pathways by interfering directly with the transcription of glucose-regulated genes, such as liver pyruvate kinase (6). Furthermore, FXR also controls the adaptive response to fasting, since FXR−/− mice exhibit transient hypoglycemia upon fasting (11).

In the present study, we investigated the role of FXR in whole-body glucose homeostasis and insulin sensitivity by performing glucose tolerance tests and hyperinsulinemic-euglycemic clamp studies in FXR−/− mice. Interestingly, a decreased peripheral insulin sensitivity was observed in these mice. In vitro studies indicated that FXR directly modulates adipocyte function. Furthermore, we found that treatment with the specific FXR agonist GW4064 improved insulin sensitivity in ob/ob mice. These findings provide new evidence to support an important role of FXR in the maintenance of normal glucose homeostasis.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma France, GW4064 from Genfit SA (Loos, France). Rabbit polyclonal anti-IRβ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse monoclonal anti-phosphotyrosine (4G10), rabbit polyclonal anti-phosphatidylinositol 3-kinase (Ser473), anti-Akt, anti-phospho-PDK1 (Ser241), and anti-PDK1 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-phospho-Akt (Ser273), anti-Akt, anti-phospho-PDK1 (Ser241), and anti-PDK1 antibodies were from Cell Signaling. Different kits were used to determine plasma concentrations of various metabolites: insulin (Mercodia AB), leptin and adiponectin (R&D systems), triglycerides, total, and HDL.
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cholesterol (Roche Applied Science), and FFAs (WAKO). Fat content in feces was measured as previously described (12).

Animals—Homozygous FXR-/- male mice of 18–20 weeks of age and sex- and age-matched wild type mice (FXR+/+) bred on the C57BL/6N genetic background (2) were housed with a 12-h light/12-h dark cycle with free access to water and a standard laboratory chow diet (UAR A03, Villemoison/Orge, France). Ob/Ob mice of 20 weeks of age were treated with the FXR agonist GW4064 (30 mg/kg of body weight/day) or with its vehicle (corn oil) for 10 days via intraperitoneal injection.

Glucose and Insulin Tolerance Tests—Mice were fasted for 6 h with free access to water. For the glucose tolerance test (GTT), glucose (1 g/kg) was administered intraperitoneally, and blood glucose was measured with the Accu-Check active® (Roche Applied Science) at 15, 30, 60, 90, and 120 min. For ITT, recombinant human insulin (Actrapid®, Novo Nordisk) was administered intraperitoneally (FXR+/+ and FXR-/- mice, 0.75 units/kg; ob/ob mice, 2 units/kg), and blood glucose was measured at 0, 30, 60, 90, and 120 min after insulin injection.

Hyperinsulinemic-Euglycemic Clamp Study—These experiments were performed in chronically catheterized, freely moving animals, exactly as described before (13).

In Vivo Insulin Stimulation—After an overnight fast, insulin (0.1 IU/kg) or saline was injected into the inferior venae cavae of anesthetized mice. After 5 min, epididymal fat pads and quadriceps muscle were dissected, frozen in liquid nitrogen, and stored at −80 °C until further analysis.

Real Time Quantitative Reverse Transcription-PCR—Total RNA was isolated from white adipose tissue (WAT) and muscle using the acid guanidinium thiocyanate/phenol/chloroform method and isolated from 3T3-L1 cells and MEFs using the Trizol reagent (Invitrogen), as previously described (4). The complete table of primers can be found in the supplemental data.

Western Blot Analysis—Tissue or cell proteins were extracted in lysis buffer, as described previously (6). For coimmunoprecipitation experiments, proteins were incubated overnight at 4 °C with the indicated antibodies in the presence of protein A-agarose. The immunoprecipitates were washed in lysis buffer, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Immunoreactive bands were revealed using an ECL detection kit (Amersham Biosciences).

Adipocyte Size Determination—The samples from epididymal adipose tissue were fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin, cut into 7-μm sections, and stained with hematoxylin. Cell size was determined using the computer-assisted analysis system (Leica Mikroskopic und System GmbH, Wetzlar, Germany).

Cell Culture—3T3-L1 cells were cultured in growth medium containing Dulbecco’s modified Eagle’s medium and 10% calf serum. The cells were differentiated by the method of Bernlohr et al. (14). The fully differentiated adipocytes were cultured in postdifferentiation medium (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 5 μM insulin) with or without GW4064 (5 μM) before experiments. Mouse embryonic fibroblasts (MEFs) were derived from 13.5-day wild-type and FXR-/- embryos. Adipocyte differentiation was initiated after 2 days at confluence. MEFs were differentiated with AmnioMAX-C100 medium (Invitrogen), 7.5% AmnioMAX-C100 supplement, 7.5% fetal calf serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 5 μM insulin. From day 3 to 8, cells were incubated with the same AmnioMAX-C100 medium with 5 μM insulin. At days 0, 4, and 8, cells were lyed and homogenized for RNA isolation or fixed in 4% paraformaldehyde and stained with Oil Red O. All experiments were performed in triplicate.

**TABLE 1**

Morphometric and metabolic parameters in FXR+/+ and FXR−/− mice

| Parameter                  | FXR+/+         | FXR−/−         |
|----------------------------|----------------|----------------|
| Body weight (g)            | 29.6 ± 0.6     | 28.5 ± 0.5     |
| Epididymal fat mass (g)    | 0.71 ± 0.06    | 0.24 ± 0.03*   |
| Liver mass (g)             | 1.35 ± 0.03    | 1.95 ± 0.14*   |
| Heart mass (g)             | 0.14 ± 0.01    | 0.16 ± 0.01    |
| Kidney mass (g)            | 0.20 ± 0.03    | 0.21 ± 0.01    |
| Feces weight (mg/day)      | 835 ± 27       | 910 ± 37       |
| Total fat (μmol/mg feces)  | 89.5 ± 3.8     | 81.0 ± 4.3     |
| Food consumption (g/day)   | 5.9 ± 0.1      | 6.0 ± 0.2      |
| Glucose (mg/dl)            | 184 ± 7        | 145 ± 8*       |
| Insulin (μg/ml)            | 1349 ± 235     | 593 ± 137ⁿ     |
| Leptin (mg/ml)             | 3.86 ± 0.91    | 0.70 ± 0.11ⁿ   |
| Adiponectin (μg/ml)        | 6.97 ± 0.27    | 7.46 ± 0.98    |
| Triglycerides (mg/dl)      | 71 ± 5         | 161 ± 27*      |
| Total cholesterol (mg/dl)  | 83 ± 3         | 162 ± 6*       |
| HDL cholesterol (mg/dl)    | 62 ± 26        | 89 ± 7⁸        |
| FFA (mmol/liter)           | 0.51 ± 0.03    | 0.71 ± 0.04⁸   |

* p < 0.001; significantly different from FXR−/− mice.  
ⁿ p < 0.01; significantly different from FXR+/+ mice.

Glucose Uptake—Fully differentiated 3T3-L1 adipocytes were starved overnight and thereafter incubated in PBS with or without recombinant insulin for 30 min. During the last 6 min, 0.5 μCi/ml 2-deoxy-D-[2,6-3H]glucose (Amersham Biosciences) and 0.1 mM 2-deoxy-D-glucose were added. The reaction was ended by adding ice-cold phosphate-buffered saline containing 20 μg/ml cytochalasin B. Cells were lysed with ice-cold 1 M NaOH, the radioactivity was measured using a scintillation counter, and the data were expressed in dpm/min/mg of protein. Results were corrected for the GLUT-nonspecific uptake by removing uptake values obtained in the presence of 10 μg/ml cytochalasin B.

Statistical Analysis—Statistical significance was analyzed using the unpaired Student’s t test or analysis of variance for clamp experiments. All values are reported as means ± S.E. Values of p < 0.05 were considered significant.

RESULTS

FXR-deficient Mice Exhibit Reduced Adipose Tissue Mass, Hypoleptinemia, and Increased Circulating FFA Levels—Body and organ weights as well as several plasma parameters were determined in chow-fed male wild-type (FXR+/+) and FXR-deficient (FXR−/−) mice under basal conditions (Table 1). Whereas total body weights were similar, organ weights varied between the two genotypes. FXR−/− mice (Fig. 1). Total weight and fat content of feces, as well as food intake, were similar in FXR+/+ and FXR−/− mice (Table 1). Thus, FXR deficiency is not associated with impaired fat absorption under chow-fed conditions.

Basal plasma glucose concentrations were significantly lower in FXR−/− mice, associated with an impaired hepatic glucose production during short term fasting (11). Plasma insulin levels were decreased in FXR−/− mice, probably reflecting an adaptive response to the relative hypoglycemia. Whereas adiponectin concentrations did not differ between both genotypes, plasma leptin concentrations were drastically decreased in FXR−/− mice, presumably reflecting the decrease in adipose tissue mass. As expected (2), cholesterol and triglyceride levels...
were increased in FXR<sup>−/−</sup> mice. Moreover, FFA plasma levels were also increased in FXR<sup>−/−</sup> mice. A similar phenotype was observed in female mice as well as in another FXR-deficient mouse model on a different genetic background (12) (data not shown).

**FXR Deficiency Leads to Peripheral Insulin Resistance**—The ability of FXR<sup>−/−</sup> mice to respond to a glucose challenge was investigated. After a single intraperitoneal bolus injection of glucose, FXR<sup>−/−</sup> mice displayed a more pronounced plasma glucose excursion than the FXR<sup>+/+</sup> mice, with a significantly larger area under the curve (+263%, p < 0.001) (Fig. 2A). The decreased clearance of plasma glucose could result from an impaired insulin secretion, a peripheral insulin resistance or an increased hepatic glucose production. In view of our earlier studies (6, 11), the latter possibility can be excluded. To address the remaining options, intraperitoneal insulin tolerance tests (ITTs) were performed. The insulin-mediated decrease of plasma glucose was less pronounced in FXR<sup>−/−</sup> than in FXR<sup>+/+</sup> mice, as indicated by the lower integrated area under the curve (−65%, p < 0.01) (Fig. 2B), suggesting that FXR deficiency alters insulin sensitivity. To directly evaluate the effect of FXR deficiency on whole-body insulin sensitivity, a 6-h hyperinsuline-mic-euglycemic clamp was performed in awake, 9-h-fasted, male FXR<sup>+/+</sup> and FXR<sup>−/−</sup> mice. The glucose infusion rate required to maintain euglycemia was ~22% lower in FXR<sup>−/−</sup> than in wild-type mice, indicative of the existence of whole-body insulin resistance. Endogenous blood glucose production rate, which mainly reflects hepatic glucose production, was suppressed to similar values by insulin infusion in both genotypes, indicating the absence of hepatic insulin resistance in FXR<sup>−/−</sup> mice. In contrast, the insulin-stimulated glucose disposal rate and metabolic clearance rate of glucose were reduced by ~20% in FXR<sup>−/−</sup> mice (Fig. 2C). These results show that FXR deficiency results in peripheral insulin resistance.

**FXR Modulates Insulin Signaling at the Level of Akt in Peripheral Insulin-sensitive Tissues**—To investigate whether alteration in insulin receptor (IR)-mediated signaling occurs in peripheral insulin-sensitive tissues, in vivo insulin stimulation was performed in FXR<sup>+/+</sup> and FXR<sup>−/−</sup> mice. Both insulin-stimulated tyrosine phosphorylation of IR and insulin substrate receptor 1 (IRS-1), as well as the recruitment of the p85α regulatory subunit of phosphoinositide 3-kinase to IRS-1 were similar between both genotypes in skeletal muscle and WAT (Fig. 3A).
FIGURE 2. FXR$^{-/-}$ mice exhibit peripheral insulin resistance. A and B, GTT and ITT. Glucose (1.0 g/kg) and insulin (0.75 IU/kg), respectively, were administered by intraperitoneal injection to 6-h-fasted mice. The inset represents the integrated area under the curve. Data are presented as means ± S.E. of FXR$^{+/+}$ (●●●) and FXR$^{-/-}$ (○○○) mice (n = 16 mice/group for GTT and n = 7 mice/group for ITT). *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with FXR$^{+/+}$ mice. C, hyperinsulinemic-euglycemic clamp analysis was performed on FXR$^{+/+}$ (filled bars) and FXR$^{-/-}$ mice (open bars). Steady-state insulin-stimulated glucose infusion rate (GIR), glucose disposal rate (Rd), glucose production rate (Ra), and metabolic clearance rate of glucose (MCR) are represented. Data are expressed as the average ± S.E. (n = 5–7 mice/group). *, p < 0.05 compared with FXR$^{+/+}$ mice.
However, FXR deficiency altered the level of insulin-stimulated phosphorylation of the serine/threonine kinase Akt/protein kinase B on serine 473 in WAT and to a lesser extent in skeletal muscle (Fig. 3B). The protein expression level of phosphoinositide-dependent protein kinase 1 (PDK1), which plays a central role in activating Akt (15), was not altered by FXR deficiency. Furthermore, the level of serine 241 phosphorylation of PDK1 that is essential for its catalytic activity did not differ between the two genotypes (16) (Fig. 3C). As previously described (16, 17), insulin stimulation did not increase Ser241-PDK1 phosphorylation. FXR deficiency did not alter the expression of several upstream negative regulators of insulin signaling, such as PTEN (18), protein-tyrosine phosphatase 1B (19), and SOCS-3 (20) (Table 2). Taken together, these results suggest that FXR may act directly at the level of Akt. Since Akt plays a key role in the regulation of the metabolic effects of insulin (21), defective Akt activation may contribute to the whole-body insulin resistance observed in FXR−/− mice.

**FXR Is Expressed in White Adipose Tissue and Differentiating Adipocytes**—Previous studies have suggested that FXR might be expressed in WAT (22, 23). In accordance with this hypothesis, both FXR mRNA and protein levels increased during 3T3-L1 adipocyte differentiation (Fig. 4A). Furthermore, FXR mRNA levels were also induced during adipogenesis of MEFs, with a peak 4 days after the induction of differentiation (Fig. 4B). FXR is expressed at low levels in WAT (50-fold lower than in liver), whereas FXR mRNA levels were undetectable in skeletal muscle in mice (Fig. 4C). Nevertheless, FXR gene expression was reduced in WAT of C57Bl/6J mice upon high fat feeding (Fig. 4D) as well as in WAT of ob/ob mice (Fig. 4E), strengthening the hypothesis of a functional role of FXR in this insulin-sensitive tissue.

**FXR Activation Improves Insulin Signaling in 3T3-L1 Adipocytes**—The effect of FXR activation with the synthetic agonist GW4064 on insulin signaling was then measured in differentiated 3T3-L1 adipocytes. As observed in WAT of FXR−/− mice, alteration of FXR activity did not alter proximal insulin signaling (Fig. 5A). However, GW4064 treatment increased the level of serine 473 Akt/protein kinase B phosphorylation and enhanced glucose uptake in 3T3-L1 adipocytes upon insulin stimulation (Fig. 5, B and C). Taken together, these results suggest that FXR directly modulates insulin signaling in the adipocyte.

**FXR Deficiency Directly Alters Adipocyte Differentiation in Vitro**—To further assess a direct role for FXR in the adipocyte, the adipogenic process in MEFs derived from FXR−/− mice was investigated in vitro. Interestingly, FXR deficiency altered the kinetics of the adipogenic pro-
TABLE 2
Expression of genes involved in glucose and lipid metabolism in skeletal muscle and white adipose tissue of FXR\textasciitilde and FXR\textasciitilde mice

| Gene                           | FXR\textasciitilde | FXR\textasciitilde | Statistical significance |
|--------------------------------|-------------------|-------------------|-------------------------|
|                                | %                 | %                 |                         |
| Skeletal muscle                |                   |                   |                         |
| Insulin signaling              |                   |                   |                         |
| PTP-1B                         | 100 ± 14          | 100 ± 17          | NS                      |
| PTEN                           | 100 ± 18          | 73 ± 18           | NS                      |
| Glucose metabolism             |                   |                   |                         |
| GLUT4                          | 100 ± 9           | 116 ± 10          | NS                      |
| GLUT1                          | 100 ± 8           | 122 ± 7           | NS                      |
| PDK4                           | 100 ± 12          | 137 ± 11          | \( p = 0.05 \)          |
| Fatty acid uptake              |                   |                   |                         |
| FATP1                          | 100 ± 16          | 223 ± 43          | \( p < 0.05 \)          |
| CD36                           | 100 ± 3           | 122 ± 10          | NS                      |
| LPL                            | 100 ± 4           | 228 ± 36          | \( p < 0.05 \)          |
| Fatty acid oxidation           |                   |                   |                         |
| CPT1b                          | 100 ± 4           | 140 ± 10          | \( p < 0.01 \)          |
| UCP-2                          | 100 ± 16          | 171 ± 15          | \( p < 0.05 \)          |
| UCP-3                          | 100 ± 4           | 159 ± 6           | \( p < 0.001 \)         |
| Fatty acid synthesis           |                   |                   |                         |
| DGAT-1                         | 100 ± 4           | 210 ± 25          | \( p < 0.05 \)          |
| SCD-1                          | 100 ± 18          | 164 ± 48          | NS                      |
| FAS                            | 100 ± 29          | 146 ± 66          | NS                      |
| Peroxisomal \( \beta \)-oxidation|                 |                   |                         |
| ACO                            | 100 ± 7           | 133 ± 10          | \( p < 0.05 \)          |
| Transcription factors          |                   |                   |                         |
| PPAR\( \alpha \)               | 100 ± 8           | 181 ± 27          | \( p < 0.05 \)          |
| PPAR\( \delta \)               | 100 ± 7           | 207 ± 13          | \( p < 0.05 \)          |
| PGC1\( \alpha \)               | 100 ± 7           | 152 ± 28          | NS                      |
| SREBP-1C                       | 100 ± 7           | 143 ± 17          | \( p < 0.05 \)          |
| Epididymal adipose tissue      |                   |                   |                         |
| Insulin signaling              |                   |                   |                         |
| PTP-1B                         | 100 ± 10          | 102 ± 11          | NS                      |
| SOCS-3                         | 100 ± 26          | 145 ± 30          | NS                      |
| PTEN                           | 100 ± 6           | 112 ± 12          | NS                      |
| Glucose metabolism             |                   |                   |                         |
| GLUT4                          | 100 ± 13          | 89 ± 13           | NS                      |
| GLUT1                          | 100 ± 8           | 216 ± 56          | \( p < 0.05 \)          |
| Fatty acid uptake              |                   |                   |                         |
| FATP1                          | 100 ± 9           | 289 ± 87          | \( p = 0.07 \)          |
| CD36                           | 100 ± 8           | 109 ± 18          | NS                      |
| LPL                            | 100 ± 7           | 192 ± 32          | \( p < 0.05 \)          |
| Fatty acid metabolism          |                   |                   |                         |
| DGAT-1                         | 100 ± 6           | 132 ± 17          | NS                      |
| FAS                            | 100 ± 7           | 67 ± 17           | NS                      |
| PEPCK                          | 100 ± 19          | 86 ± 17           | NS                      |
| HSL                            | 100 ± 10          | 46 ± 8            | \( p < 0.001 \)         |
| Adipocyte differentiation      |                   |                   |                         |
| aP2                            | 100 ± 11          | 102 ± 37          | NS                      |
| PPAR\( \gamma \)               | 100 ± 9           | 78 ± 8            | NS                      |
| c-EBP\( \alpha \)              | 100 ± 6           | 113 ± 13          | NS                      |
| c-EBP\( \beta \)               | 100 ± 14          | 260 ± 37          | \( p < 0.01 \)          |
| SREBP-1C                       | 100 ± 5           | 153 ± 18          | \( p < 0.05 \)          |
| Adipocytokines                 |                   |                   |                         |
| Leptin                         | 100 ± 18          | 18 ± 3            | \( p = 0.01 \)          |
| Adiponectin                    | 100 ± 11          | 130 ± 23          | NS                      |
| Resistin                       | 100 ± 7           | 103 ± 19          | NS                      |
| TNF\( \alpha \)                | 100 ± 11          | 49 ± 11           | \( p < 0.05 \)          |
| IL-6                           | 100 ± 25          | 90 ± 14           | NS                      |

FIGURE 4. FXR is induced during adipocyte differentiation. A and B, total RNAs were extracted before (day 0) and during (days 4 and 8) 3T3-L1 (A, top) and MEF (B) cell differentiation. FXR mRNA levels were measured by quantitative PCR analysis. Values are normalized relative to cyclophilin and are expressed (means ± S.E.) relative to those at day 0, which are arbitrarily set at 1.* , *p < 0.05; **, *p < 0.01 (n = 6). Protein extracts from 3T3-L1 cells after 0, 4, and 8 days of differentiation were subjected to Western blot analysis using FXR antibodies (A, bottom). In vitro translated-translated protein (IVT) was used as positive control. C, relative expression of FXR mRNA in different tissues of C57Bl/6J mice (n = 9). Values are normalized relative to 36B4 and are expressed (means ± S.E.) relative to those in liver, which are arbitrarily set at 100; D and E, FXR mRNA expression in epididymal WAT of C57Bl/6j mice upon high fat feeding (D) and of obese (ob/ob) or obese (ob/ob) mice (E). Values are normalized relative to those in epididymal WAT of lean and obese (ob/ob) mice (E), which are arbitrarily set to 1.* , *p < 0.05; **, *p < 0.01 compared with chow diet (n = 5–8 mice/group).

was also lower in MEFs from FXR\textasciitilde mice. Moreover, the expression of both leptin and the glucose transporter GLUT4 was significantly decreased at day 8 in MEFs from FXR\textasciitilde mice, reflecting an impaired adipogenic process.

The effect of FXR deficiency on adipocyte differentiation was further investigated using Oil Red O staining to assess triglyceride accumulation in MEFs induced to differentiate (Fig. 6, B and C). In accordance with the gene expression results, there was a decrease in triglyceride accumulation in MEFs derived from FXR\textasciitilde mice, with a reduced number of lipid droplets at day 4. In addition, there was a striking difference in the lipid droplets between both genotypes (see magnification × 40, Fig. 6C). Indeed, the size of lipid droplets was drastically reduced in MEFs from FXR\textasciitilde mice, indicating that FXR directly interferes with the lipid storage process. Altogether, these results suggest that FXR plays a role in adipocyte differentiation and function.
The PPARα-β/δ Gene Regulatory Pathways Are Activated in Skeletal Muscle of FXR-deficient Mice—To characterize the pattern of gene regulatory changes elicited by FXR-deficiency in peripheral insulin target tissues, transcriptional profiling studies were performed in periepidymal WAT and skeletal muscle from FXR+/− and FXR−/− mice (Table 2).

In WAT, the expression of the adipogenic genes (i.e. PPARγ, c-EBPα and aP2) were comparable between the two genotypes. Unexpectedly, mRNA levels of both c-EBPβ and SREBP-1c were increased in WAT from FXR−/− compared with FXR+/− mice. Adipose tissue is an endocrine organ that can regulate skeletal muscle insulin sensitivity by secreting hormones and cytokines. In accordance with decreased circulating leptin concentrations, leptin mRNA levels were drastically reduced in WAT from FXR−/− mice. In contrast, the expression of other adipocytokines (i.e. adiponectin, resistin, and interleukin-6) implicated in the regulation of insulin sensitivity was similar among groups. Tumor necrosis factor-α mRNA levels were reduced in FXR−/− mice, an effect that could not explain the peripheral insulin resistance but probably reflects the decreased fat mass of these animals. Expression of genes involved in triglyceride synthesis (i.e. diacylglycerol acyltransferase-1, fatty acid synthase, phosphoenolpyruvate carboxykinase) were not affected by the loss of FXR in WAT. More surprisingly, mRNA levels of hormone-sensitive lipase, a key enzyme in WAT lipolysis, were significantly reduced in FXR−/− compared with FXR+/− mice. In addition, the increased expression of genes involved in the uptake of FFA (i.e. lipoprotein lipase, fatty acid transport protein-1) in WAT did not correlate with the reduced adipocyte size of FXR−/− mice.

FFA and fatty acid metabolites are known to act as endogenous ligands of both PPARα and -β/δ (24). In accordance with the increased circulating FFA concentrations in FXR−/− mice, both PPARα and PPARβ/δ mRNA levels were increased in skeletal muscle of FXR−/− mice. Moreover, a broad program of known PPARα-β/δ target genes involved in cellular FFA uptake, triglyceride synthesis, and mitochondrial and peroxisomal β-oxidation was stimulated in muscle of FXR−/− mice (Table 2). These results suggest that FXR influences energy substrate partitioning (i.e. FFA versus glucose) in skeletal muscle.

FXR Agonist Treatment Enhances Insulin Sensitivity in Vivo in ob/ob Mice—Finally, the effect of pharmacological FXR agonist treatment on insulin resistance was analyzed in vivo in mice. Mice were treated for 10 days with GW4064 (30 mg/kg) administered intraperitoneally. FXR agonist treatment had no effect on glucose homeostasis in basal conditions and during a GTT C57BL/6 mice (data not shown). Therefore, genetically obese male ob/ob mice were studied. In this mouse model of insulin resistance, GW4064 treatment did not alter body weight as well as food intake. Whereas fasting blood glucose concentrations were sim-
FIGURE 6. Impaired adipocyte differentiation in MEFs derived from FXR^{−/−} mice. A, MEFs were derived from 13.5-day FXR^{+/+} and FXR^{−/−} embryos as described under “Experimental Procedures.” Total RNAs were extracted 2 days after the confluence (day 0) and during adipocyte differentiation (days 2, 4, and 8). mRNA levels were measured by Q-PCR analysis. Values are normalized relative to cyclophilin and are expressed (means ± S.E.) relative to those at day 0, which are arbitrarily set at 1. Two independent experiments were performed in triplicate. *, p < 0.05; **, p < 0.01. B and C, microscopic views of the Oil Red O-stained MEFs derived from FXR^{−/−} and FXR^{+/+} mice with magnification × 20 (B) and × 40 (C).
After GW4064 treatment, insulin concentrations decreased in the treated group, suggesting an improvement in insulin sensitivity (Table 3). Results from GTT and ITT indicated both an improved glucose clearance and an enhanced insulin sensitivity in GW4064-treated ob/ob mice compared with controls (Fig. 7, A and B). As expected, GW4064 treatment had no effect on plasma glucose concentrations in the basal (i.e., 6-h-fasted) state, i.e., during a GTT FXR treatment had no effect on plasma glucose concentrations in the basal peripheral insulin sensitivity in FXR mice. Collectively, these findings indicate that FXR plays an important role in the etiology of insulin resistance.

Strikingly, our data reveal a marked dissociation between hepatic and peripheral insulin sensitivity in FXR−/− mice that delineates the complex metabolic phenotype of the FXR−/− mice. Peripheral insulin resistance appears paradoxical, since both blood glucose and insulin levels are decreased in FXR−/− mice at the basal state. We have recently shown that FXR deficiency neither affects hepatic insulin sensitivity nor hepatic insulin signaling (6). Moreover, short-term-fasted blood glucose levels are significantly decreased in FXR−/− mice, linked to an impaired hepatic glucose production (11). Thus, in the basal (i.e., 6-h-fasted) state, the metabolic phenotype of FXR−/− mice mainly reflects the consequences of hepatic FXR-deficiency. In contrast, in the postprandial state, as mimicked by the tolerance tests and the hyperinsulinemic clamp, peripheral insulin resistance becomes manifest in FXR−/− mice.

One unresolved issue of our study is the identification of the molecular mechanisms underlying the impaired insulin-stimulated Akt activation in peripheral insulin-sensitive tissues of FXR−/− mice. Since FXR deficiency leads to a moderate increase in FFA, one attractive mechanism was based on the accumulation of biologically active fatty acid metabolites that activate serine/threonine kinases, leading to IRS-1 serine-phosphorylation and subsequent impaired IRS-1 tyrosine phosphorylation and phosphoinositide 3-kinase activity upon insulin stimulation (25, 26). However, we were unable to detect any defects in the insulin signaling pathway upstream of Akt. Moreover, gene expression of several negative regulators of insulin signaling, such as PTEN, does not differ between the two genotypes. Another hypothesis is that FXR regulates the activity of phosphatases, such as protein phosphatase 2A, that regulate Akt activity by dephosphorylating both Thr308 and Ser473 residues (27, 28). Further studies are needed to answer this question.

Our results strongly suggest that FXR directly regulates adipocyte function. Whereas FXR is not expressed in skeletal muscle (29), some studies have suggested that FXR is expressed at a low level in WAT (22, 23). Of pathophysiological interest, FXR mRNA levels are down-regulated in WAT of both genetically and diet-induced obese animals. Moreover, FXR increases progressively during adipocyte differentiation in vitro both in 3T3-L1 and in MEF cells. Importantly, adipose FXR, albeit expressed at low levels, has several functional roles. First, direct activation of FXR with a synthetic agonist in 3T3-L1 adipocytes leads to enhanced insulin signaling and insulin-stimulated glucose uptake. Second, FXR deficiency leads to an impaired adipogenic program in MEFs. Surprisingly, the expression of several adipogenic regulators in WAT of FXR−/− mice in vivo does not correlate with results obtained during the adipogenic process in vitro. One explanation is that FXR preferentially affects the kinetics of the timed expression of adipogenic transcription factors without affecting the induction levels at the end of the differentiation. Such discrepancies between in vitro and in vivo models have already been reported previously. For instance, MEFs isolated from the double knock-outs c-EBPα/c-EBPβ do not undergo adipogenic conversion, whereas neither c-EBPα nor PPARγ gene expression is altered in WAT of these mice (30).

FXR also interferes with the lipid storage process in the adipocyte. Oil red O staining clearly demonstrated that MEFs isolated from FXR−/− mice were unable to correctly accumulate triglyceride during the course of adipocyte differentiation. In accordance with this new role of FXR in the adipocyte, FXR−/− mice exhibit a decreased fat mass with a reduced adipocyte size. The molecular mechanism of such a defect in triglyceride storage in FXR−/− mice remains unclear. Indeed, the expression of genes involved in lipid accumulation in the adipocyte did not differ in WAT of both genotypes. Most intriguing is the observation that fat mass is reduced despite the increased expression of genes involved in fatty acid transport (fatty acid transport protein-1 and lipoprotein lipase), since invalidation of these genes leads to decreased adiposity in mice (31, 32). This suggests that an unidentified defect in triglyceride metabolism occurs at a step distal from fatty acid uptake in adipocytes of FXR−/− mice. Another apparent paradox is the up-regulation of SREBP-1c mRNA expression in WAT, since SREBP-1c is known to favor lipid accumulation in adipocytes in vitro (33). However, overex-

| TABLE 3 |

Effects of GW4064 treatment on metabolic parameters in ob/ob mice

|                  | Vehicle           | GW 4064 (30 mg/kg/day) |
|------------------|-------------------|------------------------|
|                  | Day 0             | Day 10                 | Day 0                  | Day 10                 |
| Weight (g)       | 66.5 ± 2.5        | 65.1 ± 1.9             | 60.3 ± 7.3             | 59.8 ± 4.9             |
| Glucose (mg/dl)  | 248 ± 18          | 279 ± 23               | 291 ± 29               | 257 ± 20               |
| Insulin (ng/ml)  | 13.1 ± 3.2        | 15.6 ± 4.4             | 13.0 ± 1.7             | 6.0 ± 0.8*             |
| CT (mg/dl)       | 310 ± 11          | 315 ± 8                | 304 ± 16               | 190 ± 8*               |
| HDL-C (mg/dl)    | 260 ± 6           | 260 ± 4                | 231 ± 14               | 160 ± 12.2             |
| TG (mg/dl)       | 122 ± 8           | 127 ± 5                | 130 ± 13               | 106 ± 16               |
| FFA (mmol/liter) | 0.57 ± 0.05       | 0.63 ± 0.05            | 0.52 ± 0.10            | 0.56 ± 0.10            |

*p < 0.05, significantly different from untreated mice.

*p < 0.001, significantly different from untreated mice.

*p < 0.01, significantly different from untreated mice.
FXR Regulates Insulin Sensitivity

pression of a constitutively active SREBP-1c in WAT resulted in reduced adiposity and severe insulin resistance, mimicking a human phenotype of lipodystrophy (34). Clearly, further studies are needed to precisely unravel the molecular mechanism of FXR function in the adipocyte.

Insulin resistance is a key factor in the development of type 2 diabetes and may be related to alterations in fat metabolism. Adipose tissue expresses and secretes a variety of bioactive peptides, known as adipocytokines, which act at both the local (autocrine/paracrine) and systemic (endocrine) level (35). Peripheral insulin resistance observed in FXR−/− mice cannot be explained by alterations in circulating levels of insulin-sensitizing adiponectin. In addition, gene expression of inflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, is not increased in FXR−/− mice. In contrast, both mRNA and plasma levels of leptin are drastically reduced in FXR−/− mice, probably reflecting the reduced adiposity. It should be noticed that food intake is similar between both genotypes, indicating that leptin sensitivity is not reduced in FXR−/− mice, at least in the hypothalamus. In addition, the observation that treatment with a FXR agonist improves insulin resistance in leptin-deficient ob/ob mice reinforces the hypothesis that FXR can modulate insulin sensitivity via a leptin-independent pathway.

Since FXR is not expressed in skeletal muscle, FXR deficiency must act indirectly on this major insulin target tissue. As underlined above, FXR−/− mice also display elevated circulating FFA levels, which may contribute to their peripheral insulin resistance. Indeed, Randle et al. (36) initially suggested that elevated plasma FFA concentrations play a key role in the development of insulin resistance through a substrate competition between glucose and FFA. Since FXR deficiency induces a lipodystrophic phenotype, one potential mechanism for the peripheral insulin resistance observed in FXR−/− mice may be that the reduced fat mass or impaired ability of adipocytes to accommodate FFA causes a redirection of the FFA flux from adipocytes to skeletal muscle. In this context, the increased gene expression of lipoprotein lipase in skeletal muscle as well as the increased postheparin lipoprotein lipase activity (37) could potentially contribute to the peripheral insulin resistance observed in FXR−/− mice. Indeed, tissue-specific overexpression of lipoprotein lipase in skeletal muscle has been shown to specifically induce muscle insulin resistance (38). Another possible explanation for the FXR deficiency-related peripheral insulin resistance may be via activation of the muscle PPAR regulatory pathways. Interestingly, a recent study has suggested that activation of skeletal muscle PPARα is directly linked to the development of insulin resistance and diabetes in mice, without altering phosphorylation of key insulin signaling molecules (39). In accordance with a crucial role of FXR in fuel partitioning, we have recently shown that FXR deficiency is associated with increased de novo lipogenesis in liver, associated with increased hepatic very low density lipoprotein production (6). This phenomenon could contribute to the increased flux of FFA to the skeletal muscle. Nevertheless, further studies are required to determine the relative contribution of each insulin target tissue to the metabolic phenotype of FXR−/− mice.

Finally, our in vivo results using a synthetic FXR agonist in a mouse model of insulin resistance validates the potential of pharmacological FXR modulation in the control of glucose homoeostasis. Indeed, treatment of ob/ob mice with a specific synthetic FXR agonist improved insulin sensitivity.

The data presented here, obtained through a variety of complementary approaches, highlight a new role for FXR in regulating adipocyte function and insulin resistance. These findings open attractive perspectives for unraveling the pathophysiology of metabolic disorders, such as type 2 diabetes, and indicate that pharmacologic modulators of FXR could provide useful novel therapeutic tools.

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FIGURE 7. FXR activation improves insulin sensitivity in ob/ob mice in vivo. 20-week old male ob/ob mice were given free access to food and water. Intraperitoneal injections of GW4064 (30 mg/kg) or vehicle (corn oil) were performed during 10 days. For GTT (A) and ITT (B), glucose (1.0 g/kg) and insulin (2 IU/kg), respectively, were administered by intraperitoneal injection after 6 h of fasting. The inset represents the integrated area under the curve. Data are means ± S.E. values. * p < 0.05 compared with untreated mice (n = 6 mice/group for GTT, and n = 4 mice/group for ITT).
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