Specific Inhibition of PTEN Expression Reverses Hyperglycemia in Diabetic Mice

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Signaling through the phosphatidylinositol 3′-kinase (PI3K) pathway is crucial for metabolic responses to insulin, and defects in PI3K signaling have been demonstrated in type 2 diabetes. PTEN (MMAC1) is a lipid/protein phosphatase that can negatively regulate the PI3K pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate, but it is unclear whether PTEN is physiologically relevant to insulin signaling in vivo. We employed an antisense oligonucleotide (ASO) strategy in an effort to specifically inhibit the expression of PTEN. Transfection of cells in culture with ASO targeting PTEN reduced PTEN mRNA and protein levels and increased insulin-stimulated Akt phosphorylation in α-mouse liver-12 (AML12) cells. Systemic administration of PTEN ASO once a week in mice suppressed PTEN mRNA and protein expression in liver and fat by up to 90 and 75%, respectively, and normalized blood glucose concentrations in *db/db* and *ob/ob* mice. Inhibition of PTEN expression also dramatically reduced insulin concentrations in *ob/ob* mice, improved the performance of *db/db* mice during insulin tolerance tests, and increased Akt phosphorylation in liver in response to insulin. These results suggest that PTEN plays a significant role in regulating glucose metabolism in vivo by negatively regulating insulin signaling. *Diabetes* 51:1028–1034, 2002

Phosphatidylinositol 3′-kinase (PI3K) is a crucial signaling enzyme whose activity is regulated by a variety of biological stimuli, including insulin (1). PI3K is composed of two subunits: the p85 regulatory subunit, containing two Src homology-2 domains, and the p110 catalytic subunit (2–4). Binding of insulin to its receptor activates the insulin receptor tyrosine kinase, resulting in autophosphorylation and phosphorylation of several substrates, including insulin receptor substrate (IRS)-1 through -4. IRS then binds to the regulatory subunit of PI3K through its Src homology domains, and this interaction activates the catalytic unit. Activated PI3K phosphorylates the 3′-position of the ring in inositol phospholipids, generating phosphatidylinositol (3,4), diphosphate, and phosphatidylinositol (3,4,5)-triphosphate (PIP3). The lipid products of PI3K initiate phosphorylation and activation of Akt, which is believed to act as a downstream mediator of many of the metabolic effects of insulin (5,6). Thus, the expression of inactive PI3K mutants or chemical agents, such as wortmannin and LY294002 that interfere with PI3K activity, inhibit Akt phosphorylation, glucose uptake, and glycogen and lipid synthesis in vitro (7,8).

PTEN (MMAC1/TEP1) is a dual-specificity protein phosphatase involved in signal transduction and tumor suppression (9,10). PTEN also has phosphoinositide 3′-phosphatase activity and is therefore capable of suppressing PI3K signaling by dephosphorylating PIP3 (11,12). Mutations in PTEN have been associated with several human cancers, and mice heterozygous for the PTEN gene have a high incidence of spontaneous tumors (13,14). Tumor cells and fibroblasts deficient in PTEN have elevated levels of PIP3 and phosphorylated Akt/protein kinase B (PKB) and are resistant to many apoptotic stimuli (15,16).

Because many of the metabolic effects of insulin are mediated through activation of PI3K and the subsequent rise in intracellular PIP3 concentrations, inhibition of a negative regulator of this pathway may enhance insulin signaling. Although the tumor-suppressive functions of PTEN have been elucidated, its physiological role in glucose metabolism in vivo is largely unknown. Inhibition of the *daf-18* gene, a homolog of PTEN in *Caenorhabditis elegans*, can partially bypass the need for DAF-2, an insulin receptor-like molecule (17,18). PTEN overexpression in vitro inhibits glucose uptake and GLUT4 transport in 3T3L1 cells, whereas microinjection of PTEN antibodies increased GLUT4 translocation (19). These results suggest that PTEN may modulate insulin signaling in vivo; however, the lethality of the PTEN null mutation has made this difficult to study. We therefore designed and characterized antisense oligonucleotides (ASOs) targeting PTEN and used them in vitro and in vivo to determine whether the inhibition of PTEN expression affects insulin signaling and glucose metabolism.

RESEARCH DESIGN AND METHODS

Oligonucleotides. A total of 80 oligonucleotides were screened for their ability to inhibit PTEN mRNA expression in T-24 bladder carcinoma cells by quantitative real-time RT-PCR. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2′-*O*-methoxyethyl groups on bases 1–5 and 16–20. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (Perkin Elmer-Applied Biosystems) and purified as described (20). Two active PTEN ASOs complementary to human and mouse PTEN mRNA (Genbank accession nos. AA017584 and AA124728, respectively), a six-base mismatch, and a control

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Received for publication 27 September 2001 and accepted in revised form 27 December 2001.

AS1, antisense oligonucleotide 1; ASO, antisense oligonucleotide; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; IRS, insulin receptor substrate; MIS, mismatch control oligonucleotide; PI3K, phosphatidylinositol 3′-kinase; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKB, protein kinase B; UC, universal control oligonucleotide.

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oligonucleotide were used in the experiments described and are designated as follows: antisense oligonucleotide 1 (AS1) (ISIS 116847: 5′-CTGCTAGCCTCT GGATTTGA-3′, beginning at position 2097 in the human RNA); AS2 (ISIS 116845: 5′-CACATAGCGCCTCTGACTGGG-3′, beginning at position 1,539); MIS (ISIS 116848: 5′-CTTCTGGCATCCGGTTTAGA-3′, a six base mismatch to AS1); and UC, a universal control (ISIS 29848: synthesized using a mix of random mixture of A, G, T, and C so that the resulting preparation represents an equimolar mixture of all possible four [19] oligonucleotides).

Cell culture. 3T3L1 murine fibroblasts (American *Type Culture* Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (growth media). Cells were grown to confluence in 12- or 24-well plates before initiating differentiation. Confluent monolayers were differentiated to the adipocyte phenotype by culturing with 500 μmol/l isobutulmethylaxanthine, 250 nmol/l dexamethasone, and 400 nmol/l insulin in growth media for 3 days, followed by growth media alone for 3 days. Following this protocol, >90% of the adipocytes express the fully differentiated phenotype by 6 days after initiation.

Fully differentiated 3T3L1 adipocytes were transfected by the addition of serum-free DMEM and FuGENE6 (Roche) following the manufacturers instructions. The final concentration of 500 nmol/l oligonucleotide and a ratio of 4 μl FuGENE6 per microgram oligonucleotide were empirically determined to maximally suppress target RNA expression. Cell media was typically refreshed 36 h after transfection.

AML12 cells (American *Type Culture* Collection), a nontransformed hepatocyte cell line from transforming growth factor-β transgenic mice, were used to demonstrate antisense-mediated PTEN protein reduction and insulin-stimulated Akt phosphorylation in vitro. The cells were maintained in 90% of 1:1 mixture of DMEM and Ham's F12 medium containing 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone, and 10% fetal bovine serum. The cells were treated with AS1 or mismatch control oligonucleotide (MIS) for 72 h using Lipofectin (Gibco) as a transfection agent per the manufacturer's instructions. Because AML12 cells require insulin for maintenance, the cells were serum- and insulin-starved for 8 h after transfection, and then 100 nmol/l insulin was added for 30 min before harvesting in lysis buffer for Western blotting.

Northern blots. RNA was prepared from cultured cells using a Qiagen RNA Easy Kit and from animal tissues homogenized in guanidinium isothiocyanate followed by cesium chloride gradients (21). Northern blots were performed as described using full cDNA probes generated by RT-PCR (22). The RNA signal was detected using a PhosphorImager (Molecular Dynamics) and normalized against the signal for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) using ImagePro software.

Western blots. Cells or tissues were harvested in lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris, pH 7.5, 1% Triton X-100, 0.5% NP-40, 0.25% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.2 mmol/l ortho-vanadate, 1 mmol/l NaF, and 1:200 dilution of protease cocktail III; (Calbiochem), and the proteins were separated by SDS-PAGE. After transfer of the proteins onto polyvinylidine fluoride membranes, the blots were reacted with antibodies to phospho-Akt (New England Biolabs), Akt (New England Biolabs), or PTEN and developed using enhanced chemiluminescence (Amersham Pharmacia).

Mice and metabolic measurements. All animal experiments were performed under the institutional American Association for the Accreditation of Laboratory Animal Care (AALAC) guidelines. Male *db/db* mice (C57BLKS/J-*m* +/+*Lepr*^{db}) and age-matched lean littermates (C57BLKS/J-*m*+/+ *Lepr*^{db}) at 10 weeks of age or male *ob/ob* (C57BL/6J-*Lep*^{ob}) at 8 weeks of age (The Jackson Laboratory) were used for all experiments. Mice were maintained on a 12-h light/dark cycle and fed ad libitum unless otherwise noted. Whole blood was obtained from the retro-orbital sinus of fed mice, and glucose was measured using a Metabolics glucose oxidase-based analyzer. Mice were weighed once a week, and food intake was monitored over a 24-h period. An insulin tolerance test was performed after a 4-h fast by intraperitoneal injection of 1 unit/kg human insulin (Lilly). Blood was drawn from the tail before insulin injection (time 0) and then 30, 60, and 90 min afterward and measured as described above. For in vivo phospho-Akt measurements, mice were fasted for 12 h and then injected with 2 units/kg insulin.

Serum glucose, triglycerides, and cholesterol concentrations were analyzed on a Johnson and Johnson Vitros 950 automated clinical chemistry analyzer, and serum insulin concentrations were quantitated using an enzyme-linked immunosorbent assay for rat insulin (Alpco).

RESULTS

Characterization of PTEN ASO in vitro. ASOs designed to be complementary to human and mouse PTEN genomic sequences were screened for suppression of PTEN mRNA expression in cells in vitro, as previously described (23). The most potent oligonucleotide from the screen, AS1 (see RESEARCH DESIGN AND METHODS), reduced PTEN mRNA levels in a concentration-dependent manner in 3T3-L1 adipocytes. Similar results were obtained with a second PTEN antisense (AS2) that hybridizes to a different region of the PTEN mRNA. A control antisense with six mismatched nucleotides (MIS) to AS1 was inactive in reducing PTEN mRNA expression. Maximal inhibition (90%) of PTEN protein expression was achieved after 72 h of oligonucleotide treatment, which is presumably indicative of the intrinsic half-life of the PTEN protein (data not shown).

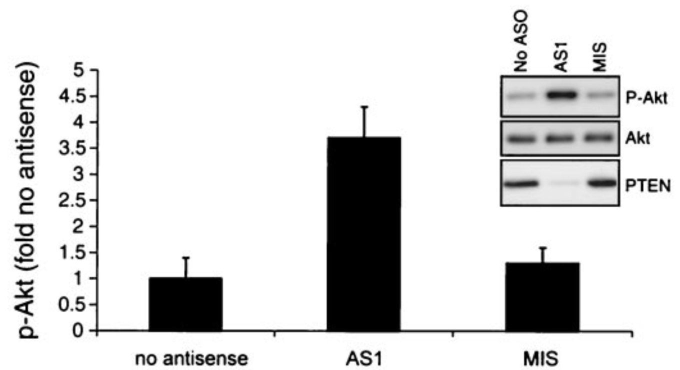

FIG. 1. Reduction of PTEN protein levels and stimulation of Akt phosphorylation by PTEN antisense treatment in vitro. AML12 cells were treated with AS1 or MIS for 72 h using Lipofectin as a transfection agent. Immunoblots of cell proteins were sequentially reacted with antibodies to phospho-Akt, -Akt, and -PTEN. Data are expressed as the fold increase in phospho-Akt intensity compared with Lipofectin-only treated cells. Graphs represent the mean of three replicates ±SD.

As described above, PIP3 is believed to initiate phosphorylation and activation of Akt, an important downstream mediator of the metabolic effects of insulin (24). We reasoned that if PTEN is indeed involved in modulating insulin signaling, inhibition of PTEN expression might increase the level of Akt phosphorylation in response to insulin. To test this, AML12 cells were treated with PTEN AS1, and the effects on PTEN protein levels and insulin-stimulated Akt phosphorylation were examined. Cells treated with PTEN AS1 resulted in a >90% reduction in PTEN protein levels (Fig. 1). Furthermore, PTEN AS1 treatment resulted in an increase in insulin-stimulated phosphorylation of Akt by ~3.5-fold, relative to untreated and MIS-treated cells (Fig. 1), whereas the Akt protein levels remained the same.

Antisense-mediated inhibition of PTEN expression in vivo. Based on the in vitro results obtained with AS1, we reasoned that inhibition of PTEN expression might improve insulin sensitivity in the *db/db* mouse, a rodent model of type 2 diabetes. First, we investigated the ability of systemically administered AS1 to reduce PTEN mRNA and protein levels in insulin-sensitive tissues. The half-life of 2′-*O*-methoxyethyl chimeric phosphorothioate oligonucleotides is ~7 to 19 days in the liver, depending on the dose (25). Therefore, *db/db* mice were treated by intraperitoneal injection once a week for 4 weeks with 10, 25, or 50 mg/kg of AS1, and PTEN mRNA levels in liver, fat, and muscle tissues were measured by Northern blotting. PTEN mRNA levels were reduced in a dose-dependent manner in

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liver extracts from treated mice relative to saline controls, with maximal inhibition occurring (88%) at the 50-mg/kg dose (Fig. 2*A*). In lean littermates dosed with 100 mg/kg of AS1, PTEN mRNA levels were also reduced by >90%, relative to saline-treated controls. There was no apparent difference in the relative levels of PTEN mRNA in untreated lean versus *db/db* mice. Moreover, neither the MIS nor another control oligonucleotide, universal control oligonucleotide (UC), affected PTEN mRNA levels significantly. Also, the mRNA levels of PTP1B and SHIP2, two other phosphatases that have the potential to inhibit insulin signaling (26,27), were not affected by PTEN AS1 treatment (Fig. 2*B*). These results demonstrate that the effect of AS1 was both PTEN target–specific and antisense sequence–specific and indicate that the metabolic effects of the PTEN antisense (described below) were primarily caused by a specific reduction in PTEN expression.

PTEN protein levels in liver samples from saline-, AS1-, and UC-treated *db/db* mice were analyzed by Western blotting. After 4 weeks of AS1 treatment, a dose-dependent decrease in PTEN protein levels in livers from *db/db* mice was observed (Fig. 2*C*). Reduction of PTEN protein levels was also observed in livers from lean littermates treated with AS1. As with the mRNA results, no difference in the relative levels of PTEN protein in control lean versus *db/db* mouse livers was apparent, nor were any effects observed by a control oligonucleotide (UC) on PTEN protein levels.

Northern analysis of other insulin-sensitive tissues demonstrated that PTEN mRNA levels were also reduced in a dose-dependent manner, relative to saline controls, in fat tissue from AS1-treated *db/db* mice, with maximal inhibition of 80% at the 50 mg/kg dose (Fig. 2*D*). A similar reduction in PTEN protein levels in fat from AS1-treated mice was also observed (data not shown). PTEN message levels appeared to be less abundant in muscle relative to liver and fat, and no consistent reduction in PTEN mRNA expression was observed in the skeletal muscle of animals treated with PTEN AS1. This result is in agreement with pharmacokinetic studies showing that accumulation of oligonucleotides in muscle after parenteral injection is relatively low (28). Interestingly, no PTEN protein was detectable on immunoblots of muscle lysates (data not shown).

Effect of inhibiting PTEN expression on glucose, insulin, and lipid concentrations in diabetic and lean mice. Having characterized the effect of AS1 on PTEN mRNA and protein expression in vivo, we next investigated the effect of inhibiting PTEN expression on hyperglycemia in *db/db* and *ob/ob* mice. Blood glucose concentrations in *db/db* mice were reduced in a dose-dependent manner over the course of a 4-week treatment with AS1 (Fig. 3*A*), becoming normalized (138 ± 5 mg/dl) at the highest dose tested (50 mg/kg). The second PTEN antisense, AS2, produced a similar reduction in serum glucose levels at the end of 4 weeks of treatment (Fig. 3*B*). In related studies, treatment of *db/db* mice with PTEN AS2 resulted in a reduction of PTEN mRNA and protein levels in liver that was comparable with that produced in animals treated with PTEN AS2 (data not shown). In contrast, neither the MIS nor UC controls affected glucose levels. Furthermore, PTEN antisense treatment had no effect on glucose concentrations in lean littermates, despite the fact

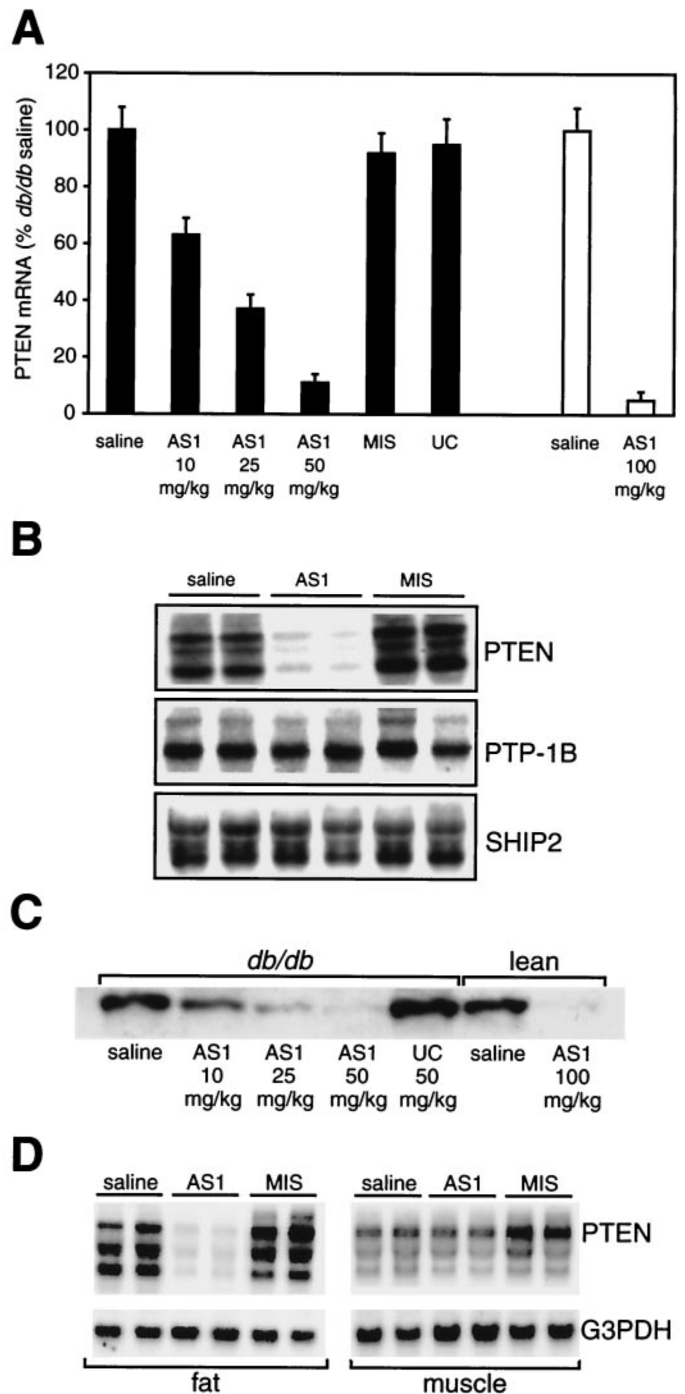

FIG. 2. PTEN antisense specifically reduces PTEN mRNA and protein levels in livers and fat from *db/db* mice. *A*: Dose-dependent reduction of PTEN mRNA levels in liver. *db/db* (black bars) and lean (white bars) mice received indicated doses of ASOs intraperitoneally once a week for 4 weeks. Total mRNA was prepared from liver and was analyzed by Northern blotting ($n = 3$ per group). The PTEN signal was normalized against the signal for G3PDH. Data are expressed as the mean percentage of mRNA levels in saline-treated *db/db* mice ±SD. *B*: Specificity of PTEN antisense. Representative Northern blots of PTEN mRNA (*A*), PTP1B mRNA (*B*), and SHIP2 mRNA (*C*) in livers from *db/db* mice treated once a week for 4 weeks with saline, 50 mg/kg AS1, or 50 mg/kg MIS. Each lane contained 25 μg of RNA from an individual animal. *C*: Reduction of PTEN protein expression in liver. PTEN immunoblots of proteins in liver lysates from mice treated for 4 weeks with indicated doses of AS1 or UC. Each lane contained 50 μg of protein. *D*: Reduction of PTEN mRNA in fat but not muscle. Representative Northern blots of PTEN and G3PDH mRNA in fat and muscle from *db/db* mice treated with saline, 50 mg/kg AS1, or 50 mg/kg MIS.

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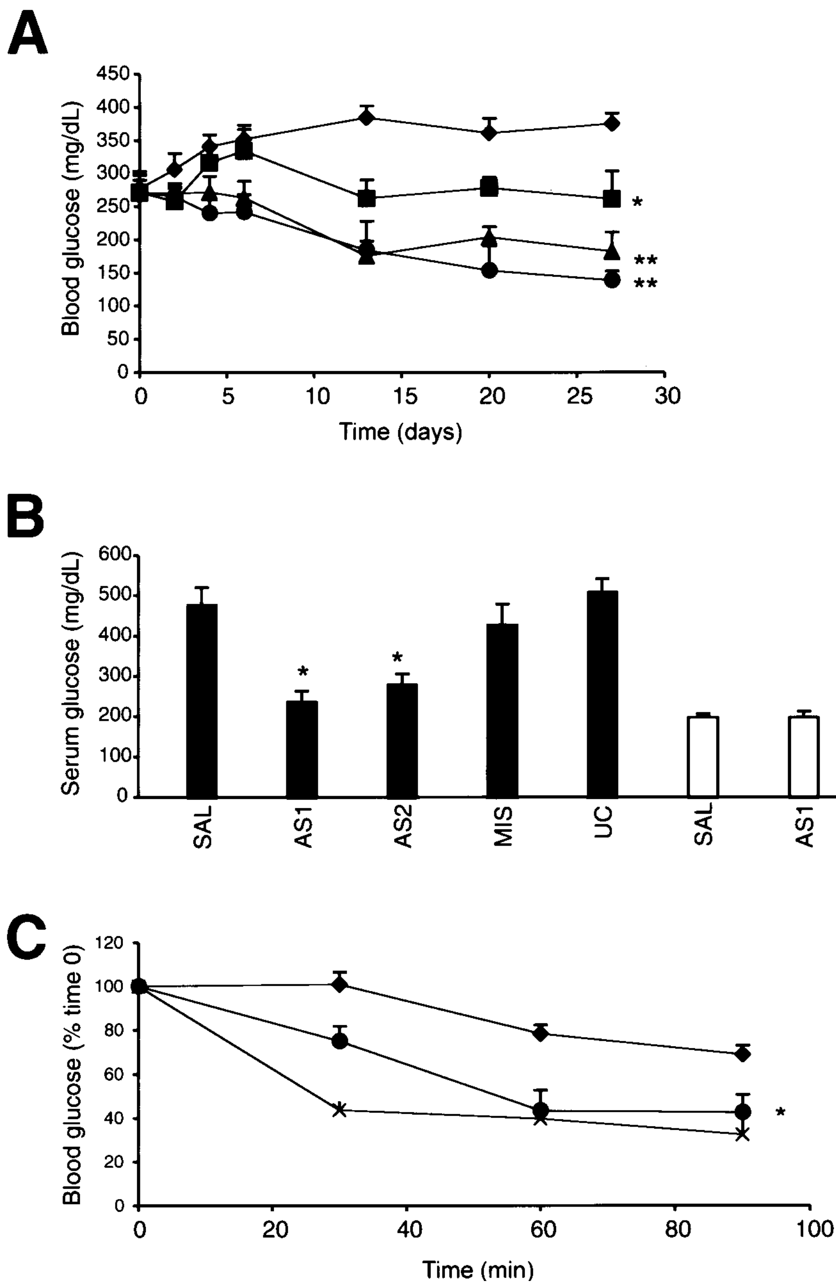

FIG. 3. Inhibition of PTEN expression lowers glucose and increases insulin sensitivity in *db/db* mice. *A*: Time- and dose-dependence of glucose-lowering effects of PTEN AS1. *db/db* mice were injected intraperitoneally once a week with saline (◆) or 10 mg/kg (■), 25 mg/kg (▲), or 50 mg/kg(●) of AS1 in saline. Mice were bled every 2 days for the first week and then once a week thereafter, 6 days after the previous dose. Values are expressed as means ± SE (n = 6–8). Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, **$P < 0.0001$, *$P < 0.01$. *B*: Specificity of the antidiabetic effect of PTEN ASOs on serum glucose concentrations. *db/db* mice (■, n = 5–6) were injected intraperitoneally with 50 mg/kg of indicated oligonucleotides for 4 weeks. Lean littermates (□, n = 5) were dosed with saline or 100 mg/kg of AS1. Statistics were performed using ANOVA, followed by Bonferroni-Dunn. Compared with saline-treated, *$P < 0.005$. *C*: Insulin tolerance test in PTEN antisense–treated mice. Mice were treated once a week for 3 weeks with saline (◆) or 50 mg/kg AS1 (●) (n = 5). Lean controls (x) were untreated. Results are expressed as the mean ±SE percentage of the glucose concentration at time 0. Statistical analysis was done using ANOVA repeated measures followed by Bonferroni-Dunn. Compared with saline-treated, *$P < 0.05$.

that PTEN mRNA and protein levels were reduced to the same extent as that of *db/db* mice.

To determine whether inhibition of PTEN expression had an effect on insulin sensitivity, an insulin tolerance test was performed in *db/db* mice treated once a week for 3 weeks with saline or AS1 (Fig. 3*C*). PTEN AS1 significantly increased sensitivity to insulin; the relative blood glucose concentrations in the AS1-treated mice were significantly lower at all time points after insulin injection compared with those in saline-treated animals. Also, inhibition of PTEN expression did not appear to cause hypoglycemia, in that glucose levels in mice remained normoglycemic in PTEN AS1–treated *db/db* and lean mice fasted for 16 h (data not shown).

Inhibition of PTEN expression also lowered serum triglyceride and cholesterol concentrations in *db/db* mice in a dose-dependent manner (Table1). Lipid concentrations were unaffected relative to lean littermates at the 50-mg/kg dose of AS1, and the control oligonucleotide had no effect. Treated *db/db* mice gained significantly more weight than saline- and control antisense–treated *db/db* mice, despite the fact that food intake was similar in all groups. However, *ob/ob* mice did not gain weight relative to saline-treated animals during their 4-week antisense treatment (see below). No significant changes in body composition (i.e., lean versus fat body mass) were observed in any mice treated with PTEN ASOs (data not shown).

The effect of inhibiting PTEN expression on hyperinsulinemia was investigated in *ob/ob* mice, which have higher circulating levels of insulin and are less hyperglycemic than *db/db* mice. Male *ob/ob* mice were injected with 50 or 20 mg/kg of AS1 on day 0 and then with either 20 or 10 mg/kg a week thereafter for 3 weeks, a dosing schedule designed to attain more moderate steady-state levels of oligonucleotide in liver. At the end of 4 weeks, PTEN

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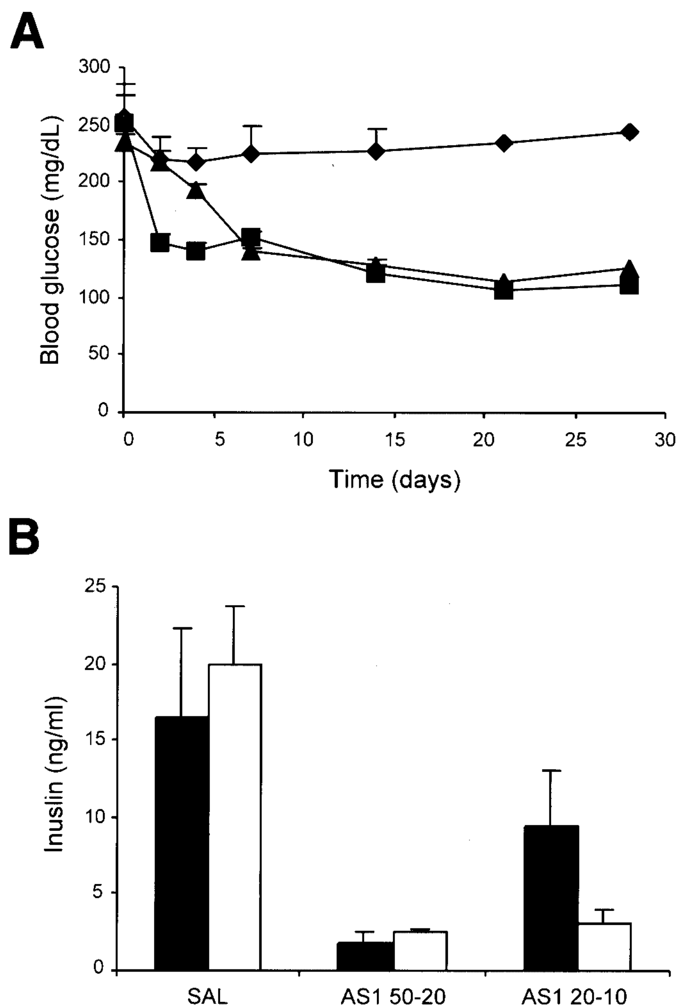

FIG. 4. Reduction of blood glucose (*A*) and serum insulin (*B*) in *ob/ob* mice treated with PTEN antisense. *A*: *Ob/ob* mice were injected intraperitoneally once a week with saline (♦) or 50 mg/kg AS1 on day 0, followed by 20 mg/kg once a week thereafter (■), or 20 mg/kg AS1 on day 0, followed by 10 mg/kg once a week thereafter (▲). Values are expressed as means ± SE (n = 8). *B*: *ob/ob* mice treated as described above were fasted for 4 h before measuring serum insulin after 2 weeks (■) and 4 weeks (□) of dosing. Values are expressed as the means ± SE (n = 6).

mRNA levels were reduced by 71% in the higher-dose group and by 49% in the lower-dose group (data not shown) relative to saline-treated animals, and blood glucose concentrations were normalized by 2 weeks in both dose groups (Fig. 4*A*). Serum insulin levels were reduced by 90% at 2 weeks in the higher-dose group and by 84% at 4 weeks in the lower-dose group (Fig. 4*B*).

Effect of inhibiting PTEN expression on Akt phosphorylation in diabetic and lean mice. If the effects of PTEN inhibition on glucose and insulin levels in diabetic mice are caused by an activation of PI3K signaling, evidence of a biochemical improvement in insulin signaling downstream of PI3K should be detectable. Because Akt activation is dependent on the products of PI3K, we reasoned that decreasing PTEN expression would result in increased levels of Akt phosphorylation in response to insulin in diabetic animals. To test this, *ob/ob* mice and their lean littermates were treated with either saline, AS1, or MIS at 50 mg/kg once a week for 2 weeks, and PTEN and phospho-Akt protein levels were determined in liver. As can be seen in Fig. 5*A*, PTEN protein expression was reduced by ~90% in livers from both lean and *ob/ob* mice treated with AS1, relative to saline-treated mice. In lean mice, neither the basal levels of Akt phosphorylation nor the sixfold increase in Akt phosphorylation in response to a bolus insulin injection were affected by PTEN antisense treatment (Fig. 5*B*). As has been previously reported in diabetic rats (29), no increase in Akt phosphorylation in response to insulin was observed in control-treated *ob/ob* mice. However, PTEN AS1 treatment appeared to restore Akt phosphorylation in response to insulin in *ob/ob* mice (Fig. 5*C*). However, PTEN AS1 treatment did not appear to affect basal levels (non–insulin-stimulated) of phosphorylated Akt in *ob/ob* mice.

DISCUSSION

The molecular defects that cause insulin resistance and hyperglycemia in type 2 diabetes have not been well defined. Impaired insulin receptor function leading to reduced activation of PI3K could be a cause of insulin resistance, or the primary defect may lie further downstream in the PI3K pathway. The results presented here indicate that PTEN, a tumor suppressor with phosphoinositide 3′-phosphatase activity, may play a role in glucose metabolism in vivo by negatively regulating insulin signaling.

Several lines of evidence indicate that PI3K activation and the subsequent rise in PIP3 concentrations are necessary for many of the metabolic responses to insulin, including Akt activation, glucose transport, and glycogen and lipid synthesis. PTEN is capable of dephosphorylating PIP3 (12), and cells in which PTEN activity has been inhibited have elevated PIP3 concentrations and higher levels of Akt phosphorylation (16). Thus, it seems logical that PTEN can regulate insulin signaling through the PI3K pathway. Indeed, Nakashima et al. (19) recently demonstrated that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 translocation in vitro, whereas microinjection of a PTEN antibody increased basal and insulin-stimulated GLUT4 translocation also in vitro. Our results demonstrating that antisense-mediated reduction of PTEN expression increased insulin-dependent Akt phosphorylation in vitro also supports the conclusion that PTEN may negatively regulate insulin signaling in cultured cells.

Moreover, using our antisense approach, we were able to determine the effect of inhibiting PTEN expression in animals, and our results suggest that PTEN plays an important role in glucose homeostasis in vivo as well. We have shown that systemic administration of PTEN antisense reduced PTEN mRNA and protein expression in a dose-dependent manner in mouse liver but had no effect on the levels of the phosphatases PTP1B and SHIP2. Similar results have recently been obtained using a Fas ASO, which reduced Fas mRNA and protein expression in hepatocytes by up to 90% after systemic injection in mice (25). We also found that systemically administered PTEN oligonucleotides are capable of reducing target expression in fat, but not muscle, and that PTEN mRNA and protein levels are much less abundant in muscle than in fat and liver. Taken together, these results indicate that the effects of the antisense were indeed specific for PTEN and suggest that the reduction of PTEN expression primarily in

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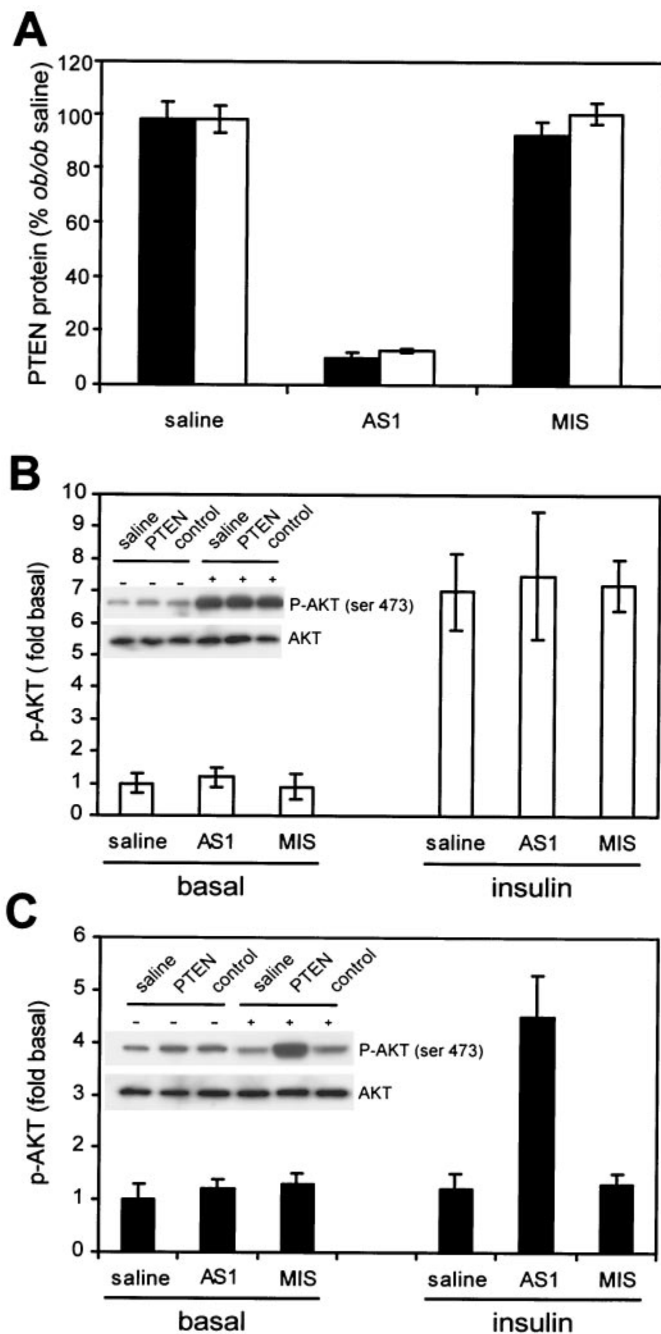

FIG. 5. Reduction of PTEN expression in *ob/ob* and lean mice and increased Akt phosphorylation in *ob/ob* mice treated with PTEN antisense. *A*: Reduction of PTEN protein levels in both *ob/ob* (■) and lean (□) mouse livers after PTEN antisense treatment. Mice (n = 4 per group) were injected for 2 weeks with saline or 50 mg/kg ASI or MIS oligonucleotides, and liver lysates were immunoblotted with antibodies to PTEN and G3DPH. Data are expressed as the mean percentage of normalized PTEN protein levels in saline-treated *ob/ob* mice ±SD. *B* and *C*: Phospho-Akt levels in livers of lean and *ob/ob* mice without (basal) or 5 min after intraperitoneal injection of 2 units/kg of insulin. Immunoblots of proteins were sequentially reacted with antibodies to phospho-Akt and Akt. Data are expressed as the fold increase in phospho-Akt intensity compared with basal levels in saline-treated mice relative to Akt protein levels. Graphs represent the means ± SD. Representative gels of pooled samples (n = 3) from each group are also shown for lean and *ob/ob* mice.

liver, with some possible contribution in fat, was sufficient to produce the observed metabolic changes in diabetic mice. Improved insulin sensitivity in liver of diabetic mice would be expected to reduce hepatic glucose production and thereby reverse hyperglycemia.

Antisense-mediated inhibition of PTEN expression normalized glucose concentration in both *db/db* and *ob/ob* mice, improved insulin sensitivity in *db/db* mice, and lowered insulin concentrations dramatically in *ob/ob* mice. The fact that inhibition of PTEN expression reversed hyperglycemia and reduced insulin resistance in diabetic mice, without affecting glucose levels in lean mice, suggests that the reduction in PTEN expression compensated for a defect in the PI3K pathway in diabetic mice. Alternatively, it is possible that inhibition of PTEN in diabetic mice may somehow compensate for defects in other pathways that are unrelated to PI3K but may contribute to insulin resistance in these animals. No detectable difference in PTEN mRNA or protein levels in lean versus *db/db* mice was observed, so it does not appear that an increase in PTEN expression levels is the primary defect in these mice. It has previously been demonstrated that IRS-associated PI3K activity is decreased significantly in *ob/ob* mouse liver (30) and that Akt/PKB activity is reduced in liver and muscle from diabetic rats and humans (29). We have demonstrated that inhibition of PTEN expression in vivo restores insulin-stimulated Akt phosphorylation in *ob/ob* diabetic mice to a level comparable with that in lean mice. We have also observed significant increases in liver Akt phosphorylation in *db/db* mice treated with PTEN antisense (data not shown). Thus, it seems logical that a reduction in PTEN expression after antisense treatment resulted in increased PI3K activity by increasing the half-life and/or effective concentration of PIP3 produced during insulin activation. This logic is consistent with the putative role of PTEN in PI3K signaling, as well as with the results of other investigators who have employed different approaches for suppressing PTEN activity in vitro (11–14,17–19). Nevertheless, we cannot rule out the possibility that the effects of PTEN inhibition on Akt phosphorylation and insulin sensitivity that we have observed in vitro and in vivo may not be directly related to increased PI3K activity and increased PIP3 levels because neither of these end points were measured directly. Although PTEN antisense treatment had no effect on insulin-dependent Akt phosphorylation or on circulating glucose levels in lean mice, serum insulin levels were decreased by 50%, suggesting that PTEN inhibition may increase insulin sensitivity in lean mice as well. Interestingly, a recent report about a Cowden's disease patient with a heterozygous PTEN mutation indicated improved insulin sensitivity, as measured by glucose clearance and hyperinsulemic-euglycemic clamp (31). However, although several groups have demonstrated an increased incidence of tumors in PTEN heterozygous mice, no changes in blood glucose concentrations have been reported. This finding may not be surprising in view of the fact that in our studies, reducing PTEN expression by 90% had no significant effect on glucose levels in lean mice.

In conclusion, our results demonstrate that suppression of PTEN expression produces a marked improvement in blood glucose concentrations and insulin sensitivity in diabetic mice and suggest that pharmacological inhibition of negative regulators of the PI3K pathway may represent

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a therapeutic approach for the treatment of type 2 diabetes.

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