Troglitazone Induces GLUT4 Translocation in L6 Myotubes

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A number of studies have demonstrated that insulin resistance in the skeletal muscle plays a pivotal role in the insulin resistance associated with obesity and type 2 diabetes. A decrease in GLUT4 translocation from the intracellular pool to the plasma membranes in skeletal muscles has been implicated as a possible cause of insulin resistance. Herein, we examined the effects of an insulin-sensitizing drug, troglitazone (TGZ), on glucose uptake and the translocation of GLUT4 in L6 myotubes. The prolonged exposure (24 h) of L6 myotubes to TGZ (10^{-5} \text{ mol/l}) caused a substantial increase in the 2-deoxy-[3H]D-glucose (2-DG) uptake without changing the total amount of the glucose transporters GLUT4, GLUT1, and GLUT3. The TGZ-induced 2-DG uptake was completely abolished by cytochalasin-B (10 \mu\text{mol/l}). The ability of TGZ to translocate GLUT4 from light microsomes to the crude plasma membranes was greater than that of insulin. Both cycloheximide treatment (3.5 \times 10^{-5} \text{ mol/l}) and the removal of TGZ by washing reversed the 2-DG uptake to the basal level. Moreover, insulin did not enhance the TGZ-induced 2-DG uptake additively. The TGZ-induced 2-DG uptake was only partially reversed by wortmannin to 80%, and TGZ did not change the expression and the phosphorylation of protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator–activated receptor; RT, reverse transcription; TGZ, troglitazone.

Insulin resistance, defined as the inability of cells or tissues to respond to physiological levels of insulin, is a characteristic condition of early-stage type 2 diabetes and obesity (1). One of the thiazolidinedione derivatives, troglitazone (TGZ), was recently applied to the treatment of type 2 diabetic patients with insulin resistance. Evidence from both in vivo and in vitro studies indicates that TGZ alters glucose, protein, and lipid metabolism (2–5). The thiazolidinediones are high-affinity ligands for the adipocyte-specific peroxisome proliferator–activated receptor (PPAR)-\(\gamma\) form (6). Several aspects of intracellular metabolism are subjected to transcriptional control by PPAR-\(\gamma\), which is much more abundant in adipocytes than in skeletal muscles (7). TGZ was recently reported to upregulate PPAR-\(\gamma\) gene expression in skeletal muscle of patients with type 2 diabetes (8). Thus, it is conceivable that thiazolidinediones regulate glucose metabolism in skeletal muscles directly.

With use of cultured adipocytes, it has been found that pioglitazone, another thiazolidinedione compound, enhances glucose uptake in 3T3-F442A preadipocytes by increasing both GLUT4 and GLUT1 contents (4) and that TGZ enhances basal glucose transport by increasing the GLUT1 level in 3T3-L1 adipocytes without changing GLUT4 content (5). Several mechanisms by which these compounds enhance insulin action have been proposed. Recently, TGZ was reported to increase the number of small adipocytes without changing the white adipose tissue mass and thus to improve the insulin resistance in obese Zucker rats (9).

In contrast, it was reported that the in vivo action of thiazolidinediones is mediated mainly through skeletal muscles in a study using the glucose clamp technique in humans (10). Moreover, Burant et al. (11) reported the antidiabetic action of TGZ in aP2/DTA mice, in which white fat and brown fat are virtually eliminated by the antidiabetic action of TGZ in aP2/DTA mice. In skeletal muscles, however, only a few in vitro studies on thiazolidinediones have been conducted. It has been reported that M3, a metabolite of TGZ, enhances glucose uptake and that the total amount of GLUT1 and GLUT4 was increased by M3 in a cultured muscle cell line, L6 cells (12). But the effect of TGZ on glucose transporters was not shown. Okuno et al. (13) showed an acute effect of TGZ on glucose metabolism in perfused rat hindlimb muscles. TGZ has also been reported to have both acute and chronic effects to improve glucose metabolism with a slight in-

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2-DG, 2-deoxy-[3H]D-glucose; \(\alpha\)-MEM, \(\alpha\)-minimal essential medium; AMPK, 5’AMP-activated protein kinase; CPM, crude plasma membrane; DTT, dithiothreitol; FBS, fetal bovine serum; KRP, HEPES-buffered Krebs-Ringer phosphate; LM, light microsome; LPS, liposaccharide; PCR, polymerase chain reaction; PI, phosphatidylinositol; PKB, protein kinase B; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator–activated receptor; RT, reverse transcription; TGZ, troglitazone.
crease in GLUT1 but no increase in GLUT4 in primary cultured tissue of skeletal muscle from obese type 2 diabetic subjects (14). In an in vivo study using a euglycemic glucose clamp in rats, Miles et al. (15) demonstrated that TGF prevents hyperglycemia-induced insulin resistance in skeletal muscles. However, it is not yet completely clear whether TGF-induced changes in glucose transporters account for the improvement of insulin resistance in obesity and type 2 diabetes.

The purpose of the present study was to analyze the effect of TGF on glucose transport in skeletal muscles and to elucidate the mechanisms underlying the improvement of insulin actions.

**RESEARCH DESIGN AND METHODS**

**Materials.** TGF and BRL49653 were obtained from Sankyo (Tokyo). Darglitazone was obtained from Pfizer (Groton, CT). α-Minimal essential medium (α-MEM) was purchased from Nihon (Koto, Japan). Fetal bovine serum (FBS) was obtained from Sanko Junyaku (Tokyo). Radioactive reagents were acquired from NEN Life Science Products (Boston, MA). Protein Ag/Agarose beads were from Pierce (Rockford, IL). Male Sprague-Dawley rats weighing 120–140 g were purchased from Shimizu Breeding Laboratories (Koto, Japan). All other chemicals were purchased from Sigma (St. Louis, MO), unless otherwise noted.

**Cell culture of L6 myoblasts and myotubes.** The rat L6 skeletal muscle cell line (a gift from Dr. Amira Klip) was maintained in α-MEM containing 10% FBS and 1% antibiotic solution (penicillin-streptomycin; Gibco, Gaithersburg, MD) in 80-cm² flasks in an atmosphere of 5% CO₂ at 37°C, as reported by Mitsumoto and Klip (16). L6 cells were rendered quiescent in α-MEM containing 2% FBS for 5–6 days to promote fusion into myotubes. The percentage of myotube formation was determined as the percentage of nuclei present in multinucleated myotubes by phase-contrast microscopy. In the present experiment, ~80–90% of the myotubes fused into myotubes.

**2-Deoxy-[3H]glucose uptake.** The cells were grown in six-well plates (Corning, Corning, NY). After the indicated period of incubation with or without TGF in α-MEM, the cells were incubated without FBS for 5 h again in the presence or absence of TGF. The cells were then rinsed with KRPH (250 mmol/l sucrose, 5 mmol/l KCl, 1.3 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, and 30 mmol/l HEPES [pH 7.4]). The 10 μmol/l 2-deoxy-[3H]-glucose (2-DG) (1 μCi/ml) uptake was measured over a 10-min period under conditions in which the uptake was linear. The uptake measurement was made in triplicate. Nonspecific uptake was determined in the presence of 10 μmol/l cytochala- sine-B and was subtracted from the total uptake. The uptake of 2-DG was terminated after 10 min by rapidly aspirating off the radioactive incubation medium and washing the cells three times in ice-cold phosphate-buffered saline. The radioactivity associated with the cells was determined by cell lysis in 0.5 N NaOH with neutralization by the addition of 0.5 N HCl, followed by liquid scintillation (Scintisol EX-H; Dojin Chemicals, Kumamoto, Japan). Aliquots from each well were used to determine the protein concentration using the BCA Protein assay kit (Pierce). In experiments in which the effect of insulin was examined, 10⁻⁷ mol/l insulin was added to the incubation mixture in KRPH for 20 min before transport studies. Nonspecific uptake and absorption were always <15% of the total uptake.

**Subcellular fractionation.** The subcellular fractionation of myotubes was carried out by the method of Mitsumoto and Klip (16) with slight modification. The cells from 10-cm dishes were gently scraped, centrifuged (700g for 10 min), and placed on ice. All subsequent steps were carried out at 4°C. The cells were resuspended in buffer I (250 mmol/l sucrose, 5 mmol/l NaNO₃, 2 mmol/l EGTA, 200 μmol/l phenylmethylsulfonyl fluoride [PMSF], 1 μmol/l pepstatin A, 1 μmol/l aprotinin, and 20 mmol/l HEPES [pH 7.4]) and then homogenized using 20 strokes of a Dounce homogenizer. This homogenization was sufficient for the almost-complete cell breakage of myotubes, as judged by phase-contrast microscopy. The homogenate was centrifuged at 700g for 5 min to remove nuclei and unbroken cells. The supernatant was centrifuged at 31,000g for 60 min to pellet the crude plasma membrane (CPM). The light microsomes (LMS) were collected from the 31,000g supernatant by centrifugation at 190,000g for 60 min. Both the CPM and LMs pellets were suspended in buffer I and frozen at –80°C.

**Gel electrophoresis and immunoblotting.** Proteins (20 μg) were separated in 10% SDS-PAGE and transferred to a polyvinylidene fluoride transfer membrane (Vest Life Science Products) in 25 mmol/l Tris, 102 mmol/l glycine, and 20% methanol as described (17). After transfer, the membrane was blocked in 4% nonfat milk. The first antibodies used for the detection of GLUT1, GLUT3, and GLUT4 were generated against the COOH-terminus deduced from each transporter (gifts from Drs. S.W. Cushman and L.A. Simpson). For the detection of protein kinase C (PKC), α-phosphorylated PKB, we used an anti-PKB or α-phosphorylated (Ser473) PKB antibody, respectively (New England BioLabs, Beverly, MA). For the detection of protein kinase C (PKC-α, PKC-β2, and PKC-ζ, we used an anti-PKC-α (Transduction Laboratories, Lexington, KY), anti–PKC-β2 (Gibco BRL), and anti–PKC-ζ (Gibco BRL) antibody, respectively. The results were then visualized with horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence kit (Amersham, Amersham, U.K.).

**Calculation of the number of glucose transporters.** To elucidate the mechanisms underlying the improvement of insulin resistance in obesity and type 2 diabetes. The amount of each transporter in the myotubes was then calculated from the results of the Western blotting.

**RNA isolation and Northern blot analysis.** Total RNA was isolated from the cells by extraction with Trizol (Gibco BRL) and prepared according to the manufacturer’s instructions. Typically, 50 μg total RNA was isolated from cells cultured in a 10-cm dish. For Northern blot analysis, 40 μg total RNA was separated by electrophoresis in 1.2% agarose and 2.0 mol/l formaldehyde gels and transferred to a nitrocellulose membrane (Hybond, Piscataway, NJ). After ultraviolet crosslinking, the filters were prehybridized, hybridized, and subjected to analysis as described elsewhere. The Northern blot analysis was performed using [32P]-labeled rat GLUT4 and GLUT1 cDNA fragments as probes (30). The mRNA levels were normalized to the 28S ribosomal RNA levels in the cells to correct for differences in the amount of RNA applied. The mRNA levels (arbitrary units) are expressed in relation to those of the control cells.

**5'-AMP-activated protein kinase activity assay.** L6 myotubes were incubated in the absence or presence of TGF (10⁻⁷ mol/l) for 24 h. Rat epidermal growth factors were dissected and incubated in Krebs-Ringer bicarbonate buffer as described (21). L6 myotubes and muscles were then homogenized in ice-cold lysis buffer (1:100 wt/vol) containing 20 mmol/l Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/l NaCl, 250 mmol/l sucrose, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 2 mmol/l dithiothreitol (DTT), 4 mg/ml leupeptin, 50 μg/ml trypsin inhibitor, 0.1 mmol/l benzamidine, and 0.5 mmol/l PMSF and then centrifuged at 14,000g for 20 min at 4°C. Supernatants (200 μg protein) were immunoprecipitated with an antibody raised using a synthetic peptide of the rat 5'-AMP-activated protein kinase (AMPK) α subunit (residues 490–514) from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitates were washed twice in lysis buffer and two times in buffer containing 240 mmol/l HEPES (pH 7.4) and 480 mmol/l NaCl. The kinase reaction was carried out in 40 mmol/l HEPES (pH 7.0), 0.2 mmol/l AMP, 80 mmol/l NaCl, 0.8 mmol/l DTT, 5 mmol/l MgCl₂, 0.2 mmol/l ATP (2 μCi [γ⁻³²P]ATP), and 0.1 mmol/l SAMS peptide (23) for 20 min at 30°C. Reaction products were spotted on Whatman P81 filter paper, and then the papers were extensively washed in 1% phosphoric acid. The radioactivity in the papers was measured with a scintillation counter. Kinase activity was expressed as incorporated ATP (picomoles) per immunoprecipitated protein (milligrams) per minute.

**Reverse transcription–polymerase chain reaction of PPAR-γ.** Reverse transcription (RT) was performed on 2 μg RNA using the Superscript Pre-amplification System (Gibco BRL) according to the manufacturer’s instructions. The cDNA was amplified by polymerase chain reaction (PCR) using a pair of primers to amplify the PPAR-γ fragment as follows (nucleotide positions according to GenBank): 5'-GGATCTAGGACCCAGGATTCCTC3' (residues 1120–1143, sense strand) and 5'-GGGTTCTCTACACTGAAATATGAC3' (residues 1252–1275, antisense strand).

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance was tested with a one-way analysis of variance followed by Duncan’s multiple range test and Wilcoxon’s signed-rank test, as appropriate, and differences were accepted as significant at the P < 0.05 level.

**RESULTS**

**Effect of TGF on 2-DG uptake.** We examined the effect of TGF (10⁻⁷ mol/l) on the 2-DG uptake in L6 myotubes over a 30-h time course. As shown in Fig. 1A, TGF increased the 2-DG uptake in a time-dependent fashion, and
the uptake was maximal after 15 h (0 h, 0.25 ± 0.13 vs. 30 h, 2.95 ± 0.26 nmol/min/well, well). The dose-response curve (Fig. 1B) showed a maximum response at concentrations >7.5 × 10⁻⁶ mol/l. This TGZ-stimulated glucose uptake was completely blocked by adding cytochalasin-B (10 μmol/l) to the incubation buffer just before the 2-DG assay (Fig. 1B). TGZ treatment had no significant effect on cell number or protein content in each well (data not shown). There were no obvious differences in cell morphology after the treatment.

Effects of TGZ on glucose transporter protein and mRNA levels. We next investigated whether the TGZ-induced 2-DG uptake was accompanied by an increase in the total amount of glucose transporters in the myotubes. It has been reported that L6 myotubes contain three isoforms of glucose transporters: GLUT4, GLUT1, and GLUT3 (24). After incubating the cells with TGZ (10⁻⁵ mol/l) for 24 h, we assessed the total amount of GLUT4, GLUT1, and GLUT3 protein by Western blotting using the specific antibody against each transporter (Fig. 2A and B). TGZ did not affect the amount of each transporter in the myotubes.

To analyze the relative contribution of the three isoforms of glucose transporters to 2-DG uptake in L6 cells, we performed Western blot analysis using the plasma membranes from rat adipocytes for GLUT4 and GLUT1 and the rat brain for GLUT3 as standards (the left lanes in Fig. 2A). The amount of each glucose transporter in the standard membrane was estimated by a cytochalasin-B binding study and photo-affinity labeling with [³H]ATB-BMPA as described in RESEARCH DESIGN AND METHODS. The amounts of GLUT4 and GLUT1 in the adipocytes and GLUT3 in the brain were calculated from the results of a cytochalasin-B binding study and photo-affinity labeling with [³H]ATB-BMPA as described in RESEARCH DESIGN AND METHODS. The amount of each glucose transporter in L6 myotubes was then calculated from the ratio in the Western blots. Comparable results were obtained by Northern blotting (Fig. 2C and D).

FIG. 1. Effects of TGZ on 2-DG uptake in L6 myotubes. A: Time course of the TGZ-induced stimulation of 2-DG uptake in L6 myotubes. L6 myotubes were incubated in the presence of TGZ (10⁻³ mol/l) for 0 to 30 h (●). Control cells (○) were incubated with vehicle alone (DMSO 0.1%) for 30 h. The 2-DG uptake was then determined in triplicate as described in RESEARCH DESIGN AND METHODS. The values are the means ± SE of four independent experiments. B: Dose-dependent effects of TGZ on 2-DG uptake in L6 myotubes. L6 myotubes were incubated for 24 h in the presence of various concentrations of TGZ (0 to ~10⁻³ mol/l) (○). The 2-DG uptake was then determined as described in RESEARCH DESIGN AND METHODS. The ● shows the result of TGZ-induced (10⁻³ mol/l) 2-DG uptake measured in the presence of cytochalasin-B (10 μmol/l). The values are means ± SE of four independent experiments.

FIG. 2. Effect of TGZ on the amounts of GLUT4, GLUT1, and GLUT3 glucose transporters in the total cell lysates and the amounts of GLUT4, GLUT1, and GLUT3 mRNA levels in L6 myotubes. A: L6 myotubes were incubated for 24 h in the presence or absence of TGZ (10⁻² mol/l). Total cell lysates (20 μg) were subjected to electrophoresis in 10% SDS-PAGE and Western blotting. As standards for the quantification of each transporter, rat adipocyte plasma membranes for GLUT4 and GLUT1 and rat brain for GLUT3 were applied in the left lanes, respectively. The membranes were immunoblotted with anti-GLUT4, anti-GLUT1, or anti-GLUT3 antibodies. Typical results of four independent experiments are shown. B: Quantitation of GLUT4, GLUT1, and GLUT3. The signals in immunoblots were quantitated by an image analyzer and normalized using the adipocytes and the brain as standards. The amounts of GLUT4 and GLUT1 in the adipocytes and GLUT3 in the brain were calculated from the results of a cytochalasin-B binding study and photo-affinity labeling with [³H]ATB-BMPA as described in RESEARCH DESIGN AND METHODS. The amount of each glucose transporter in L6 myotubes was then calculated from the ratio in the Western blots. The values are the means ± SE of four independent experiments. C: L6 myotubes were incubated for 24 h in the presence or absence of TGZ (10⁻² mol/l). Total RNA was isolated as described in RESEARCH DESIGN AND METHODS, and the RNA samples (40 μg) were subjected to a Northern blot analysis to determine the levels of GLUT4, GLUT1, and GLUT3 mRNA. Typical results of three independent experiments are shown. D: Bar graphs show the mRNA levels (mean ± SE) normalized to the 28S ribosomal RNA level in the cells to correct for differences in the amount of RNA applied. The mRNA levels (arbitrary units) are expressed in relation to those of the control cells.
Effects of cycloheximide and the removal of TGZ by washing on 2-DG uptake. To examine whether the change in the TGZ-treated cells was caused by a protein synthesis-dependent process, we studied the effect of cycloheximide \((3.5 \times 10^{-6} \text{ mol/l})\) on TGZ-induced 2-DG uptake. In the presence of cycloheximide, the effect of TGZ was completely abolished (Fig. 4A: control, \(0.9 \pm 0.2\); TGZ, \(2.7 \pm 0.2\); TGZ and cycloheximide, \(1.0 \pm 0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{well}^{-1}\)). The 2-DG uptake in the cells treated with cycloheximide alone \((0.5 \pm 0.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{well}^{-1})\) was significantly lower than that in the control cells \((P < 0.05)\). The removal of TGZ by the washing procedure completely reversed the 2-DG uptake to the basal level after further incubation without TGZ for 24 h (Fig. 4A). Moreover, the reversed 2-DG uptake of the cells was restimulated by insulin (Fig. 4A). Thus, the TGZ-stimulated GLUT4 translocation was reversible, suggesting that GLUT4, which was translocated to the plasma membranes by TGZ, could enter the recycling compartment again.

Additive effect of insulin and reversal effect of wortmannin on 2-DG uptake. To clarify the mechanism of the enhancement of GLUT4 translocation to the plasma membrane by TGZ, we first studied whether insulin enhances the TGZ-stimulated 2-DG uptake additively. As shown in Fig. 4B, the 2-DG uptake in TGZ-treated cells \((10^{-5} \text{ mol/l}, 24 \text{ h})\) was not further enhanced by insulin \((10^{-7} \text{ mol/l}, 20 \text{ min})\), thus indicating the likelihood that a complete insulin-dependent transporter translocation has already occurred.

We next examined whether the TGZ-induced 2-DG uptake was reversed by wortmannin, which is a specific inhibitor for phosphatidylinositol (PI) 3-kinase that blocks the insulin-signaling pathway. After treatment of the cells with TGZ \((10^{-5} \text{ mol/l})\) for 24 h, wortmannin \((5 \times 10^{-8} \text{ mol/l})\) was added to the culture medium for 1 h, and the 2-DG uptake was then measured. In the TGZ-treated cells, 2-DG uptake was only partially reversed by wortmannin to \(-80\%
(\text{Fig. 4C}). In a parallel experiment, the 2-DG uptake in insulin-treated cells \((10^{-7} \text{ mol/l}, 20 \text{ min})\) was completely reversed to the basal level by wortmannin within 1 h.

Effects of TGZ on the protein level of PKB, serine-phosphorylated PKB, and PKCs. To examine whether TGZ \((10^{-5} \text{ mol/l})\) affects the post–PI 3-kinase step in the insulin-signaling system for glucose transport, we examined the levels of PKB, phosphorylated (Ser473) PKB, and PKCs \((\text{PKC-}\alpha, \text{PKC-}\beta, \text{PKC-}\zeta)\) in the total cell lysate using Western blots. As shown in Fig. 5A, the protein level of PKB was not changed by the TGZ treatment. The levels of phosphorylated PKB in the basal and insulin-stimulated \((10^{-7} \text{ mol/l})\) states were not changed by TGZ, although insulin alone significantly increased the phosphorylation of PKB. As shown in Fig. 5A (upper panel), the mobility of PKB was shifted in parallel with its phosphorylation. Figure 5B shows that the protein levels of \(\text{PKC-}\alpha, \text{PKC-}\beta,\) and \(\text{PKC-}\zeta\) were not changed by the TGZ treatment \((10^{-5} \text{ mol/l}, 24 \text{ h})\).

Effects of TGZ on AMPK activity. Recent studies \((21,25)\) have provided evidence that AMPK is involved in enhancing glucose transport by an insulin-independent

**FIG. 3.** Effect of TGZ on the subcellular distribution of GLUT4 in L6 myotubes. L6 myotubes were incubated in the absence (Cont) or presence of TGZ \((\text{TGZ}: 10^{-5} \text{ mol/l}, 24 \text{ h})\) or insulin \((\text{INS}: 10^{-7} \text{ mol/l}, 20 \text{ min})\). Subcellular membrane fractions of the L6 myotubes were prepared by sucrose velocity gradient centrifugation. The CPM and LM fractions \((20 \mu \text{g})\) were subjected to SDS-PAGE and Western blotting using anti-GLUT4 antibody. This photograph shows typical results of three independent experiments. The lower panel shows the quantification of GLUT4 protein levels in CPM and LM by densitometry scanning. The levels (arbitrary units, mean ± SE) are expressed in relation to those of GLUT4 in the CPM fraction of the control cells. \(*P < 0.05\) comparing the levels to Cont. \(*P < 0.05\) comparing the levels to INS.
signaling mechanism in skeletal muscle. AMPK is a het-
erotrimeric protein consisting of one catalytic subunit (α)
and two noncatalytic subunits (β and γ) (26). Two iso-
forms of the α subunit have been identified (α1 and 
α2) in mammalian skeletal muscle. The α2 isoform is highly ex-
pressed in skeletal muscle, heart, and liver, whereas the 
α1 isoform is widely distributed in many tissues (27). A recent 
report has shown that the activity of AMPK α2 is closely 
correlated with glucose transport activity in rat skeletal
muscle (28). To examine whether AMPK α2 is involved in 
the TGZ-induced increase in glucose transport, we deter-
mined AMPK α2 activity in L6 myotubes. Figure 6 shows 
that TGZ (10^{-2} mol/l, 24 h) did not change AMPK α2 activ-
ity in L6 myotubes. Furthermore, AMPK activity in L6 myo-
tubes was 1/70 of that in nonstimulated rat skeletal muscle.

FIG. 4. A: Effects of cycloheximide and the removal of TGZ by a washing procedure on 2-DG uptake in L6 myotubes. The cells were incubated in the absence (Cont) or presence of TGZ (TGZ, 10^{-2} mol/l) without or with cycloheximide (CHX, 3.5 × 10^{-6} mol/l) for 24 h; Cont, TGZ, CHX, TGZ+CHX, respectively. As an internal control, the result for insulin-
treated (10^{-7} mol/l, 20 min) cells is also shown (INS). After their in-
cubation with TGZ (10^{-2} mol/l) for 24 h, the cells were washed three 
times with fresh α-MEM to remove TGZ. The cells were incubated for a 
further 24 h, and the 2-DG uptake was determined in the absence or 
presence of insulin (INS, 10^{-7} mol/l) for 20 min; Cont, INS, TGZ, 
TGZ+INS, respectively. The values are the means ± SE of four independent exper-
iments. *P < 0.01 comparing the levels to Cont. **P < 0.05 comparing the levels to Cont. †P < 0.001 comparing the levels to Cont. ‡P < 0.05 comparing the levels to Cont.

B: Additive effect of insulin on TGZ-induced 2-DG uptake in L6 myotubes. L6 myo-
tubes were incubated in the absence or presence of TGZ (TGZ, 10^{-2} mol/l) for 24 h. The basal and insulin-stimulated (10^{-7} mol/l, 20 min) 2-DG uptake was determined in the absence or presence of insulin (INS, 10^{-7} mol/l) for 24 h; Cont, INS, TGZ, TGZ+INS, respectively. The values are the means ± SE of four independent experi-
ments. *P < 0.01 comparing the levels to Cont. **P < 0.05 comparing the levels to Cont. †P < 0.001 comparing the levels to Cont. ‡P < 0.05 comparing the levels to Cont.

C: Reversal effect of wortmannin on insulin- and TGZ-induced 2-DG uptake in L6 myo-
tubes. After the incubation of L6 myotubes with TGZ (TGZ, 10^{-3} mol/l) for 24 h or with insulin for 20 min (INS), wortmannin (WOR, 5 × 10^{-8} mol/l) was or was not added to the cells. The cells were incubated for another 1 h, and the 2-DG uptake was determined as described in RESEARCH DESIGN AND METHODS. The values are the means ± SE of four independent experiments. *P < 0.01 comparing the levels to Cont. **P < 0.05 comparing the levels to Cont. †P < 0.001 comparing the levels to Cont. ‡P < 0.05 comparing the levels to Cont.

FIG. 5. A: Effects of TGZ and insulin on the levels of protein and phosphorylation of PKB. L6 myotubes were incubated in the absence or presence of TGZ (TGZ, 10^{-2} mol/l) for 24 h and then stimulated by insulin (INS, 10^{-7} mol/l) for 1 min. The cells were solubilized with Laemmli’s sample buffer, immediately boiled for 3 min, and then subjected to SDS-PAGE and Western blotting. The immunoblotting was performed using anti-PKB antibody (α-PKB, upper panel) or antiphosphorylated PKB antibody (α-pPKB, lower panel), horseradish peroxidase–conju-
gated secondary antibodies, and an enhanced chemiluminescence kit. The results are representative blots of three independent experiments. B: Effects of TGZ on the protein level of PKCs. L6 myotubes were incubated in the absence or presence of TGZ (TGZ, 10^{-2} mol/l) for 24 h. Total cell lysates (20 μg) were subjected to electrophoresis in 7.5% SDS-PAGE gel and Western blotting. The membranes were immuno-
blotted with anti–PKC-λ, anti–PKC-β2, or anti–PKC-ζ antibodies. Typical results of four independent experiments are shown. Cont, control.

2-DG uptake of other thiazolidinediones. We investigat-
ed the effects of two more thiazolidinediones, darglitazone
and BRL49653, on glucose uptake in L6 myotubes. Figure 7 shows that TGZ, darglitazone, and BRL49653 increased the 2-DG uptake in L6 myotubes in a dose-dependent manner. The rank order of potency for the glucose uptake in L6 myotubes was as follows: TGZ > darglitazone > BRL49653.

Detection of PPAR-γ mRNA in L6 myotubes. Figure 8 shows the result of the RT-PCR using a primer to amplify the sequences of PPAR-γ cDNA. PPAR-γ mRNA was detected in L6 myotubes only when PCR was performed for 35 cycles, whereas in adipocytes (fat), it was detected after both 25 and 35 cycles. The mRNA level of PPAR-γ was not enhanced by the TGZ treatment (10⁻⁵ mol/l, 24 h). PPAR-γ protein was not detected by Western blot analysis in contrast with the adipocytes (data not shown).

Effect of α-tocopherol on 2-DG uptake. The action of TGZ may be attributable to the similarity of its molecular structure to that of α-tocopherol (29,30). Figure 9 shows that α-tocopherol (10⁻⁵ mol/l, 24 h) did not increase glucose uptake in L6 myotubes.

DISCUSSION

A number of studies have demonstrated that insulin resistance in skeletal muscles plays a role in the insulin resistance of obesity and type 2 diabetes. A decrease in GLUT4 translocation from LMs to the plasma membranes has been implicated as a possible cause of insulin resistance, as has the reduced kinase function of the insulin receptor. Investigations of the mechanisms underlying insulin resis-
tance in type 2 diabetes have revealed tissue-specific regulation of GLUT4 with decreased gene expression in adipose cells but not in skeletal muscle (31). This has led to the hypothesis that alterations in the trafficking of the GLUT4 vesicle or in the exposure or activation of the GLUT4 transporter may cause insulin resistance in skeletal muscle in obesity and diabetes. In a human study, TGZ has been reported to increase peripheral glucose disposal, mainly in skeletal muscles, but to have no significant effect on hepatic glucose production (10). Moreover, Burant et al. (11) reported the antidiabetic action of TGZ in aP2/DTA mice, in which white fat and brown fat are virtually eliminated by the fat-specific expression of diphtheria toxin A chain. These results suggest that TGZ is one of the keys to improving insulin resistance in skeletal muscle. To investigate this hypothesis, we tested the effect of TGZ on glucose uptake and changes in glucose transporters in L6 myotubes.

In the present study, the TGZ-stimulated 2-DG uptake was completely blocked by cytochalasin-B, suggesting that this effect was caused by changes in glucose transporters in the plasma membrane and not by an increase in simple diffusion across the membrane. The total amount of glucose transporters, including GLUT1, GLUT3, and GLUT4, was not changed by the TGZ treatment. We have also shown that GLUT4 was expressed much more intensely than GLUT1 and GLUT3 in L6 myotubes. The ability of TGZ to translocate GLUT4 from LM to the CPM was more augmented than that of insulin. In another study (12), the short-term (2-h) exposure of fully differentiated L6 myotubes to TGZ had no effect on glucose transport activity, but the long-term (72-h) treatment of myotubes with TGZ resulted in a doubling of glucose transport in the absence of insulin. This is compatible with the present results. However, the authors of that study did not refer to the changes of glucose transporters in their conditions. Recently, Cooksey et al. (32) reported that transgenic mice overexpressing the rate-limiting enzyme for hexosamine synthesis, glutamine:fructose-6-phosphate amidotransferase, had hexosamine-induced insulin resistance because of a decrease in the GLUT4 translocation in the skeletal muscle and that TGZ improved the glucose disposal in those transgenic mice. These reports further support the hypothesis that TGZ directly acts on skeletal muscles and affects the translocation of GLUT4.

As shown in Fig. 4B, exposure of the cells to TGZ resulted in an increase in basal glucose uptake. More important, there was no further increase in insulin-stimulated glucose transport, thus indicating the likelihood that complete insulin-dependent transporter translocation in the newly steady state was already achieved and that the signaling pathway activated by TGZ converges with that activated by insulin. In the presence of insulin, GLUT4 is recycled and a new steady state is achieved in rat adipose cells (33), increasing exocytosis, although it is still unclear whether insulin also affects the endocytosis of GLUT4. Acting as an inhibitor of the lipid kinase PI 3-kinase (34), wortmannin blocks the insulin-stimulated translocation of GLUT4 from its basal compartment to the plasma membrane (35). In the present study, wortmannin reversed the insulin-stimulated 2-DG uptake to the basal level when added after full stimulation by insulin (Fig. 4C). The addition of wortmannin to the TGZ-stimulated cells, however, did not reverse the uptake to the basal level. In addition, the amount of PKB and its phosphorylated state (Ser 473) were not changed by the TGZ treatment (Fig. 5A). TGZ did not affect the protein levels of each isoform of PKC (Fig. 5B). Although the site of action on TGZ is still not clear, one possibility is that TGZ acts at a step beyond PI 3- kinase, PKB, and PKC in which GLUT4 endocytosis is enhanced according to the present results. Another possibility is that TGZ reduces the endocytosis of GLUT4 and thus allows the accumulation of GLUT4 in the plasma membrane. The latter mechanisms have been described in several recent articles. The disassembly of clathrin lattices by potassium depletion results in the accumulation of GLUT4 at the cell surface (36), and the expression of a dominant interfering dynamin mutant in rat (37) and 3T3-L1 (38) adipocytes inhibits GLUT4 endocytosis. Further study will be necessary to elucidate the precise site of action of TGZ with regard to the exocytosis and endocytosis steps.

The antioxidant action of TGZ, which is attributable to the similarity of its molecular structure to that of B-tocopherol, is considered to be of benefit in preventing diabetic vascular complications, in addition to having hypoglycemic and hypolipidemic effects (29,30). We studied 2-DG uptake in the presence of B-tocopherol. Figure 9 shows that B-tocopherol (10⁻⁵ mol/l, 24 h) did not increase glucose uptake in L6 myotubes. Various thiazolidinediones increased glucose uptake in L6 myotubes as shown in Fig. 7. We believe that the structure of the thiazolidinedione is needed for enhancing glucose uptake in L6 myotubes, but that of B-tocopherol is not.

The onset of the augmentation of the basal glucose transport by TGZ was slow; 6–12 h passed before significant effects were observed, suggesting that the effect requires the synthesis of new proteins. To examine this possibility, we incubated L6 cells together with cycloheximide (3.5 × 10⁻⁶ mol/l) and TGZ for 24 h. Figure 4A illustrates that the addition of the protein synthesis inhibitor cycloheximide prevented the TGZ-induced 2-DG uptake. Furthermore, the washing procedure to remove TGZ from the cells completely reversed the 2-DG uptake to the level of the control cells, and the uptake could be restimulated by insulin. Thus, the increase of glucose uptake with time apparently reflects the ongoing synthesis of a new protein that affects GLUT4 translocation. Further study will be necessary to find the protein(s).

The recent discovery that thiazolidinediones are synthetic ligands for PPAR-γ reveals a potential mechanism by which TGZ could regulate gene expression. PPAR-γ mRNA levels are lower in skeletal muscle biopsies and cultured muscle than in adipose tissue (39,40), although PPAR-γ is expressed in skeletal muscles (39–42) and provides a potential target for TGZ action. TGZ treatment has also been reported to increase mRNA levels for PPAR-γ in skeletal muscles (8). In fact, PPAR-γ mRNA was detected in L6 myotubes only when RT-PCR was performed in our experiments (Fig. 8), and the mRNA level of PPAR-γ was not enhanced by the TGZ treatment (10⁻⁵ mol/l, 24 h).

The rank order for clinical efficacy has been reported to be BRL49653 > darglitazone > TGZ (43–45). It has also been reported that BRL49653 has high affinity for PPAR-γ among thiazolidinediones (6), and the potency of thiazolidinediones as PPAR-γ agonists correlates with their anti-
diabetic efficacy in vivo (46,47). In our experiments, the rank order of potency for the glucose uptake in L6 myotubes was as follows: TGZ > darglitazone > BRL49653 (Fig. 7), which is inconsistent with the clinical efficacies and affinities for PPAR-γ reported previously. Recently, one of the thiazolidinediones has been reported to possess strong action of lowering blood glucose in spite of its low binding affinity for PPAR-γ (48). This effect is partly explained by unique partial agonism of coactivator recruitment to PPAR-γ. Thiazolidinedione action in skeletal muscle (L6 myotubes) may include activation of PPAR-γ coactivators. Another possibility is that the bioavailability is different among such thiazolidinediones in skeletal muscle or in L6 myotubes. For example, the intracellular concentration of each drug could be different from the extracellular concentration. It is also possible that the thiazolidinediones activate an alternative signal transduction pathway and increase glucose uptake through PPAR-γ-independent mechanisms in skeletal muscle.

In our study, thiazolidinediones (TGZ, BRL49653, and darglitazone) increased glucose transport in L6 myotubes in the absence of insulin. This observation is consistent with previous in vitro results obtained using the L6 cell (12,49), 3T3-L1 adipocyte (50), and cardiomyocyte (51). However, many studies have shown that insulin is necessary for thiazolidinediones to be effective in vivo. Insulin lowers plasma glucose levels both by stimulating glucose uptake into muscle and adipose tissue and by inhibiting hepatic glycogen breakdown and gluconeogenesis. Insulin is also necessary for lipid metabolism. Therefore, it may be reasonable that increased glucose transport by thiazolidinediones is not sufficient to ameliorate the metabolic perturbations under conditions of insulin deficiency such as type 1 diabetes or in animal models of streptozocin-induced diabetes.

AMPK is involved in enhancing glucose transport by an insulin-independent signaling mechanism in skeletal muscle (21). A recent report showed that the activity of AMPKα2 is closely correlated with glucose transport activity in rat skeletal muscle (28). Figure 6 shows that TGZ did not change AMPKα2 activity in L6 myotubes and that the magnitude of the activities was 1/70 that of skeletal muscle of rat per same protein content. The result suggests that AMPK has no major role in TGZ-induced glucose uptake in L6 cells.

According to one report (52), in L6 myotubes, cytokines and lipopolysaccharide (LPS) significantly stimulated nitric oxide (NO) production and induced inducible NO synthase (iNOS) protein and mRNA expression. Cytokines and LPS markedly increased basal glucose transport, but inhibited insulin-stimulated glucose transport. It has been suggested that cytokines/LPS exposure significantly increased GLUT1 transporter protein levels but decreased GLUT4 transporter protein levels by inducing iNOS expression and NO production in L6 cells. Another study (53) found that TGZ upregulated cytokine-stimulated NO synthase in vascular smooth muscle cells, but TGZ alone did not stimulate it. In our study, L6 myotubes were not exposed to cytokines. TGZ did not change the protein levels of GLUT4 and GLUT1. TGZ increased basal glucose transport and did not inhibit insulin-stimulated glucose transport. Thus, we consider the NO pathway not to be involved in the TGZ-induced glucose uptake in L6 myotubes.

In summary, prolonged TGZ treatment stimulates glucose transport in L6 myotubes by activating GLUT4 translocation from the LM to plasma membrane without changing the total amount of GLUT4. Synthesis of protein(s) in the GLUT4 translocation machinery may play an important role in activating transport, although further investigation is necessary to identify the protein(s). The effects of TGZ on GLUT4 translocation may include a new mechanism for improving glucose transport in skeletal muscle.

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