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Phosphatase and Tensin Homolog Deleted on Chromosome 10 Suppression Is an Important Process in Peroxisome Proliferator-Activated Receptor-γ Signaling in Adipocytes and Myotubes

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ABSTRACT

Peroxisome proliferator-activated receptor-γ (PPARγ) activation enhances insulin sensitivity in type 2 diabetes mellitus. However, downstream mediators of PPARγ activation in adipocytes and myotubes, the most important cell types involved in glucose homeostasis, remained unclear. Here we show by using two synthetic PPARγ agonists (rosiglitazone and KR-62776, a novel PPARγ agonist) that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a key downstream mediator of PPARγ signaling. The PPARγ agonists down-regulated PTEN expression, resulting in glucose uptake increase in differentiated 3T3-L1 adipocytes and C2C12 skeletal muscle cells. In both cells, PTEN knockdown increased glucose uptake, whereas overexpression abolished the agonist-induced effects. The effects of PPARγ agonists on PTEN expression and glucose uptake disappeared by pretreatment with a PPARγ antagonist or by knockdown of PPARγ expression. In vivo treatment of the agonists to C57BL/6J-*ob*/*ob* mice resulted in the reduction of PTEN level in both adipose and skeletal muscle tissues and decreased plasma glucose levels. Thus, these results suggest that PTEN suppression is a key mechanism of the PPARγ-mediated glucose uptake stimulation in insulin-sensitive cells such as adipocytes and skeletal muscle cells, thereby restoring glucose homeostasis in type 2 diabetes.

Peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors (Cantley and Neel, 1999). There are three distinct isoforms of PPAR, termed as α, δ/β, and γ. Among these isoforms, PPARγ is involved in a variety of important functions including adipocyte differentiation, lipid and glucose metabolism, and inflammation (Rosen and Spiegelman, 2001). So far, several PPARγ agonists were shown to have potent antihyperglycemic activity in vivo, thus providing an effective therapy for the treatment of type 2 diabetes mellitus (Dey et al., 2003). However, the exact mechanism of PPARγ activation in stimulating glucose uptake in cellular level is not yet clearly defined.

Type 2 (noninsulin-dependent) diabetes mellitus is a polygenic disorder characterized by reduced insulin secretion and insulin resistance (Virkamäki et al., 1999; Pessin and Saltiel, 2000). Peripheral insulin resistance in type 2 diabetes may be at least partially caused by defects in insulin signaling pathways. Among intracellular molecules constituting insulin signaling pathways, phosphoinositide-3 kinase (PI-3 kinase) acts as a critical signaling molecule triggering a number of insulin-stimulated effects, including glucose uptake, glycogen synthesis, and cell differentiation. On the other hand, a tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) that dephosphorylates $PI(3,4,5)P_3$ functions as a negative regulator of PI-3 kinase action. The involvement of PTEN in insulin action has been investigated previously in many studies. For example, overexpression of PTEN inhibited the glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes (Nakashima et al., 2000), whereas endogenous PTEN does not seem to contribute to the glucose transport activity in normal condition (Mosser et al., 2001; Ono et al., 2001). Furthermore, in vivo inhibition of PTEN expression by antisense oligonucleotide resulted in the

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; BADGE, bisphenol A diglycidyl ether; PTEN, phosphatase and tensin homolog deleted on chromosome 10; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATCC, American Type Culture Collection; KRP, Krebs-Ringer phosphate; PCR, polymerase chain reaction; *ob*/*ob* mice, C57BL/6J-*ob*/*ob* mice; GW9662, 2-chloro-5-nitrobenzanilide; PI-3, phosphoinositide-3.

normalization of blood glucose level in diabetic animal models, suggesting that PTEN plays an important role in glucose homeostasis in vivo (Butler et al., 2002). Recently, specific suppression of PTEN in adipose and muscle tissues of mice was shown to protect against insulin resistance and diabetes (Komazawa et al., 2004; Kurlawalla-Martinez et al., 2005; Wijesekara et al., 2005).

PPARγ expression is largely limited to adipocytes, primarily regulating adipocyte differentiation. A recent study has demonstrated that PPARγ also plays a role in regulating glucose uptake in skeletal muscle cells by using PPARγ sense and antisense constructs (Verma et al., 2004). Furthermore, muscle-specific PPARγ gene deletion in mice caused insulin resistance, supporting the role of skeletal muscle PPARγ in glucose homeostasis (Hevener et al., 2003). Because PTEN has been shown to play an important role in glucose homeostasis, we focused in the present study to elucidate the possible involvement of PTEN in PPARγ-mediated glucose homeostasis by using selective PPARγ agonists, rosiglitazone and KR-62776, a novel PPARγ agonist (patent PCT/KR2005/001066; Fig. 1).

Here, we demonstrate that two structurally different PPARγ agonists seem to inhibit PTEN expression, resulting in the increased glucose uptake. The effects of the agonists are mediated by PPARγ activation because bisphenol A diglycidyl ether (BADGE), a PPARγ antagonist or PPARγ knockdown reversed the agonist effects. On the other hand, overexpression of PTEN masked the stimulatory effects of the agonists on glucose uptake in differentiated 3T3-L1 adipocytes and C2C12 myotubes. The down-regulation of PTEN by PPARγ activation in adipose and skeletal muscle tissues was further confirmed in vivo. To our knowledge, this is the first report showing that PPARγ activation suppresses PTEN expression, subsequently increasing glucose uptake in insulin-sensitive and major cell types responsible for PPARγ action, namely adipocytes and skeletal muscle cells.

Materials and Methods

Materials. C2C12 skeletal muscle cells and 3T3-L1 preadipocytes were obtained from the ATCC (Manassas, VA). The following reagents were obtained: anti-PTEN (Cell Signaling Technology, Danvers, MA); actin and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); [^{3}H]2-deoxy-glucose and nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK); Supersignal West Dura Extended Duration Substrate Kit (Pierce, Rockford, IL); and BADGE, insulin, 2-deoxy-glucose, phenylmethylsulfonyl fluoride, and aprotinin (Sigma Chemical Co., St. Louis, MO). KR-62776, 1-(*trans*-methylimino-*N*-oxy)-3-phenyl-6-(3-phenylpropoxy)-1*H*-indene-2-carboxylic acid ethyl ester, and rosiglitazone were synthesized by the Korea Research Institute of Chemical Technology (Daejeon, Korea).

Cell Cultures. C2Cl2 cells (ATCC mouse skeletal muscle cell line) were grown and maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 environment and then differentiated by the addition of the same medium containing 2% horse serum for 4 days. 3T3-L1 cells (ATCC mouse adipocyte) were grown and maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antibiotics in a 5% CO_2 environment. 3T3-L1 cells differentiated to mature adipocytes by the same medium containing 20 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone for 3 days, were replaced with medium containing 20 μg/ml insulin for 2 days, and then were cultured for 2 days in culture medium. MCF-7 (ATCC human breast cancer cell) cells were cultured in Dulbecco's minimum essential medium supplemented with 10 μg/ml insulin, 10% fetal bovine serum, and 1% antibiotics.

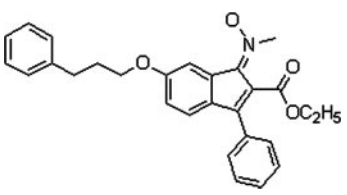


Fig. 1. Chemical structure of KR-62776.

Glucose Uptake Assays. Fully differentiated C2C12 cells (2×10^5 cells/ml) or 3T3-L1 adipocytes (2×10^4 cells/ml) were treated with or without BADGE (20 μM) for 1 h and then stimulated with either KR-62776 or rosiglitazone in the absence or presence of insulin (100 nM) for 2 days. After washing twice with Krebs-Ringer phosphate (KRP) buffer, cells were treated with 0.2 μCi/well [^{3}H]2-deoxy-glucose in KRP buffer supplemented with 100 μM 2-deoxy-glucose and the compounds for 30 min at 37°C. After washing twice with KRP buffer, cells were air-dried, solubilized with 500 μl of 0.1 N NaOH for 2 h, and 400 μl of lysate was mixed with 40 μl of 1 N HCl (for neutralizing) and 4 ml of scintillation cocktail/vial, and then the radioactivity was counted. Similar procedures were used to determine the effect of a PI-3 kinase inhibitor (50 nM wortmannin) on the agonist-induced glucose uptake.

Western Blot Analysis. Cultured cells were lysed in a lysis buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. After centrifugation of cell lysates at 12,000 rpm, 10 μg of total protein was loaded into 8% SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane. Protein bands were visualized using chemiluminescent reagents (Pierce) and quantified using the UN-SCAN-IT gel 5.1 software (Silk Scientific, Orem, UT). Relative abundance of protein was calculated after normalization to GAPDH or actin.

Reverse Transcription-Polymerase Chain Reaction and Real-Time Quantitative Polymerase Chain Reaction Analysis. Total RNA was isolated from compounds-treated cells and tissues using easy-BLUE Total RNA extraction Kit (iNtRON Inc., Kyungki-Do, Korea). Reverse transcription of total RNA (1 μg) was performed using AccuPower RT PreMix (Bioneer Inc., Daejeon, Korea). Polymerase chain reaction (PCR) primers for amplification of PTEN were designed based on the sequences obtained: sense, 5′-AGG GAC CGA CTG TAA TG-3′; antisense, 5′-AGT GCC ACG GGT CTG TAA TC-3′. Reverse transcription-PCR conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a 10-min extension reaction at 72°C. Aliquots of the PCR reactions were checked by melting curve analysis as provided by the Roter-Gene 3000 system (Corbett Research, Mortlake, NSW, Australia). The instrument settings were the following: PTEN, denaturing at 95°C for 15 min with 40 repeated cycles of denaturing at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Relative abundance of mRNA was calculated after normalization to GAPDH.

Plasmid Construction and Transfection for PTEN Overexpression. The expression plasmid encoding the mouse PTEN protein was cloned by reverse transcription-PCR using the total RNAs obtained from C2C12 and 3T3-L1 cells. PTEN primers were designed using known mouse PTEN gene sequences (GenBank accession no. AK076980) corresponding to nucleotides 748 to 768 (5′-catcctgcagaagacgcctcg-3′) of the coding strand and to nucleotides 1997 to 2038 (5′-ccttttgatgaagatcagcattcacaaattacaaaagtctga-3′) of the noncoding strand of PTEN gene. After amplification with Taq polymerase, products were cloned into pTARGET mammalian expression vector (Promega, Madison, WI). Transfection conditions were optimized using CMV-β-galactosidase (Clontech Laboratories Inc., Mountain

View, CA). After transfection of the plasmid, PTEN expression was analyzed by Western blot or reverse transcription-PCR using whole-cell extracts.

Transfection of Antisense Oligodeoxynucleotide for PTEN Knockdown in C2C12 Cells. The antisense phosphorothioate-modified oligodeoxynucleotide was a 20-mer complementary to human PTEN mRNA (5′-CTGCTAGCCTCTGCATTTGA-3′, beginning at position 2097; GenBank accession no. AA017584). The scrambled oligodeoxynucleotide was 5′-CTTCTGGCATCCGGTTTAGA-3′. Differentiated C2C12 cells at 90% confluence were treated with a transfection cocktail containing 500 nM PTEN antisense oligodeoxynucleotide, 2 μl of LipofectAMINE reagent, and 6.8 μl of Plus reagent (both from Invitrogen, Carlsbad, CA). After 3 h, the medium was removed, and then 800 μl of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum was added to each dish. PTEN expression was analyzed by Western blot or reverse transcription-PCR using whole-cell extracts.

Transfection of Stealth RNAi for PTEN Knockdown in 3T3-L1 Cells. The PTEN Stealth Select RNAi oligonucleotide (Target Accessions no. NM 016335) was synthesized by Invitrogen. The Stealth RNAi negative control duplex was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT Fluorescent oligonucleotide; Invitrogen) and was estimated to be 20 to 30%. The Stealth RNAi molecules were transfected into differentiated 3T3-L1 adipocytes using LipofectAMINE 2000 by following Invitrogen's protocols. The final concentrations of 100 nM PTEN Stealth Select RNAi oligonucleotide was empirically determined to maximally suppress PTEN RNA expression, and the Stealth RNAi oligonucleotide was transfected to the cells 48 h before the treatment of compounds. The ability of the Stealth RNAi oligonucleotide to knock down PTEN expression was analyzed by Western blot and reverse transcription-PCR on whole-cell extracts.

Transfection of Stealth RNAi for PPARγ Knockdown in 3T3-L1 and C2C12 Cells. The PPARγ Stealth Select RNAi oligonucleotide (Target Accession nos. NM 138712.1, M015869.2, NM138711.1, and NM 005037.3) was synthesized by Invitrogen. The Stealth RNAi negative control duplex (Invitrogen) was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT Fluorescent oligonucleotide; Invitrogen) and estimated to be 20 to 30% in 3T3-L1 cells and 40% in C2C12 cells. The Stealth RNAi molecules were transfected into differentiated 3T3-L1 and C2C12 cells using LipofectAMINE 2000 by following Invitrogen's protocols. The final concentrations of 100 and 50 nM PPARγ Stealth Select RNAi oligonucleotide were selected for 3T3-L1 cells and C2C12 cells, respectively, and the Stealth RNAi oligonucleotides were transfected to the cells 48 h before the treatment of compounds. The ability of the Stealth RNAi oligonucleotide to knock down PPARγ expression was analyzed by Western blot and reverse transcription-PCR on whole-cell extracts.

Animals and Drug Administration. Eight- to nine-week-old male C57BL/6J mice and male C57BL/6J-*ob*/*ob* mice (*ob*/*ob* mice) (Orient Co., Kyungki-do, Korea) were housed in plastic cages on a 12-h light/dark cycle with free access to water and food. Each mouse was treated with 50 mg/kg/day KR-62776 (diluted saline plus 0.2% Tween 80 solution) or 10 mg/kg/day rosiglitazone, i.p., twice per day for 9 days. The animals were sacrificed, and the muscle and adipose tissues were quickly removed and then frozen in liquid nitrogen. Plasma concentration of glucose was measured by a colorimetric assay using an automatic biochemical analyzer, the Selectra 2 (Vital Scientific NV, Dieren, The Netherlands).

Statistical Analysis. The results are expressed as means ± S.E.M. Statistical differences between corresponding groups were determined by paired or unpaired Student's *t* test or analysis of variance. $P < 0.05$ was considered to be significant.

Results

PPARγ Agonists Stimulate Glucose Uptake. To investigate the role of PPARγ activation in glucose uptake, we determined the 2-deoxyglucose uptake activities of KR-62776 and rosiglitazone in differentiated 3T3-L1 adipocytes and C2C12 myotubes. The potencies of KR-62776 and rosiglitazone for PPARγ were estimated in transactivation assay as EC_{50} values of 50 and 180 nM, respectively (results not shown). In 3T3-L1 adipocytes, insulin (100 nM) treatment alone increased glucose uptake by approximately 250%, and KR-62776 (10 μM) increased insulin-stimulated glucose uptake by almost 380% (Fig. 2A). Comparable effects were also detected by rosiglitazone, although the effect elicited by rosiglitazone was less potent than that by KR-62776. Similar

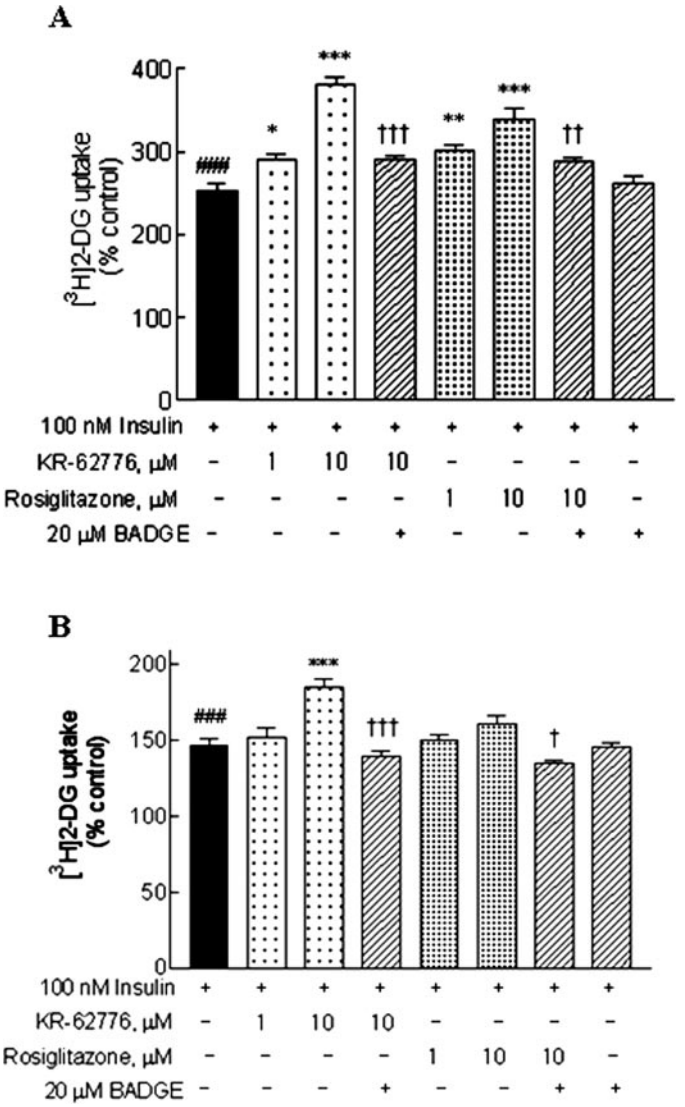


Fig. 2. Effects of KR-62776 and rosiglitazone on glucose uptake in differentiated 3T3-L1 and C2C12 cells. Fully differentiated 3T3-L1 (A) and C2C12 cells (B) were treated with or without BADGE (20 μM) for 1 h, stimulated with either KR-62776 or rosiglitazone in the presence of insulin (100 nM) for 48 h, and then glucose uptake was measured. Values are means ± S.E.M. of two different preparations with triplicate experiments. ###, $P < 0.001$ versus control group; *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$; ††, $P < 0.01$, and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group.

results were observed in C2C12 myotubes, although overall increase of glucose uptake was less than in adipocytes (Fig. 2B). The agonist effects on glucose uptake in the presence of insulin were blocked by BADGE (20 μM), a known PPARγ antagonist. In addition, PPARγ knockdown by RNAi (approximately 50% reduction compared with control) prevented the agonist-induced glucose uptake in both cell types (Fig. 3, A and B). These results suggest that the stimulatory effects of the agonists on glucose uptake are mediated by PPARγ activation.

PTEN Is Down-Regulated by PPARγ Activation. To determine the signaling mechanism of the PPARγ agonists on glucose uptake, we examined the effect of KR-62776 on PTEN expression, a negative regulator of PI-3 kinase activity, by Western blot. PTEN protein expression was reduced by approximately 60 and 80% with 10 μM KR-62776 in 3T3-L1 and C2C12 cells, respectively (Fig. 4, A and B). Similar but lesser suppressive effects by rosiglitazone were also observed. The reduction of PTEN protein by both KR-62776 and rosiglitazone was reversed by BADGE, a PPARγ antagonist as shown in Fig. 4. Furthermore, PPARγ knockdown by Stealth RNAi abolished the suppressive effects of the agonists on PTEN expression, supporting that the agonist effects are mediated via PPARγ activation (Fig. 5, A and B). Negative control Stealth RNAi treatment had no influence on the agonist actions.

To confirm that the down-regulation of PTEN protein expression by the agonists is mediated via transcriptional control, we examined the effect of KR-62776 on the PTEN mRNA levels by real-time PCR. As with the protein results, KR-62776 treatment significantly decreased PTEN mRNA levels to approximately 60% in both 3T3-L1 and C2C12 cells, which was reversed by BADGE pretreatment (Fig. 6, A and B),

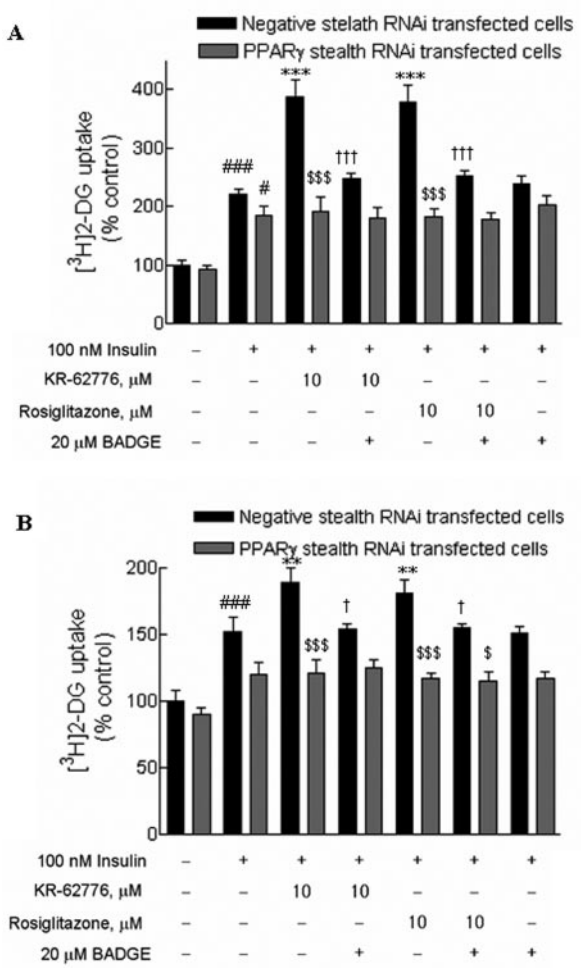


Fig. 3. Effects of PPARγ knockdown on KR-62776- and rosiglitazone-induced glucose uptake in differentiated 3T3-L1 and C2C12 cells. The differentiated 3T3-L1 (A) and C2C12 cells (B) transfected with either negative control Stealth RNAi oligonucleotide or PPARγ Stealth RNAi oligonucleotide were incubated with or without KR-62776 or rosiglitazone for 3 h. After 2 days of incubation with 100 nM insulin, glucose uptake assay was carried out. Values are means ± S.E.M. of two different preparations with triplicate experiments. #, $P < 0.05$ and ###, $P < 0.001$ versus control group; **, $P < 0.01$ and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$ and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group; $, $P < 0.05$ and $$$, $P < 0.001$ versus negative stealth RNAi-transfected group.

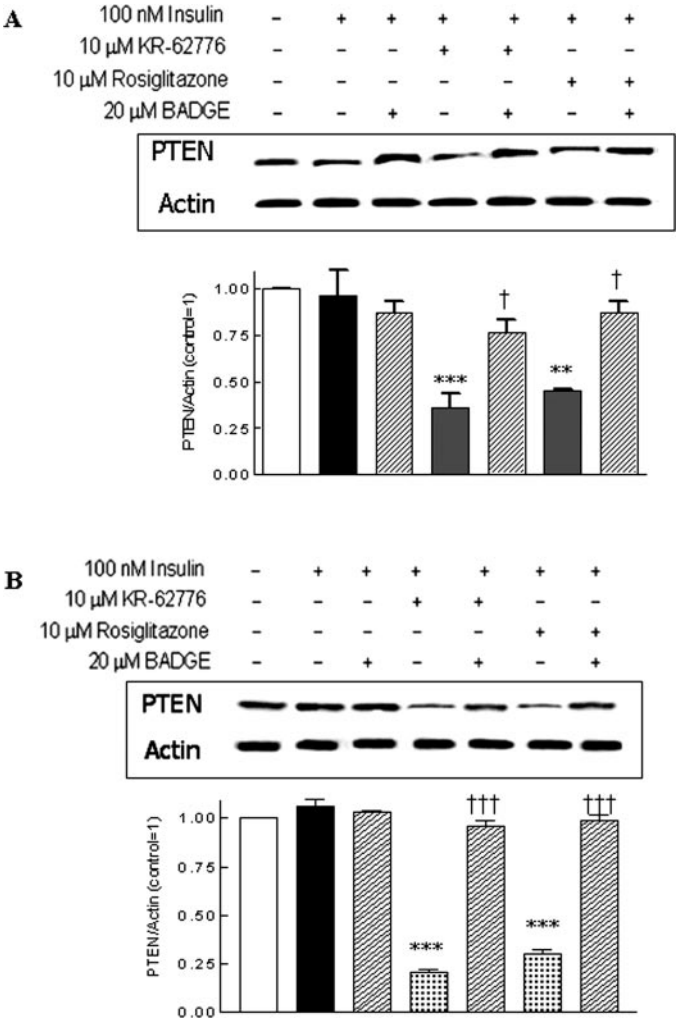


Fig. 4. Effects of KR-62776 and rosiglitazone on PTEN protein expression in differentiated 3T3-L1 and C2C12 cells. Fully differentiated 3T3-L1 (A) and C2C12 cells (B) were treated with or without BADGE (20 μM) for 1 h and then stimulated with either KR-62776 or rosiglitazone in the presence of insulin (100 nM) for 48 h. Protein expression was determined by Western blot, and relative abundance of protein was calculated after normalization to actin. Western blot results are the representative of three experiments performed on different experimental days. Densitometric analysis was carried out by using the UN-SCAN-IT gel 5.1 software (Silk Scientific), and values are means ± S.E.M. of three different experiments. **, $P < 0.01$ and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$ and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group.

suggesting that down-regulation of PTEN expression by KR-62776 is mediated by PPARγ-induced transcriptional suppression. Similar results were obtained by rosiglitazone treatment with lesser extent than that of KR-62776 (30% inhibition of PTEN mRNA levels), strongly supporting that PTEN suppression by the agonists is a PPARγ-dependent mechanism of action.

Overexpression of PTEN Abolishes the PPARγ-Induced Glucose Uptake. To confirm that a key mechanism of PPARγ-mediated glucose uptake is PTEN suppression, we overexpressed PTEN in differentiated 3T3-L1 and C2C12 cells and examined the effects of PPARγ agonists on glucose uptake. Transfection of PTEN expression plasmid to 3T3-L1 cells resulted in the increased PTEN expression by 3-fold over wild type in both protein and mRNA levels (Fig. 7A). The stimulatory effects of KR-62776 or rosiglitazone on glucose uptake were blocked in PTEN-overexpressed 3T3-L1 cells (Fig. 7B), supporting further that PTEN suppression is critical for the PPARγ-activated glucose uptake. Similar effects were observed in C2C12 cells with PTEN overexpression (Fig. 7, C and D).

On the other hand, transfection of Stealth RNAi or antisense oligonucleotide resulted in the reduction of both PTEN protein and mRNA expressions by approximately 80% in 3T3-L1 cells and 60% in C2C12 cells (Fig. 8, A and C). Glucose uptake was increased in PTEN knockdown cells both in the absence and presence of insulin (Fig. 8, B and D), suggesting that endogenous PTEN may down-regulate the glucose uptake activity. KR-62776 and rosiglitazone were ineffective for further enhancing glucose uptake in PTEN knockdown 3T3-L1 and C2C12 cells (Fig. 8, B and D), probably caused by the maximum increase of glucose uptake by

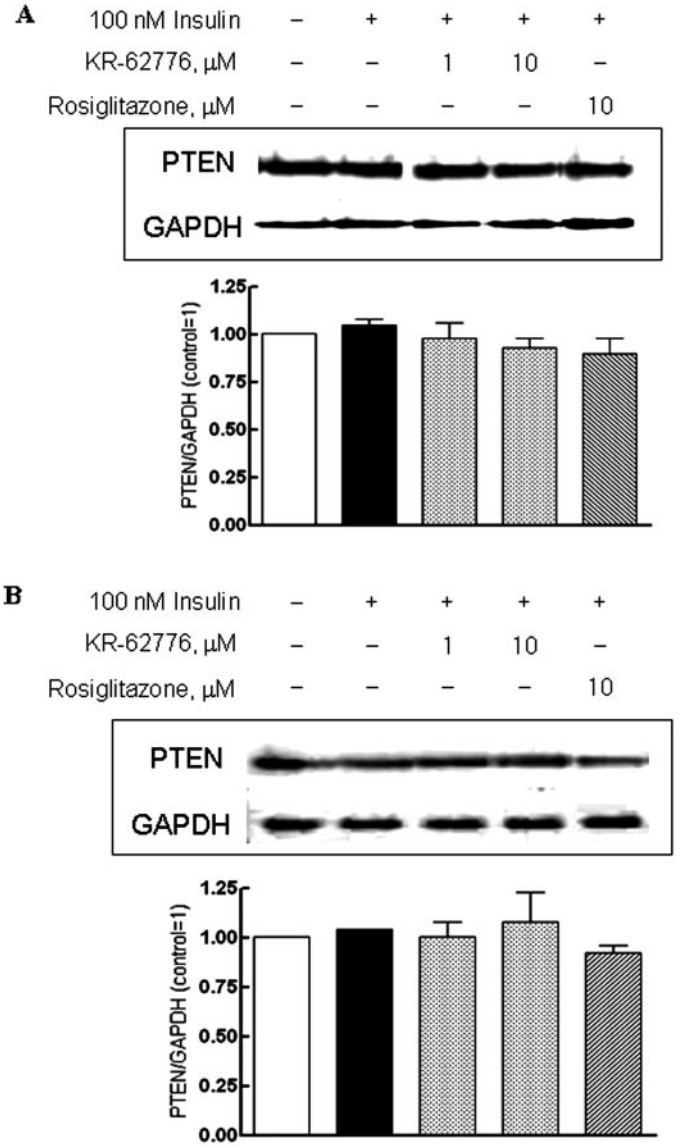


Fig. 5. Effects of PPARγ knockdown on KR-62776- and rosiglitazone-induced PTEN suppression in differentiated 3T3-L1 and C2C12 cells. The differentiated 3T3-L1 (A) and C2C12 cells (B) transfected with either negative control Stealth RNAi oligonucleotide or PPARγ Stealth RNAi oligonucleotide were incubated with or without KR-62776 or rosiglitazone for 3 h. After 2 days of incubation with 100 nM insulin, PTEN protein expression was determined by Western blot, and relative abundance of protein was calculated after normalization to GAPDH. Values are means ± S.E.M. of three different experiments.

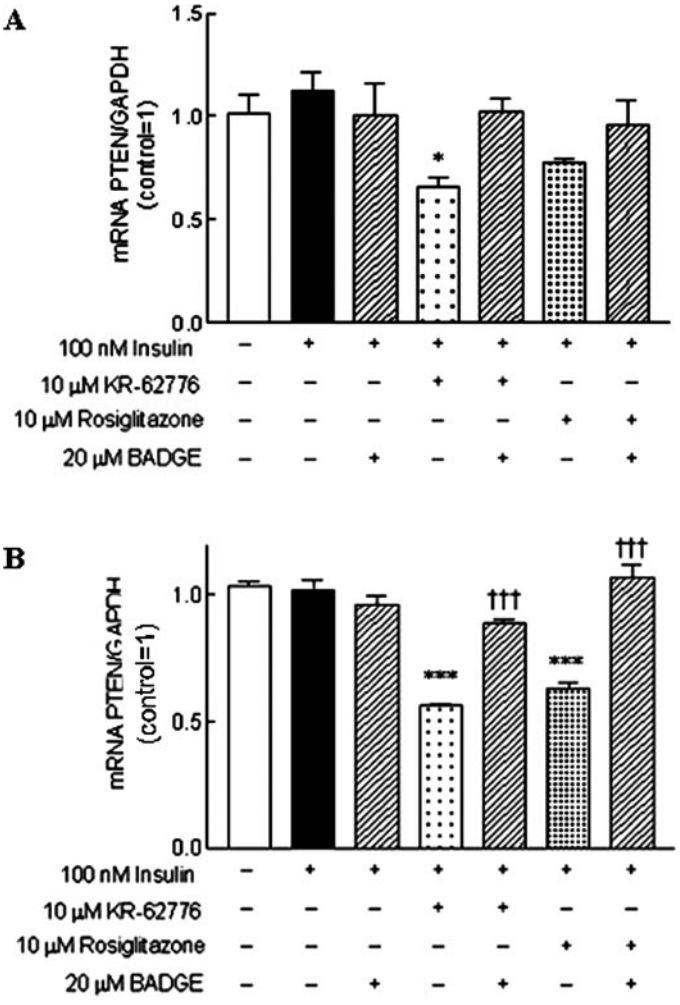
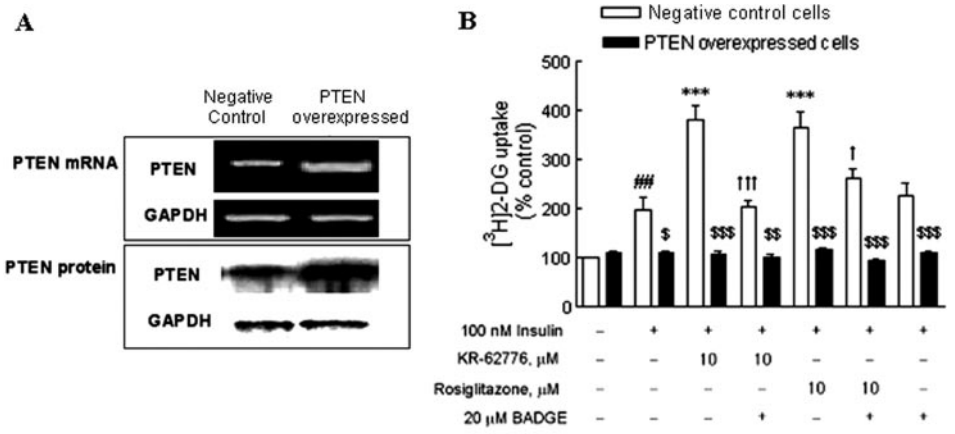


Fig. 6. Effects of KR-62776 and rosiglitazone on PTEN mRNA expression in differentiated 3T3-L1 and C2C12 cells. Fully differentiated 3T3-L1 (A) and C2C12 cells (B) were treated with or without BADGE (20 μM) for 1 h and then stimulated with either KR-62776 or rosiglitazone in the presence of insulin (100 nM) for 48 h. The PTEN mRNA was determined using real-time PCR analysis, and relative abundance of mRNA was calculated after normalization to GAPDH. Values are means ± S.E.M. of three different experiments. *, $P < 0.05$ and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group.



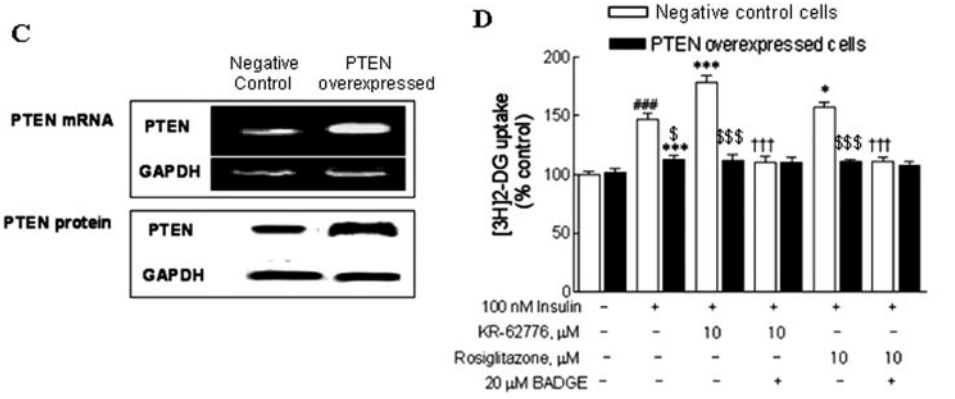
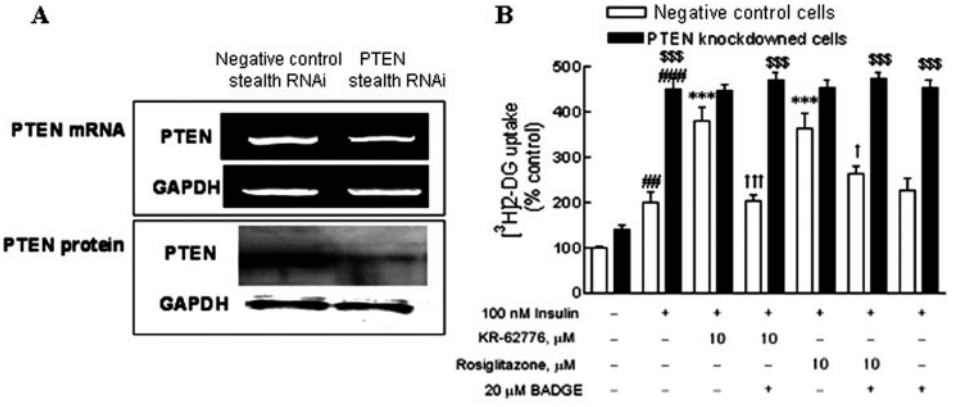


Fig. 7. Effects of PTEN overexpression on KR-62776- and rosiglitazone-induced glucose uptake in differentiated 3T3-L1 and C2C12 cells. A and C, after transfection of PTEN expression plasmids, PTEN protein and mRNA expressions were determined. B and D, BADGE (20 μM) was pretreated with PTEN overexpressed 3T3-L1 and C2C12 cells for 1 h followed by KR-62776 or rosiglitazone treatment for 3 h. After 2 days of incubation with 100 nM insulin, glucose uptake assay was carried out. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. ##, $P < 0.01$ and ###, $P < 0.001$ versus control group; *, $P < 0.05$ and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$ and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group; $, $P < 0.05$, $$, $P < 0.01$, and $$$, $P < 0.001$ versus negative control group.



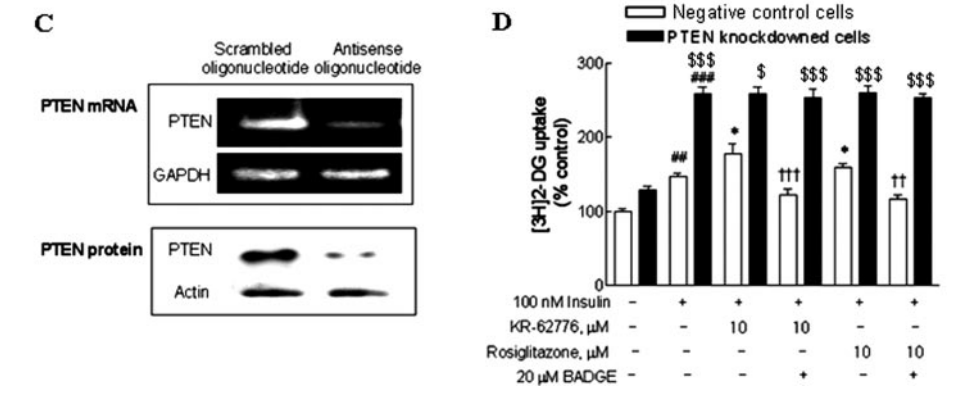


Fig. 8. Effects of PTEN knockdown on KR-62776- and rosiglitazone-induced glucose uptake in differentiated 3T3-L1 and C2C12 cells. A and C, after transfection of PTEN stealth RNAi or antisense oligonucleotide, PTEN protein and mRNA expressions were determined. B and D, BADGE (20 μM) was pretreated with PTEN knockdown 3T3-L1 and C2C12 cells for 1 h followed by KR-62776 or rosiglitazone treatment for 3 h. After 2 days of incubation with 100 nM insulin, glucose uptake assay was carried out. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. ##, $P < 0.01$ and ###, $P < 0.001$ versus control group; *, $P < 0.05$ and ***, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$; ††, $P < 0.01$, and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group. $, $P < 0.05$ and $$$, $P < 0.001$ versus negative control group.

Stealth RNAi or antisense oligonucleotide-induced down-regulation of PTEN expression.

PPARγ-Mediated Glucose Uptake Is Suppressed by a PI-3 Kinase Inhibitor Wortmannin. To further prove the critical role of PTEN suppression by PPARγ agonists in glucose uptake, a PI-3 kinase inhibitor was used to abrogate the effect of the decreased PTEN expression by the agonists. As expected, wortmannin, a PI-3 kinase inhibitor, had an inhibitory effect on glucose uptake induced by either KR-62776 or rosiglitazone in both 3T3-L1 and C2C12 cells (Fig. 9, A and B). These results provided further evidence that PTEN suppression is a significant factor for the PPARγ-mediated stimulation of glucose uptake in both cells.

PPARγ-Mediated Regulation of PTEN in Other Cell Types. On the contrary to the present results, up-regulation of PTEN by PPARγ activation was reported previously in several cancer cells, including MCF-7 breast cancer cells (Patel et al., 2001). We reassessed the effects of both agonists on PTEN expression in MCF-7 cells under our experimental conditions. Consistent with previous findings, PPARγ activation by either KR-62776 or rosiglitazone resulted in the increased PTEN expression in MCF-7 cells (Fig. 10). These results may imply that PPARγ activation has dual effects on PTEN expression depending on the cell types: up-regulation leading to apoptosis in cancer cells, and down-regulation leading to glucose uptake in adipocytes and muscle cells.

PPARγ-Mediated Regulation of PTEN in *ob/ob* Mice. We further investigated in vivo study to determine whether PTEN suppression is involved in the PPARγ-induced glucose uptake by using *ob/ob* mice, a well-known type 2 diabetes animal model. PTEN expression in C57BL/6J (lean) mice and *ob/ob* mice was not statistically different in adipose and muscle tissues. It is noteworthy that PTEN expressions in muscle and adipose tissues of *ob/ob* mice were reduced by both KR-62776 and rosiglitazone treatments, with concurrent lowering of plasma glucose levels (Fig. 11). Concomitant with the decreased PTEN expression, increase in PPARγ level was detected (results not shown). On the other hand, the agonist effects on C57BL/6J mice were minimal with regard to PTEN expression and plasma glucose levels. Together, these results are consistent with in vitro observation using differentiated 3T3-L1 and C2C12 cells, and therefore PTEN suppression seems to be an important mechanism for PPARγ-mediated glucose homeostasis in muscle and adipose tissues in vivo.

Discussion

Insulin signaling pathway is critical for maintaining normal glucose homeostasis, thus causing the insulin-resistant state and type 2 diabetes by its improper regulation. PTEN, a recently identified tumor suppressor gene with 3′-phosphatase activity, seems to be involved in the metabolic action of insulin based on the several studies (Nakashima et al., 2000; Mosser et al., 2001; Ono et al., 2001; Butler et al., 2002; Komazawa et al., 2004; Kurlawalla-Martinez et al., 2005; Wijesekara et al., 2005). The current study defines the crucial role of PTEN in the PPARγ-induced glucose uptake in C2C12 and 3T3-L1 cells, two major cell types involved in glucose homeostasis, which indicates that the reduction of PTEN expression may be a primary mechanism of the

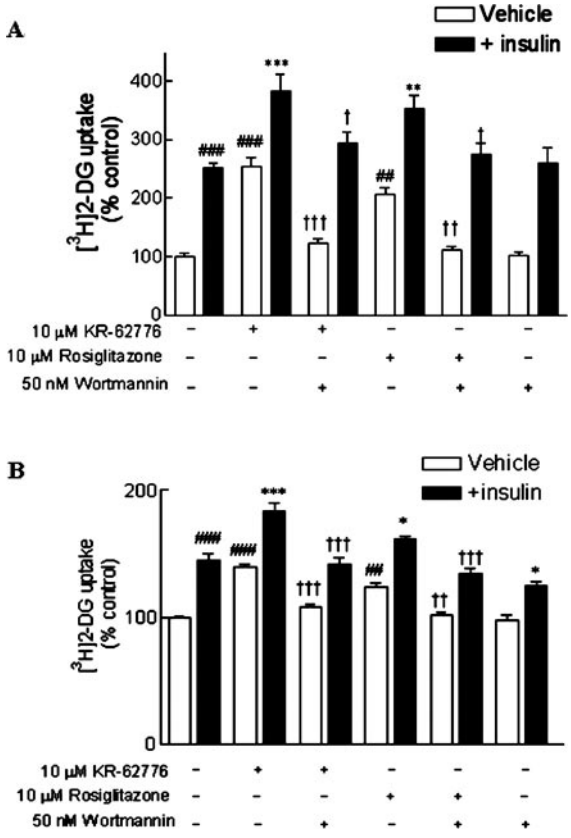


Fig. 9. Effects of wortmannin, a PI-3 kinase inhibitor, on KR-62776- and rosiglitazone-induced glucose uptake in differentiated 3T3-L1 and C2C12 cells. Fully differentiated 3T3-L1 (A) and C2C12 cells (B) were treated with or without wortmannin (50 nM) for 1 h, stimulated with either KR-62776 or rosiglitazone in the presence of insulin (100 nM) for 48 h, and then glucose uptake assay was carried out. Values are means ± S.E.M. of two different preparations with quadruplicate experiments. ##, $P < 0.01$ and ###, $P < 0.001$ versus control group; ∗, $P < 0.05$; ∗∗, $P < 0.01$, and ∗∗∗, $P < 0.001$ versus insulin (100 nM)-treated group; †, $P < 0.05$; ††, $P < 0.01$, and †††, $P < 0.001$ versus 10 μM KR-62776- or rosiglitazone-treated group.

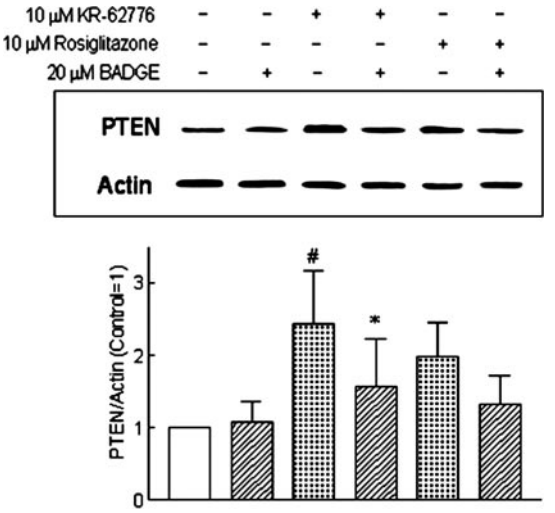


Fig. 10. Effects of KR-62776 and rosiglitazone on PTEN protein expression in MCF-7 breast cancer cells. Cells were treated with or without BADGE (20 μM) for 1 h and then stimulated with either KR-62776 or rosiglitazone for 24 h. Protein expression was determined by Western blot, and relative abundance of protein was calculated after normalization to actin. Values are means ± S.E.M. of three different experiments. #, $P < 0.05$ versus control group; ∗, $P < 0.05$ versus 10 μM KR-62776- or rosiglitazone-treated group.

PPARγ activation for the observed increase in glucose uptake in the cells. Furthermore, the decreased PTEN levels in adipose and muscle tissues with the reduction of plasma glucose levels were observed upon treatment of two structurally different PPARγ agonists to *ob*/*ob* mice, confirming the contribution of PTEN down-regulation for PPARγ-induced insulin sensitization in vivo.

Although the expression of PPARγ in adipocytes has been well documented, the role of PPARγ in skeletal muscle cells has been unclear. In the present study, we observed that PPARγ expression was significantly increased in the process of differentiation of C2C12 skeletal muscle cell line, implying that PPARγ might play a role in skeletal muscle cell differentiation. Treatment of either KR-62776 or rosiglitazone to differentiated C2C12 and 3T3-L1 cells enhanced basal and insulin-stimulated glucose uptake, which was accompanied with PTEN suppression, and the effects of the agonists were inhibited either by PTEN overexpression or by a PI-3 kinase inhibitor. In parallel with the suppression of PTEN expression, Akt phosphorylation was enhanced by KR-62776 or rosiglitazone, implying that indeed PI-3 kinase activity was increased by suppression of PTEN expression (data not shown). All of the effects were more marked with KR-62776 than rosiglitazone, correlating with their transactivation potencies for PPARγ. The effects on PTEN expression by two different PPARγ agonists were blocked by pretreatment of BADGE and by PPARγ knockdown, both of which inhibit PPARγ activation, suggesting that down-regulation of PTEN with concurrently increased Akt phosphorylation by the agonists is mediated by PPARγ activation.

The involvement of PPARγ in the regulation of PTEN has been reported in several cell types, including cancer cells and inflammatory cells. In human pancreatic cancer cells (AsPC-1), several known PPARγ agonists, including thiazolidinediones, increased PTEN expression, which was antagonized by the PPARγ antagonist GW9662 (Farrow and Evers, 2003). In addition, activation of PPARγ by rosiglitazone up-regulated PTEN expression in human macrophages, Caco-2 colorectal cancer cells, and MCF-7 breast cancer cells (Patel et al., 2001). All of these results suggest that PPARγ activation has tumor suppression and anti-inflammatory activities by up-regulating PTEN expression. On the other hand, the effects of PPARγ activation on PTEN expression in insulin-sensitive adipocytes and skeletal muscle cells have not been reported. In contrast to the previous results on cancer cells and macrophages, we found the opposite effects showing that PPARγ activation down-regulated PTEN mRNA and protein levels, leading to Akt activation and glucose uptake in differentiated

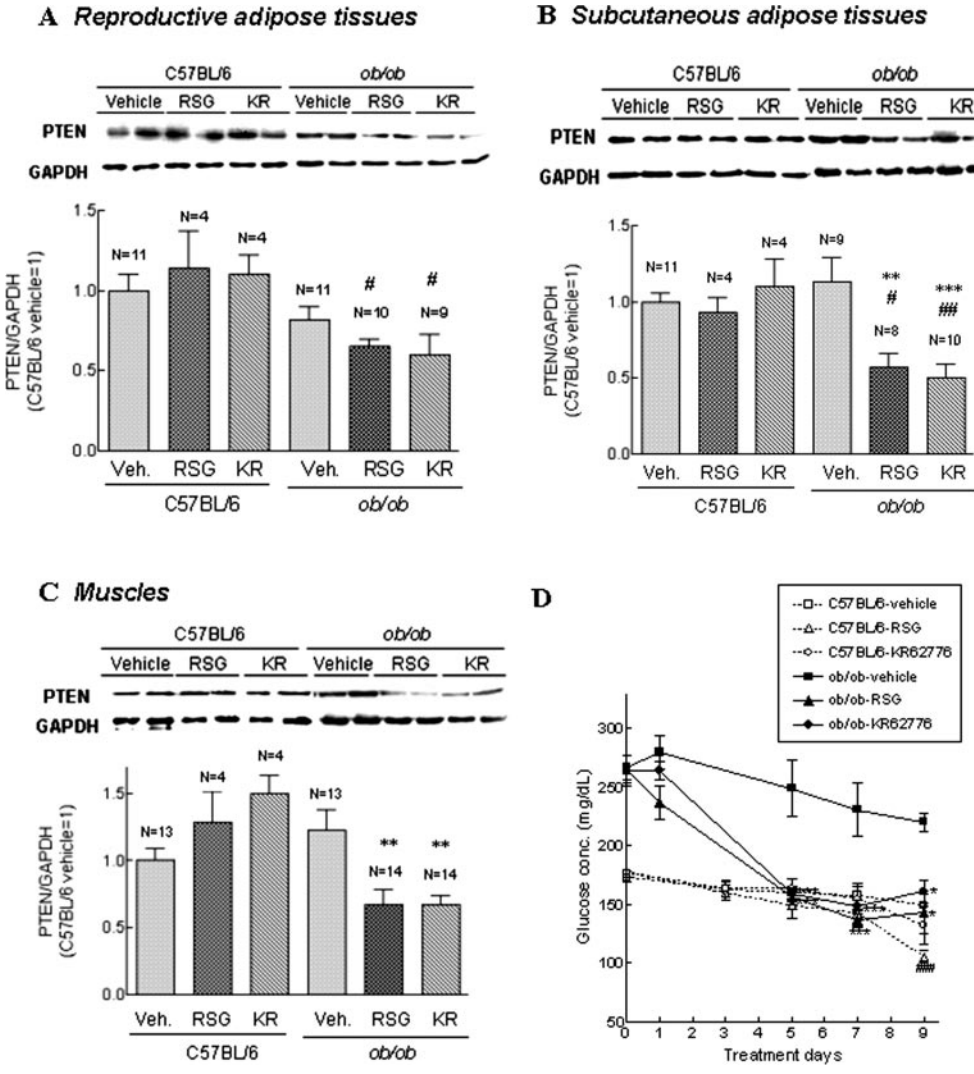


Fig. 11. Effects of KR-62776 and rosiglitazone on PTEN expression in reproductive adipose tissues (A), subcutaneous adipose tissues (B) and muscles (C), and on the plasma glucose levels (D) of C57BL/6J and *ob*/*ob* mice. Each mouse was treated with 50 mg/kg/day KR-62776 (diluted saline plus 0.2% Tween 80 solution) or 10 mg/kg/day rosiglitazone, i.p., twice per day for 9 days. Plasma glucose levels were determined at the indicated days, and PTEN protein expression was determined by Western blot at the end of the experiments. Values are means ± S.E.M. of 4 to 13 experiments. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ versus C57BL/6J vehicle group; **, $P < 0.01$ and ***, $P < 0.001$ versus ob/ob vehicle group.

C2C12 cells. Similar phenomenon by the agonists treatment was observed in 3T3-L1 adipocytes, indicating that PTEN suppression is critical for stimulating glucose uptake by PPARγ activation in both cell types. The promoter region of PTEN is known to contain PPAR responsive element sequence (Patel et al., 2001); thus, the activated PPARγ may bind to the sequence, leading to the regulation of PTEN expression. Clear explanation for the dual effects of PPARγ activation on PTEN regulation is unknown at present, but it is possible that differences in cell types, comprising different coactivators or corepressors and other experimental conditions, may account for the discordance between the results.

One mechanism for the short-term regulation of PTEN involves covalent modifications, including phosphorylation. The 50 amino acid C-terminal domain of PTEN (referred as the tail region) contains the phosphorylation sites at Ser380, Thr382, and Thr383. It was shown previously that phosphorylation of the PTEN tail increased the stability of the protein with little effect on phosphatase activity by masking it from proteolytic degradation (Vazquez et al., 2000; Birle et al., 2002). Although we have not determined the PTEN phosphorylation in the present study, it is possible that PTEN phosphorylation may be decreased by KR-62776 due to the consequence of decreased PTEN expression via transcriptional regulation. Alternatively, decreased PTEN phosphorylation may be induced by KR-62776, probably via regulation of PPARγ-dependent kinase or phosphatase, and that resultant instability of PTEN may result in the decreased PTEN protein levels. One possible candidate that mediates PTEN phosphorylation is casein kinase 2 (Torres and Pulido, 2001). Clearly, much is still to be understood about whether the suppressive effect of PPARγ on PTEN is direct or indirect via phosphorylation regulation.

Despite many studies on the role of PTEN in adipocytes by using different approaches for modulating PTEN activity, the functional significance of endogenous PTEN in glucose uptake is somewhat controversial. Ono et al. (2001) reported that endogenous PTEN is not important in the regulation of glucose transport in 3T3-L1 adipocyte. Likewise, studies using various PTEN mutants revealed that endogenous PTEN may not modulate GLUT4 translocation in rat primary adipocytes under normal conditions (Mosser et al., 2001). On the other hand, the results using anti-PTEN antibodies to interfere with endogenous PTEN activity indicated that basal and insulin-stimulated GLUT4 translocation was potentiated in 3T3-L1 adipocytes, implying that endogenous PTEN is functionally active for GLUT4 translocation (Nakashima et al., 2000). In keeping with this idea that PTEN seems to be constitutively active, the observation in the present study also raises the possibility that endogenous PTEN negatively regulates PI-3 kinase activity in C2C12 and 3T3-L1 cells and that the releasing suppressive activity of PTEN by PPARγ-mediated down-regulation leads to the stimulation of PI-3 kinase-Akt pathway.

In summary, our data demonstrate that PPARγ activation in skeletal muscle cells and adipocytes results in the down-regulation of PTEN expression, subsequently up-regulating glucose uptake. All of the effects of KR-62776 described in the present study were also observed with the alternative PPARγ agonist rosiglitazone, lending further support for the role of PPARγ activation in the two cell types. Taking previous results showing up-regulation of PTEN by PPARγ agonists in cancer cells into consideration, it may be reasonable to generalize that PPARγ activation has dual effects on PTEN expression depending on the cell types: up-regulation leading to apoptosis in cancer cells, and down-regulation leading to glucose uptake in muscle cells and adipocytes. We believe that these data represent the first evidence of a PPARγ-dependent down-regulation of PTEN expression in C2C12 and 3T3-L1 cells, thereby up-regulating insulin signaling. Furthermore, the suppression of PTEN expression was also observed in vivo by administration of the two agonists. Although the relative contribution of PTEN suppression for insulin sensitization by PPARγ activation is unclear at present, our findings provide a new insight for the mechanism of PPARγ-mediated improvement of insulin sensitivity. In addition, KR-62776, a new PPARγ agonist, may provide a good lead compound for the development of therapeutic agents for the treatment or prevention of type 2 (non-insulin-dependent) diabetes mellitus.

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