Type 2 diabetes is characterized by a progressive resistance of peripheral tissues to insulin. Recent data have established the lipid phosphatase SH2 domain–containing inositol phosphatase 2 (SHIP2) as a critical negative regulator of insulin signal transduction. Mutations in the SHIP2 gene are associated with type 2 diabetes. Here, we used hyperglycemic and hyperinsulinemic KKAy mice to gain insight into the signaling events and metabolic changes triggered by SHIP2 inhibition in vivo. Liver-specific expression of a dominant-negative SHIP2 mutant in KKAy mice increased basal and insulin-stimulated Akt phosphorylation. Protein levels of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase were significantly reduced, and consequently the liver produced less glucose through gluconeogenesis. Furthermore, SHIP2 inhibition improved hepatic glycogen metabolism by modulating the phosphorylation states of glycogen phosphorylase and glycogen synthase, which ultimately increased hepatic glycogen content. Enhanced glucokinase and reduced pyruvate dehydrogenase kinase 4 expression, together with increased plasma triglycerides, indicate improved glycolysis. As a consequence of the insulin-mimetic effects on glycogen metabolism, gluconeogenesis, and glycolysis, the liver-specific inhibition of SHIP2 improved glucose tolerance and markedly reduced prandial blood glucose levels in KKAy mice. These results support the attractiveness of a specific inhibition of SHIP2 for the prevention and/or treatment of type 2 diabetes. Diabetes 56:2235–2241, 2007

A key event in insulin signal transduction is the increase in phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3], which recruits and activates downstream effectors at the plasma membrane (1). The lipid phosphatase SH2 domain–containing inositol phosphatase 2 (SHIP2) is expressed in insulin target tissues and dephosphorylates PI(3,4,5)P3 at the D5 position (2,3). Activating mutations in the SHIP2 gene contribute to the genetic susceptibility to type 2 diabetes in humans (4). Mice lacking the SHIP2 gene (Inppl1−/− mice) are viable and have normal glucose and insulin levels with normal insulin and glucose tolerances. However, Inppl1−/− mice are protected from diet-induced obesity, hyperglycemia, and insulin resistance (5). The function of SHIP2 in differentiated L6 myotubes and 3T3-L1 adipocytes was extensively studied by inhibition of the endogenous SHIP2 via overexpression of a dominant-negative SHIP2 mutant (dnSHIP2). SHIP2 inhibition increases phosphorylation of Akt (also known as protein kinase B) and glycogen synthesis in both cell lines (6,7). The liver-specific overexpression of dnSHIP2 in db/db mice results in dramatically decreased fasting glucose levels due to the reduced expression of the gluconeogenic genes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (8). However, the strong reduction in fasting blood glucose concentration confounds the interpretation of an improved glucose tolerance test in this model. Furthermore, an effect on prandial blood glucose was not reported to date.

In the liver, in addition to gluconeogenesis, several other pathways are involved in glucose metabolism and are dominantly regulated by insulin. First, glycolysis is enhanced upon insulin stimulation via an increase in glucokinase and inhibition of pyruvate dehydrogenase kinase 4 (PDK4) gene expression (9,10). Second, insulin promotes...
glocygen accumulation by activation of glycojen synthesis, through stimulation of glycojen synthase and reduction of glycojen breakdown, via inhibition of glycojen phosphorylase. The activities of both enzymes are regulated by changes in their phosphorylation states (11), and it has been shown recently that Akt also plays an important role in mediating the inactivation of hepatic glycojen phosphorylase and activation of glycojen synthase (12).

Many of the above-mentioned effectors of insulin in the liver appear to be deregulated either at the phosphorylation/activity level or at the level of gene transcriptions in type 2 diabetes, raising the possibility that these malfunctions could be corrected by SHIP2 inhibition (10,13,14). The consequences of SHIP2 inhibition in the pathophysiology of type 2 diabetes have been studied in db/db mice but mechanistically are not fully understood. To gain further insight into the signaling events and metabolic changes triggered by SHIP2 inhibition in the liver, we used hyperglycemic and hyperinsulinnic KKA\textsuperscript{y} mice, a second disease-relevant animal model.

**RESEARCH DESIGN AND METHODS**

**Regents.** The following antibodies were used: polyclonal anti-phospho (Ser\textsuperscript{Thr}-Akt, anti-phospho (Ser\textsuperscript{Thr})-glycojen synthase, and anti-Akt (Cell Signaling Technology, Beverley, MA); anti-glucokinsase, anti-G6Pase, anti-SHIP2, anti-PDK4, anti-actin, and anti-PEPCk (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-\(\beta\)-galactosidase (βGal) (Rockland, Gilbertsville, PA). The anti-phospho (Ser\textsuperscript{Thr})-glycojen phosphorylase antibody was kindly provided by the group of Patricia T.W. Cohen (University of Dundee, Dundee, U.K.). All other regents were of analytical grade and purchased from Sigma-Aldrich (Gillingham, U.K.) or Roche (Mannheim, Germany).

**Adenovirus production and purification.** SHIP2 cDNA (ref. seq. NM_001567) was amplified from HepG2-derived cDNA samples and cloned into the pcR2-T-TOPO (Invitrogen, Carlsbad, CA); and anti-\(\beta\)-galactosidase (βGal) (Rockland, Gilbertsville, PA). The anti-phospho (Ser\textsuperscript{Thr})-glycojen phosphorylase antibody was kindly provided by the group of Patricia T.W. Cohen (University of Dundee, Dundee, U.K.). All other regents were of analytical grade and purchased from Sigma-Aldrich (Gillingham, U.K.) or Roche (Mannheim, Germany).

**Results**

Liver-specific expression of dnSHIP2 increases hepatic but not muscle Akt phosphorylation in KKA\textsuperscript{y} mice. A phosphatase-defective mutant of human SHIP2 that acts in a dominant-negative manner (dnSHIP2) was constructed as previously described for rat SHIP2 (7). dnSHIP2 was expressed via adenovirus-mediated gene transfer. Ad5-βGal was used as a control. Injection of adenoviruses into mice via the tail vein exclusively results in infection of the liver (17), where the transgene is detectable for up to 14 days postinfection (8). KKA\textsuperscript{y} mice were injected with either Ad5-βGal or Ad5-dnSHIP2 at day 1 of the experiment. At day 5, fasted animals were administered with insulin or vehicle and killed after 5 min. Livers and quadriceps muscles were removed. Overexpression of dnSHIP2 was observed in the liver and was 14-fold higher than endogenous SHIP2 levels (Fig. 1A). dnSHIP2 expression increased hepatic Akt phosphorylation up to 175 ± 22% in animals without insulin treatment. Stimulation with insulin increased the phosphorylation of Akt up to 297 ± 34% over the basal level in the βGal group. In the dnSHIP2 group, the action of insulin was further increased up to 383 ± 23% (Fig. 1A and B). Neither βGal (data not shown) nor dnSHIP2 overexpression was observed in the muscle, and consequently no difference in Akt phosphorylation was detected (Fig. 1C and D).

**SHIP2 inhibition represses hepatic gluconeogenesis.** To test whether hepatic gluconeogenesis is affected by SHIP2 inhibition, we analyzed the expression of two key gluconeogenic proteins in the fasted state. A significant reduction of PEPCk and G6Pase protein levels was detected in the dnSHIP2-expressing animals (Fig. 2A and B). Next, we analyzed whether the inhibition of these gluconeogenic enzymes impaired the capacity of the liver to produce glucose from pyruvate. For this, fasted animals were subjected to a pyruvate challenge test in a separate experiment. The dnSHIP2 group showed a marked reduction in glucose production (area under the curve [AUC]_{10-180min} was reduced by 55%), further confirming the inhibition of hepatic gluconeogenesis (Fig. 2C).
whether this results in increased hepatic glycogen content, a separate experiment was performed. Ad libitum–fed animals were killed, and hepatic glycogen content was measured. In agreement with the phosphorylation states of glycogen synthase and glycogen phosphorylase, a significant increase in hepatic glycogen content was detected in the dnSHIP2 group (Fig. 3).

SHIP2 inhibition increases glycolysis and serum triglycerides. To examine whether the inhibition of SHIP2 has an impact on glycolysis, we analyzed protein levels of glucokinase and PDK4 in the prandial state. The animals were fed ad libitum until day 5, when they were killed. In the dnSHIP2 group, the protein levels of glucokinase were significantly increased and the protein levels of PDK4 significantly decreased (Fig. 4A and B). An increased glycolysis should result in an elevated substrate supply for fatty acid synthesis and may increase serum triglyceride levels. Therefore, in a separate experiment, serum triglycerides were measured in ad libitum–fed animals. The dnSHIP2 animals showed increased serum triglycerides (βGal 2.7 ± 0.2 vs. dnSHIP2 3.6 ± 0.3 mmol/l, P = 0.057), which may represent an elevated hepatic fatty acid synthesis (Fig. 4C).

Liver-specific expression of dnSHIP2 improves glucose tolerance with reduced insulin concentrations. To assess the impact of hepatic SHIP2 inhibition on glucose tolerance of KKA mice, the animals were fasted and an OGTT performed. No significant effect of hepatic dnSHIP2 overexpression on fasting blood glucose was observed (βGal 7.9 ± 0.3 vs. dnSHIP2 7.5 ± 0.7 mmol/l). However, dnSHIP2-expressing animals exhibited a marked improvement of glucose tolerance (AUC0–180min reduced by 52%) (Fig. 5A). Directly after the OGTT, the insulin levels were significantly reduced in the dnSHIP2 group (βGal 31.4 ± 2.7 vs. dnSHIP2 23.7 ± 2.9 ng/ml) (Fig. 5B). Thus, hepatic SHIP2 inhibition improves glucose tolerance with reduced insulin levels.

Hepatic SHIP2 inhibition normalizes prandial blood glucose and reduces insulin levels. KKA mice were fed ad libitum, and prandial blood glucose was measured at days 1, 3, 4, and 5. Throughout the experiment, no significant difference in food intake was observed (data not shown). However, a normalization of prandial blood glucose was seen beginning at day 3 of the experiment in the dnSHIP2 group (12.8 ± 1.1 mmol/l) compared with the βGal controls (21.4 ± 2.0 mmol/l). Overall, the prandial glucose levels were significantly reduced by 42% (AUCday 3 to day 5) (Fig. 6A). At day 4 of the study, a significant and marked reduction in plasma insulin was observed.
FIG. 2. SHIP2 inhibition represses G6Pase and PEPCK protein expression and reduces hepatic glucose output after a pyruvate challenge. KKAy mice were injected with Ad5-βGal or Ad5-dnSHIP2 via the tail vein. At day 4 postinfection, the animals were starved for 16 h and killed. Liver tissue was homogenized and lysates subjected to immunoblot analysis using anti-G6Pase (A) and anti-PEPCK (B) antibodies. Superscript numbers refer to the respective animal number of each group. The bar graphs show the densitometric analysis of the respective blots (n = 6 in each group). *P < 0.05 vs. βGal control.

C: Pyruvate challenge test. Animals were fasted from day 7 for 16 h and intraperitoneally injected with 1.5 g pyruvate/kg body wt. *P < 0.05 vs. the corresponding values in βGal-expressing mice, provided by ANOVA.

FIG. 3. SHIP2 inhibition reduces phosphorylation of glycogen synthase (pGS) and glycogen phosphorylase (pGP) and increases hepatic glycogen content. KKAy mice were injected with Ad5-βGal or Ad5-dnSHIP2 via the tail vein at day 1, and animals were fed ad libitum. At day 5, the animals were killed. Liver tissue was homogenized and lysates subjected to immunoblot analysis using anti–phospho (Ser641)-glycogen synthase (A) and anti–phospho (Ser14)-glycogen phosphorylase (B) antibodies. Superscript numbers refer to the respective animal number of each group. The bar graphs show the densitometric analysis of the respective blots (n = 12 in each group). *P < 0.05 vs. βGal control.

C: Hepatic glycogen content of ad libitum–fed animals at day 6 (n = 14 per group). Values represent median ± variance. *P < 0.05 vs. βGal-expressing animals.
shown in the dnSHIP2-expressing group (βGal 19.7 ± 4.8 vs. dnSHIP2 8.4 ± 2.8 ng/ml) (Fig. 6B).

DISCUSSION
We used KKAy mice to study the impact of liver-specific SHIP2 inhibition on metabolic parameters and on key enzymes of gluconeogenesis, glycolysis, and glycogen metabolism. The KKAy mouse is a well-established model of type 2 diabetes characterized by obesity, hypertriglyceridemia, hyperglycemia, and hyperinsulinemia (18).

KKAy mice with hepatic SHIP2 inhibition exhibited an increase in both basal and insulin-induced Akt phosphorylation after an overnight fast. In contrast, in db/db mice it has been shown that SHIP2 inhibition ameliorates the insulin-induced but not the basal phosphorylation of Akt after an overnight fast (8). These differences could be explained by a different sensitivity of the two mouse models to SHIP2 inhibition due to the specific pathophysiology. On the other hand, the efficiency of SHIP2 inhibition could be different because of dissimilar expression levels of dnSHIP2 (5-fold over endogenous in db/db mice [8] and 14-fold in KKAy mice). Although in these two studies SHIP2 genes from different species were used to generate dnSHIP2 (human in our study vs. rat in the study of Fukui et al. [8]), it seems unlikely that this has functional consequences, since both proteins share 95% homology overall and 99.8% identity in their functional domains.

The observation of an insulin-mimetic effect of hepatic SHIP2 inhibition is consistent with our mechanistic stud-
ies in vitro using C3A hepatoma cells that showed increased phosphorylation of Akt in the absence of insulin (Supplementary Fig. 1, available in an online appendix at http://dx.doi.org/10.2337/db06-1660). In line with this, the reduction of insulin levels that we observed in KKAy mice is believed to be secondary to the reduction of blood glucose concentrations and not due to improved insulin sensitivity. Together, our data point toward an insulin-mimetic effect of hepatic SHIP2 inhibition.

We showed here for the first time that SHIP2 inhibition affects hepatic glycogen metabolism in vivo, as a consequence of increased Akt phosphorylation in the prandial state (Supplementary Fig. 2). Recent studies have shown that overexpression of a constitutively active form of Akt in rat hepatocytes induces glycogen synthase activity and inhibits glycogen phosphorylase activity (12). Our data provide in vivo evidence that increased Akt activity indeed influences both branches of glycogen metabolism, since in our study hepatic SHIP2 inhibition led to decreased phosphorylation of glycogen synthase and glycogen phosphorylase. Consistently, we detected a significant increase in hepatic glycogen content. Although it has been shown recently that Akt signaling is not absolutely required for the maintenance of hepatic glycogen stores, we provide evidence that activation of Akt is sufficient to improve glycogen metabolism in the diabetic state (19).

Furthermore, the increased glucokinase and decreased PDK4 protein levels observed after SHIP2 inhibition should enhance glycolysis. In line with this, a rise in plasma triglycerides was observed in both the fasted (data not shown) and the fed states, although the protein levels of fatty acid synthase were similar between the groups under both conditions (data not shown). Therefore, the changes in PEPCK, G6Pase, glucokinase, and PDK4 protein levels may divert substrates toward fatty acid synthesis. In this context, it is worth noting that all changes in gene expression were detected in both fasted and fed states.

We showed that gluconeogenesis is markedly reduced in dnSHIP2-expressing KKAy animals due to a reduction of PEPCK and G6Pase protein levels. This is in agreement with the inhibition of PEPCK and G6Pase gene expression and decreased fasting blood glucose levels observed in db/db mice (8). Because the transcription factor forkhead box class O (FoxO1) is a key activator of PEPCK and G6Pase gene expression (20) and because FoxO1 is inhibited and degraded upon phosphorylation by Akt (21,22), we attempted to examine the phosphorylation states of FoxO1 using antibodies for the three different Akt phosphorylation sites (Thr24, Ser256, and Ser319) (21). However, none of the tested antibodies detected the phosphorylated FoxO1 protein in the liver lysates from KKAy mice, although at least the phospho-(Ser256)–specific FoxO1 antibody (Cell Signaling Technology) detected phosphorylated FoxO1 in mouse liver lysates in another study (19). In the βGal control group, a phosphorylation of FoxO1 is not expected because of the insulin resistance of the liver. We speculate that in the dnSHIP2-expressing animals, the persistent activation of Akt may lead to continuous phosphorylation and degradation of FoxO1; therefore, no phosphorylation could be detected. Understanding this aspect will require additional work focusing on FoxO1 regulation.

Despite the reduction of hepatic gluconeogenesis, we could not detect a significant decrease in the fasting blood glucose levels in KKAy mice. The reason for this discrepancy is unknown but may be explained by differences of the two mouse models. The db/db mice had tremendously elevated fasting glucose levels, representing their pronounced diabetic state, while KKAy mice displayed only elevated fasting glucose levels, representing their pronounced diabetic state, while KKAy mice displayed only slightly elevated fasting glucose levels (8). Furthermore, in db/db mice the interpretation of an improved glucose tolerance was confounded by the strong reduction in fasting blood glucose concentration. Rather, the glucose AUC is unchanged when the values are determined in reference to the baseline blood glucose of each animal group (8). However, we clearly demonstrated an improved glucose tolerance in KKAy mice (glucose AUC −52%) with unchanged fasting glucose levels.

Our data are highly consistent with previous reports that relate to an improvement of the hepatic insulin signaling pathway. First, liver-specific PTEN deletion in mice leads to increased phosphorylation of Akt, enhanced glycogen storage, suppressed PEPCK and G6Pase gene expression, and improved glucose tolerance (23). Second, overexpression of a constitutively active Akt in C57BL/6 mice reduces blood glucose and insulin levels and increases hepatic...
glycogen content and plasma triglycerides; this phenotype is again very similar to our data but much more pronounced because of the constitutive activity of Akt (24).

A major new finding of our study is the marked reduction of prandial blood glucose levels. Taken together, both the improved glucose tolerance and the reduction of prandial blood glucose levels in KKAy mice with hepatic SHIP2 inhibition can be explained by an improved glucose disposal by the liver. For this, SHIP2 inhibition reverses changes of gene expression and protein phosphorylation that are associated with type 2 diabetes (10,13,14) and ultimately leads to increased glycolysis and glycogen storage with reduced gluconeogenesis. Our data extend the knowledge of how SHIP2 inhibition leads to improved glucose metabolism and support the attractiveness of a specific inhibition for the prevention and/or treatment of type 2 diabetes.

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