Association of SH2-Containing Inositol Phosphatase 2 With the Insulin Resistance of Diabetic *db/db* Mice

Hiroyuki Hori,[1] Toshiyasu Sasaoka,[2] Hajime Ishihara,[1] Tsutomu Wada,[1] Shihou Murakami,[1] Manabu Ishiki,[1] and Masashi Kobayashi[1]

SH-2−containing inositol 5′-phosphatase 2 (SHIP-2) is a physiologically important lipid phosphatase that functions to hydrolyze phosphatidylinositol (PI) 3-kinase product PI(3,4,5)P3 to PI(3,4)P2 in the negative regulation of insulin signaling. We investigated whether SHIP-2 is associated with the insulin resistance of diabetic *db/db* mice. The amount of SHIP-2 protein was elevated in quadriceps muscle and epididymal fat tissue, but not in the liver, of *db/db* mice relative to that in control *db/*+m mice. In accordance with the enhanced expression of SHIP-2, its localization at the membrane preparation was increased in the skeletal muscle and fat tissue of *db/db* mice. Insulin stimulation of PI 3-kinase activity was modestly decreased in skeletal muscle, fat tissue, and liver of *db/db* mice compared with that of *db/*+m mice. In addition to the modest decrease at the level of PI 3-kinase, the activity of Akt and protein kinase C (PKC)-ζ/λ, which are downstream molecules of PI 3-kinase, was more severely reduced in the skeletal muscle and fat tissue, but not in liver of *db/db* mice. Treatment with the insulin-sensitizing agent rosiglitazone decreased the elevated expression of SHIP-2 in the skeletal muscle and fat tissue of *db/db* mice. Insulin-induced Akt activation and PKC-ζ/λ phosphorylation were restored to the control level, although insulin-stimulated PI 3-kinase activation was minimally affected in the skeletal muscle and fat tissue of *db/db* mice. These results indicate that SHIP-2 is a novel molecule associated with insulin resistance in the skeletal muscle and fat tissue, and that insulin-induced activity of the downstream molecules of PI 3-kinase is decreased, at least in part, by the elevated expression of SHIP-2 in diabetic *db/db* mice. *Diabetes* 51:2387–2394, 2002

The activated insulin receptor phosphorylates the tyrosine residues of the insulin receptor substrate (IRS) family (1–3). IRS propagates insulin signals to the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, which activates the p110 catalytic subunit (1–3). Insulin-induced PI 3-kinase activation is known to be important for exerting a variety of insulin-induced metabolic actions, including glucose uptake and glycogen synthesis (1–3). PI 3-kinase functions as a lipid kinase that preferentially phosphorylates PI(4,5)P2 to PI(3,4,5)P3 in vivo (4,5). PI(3,4,5)P3 can be subsequently hydrolyzed to PI(3,4)P2 by PI 5′-phosphatase(s) (6,7). In fact, insulin treatment increases the amount of PI(3,4,5)P3 and PI(3,4)P2 in the cell, which can serve as lipid second messengers and possibly relay the signal to downstream target molecules, including Akt and atypical protein kinase C (PKC) for the metabolic actions of insulin (5,8–10). Recently, we and others have cloned a novel 5′-phosphatase named SH-2−containing inositol 5′-phosphatase 2 (SHIP-2), which is predominantly expressed in target tissues of insulin (11,12). Overexpression of SHIP-2 inhibits insulin-induced glucose uptake and glycogen synthesis via its 5′-phosphatase activity in 3T3-L1 adipocytes and L6 myotubes (13,14). Targeted disruption of the SHIP-2 gene in mice results in increased insulin sensitivity with no effect on biological systems other than insulin signaling (15). These previous findings indicate that SHIP-2 is the physiologically important negative regulator relatively specific to insulin signaling, and that PI(3,4,5)P3 rather than PI(3,4)P2 is important for in vivo activation of Akt and atypical PKC.

Although the physiological importance of SHIP-2 is clear in insulin signaling, it is still unknown whether SHIP-2 is associated with the insulin resistance of type 2 diabetes. Previous studies on insulin-induced Akt activation in animal models of insulin resistance and diabetic subjects have shown contradictory results regarding the possible involvement of Akt in impaired glucose homeostasis (16–22). Although more studies are needed to clarify further whether Akt is in fact responsible for insulin resistance, it has been reported that in diabetic Goto-Kakizaki (GK) rats, insulin-induced Akt activation and glucose uptake are impaired in the skeletal muscle, whereas insulin signaling at the level of IRS-1/PI 3-kinase is intact (18). Furthermore, in Zucker diabetic fatty (ZDF) rat, insulin-induced phosphorylation and kinase activity of Akt has been reported to be severely suppressed, despite the increased PI 3-kinase

From the [1]First Department of Internal Medicine, Toyama Medical & Pharmaceutical University, Toyama, Japan; and the [2]Department of Clinical Pharmacology, Toyama Medical & Pharmaceutical University, Toyama, Japan.

Address correspondence and reprint requests to Dr. Toshiyasu Sasaoka, Department of Clinical Pharmacology, Toyama Medical & Pharmaceutical University, 2630 Sugitani, Toyama, 930-0194, Japan. E-mail: tsasaoka-tym@umin.ac.jp.

Received for publication 19 November 2001 and accepted in revised form 6 May 2002.

DTT, dithiothreitol; ECL, enhanced chemiluminescence; HES, HEPES EDTA sucrose; IRS, insulin receptor substrate; PI, phosphatidylinositol; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PVDM, polyvinylidene difluoride membrane; SHIP-2, SH-2−containing inositol 5′-phosphatase 2.

activity (19). Concerning the possible involvement of atypical PKC in the state of insulin resistance, insulin-induced PKC-ζ/λ activation and glucose transport have been found to be profoundly decreased in skeletal muscle and adipocyte of GK rats (23,24). Along this line, insulin completely failed to activate PKC-ζ/λ, regardless of only a modest decrease in insulin-induced PI 3-kinase activity in the skeletal muscle of high fat−fed rats (25).

These previous reports suggest that the molecular mechanism underlying insulin resistance in animal models of type 2 diabetes is involved, at least in part, at the step between PI 3-kinase and its downstream molecules, Akt and PKC-ζ/λ. In view of this, we hypothesized that there is possible involvement of SHIP-2 in insulin resistance. The *db/db* mouse is an insulin-resistant diabetic model caused by the defect of long-form leptin receptor gene (26). Previous studies with *db/db* mice have shown that insulin-induced Akt activation and glucose uptake in skeletal muscles and fat tissues are decreased compared to control littermates (27). To clarify the association of SHIP-2 with the insulin resistance of *db/db* mice, we examined the change in the amount of expressed SHIP-2 protein in skeletal muscle, fat tissue, and liver of *db/db* mice compared to that of control *db/+*m mice. Furthermore, we tested whether treatment with an insulin-sensitizing thiazolidinedione, rosiglitazone, affects the level of SHIP-2 and ameliorates insulin signaling at the level distal to PI 3-kinase, thereby resulting in the restoration of insulin-induced Akt and PKC-ζ/λ activation. In this study, we showed that SHIP-2 is an important molecule associated with the insulin resistance of diabetic *db/db* mice.

RESEARCH DESIGN AND METHODS

Materials. Human crystal insulin was provided by Novo Nordisk Pharmaceutical (Copenhagen, Denmark). [γ-^{32}P]ATP (111 TBq/mmol) was purchased from NEN Life Science Products (Boston, MA). Two polyclonal anti–SHIP-2 antibodies were described previously (12). A polyclonal anti-Ser (21/9) phosphospecific GSK-3 antibody, a polyclonal anti-Thr (410/403) phosphospecific PKC-ζ/λ antibody, and the Akt kinase assay kit were obtained from New England Biolabs (Beverly, MA). A polyclonal anti-Akt antibody and a polyclonal anti-PKC-ζ/λ antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal anti-phosphotyrosine antibody (PY20) and a monoclonal anti-p85 antibody of PI 3-kinase were obtained from Transduction Laboratories (Lexington, KY). Enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other routine reagents were analytical grade and were purchased from Sigma Chemicals (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan).

Animals. Male C57BL/KsJ-*db/db* mice and their lean heterozygote littermates (*db/+*m) and C57BL/6J were purchased from Clea Japan (Tokyo, Japan) at age 6 weeks and maintained under standard light (12 h light/dark) and temperature conditions. These mice were caged in groups of four and were provided with food and water ad libitum, and were used for the experiments at approximately ages 8–12 weeks. The mice group with a high-fat diet were fed for 2 weeks with a diet containing 32% safflower oil, 33.1% casein, 0.5% DL-methionine, 17.6% sucrose, 1.4% vitamin, 9.8% mineral mixture, and 5.6% cellulose powder (28). The treatment group of *db/db* and *db/+*m mice received rosiglitazone, provided from SmithKline Beecham, in their food at a level of 0.005%, which resulted in the ingestion of 10 mg · kg^{-1} · day^{-1} for 2 weeks.

Preparation of tissue lysates and total membrane fraction. Food was withdrawn 12 h before the experiments, and mice were intraperitoneally injected with saline or insulin (5 mU/g body wt) in saline. After 10 min, the mice were killed, and their quadriceps muscle, epididymal fat tissue, and liver were rapidly excised. Then 50 mg of these tissues were homogenized using a polytron at half maximum speed (15,000 rpm) for 1 min on ice in 500 μl of a homogenization buffer containing 20 mmol/l Tris, 5 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 2 mmol/l Na_3VO_4, 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.5). The tissue lysates were solubilized by continuous stirring for 1 h at 4°C, and centrifuged for 10 min at 14,000*g*. The supernatants were used for the immunoblot analysis (23,29). For total membrane preparation, the tissue samples were homogenized with the polytron homogenizer in HEPES EDTA sucrose (HES) buffer containing 20 mmol/l HEPES, 5 mmol/l EDTA, 250 mmol/l sucrose, 50 nmol/l okadaic acid, 1 mmol/l Na_3VO_4, 2 μg/ml pepstatin, 1 mmol/l PMSF, 10 μg/ml aprotinin, and 2 μg/ml leupeptin (pH 7.5) at 4°C. The relative volumes of the tissues to HES buffer were 1:3 for the adipose tissue and 1:10 for skeletal muscle and liver. The homogenate was centrifuged at 1,200*g* at −4°C for 15 min, and the fat cake and pellet were discarded. The infranatant was then centrifuged at 220,000*g* for 90 min at 4°C. The pellet containing the total membrane fraction was resuspended in the HES buffer and subjected to immunoblot analysis (23,29). Blood samples were also collected at the time the mice were killed. Serum glucose was measured by an automated glucose analyzer (A&T, Tokyo, Japan) and serum insulin was detected by an enzyme-linked immunosorbent assay kit (MIA Institute of Biological Science, Yokohama, Japan). All procedures were approved by the Committee of Animal Experiment at Toyama Medical & Pharmaceutical University.

Western blotting. The equal protein amount of the samples was separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDMs) using a Bio-Rad Transblot apparatus. The membranes were blocked in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween 20, and 2.5% BSA or 5% non-fat milk (pH 7.5) for 2 h at 20°C. The membranes were then probed with the specified antibodies for 2 h at 20°C or for 16 h at 4°C. After washing the membranes in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, and 0.1% Tween 20 (pH 7.5), blots were incubated with horseradish peroxidase−linked second antibody and then examined by enhanced chemiluminescence (ECL) detection using an ECL reagent according to the manufacturer's instructions (Amersham) (13,14).

PI 3-kinase assay. The tissue lysates (0.5 mg protein) were immunoprecipitated with anti-phosphotyrosine antibody (PY20) coupled to protein G-sepharose for 16 h at 4°C. The immune complex was washed twice with PBS containing 1% NP-40, 100 μmol/l Na_3VO_4, and 1 mmol/l dithiothreitol (DTT); twice with 100 mmol/l Tris-HCl containing 500 mmol/l $LiCl_2$, 100 μmol/l Na_3VO_4, and 1 mmol/l DTT (pH 7.5); and twice with 10 mmol/l Tris containing 0.1 mol/l NaCl, 1 mmol/l EDTA, and 1 mmol/l DTT (pH 7.5). The pellet was resuspended in 10 μl Tris-NaCl buffer. The PI 3-kinase reaction was started by adding 20 μl of PI solution containing 0.5 mg PI/ml, 50 mmol/l HEPES, 1 mmol/l NaH_2PO_4, and 1 mmol/l EGTA (pH 7.6) at 20°C, followed by the addition of 10 μl of a reaction mixture containing 250 μmol/l [γ-^{32}P]ATP (0.37 MBq/tube), 100 mmol/l HEPES, and 50 mmol/l $MgCl_2$ (pH 7.6) for 5 min. The reaction was stopped by the addition of 15 μl of 8N HCl. The products were extracted by adding 130 μl chloroform-methanol (1:1) followed by centrifugation. The organic phase was removed and spotted on a silica gel thin-layer chromatography plate (Merck), and the plates were developed and dried. The phosphorylated inositol was visualized by autoradiography and quantitated with the BAS 2000 image analyzer (Fuji Film, Tokyo, Japan) (13,14).

Akt activity assay. The Akt kinase assay was performed using an Akt kinase assay kit (New England Biolabs, Beverly, MA). In brief, the tissue lysates were immunoprecipitated with immobilized anti-Akt antibody for 3 h at 4°C. The precipitates were washed twice with the cell lysis buffer and twice with a kinase buffer containing 25 mmol/l Tris, 5 mmol/l β-glycerophosphate, 2 mmol/l DTT, 0.1 mmol/l Na_3VO_4, and 10 mmol/l $MgCl_2$ (pH 7.5). Then 40 μl of the pellets were suspended with 200 μmol/l ATP and 1 μg GSK-3 fusion protein and incubated for 30 min at 30°C. The reaction was terminated by adding SDS sample buffer containing 187.5 mmol/l Tris, 6% wt/vol SDS, 30% glycerol, 150 mmol/l DTT, and 0.03% wt/vol bromophenol blue (pH 6.8). The samples were then separated by 12% SDS-PAGE and transferred onto PVDMs. The membranes were blocked and probed with anti–Ser$^{21/9}$-phosphospecific GSK-3 antibody for 16 h at 4°C. After the membranes were washed, the blots were incubated with horseradish peroxidase−linked second antibody then examined by ECL using ECL reagent according to the manufacturer's instructions (Amersham) (13,14).

Statistical analysis. Data are presented as means ± SE. *P* values were determined by a paired *t* test or Bonferroni test with ANOVA; $P < 0.05$ was considered statistically significant.

RESULTS

Change in amount of SHIP-2 protein in skeletal muscle, fat tissue, and liver of *db/db* mice and high-fat−fed *db/+*m mice. The *db/db* mouse is known to be an insulin-resistant diabetic animal model (27). We examined the expression level of SHIP-2 in skeletal muscle, epididymal fat tissue, and liver of *db/db* and *db/+*m mice. SHIP-2 was expressed in the quadriceps muscle, epididymal fat

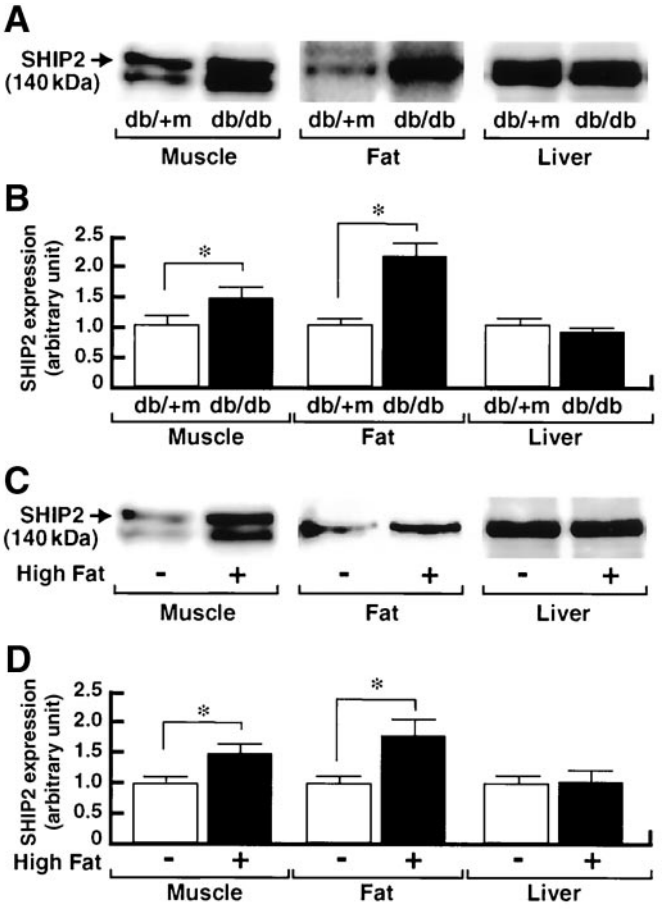

FIG. 1. Change in the amount of SHIP-2 protein in the skeletal muscle, fat tissue, and liver of *db/db* mice and high fat–fed mice. Tissue samples obtained from the quadriceps muscle, epididymal fat tissue, and liver of *db/db* mice and *db*/+m mice (*A*), or *db*/+m mice with high-fat diet and *db*/+m mice with normal diet (*C*) were subjected to immunoblot analysis with anti–SHIP-2 antibody. Representative results are shown. The amount of SHIP-2 was quantitated by densitometry. Results are means ± SE of four separate experiments, and the amount of SHIP-2 in each tissue of *db*/+m mice or *db*/+m mice with normal diet is assigned a value of 1. *$P < 0.05$ vs. the amount of SHIP-2 in *db*/+m mice (*B*) or in *db*/+m mice with normal diet (*D*) by Bonferroni test.

tissue, and liver of *db*/+m mice age 8 weeks. Similar amounts of SHIP-2 were also expressed in these tissues in C57BL/6J mice age 8 weeks (data not shown). Interestingly, the amount of SHIP-2 protein was increased in the skeletal muscle and fat tissue, but not in the liver of *db/db* mice compared with *db*/+m mice (Fig. 1*A*). Thus the amount of expressed SHIP-2 protein in the skeletal muscle and fat tissue of *db/db* mice was significantly increased by 45.4 ± 7.9 and 105.1 ± 13.5%, respectively, compared with *db*/+m mice (Fig. 1*B*). The increase in SHIP-2 expression was already seen in these tissues of *db/db* mice at age 4 weeks (data not shown). The amount of expressed SHIP-2 protein in *db*/+m mice fed the high-fat diet was examined to investigate whether the increase seen was limited to *db/db* mice. The amount of expressed SHIP-2 protein was also increased in the skeletal muscle and fat tissue of *db*/+m mice with insulin resistance induced by a high-fat diet (Fig. 1*C*). The expression of SHIP-2 was increased by 52.3 ± 6.5 and 81.4 ± 8.9%, respectively, compared with control *db*/+m mice (Fig. 1*D*).

Amount of SHIP-2 protein in the membrane preparation in *db/db* and *db*/+m mice. After ligand stimulation,

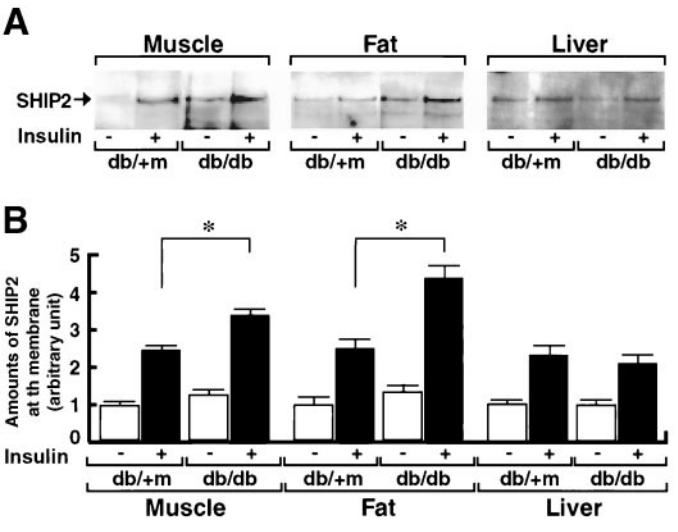

FIG. 2. Insulin-induced membrane localization of SHIP-2 in *db/db* mice and *db*/+m mice. *A*: Total membrane preparations obtained from the quadriceps muscle, epididymal fat tissue, and liver of *db/db* mice and *db*/+m mice were subjected to immunoblot analysis with anti–SHIP-2 antibody. Representative results are shown. *B*: The amount of membrane that localized SHIP-2 was quantitated by densitometry. Results are means ± SE of four separate experiments, and the amount of SHIP-2 in each tissue of *db*/+m mice is assigned a value of 1. *$P < 0.05$ vs. the amount of SHIP-2 in *db*/+m mice by Bonferroni test.

SHIP-2 translocates to the plasma membrane where SHIP-2 can access its substrate PI(3,4,5)P3 for hydrolysis to PI(3,4)P2 (30,31). We next compared the amount of SHIP-2 in the total membrane preparations in the skeletal muscle, fat tissue, and liver of *db/db* mice with that of *db*/+m mice. Insulin stimulated translocation of SHIP-2 to the membrane preparation in all three tissues of *db*/+m mice. The amount of membrane-associated SHIP-2 was increased in the skeletal muscle and fat tissue, but not in the liver of *db/db* mice compared with *db*/+m mice (Fig. 2*A*). The amount of SHIP-2 located at the membrane was increased by 40.6 ± 7.9% in the skeletal muscle and 82.3 ± 8.4% in the fat tissue of *db/db* mice, respectively, compared with *db*/+m mice. Thus, the increase of SHIP-2 at the total membrane preparation is well correlated with the elevated amount of total SHIP-2 (Fig. 2*B*).

Insulin stimulation of PI 3-kinase activity, Akt activity, and PKC-ζ/λ phosphorylation in *db/db* mice and *db*/+m mice. Insulin-induced PI 3-kinase activation is the key for the biological action of insulin (1–3). Insulin stimulation increased PI 3-kinase activity by 2.6 ± 0.1-fold in the skeletal muscle, 2.3 ± 0.3-fold in the fat tissue, and 2.8 ± 0.7-fold in the liver of *db*/+m mice. Insulin-induced PI 3-kinase activity was decreased by 38.1 ± 6.4, 48.2 ± 4.2, and 54.3 ± 9.7%, respectively, in *db/db* mice. Therefore, the moderate decrease in insulin-induced PI 3-kinase activation was seen in all three tissues of *db/db* mice compared with *db*/+m mice (Figs. 3*A* and 4). Akt and atypical PKC are downstream target molecules of PI 3-kinase, which are important for further transmission of the metabolic signaling of insulin (8,32–34). Because the expression of SHIP-2 was enhanced in the skeletal muscle and fat tissue of *db/db* mice, we examined how insulin-stimulated activity of Akt and PKC-ζ/λ was affected in *db/db* mice compared with *db*/+m mice. Insulin induced Akt activation by 2.8 ± 0.3-fold in the skeletal muscle,

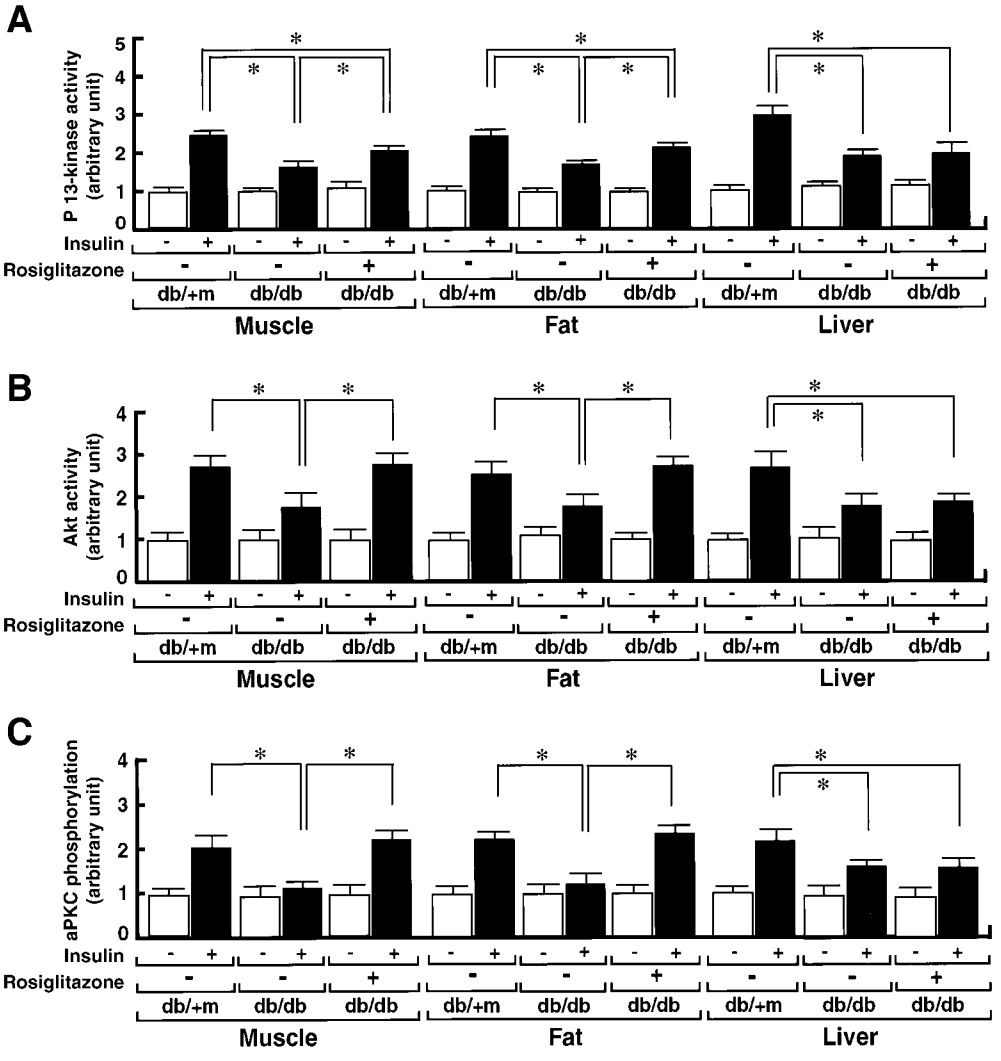

FIG. 3. Insulin stimulation of PI 3-kinase activity, Akt activity, and PKC-ζ/λ phosphorylation in *db/db* mice and *db*/+m mice, and the effect of rosiglitazone. After insulin stimulation, tissue lysates were obtained from the quadriceps muscle, epididymal fat tissue, and liver of *db/db* mice and *db*/+m mice. *A*: The tissue lysates were immunoprecipitated with anti-phosphotyrosine antibody. The washed precipitates were assayed for PI 3-kinase activity with PI as a substrate, and the labeled PI(3)P product was resolved by thin-layer chromatography and visualized by autoradiography. The labeled PI(3)P was quantitated by densitometry. *B*: The tissue lysates were immunoprecipitated with anti-Akt antibody. The washed precipitates were assayed for Akt activity with GSK-3 as the substrate. The samples were then separated by SDS-PAGE and immunoblotted with anti–$Ser^{21/9}$-phosphospecific GSK3 antibody. The amount of phosphorylated GSK3 was quantitated by densitometry. *C*: The tissue lysates were subjected to SDS-PAGE and immunoblotted with anti–$Thr^{410/403}$ phosphospecific PKC-ζ/λ antibody. The amount of phosphorylated PKC-ζ/λ was quantitated by densitometry. These results are means ± SE of four separate experiments, and the basal level in each tissue of *db*/+m mice is assigned a value of 1. *$P < 0.05$, compared by Bonferroni test.

2.6 ± 0.4-fold in the fat tissue, and 2.8 ± 0.6-fold in the liver of *db*/+m mice. Insulin-induced Akt activation was decreased by 59.0 ± 3.2, 65.0 ± 4.4, and 58.3 ± 5.2%, respectively, in *db/db* mice. Thus, insulin-induced Akt activation in the skeletal muscle and fat tissue, but not in the liver, was more severely affected than PI 3-kinase in *db/db* mice (Figs. 3*B* and 4). On the other hand, insulin increased atypical PKC phosphorylation by 2.2 ± 0.4-fold in the skeletal muscle, 2.3 ± 0.3-fold in the fat tissue, and 2.2 ± 0.5-fold in the liver of *db*/+m mice. PKC-ζ/λ phosphorylation was decreased by 88.5 ± 3.6, 86.3 ± 4.0, and 51.0 ± 5.5%, respectively, in *db/db* mice. Apparently, insulin-induced phosphorylation of PKC-ζ/λ in the skeletal muscle and fat tissue, but not in the liver, was decreased more markedly than PI 3-kinase in *db/db* mice (Figs. 3*C* and 4). The amount of the p85 regulatory subunit of PI 3-kinase, PKB, and PKC-ζ/λ in the skeletal muscle, fat tissue, and liver was similar between *db/db* mice and *db*/+m mice (data not shown). These results indicate that the activity of molecules downstream of PI 3-kinase in the skeletal muscle and fat tissue of *db/db* mice is decreased, at least in part, by increased expression of SHIP-2.

Effect of rosiglitazone on the amount of SHIP-2 protein in *db/db* mice and *db*/+m mice. The *db/db* mice in this study were obese, hyperglycemic, and hyperinsulinemic compared with the control *db*/+m mice. The 2-week treatment with rosiglitazone resulted in a reduction in serum glucose and insulin levels in *db/db* mice, but did not affect these levels in *db*/+m mice. Rosiglitazone treatment did not affect body weight in either *db/db* mice or *db*/+m mice (Table 1). We next examined whether rosiglitazone affects the amount of SHIP-2 protein in these tissues of *db/db* mice and *db*/+m mice. Rosiglitazone treatment had a tendency (which was not statistically

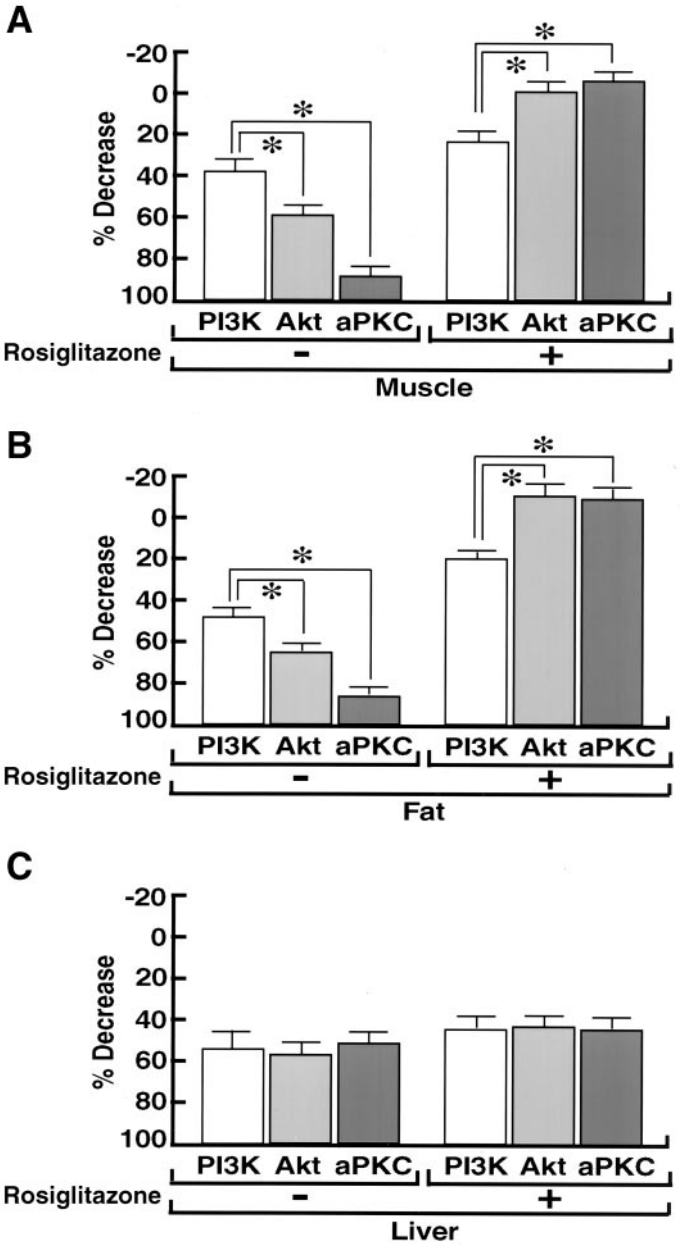

FIG. 4. Comparison of the change of PI 3-kinase activity, Akt activity, and PKC-ζ/λ phosphorylation in *db/db* mice. The results with insulin-induced PI 3-kinase activity, Akt activity, and PKC-ζ/λ phosphorylation in the skeletal muscle (*A*), fat tissue (*B*), and liver (*C*) of *db/db* mice without or with rosiglitazone treatment were expressed as the percent decrease compared with those of *db*/+m mice. Results are means ± SE of four experiments. **P* < 0.05 vs. the decrease in PI 3-kinase activity in *db*/+m mice by paired *t* test.

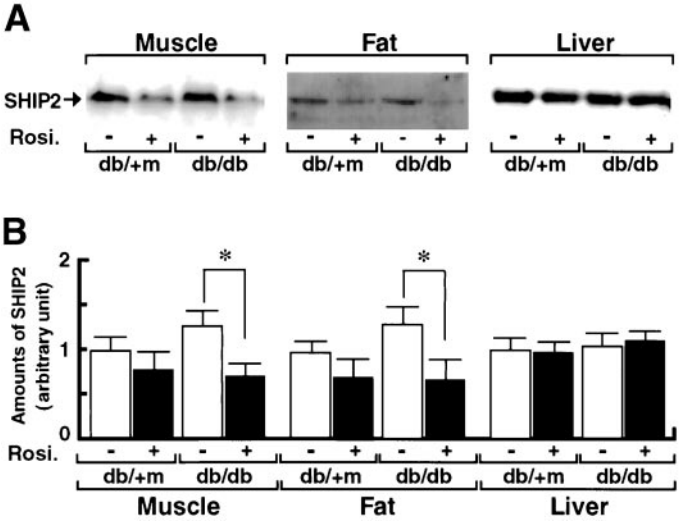

FIG. 5. Effect of rosiglitazone on the amount of SHIP-2 protein in *db/db* mice and *db*/+m mice. *A*: Rosiglitazone was administered for 2 weeks in the case of treated mice. Tissue samples obtained from the quadriceps muscle, the epididymal fat tissue, and the liver of *db/db* mice and *db*/+m mice were subjected to immunoblot analysis with anti–SHIP-2 antibody. Representative results are shown. *B*: The amount of SHIP-2 was quantitated by densitometry. Results are means ± SE of four separate experiments, and the amount of SHIP-2 in each tissue of *db*/+m is assigned a value of 1. **P* < 0.05 vs. the amount of SHIP-2 without rosiglitazone treatment by Bonferroni test.

significant) to decrease the amount of SHIP-2 in the skeletal muscle and fat tissue of *db*/+m mice. Importantly, treatment with rosiglitazone significantly reduced the amount of SHIP-2 protein in the skeletal muscle and fat tissue, but not in the liver, of *db/db* mice (Fig. 5*A*). The amount of SHIP-2 protein was decreased by 46.1 ± 4.3% in the skeletal muscle and 50.2 ± 5.1% in the fat tissue of *db/db* mice (Fig. 5*B*).

Effect of rosiglitazone on insulin-induced PI 3-kinase activation, Akt activation, and PKC-ζ/λ phosphorylation in *db/db* mice. Because rosiglitazone treatment decreased the amount of elevated SHIP-2 in the skeletal muscle and fat tissue of *db/db* mice, we further examined the effect of rosiglitazone on insulin-induced activation of Akt and PKC-ζ/λ compared with PI 3-kinase. Rosiglitazone treatment did not significantly affect insulin-induced PI 3-kinase activity, Akt activity, or PKC-ζ/λ phosphorylation in all three tissues of *db*/+m mice (data not shown). On the other hand, treatment with rosiglitazone partially restored insulin-induced PI 3-kinase activation in the skeletal muscle and fat tissue, but not in the liver, of *db/db* mice. It is interesting that, in accordance with the reduction in the amount of SHIP-2 by rosiglitazone, insulin-induced Akt activation and PKC-ζ/λ phosphorylation were completely restored to the control level in the skeletal muscle and fat tissue, but not in the liver, of *db/db* mice (Figs. 3 and 4). The amount of the p85 regulatory subunit of PI 3-kinase, Akt, and PKC-ζ/λ in the skeletal muscle, fat tissue, and liver was not affected by treatment with rosiglitazone in either *db/db* mice or *db*/+m mice (data not shown). These results further indicate that SHIP-2 is an important factor associated with the insulin resistance in *db/db* mice.

DISCUSSION

It is reported that targeted disruption of the SHIP-2 gene in mice resulted in increased insulin sensitivity without affecting biological systems other than insulin signaling (15). As the molecular mechanism by which SHIP-2 is involved in the regulation of insulin signaling, we have reported that overexpression of SHIP-2 inhibits insulin-induced activation of Akt and atypical PKC via the 5′-phosphatase activity of SHIP-2, and that PI(3,4,5)P3 rather than PI(3,4)P2 is important for further transmission of insulin signaling via PI 3-kinase in 3T3-L1 adipocytes and L6 myotubes (13,14). These previous findings indicated that SHIP-2 is a physiologically important negative regulator of insulin signaling. In the present study, we demonstrate

TABLE 1
Effect of rosiglitazone on body weight, serum glucose, and insulin in *db/db* and *db*/+m mice

Experimental groups	*n*	Body weight (g)	Glucose (mg/dl)	Insulin (ng/ml)
Untreated *db*/+m mice	8	23.8 ± 1.6	125.9 ± 20.4	0.6 ± 0.1
Rosiglitazone-treated *db*/+m mice	8	22.3 ± 1.3	115.0 ± 19.4	0.5 ± 0.1
Untreated *db/db* mice	8	38.4 ± 3.7*	355.7 ± 50.9*	7.0 ± 0.5*
Rosiglitazone-treated *db/db* mice	8	37.4 ± 4.6	182.1 ± 21.1†	4.7 ± 0.4†

Data are means ± SE. *$P < 0.05$ vs. *db*/+m mice; †$P < 0.05$ vs. untreated *db/db* mice.

that the amount of SHIP-2 protein is increased in the skeletal muscle and fat tissue of obese, insulin-resistant, diabetic *db/db* mice. In association with the increased amount of SHIP-2 protein, insulin signaling appears to be impaired, at least in part, at the level distal to PI 3-kinase in the activation of Akt and atypical PKC. Taken together, our findings appear to indicate that SHIP-2 is not only involved in the physiological regulation of insulin signaling, but is also associated with the state of insulin resistance. However, we cannot rule out the possibility that a primary defect exists at the level of phosphoinositide dependent kinase 1 or the activation of Ser/Thr phosphatase, such as PP2A, in the regulation of Akt and atypical PKC in *db/db* mice. The increased SHIP-2 expression seen in the skeletal muscle and fat tissue was not limited to obese, hyperglycemic, insulin-resistant *db/db* mice. The increased amount of SHIP-2 was also observed in mice with insulin resistance induced by a high-fat diet. Therefore, the increased expression of SHIP-2 might be a relatively common phenomenon seen in insulin resistance. Further investigations, including possible alteration of SHIP-2 expression in human type 2 diabetes, are required to clarify this issue.

It is known that the total cellular 5′-phosphatase activity of SHIP-2 is not altered by ligand stimulation (30,31). Instead, SHIP-2 translocates from cytosol to plasma membrane compartment upon ligand stimulation, whereby SHIP-2 is able to access to its substrate PI(3,4,5)P3 for hydrolysis to PI(3,4)P2 (30,31). Based on these previous reports, it is possible that the function of SHIP-2 is also affected by the altered localization of SHIP-2 in *db/db* mice. However, this does not appear to be the case, as the amount of SHIP-2 in the total membrane preparation was elevated in parallel with the increased amount of total SHIP-2 in *db/db* mice compared with control *db*/+m mice. Because the amount of SHIP-2 in the membrane is normal or supernormal after insulin stimulation, the insulin-induced process of the translocation of SHIP-2 does not appear to be impaired in the skeletal muscle and fat tissue of *db/db* mice. Taken together, the increased amount of total SHIP-2 appears to be a predominant alteration seen in the skeletal muscle and fat tissue of *db/db* mice. Although more studies will be required to examine the possible functional alteration of SHIP-2 in the state of insulin resistance, this notion is further supported by the fact that rosiglitazone treatment decreased the elevated expression of SHIP-2 together with improved insulin signaling. Importantly, rosiglitazone treatment efficiently improved insulin-induced activation of Akt and PKC-ζ/λ, whereas the decreased PI 3-kinase activity in the skeletal muscle and fat tissue of *db/db* mice was only moderately restored by the treatment. These findings further indicated that SHIP-2 is associated with impaired insulin signaling at the level of Akt and PKC-ζ/λ activation, an association that is at least in part independent of the PI 3-kinase in the state of insulin resistance. In addition, they demonstrated that SHIP-2 is a novel target molecule of thiazolidinediones in the treatment of insulin resistance. Rosiglitazone also appeared to decrease the amount of SHIP-2 in the skeletal muscle and fat tissue of control *db*/+m mice, although this decrease was not statistically significant. In contrast to the results with *db/db* mice, the possible decrease in the amount of SHIP-2 did not significantly affect the metabolic parameters in *db*/+m mice. Although the reason is uncertain, the effect of the reduction of SHIP-2 amounts by treatment with rosiglitazone is likely to have great implication for improving the metabolic parameters in mice with insulin resistance.

It is uncertain which factor(s) implicated in insulin resistance regulates SHIP-2 expression in the skeletal muscle and fat tissue of *db/db* mice. Enhanced expression of SHIP-2 may be a compensatory mechanism to limit insulin signaling against chronic hyperinsulinemia seen in *db/db* mice, as SHIP-2 is the physiological negative regulator of insulin signaling (13–15). Alternatively, it is also possible to consider the more direct implication of SHIP-2 in insulin resistance. In type 2 diabetes, hyperglycemia contributes to the development of insulin resistance (1,2,19,20). It has been reported that insulin-induced PI 3-kinase activity is relatively reduced in soleus muscle, but is not impaired in EDL muscle of GK rats, whereas Akt activity and glucose transport are severely impaired in both types of muscles (18). It should be noted that Akt activity was fully restored to control levels concomitantly with improved glycemic control (18). In addition, insulin-induced Akt activity has been reported to be severely suppressed, whereas PI 3-kinase activity is increased in ZDF rat liver (19). Interestingly, the restoration to normoglycemia by sodium-dependent glucose transporter inhibitor normalized impaired insulin-induced Akt activation (19). These reports indicate that steps in insulin signaling downstream of PI 3-kinase appear to be sensitive to changes in the glycemic milieu, although the mechanism by which this occurs is unknown. Along this line, SHIP-2 appears to be a candidate factor associated with the insulin resistance with hyperglycemia. To clarify this issue, we incubated the cells in medium containing high glucose concentrations for 24 h and found an increased expression of SHIP-2 in 3T3-L1 adipocytes. Treatment with rosiglitazone partially reduced the elevated amount of SHIP-2 (K. Fukui, T. Sasaoka, and M. Kobayashi, unpublished observations). Therefore, hyperglycemia appears to partially affect the expression of SHIP-2 in skeletal muscle and fat tissue of *db/db* mice.

On the other hand, it has been reported that decreased effects of insulin-induced glucose transport in the skeletal muscle and adipocytes of GK-diabetic rats are attributable to markedly diminished phosphorylation and activation of PKC-ζ/λ, at least in part, independent of PI 3-kinase activity (23,24). Thiazolidinediones enhance insulin-induced glucose transport in the skeletal muscle and adipocyte of diabetic GK rats through increased phosphorylation and activation of PKC-ζ/λ (23,24). Improvement in insulin-induced PKC-ζ/λ activation occurs after rosiglitazone treatment, despite only modest decreases in serum glucose levels (23,24). Thus, even though serum glucose levels in diabetic *db/db* mice were markedly improved by treatment with rosiglitazone in the current study, we may need to consider additional factors other than hyperglycemia when determining the effect of rosiglitazone on the restoration of PKC-ζ/λ activation via the change of SHIP-2 expression. In view of this, recent research has focused on the role of adipocytokines secreted from adipocytes (1). Leptin, tumor necrosis factor-α, plasminogen-activator inhibitor type 1, adipsin, resistin, and adiponectin are adipocytokines known to be implicated in the insulin resistance (1). Clearly these are interesting candidates that are possibly implicated in the regulation of SHIP-2 expression. Future studies are needed to clarify the mechanism by which the expression of SHIP-2 is regulated in the state of insulin resistance.

In summary, the amount of SHIP-2 protein was increased in the skeletal muscle and fat tissue of *db/db* mice. Insulin-induced activity of molecules downstream of PI 3-kinase appeared to be decreased, at least in part, by the elevated expression of SHIP-2. Rosiglitazone ameliorated the elevated expression of SHIP-2 with marked improvement of insulin-induced Akt and PKC-ζ/λ activation. Because SHIP-2 appears to be associated with the insulin resistance, in addition to its physiological role in the negative regulation of insulin signaling, SHIP-2 would be a potential therapeutic target molecule for the treatment of insulin resistance in type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (T.S.).

We thank Drs. Takayoshi Sugihara, Hiroshi Tsuneki, Kosei Kikuchi, Mika Kishida, and Ikuko Kimura for their technical assistance and comments in the tissue preparation of animals.

REFERENCES

1. Saltiel AR: New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* 104:517–529, 2001
2. Virkamäki A, Ueki K, Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931–943, 1999
3. Czech MP, Corvera S: Signaling mechanisms that regulate glucose transport. *J Biol Chem* 274:1865–1868, 1999
4. Stephens LR, Hughes KT, Irvine RF: Pathway of phosphatidylinositol(3,4,5)-triphosphate synthesis in activated neutrophils. *Nature* 351:33–39, 1991
5. Funaki M, Katagiri H, Kanda A, Anai M, Nawano M, Ogihara T, Inukai K, Fukushima Y, Ono H, Yazaki Y, Kikuchi M, Oka Y, Asano T: p85/p110-type phosphatidylinositol kinase phosphorylates not only the D-3, but also the D-4 position of the inositol ring. *J Biol Chem* 274:22019–22024, 1999
6. Toker A, Cantley LC: Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 387:673–676, 1997
7. Majerus PW, Kisseleva MV, Norris FA: The role of phosphatases in inositol signaling reactions. *J Biol Chem* 274:10669–10672, 1999
8. Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese RV: Protein kinase C-ζ as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes: potential role in glucose transport. *J Biol Chem* 272:30075–30082, 1997
9. Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PRJ, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT: Protein kinase B: kinases that mediate phosphatidylinositol 3,4,5-triphosphate-dependent activation of protein kinase B. *Science* 279: 710–714, 1998
10. Stokoe D, Stephens LR, Copeland T, Gaffney PRJ, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT: Dual role of phosphatidylinositol-3,4,5-triphosphate in the activation of protein kinase B. *Science* 277:567–570, 1997
11. Pesesse X, Deleu S, Smedt FD, Drayer L, Erneux C: Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem Biophys Res Commun* 239:697–700, 1997
12. Ishihara H, Sasaoka T, Hori H, Wada T, Hirai H, Haruta T, Langlois WJ, Kobayashi M: Molecular cloning of rat SH2-containing inositol phosphatase 2 (SHIP-2) and its role in the regulation of insulin signaling. *Biochem Biophys Res Commun* 260:265–272, 1999
13. Wada T, Sasaoka T, Funaki M, Hori H, Murakami S, Ishiki M, Haruta T, Asano T, Ogawa W, Ishihara H, Kobayashi M: Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3–L1 adipocytes via its 5′-phosphatase catalytic activity. *Mol Cell Biol* 21:1633–1646, 2001
14. Sasaoka T, Hori H, Wada T, Ishiki M, Haruta T, Ishihara H, Kobayashi M: SH2-containing inositol phosphatase 2 negatively regulates insulin-induced glycogen synthesis in L6 myotubes. *Diabetologia* 44:1258–1267, 2001
15. Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Marchand-Brustel YL, Erneux C, Hue L, Schurmans S: The lipid phosphatase SHIP-2 controls insulin sensitivity. *Nature* 409:92–97, 2001
16. Kim YB, Zhu JS, Zierath JR, Shen HQ, Baron AD, Kahn BB: Glucosamine infusion in rats rapidly impairs insulin stimulation of phosphoinositide 3-kinase but does not alter activation of Akt/protein kinase B in skeletal muscle. *Diabetes* 48:310–320, 1999
17. Kim YB, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB: Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* 104:733–741, 1999
18. Song XM, Kawano Y, Krook A, Ryder JW, Efendic S, Roth RA, Wallberg-Henriksson H, Zierath JR: Muscle fiber type-specific defects in insulin signal transduction to glucose transport in diabetic GK rats. *Diabetes* 48:664–670, 1999
19. Nawano M, Ueta K, Oku A, Arakawa K, Saito A, Funaki M, Anai M, Kikuchi M, Oka Y, Asano T: Hyperglycemia impairs the insulin signaling step between PI 3-kinase and Akt/PKB activations in ZDF rat liver. *Biochem Biophys Res Commun* 266:252–256, 1999
20. Kurowski TG, Lin Y, Luo Z, Tsichlis PN, Buse MG, Heydrick SJ, Ruderman NB: Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphatidylinositol 3-kinase in rat skeletal muscle. *Diabetes* 48:658–663, 1999
21. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1286, 1998
22. Carvalho E, Eliasson B, Wesslau C, Smith U: Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from type II diabetic subjects. *Diabetologia* 43:1107–1115, 2000
23. Kanoh Y, Bandyopadhyay G, Sajan MP, Standaert ML, Farese RV: Thiazolidinedione treatment enhances insulin effects on protein kinase C-ζ/λ activation and glucose transport in adipocytes of non diabetic and Goto-Kakizaki type II diabetic rats. *J Biol Chem* 275:16690–16696, 2000
24. Kanoh Y, Bandyopadhyay G, Sajan MP, Standaert ML, Farese RV: Rosiglitazone, insulin treatment, and fasting correct defective activation of protein kinase C-ζ/λ by insulin in vastus lateralis muscles and adipocytes of diabetic rats. *Endocrinology* 142:1595–1605, 2001
25. Tremblay F, Lavigne C, Jacques H, Marette A: Defective insulin-induced Glut4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (ζ/λ) activities. *Diabetes* 50:1901–1910, 2001

26. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763–770, 1998
27. Shao J, Yamashita H, Qiao L, Friedman JE: Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Lepr$^{db/db}$ mice. *J Endocrinol* 167:107–115, 2000
28. Ikemoto S, Thompson KS, Takahashi M, Itakura H, Lane MD, Ezaki O: High fat diet-induced hyperglycemia: prevention by low level expression of a glucose transporter (Glut4) minigene in transgenic mice. *Proc Natl Acad Sci U S A* 92:3096–3099, 1995
29. Tang Y, Osawa H, Onuma H, Nishimiya T, Ochi M, Makino H: Improvement in insulin resistance and the restoration of reduced phosphodiesterase 3B gene expression by pioglitazone in adipose tissue of obese diabetic KKAy mice. *Diabetes* 48:1830–1835, 1999
30. Taylor V, Wong M, Brandts C, Reilly L, Dean NM, Cowsert LM, Moodie S, Stokoe D: 5′ phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells. *Mol Cell Biol* 20:6860–6871, 2000
31. Pesesse X, Dewaste V, Smedt FD, Laffargue M, Giuriato S, Moreau C, Payrastre B, Erneux C: The src homology 2 domain containing inositol 5-phosphatase SHIP-2 is recruited to the epidermal growth factor (EGF) receptor and dephosphorylates phoshatidylinositol 3,4,5-triphosphate in EGF-stimulated Cos-7 cells. *J Biol Chem* 276:28348–28355, 2001
32. Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U, Kasuga M: Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not glucose transport. *Mol Cell Biol* 18:3708–3717, 1998
33. Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR, Klip A: Protein kinase B/Akt participates in Glut4 translocation by insulin in L6 myotubes. *Mol Cell Biol* 19:4008–4018, 1999
34. Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, Miyake K, Sano W, Akimoto K, Ohno S, Kasuga M: Requirement of atypical protein kinase Cλ for insulin stimulation of glucose uptake but not for Akt activation in 3T3–L1 adipocytes. *Mol Cell Biol* 18:6971–6982, 1998