Troglitazone Not Only Increases GLUT4 but Also Induces Its Translocation in Rat Adipocytes

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Thiazolidinediones, insulin-sensitizing agents, have been reported to increase glucose uptake along with the expression of glucose transporters in adipocytes and cardiomyocytes. Recently, we have further suggested that the translocation of GLUT4 is stimulated by thiazolidinediones in L6 myocytes. However, the direct effects of thiazolidinediones on translocation of glucose transporters have not yet been determined. In this study, using hemagglutinin epitope-tagged GLUT4 (GLUT4-HA), we provide direct evidence of the effect of troglitazone on the translocation of GLUT4 in rat epididymal adipocytes. Primary cultures of rat adipocytes were transiently transfected with GLUT4-HA and over-expressed eightfold compared with endogenous GLUT4 in transfected cells. A total of 24 h of treatment with troglitazone (10−4 mol/l) increased the cell surface level of GLUT4-HA by 1.5 ± 0.03–fold (P < 0.01) without changing the total amount of GLUT4-HA, whereas it increased the protein level of endogenous GLUT4 (1.4-fold) without changing that of GLUT1. Thus, the direct effect on the translocation can be detected apart from the increase in endogenous GLUT4 content using GLUT4-HA. Troglitazone not only increased the translocation of GLUT4-HA on the cell surface in the basal state but also caused a leftward shift in the dose-response relations between GLUT4-HA translocation and insulin concentration in the medium (ED50 from ~0.1 to 0.03 nmol/l). These effects may partly contribute to the antidiabetic activity of troglitazone in patients with obesity and type 2 diabetes. Diabetes 50: 2296–2300, 2001

Thiazolidinediones, insulin-sensitizing agents, improve hyperglycemia and hyperinsulinemia by increasing insulin responsiveness and/or sensitivity in obese, type 2 diabetic, and glucose-intolerant patients (1,2). These compounds increase peripheral glucose uptake, while decreasing the serum insulin level and gluconeogenesis in rodent models of type 2 diabetes (3–5). Several mechanisms by which these compounds enhance insulin action have been proposed. Under in vivo conditions, it has been suggested that thiazolidinediones improve insulin resistance by enhancing adipocyte differentiation and increasing the number of small adipocytes in obese Zucker rats (6) as well as correcting the deficit in glucose transporters in KKAy diabetic mice (7). Young et al. (8) reported that repeat treatment of thiazolidinedione enhances insulin action in white adipocytes by increasing insulin binding and cell-surface GLUT4. In in vitro studies, it has been reported that thiazolidinediones increased glucose uptake in 3T3-F442A and 3T3-L1 adipocytes by increasing GLUT4 and/or GLUT1 content (9,10). In mature adipocytes, troglitazone has also been reported to increase GLUT4 mRNA levels (11). Furthermore, thiazolidinediones have been suggested to modulate intrinsic activity and translocation of glucose transporters (12). Recently, we have further suggested that the translocation of GLUT4 is stimulated by thiazolidinediones in L6 myocytes (13). However, the direct effects of thiazolidinediones on the translocation of glucose transporters have not yet been verified. Because the total cellular GLUT4 content was increased by troglitazone in adipocytes, it was difficult to determine the direct effect of troglitazone on the translocation of GLUT4 by the membrane fractionation method.

In the present study, to investigate the direct effect of troglitazone on translocation of GLUT4, we transfected hemagglutinin epitope-tagged GLUT4 (GLUT4-HA) into rat adipocytes, incubated the cells in the presence or absence of troglitazone for 24 h, and detected the cell-surface GLUT4-HA. This system was chosen because troglitazone did not change the expression of GLUT4-HA inserted into the expression plasmid. Moreover, it is possible to examine the direct effect of GLUT4-HA on translocation in physiologically relevant cells. We directly demonstrated for the first time that troglitazone increases the translocation of GLUT4 in adipocytes.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco’s modified Eagle’s medium (DMEM) was obtained from GibCO/BRL (Gaithersburg, MD). Troglitazone was a generous gift from Sankyo (Tokyo), and all other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted.

Animals and adipose cell preparation. Male Sprague-Dawley rats (180–200 g: Shimizu Breeding Laboratories, Kyoto, Japan) fed ad libitum were used throughout the study. The rats were housed under controlled temperature (25°C) and lighting (12-h light-dark, lights on at 8:00 A.M.) conditions. The rats were anesthetized with a gas mixture of 70% CO2 and 30% O2 and decapitated. All experiments were approved by the Institutional Animal Care and Use Committee of Kyoto University. The epididymal fat pads were removed,

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BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; GLUT4-HA, hemagglutinin epitope-tagged GLUT4; HA, hemagglutinin epitope; KRBH, Krebs-Ringer bicarbonate HEPES buffer.

DIABETES, VOL. 50, OCTOBER 2001
minced, and digested with collagenase (type I; Cooper Biochemical, Freehold, NJ). The preparation of the cells was carried out at 37°C in a Krebs-Ringer bicarbonate HEPES buffer (KRBH), pH 7.4, containing 10 mmol/l NaHCO₃, 30 mmol/l HEPES, 1% (w/v) bovine serum albumin (BSA) (fraction V; Intergen, Purchase, NY) and 200 mmol/l adenosine as previously described (14). The cells were then washed three times with KRBB and twice with DMEM supplemented with 5% (w/v) BSA, 200 mmol/l NaHCO₃ and 75 μmol/l aminonucleoside. All incubations were carried out at 37°C in DMEM at a cytotic of ~20% containing the indicated reagents. Troglitazone was dissolved in DMSO, and all cells were exposed to a final concentration of DMSO of 0.1%.

**Plasmid constructs.** pCIS2 is an expression vector that contains a cytomegalo-virus promoter and enhancer with a genetic intron located upstream from the influenza virus hemagglutinin epitope (HA)-1 inserted in the first exofacial loop of human GLUT4. These vectors were kindly supplied by Drs. M.J. Quon and S.W. Cushman.

**Transfection of rat adipocytes.** Isolated rat adipocytes were washed twice with DMEM and resuspended in a cytotic of 40%. A total of 200 μl of the cell suspension was added to 200 μl of the DMEM containing 100 μg of carrier DNA (sheared herring sperm DNA; Boeringer Manheim, Mannheim, Germany) and 0.5 μg of the expression plasmids. Electroporation was performed in a 0.4-cm gap cuvettes (Bio-Rad Laboratories, Richmond, CA) with a double electric shock (800 V, 25 μF, 200 V, and 1,050 μF) using an Easyject electroporator (Equibio, Kent, U.K.). After the electroporation, the cells were washed once in DMEM, pooled in groups of 4–10 cuvettes, and cultured in DMEM containing 3.5% BSA with or without troglitazone (10⁻⁴ mol/l) for 16–24 h at 37°C in a humidified atmosphere of 5% CO₂.

**Assay for cell-surface GLUT4-HA.** After 16–24 h incubation, the transfected adipocytes were washed in a KRBB containing 5% BSA. Samples corresponding to the cells from one cuvette were distributed into 1.5-ml microcentrifuge tubes. After incubation with 60 nmol/l of insulin for 30 min at 37°C, KCN (potassium cyanide) (final concentration, 2 mmol/l) was added to prevent GLUT4 redistribution. Then, cell-surface GLUT4-HA was detected using a monoclonal anti-HA antibody conjugated with ¹²⁵I-labeled sheep anti-mouse IgG (Amersham Pharmacia Biotech). The transfection efficiency was ~5%, as estimated by immunofluorescence. The efficiency was comparable with previous studies (16–18). Cells transfected with empty vector pCIS2 were used to determine nonspecific binding of the antibodies. The lipid weight from a 200-μl aliquot of cells was determined as described (19) and used to normalize the data for each sample.

**Immunoblotting.** Total cell lysates were prepared from the adipocytes incubated with or without troglitazone (10⁻⁴ mol/l) for 24 h in DMEM. After the protein concentration had been determined, dithiothreitol was added to a final concentration of 100 mmol/l. The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (New England Nuclear Life Science Products, Boston, MA). Western blotting was performed using a polyclonal antibody raised against the COOH-termini of GLUT4 (14), GLUT1 (14) (kind gifts from Dr. S.W. Cushman), or a monoclonal anti-HA antibody (HA 11; Berkeley Antibody, Richmond, CA). Bound antibodies were visualized with secondary antibodies conjugated to horseradish peroxidase using enhanced chemiluminescence, as described by the manufacturer (Amer sham International, Buckinghamshire, U.K.), and autoradiography film (Konica, Tokyo). The densities of bands on the film were measured using a scanning densitometer. To verify a quantitation of blots, we made standard curves of the blots using anti-GLUT4 (Fig. 1B, left lane). In the pCIS2/GLUT4-HA–transfected cells (Fig. 1B, right lane), GLUT4-HA was detected.

To quantify the relative amount of GLUT4-HA compared with endogenous GLUT4 in the adipocytes, total cell lysates were analyzed with a Western blot using a polyclonal antibody against the COOH-terminus of GLUT4. The antibody recognizes both endogenous GLUT4 and GLUT4-HA. In pCIS2/GLUT4-HA–transfected cells, electrophoretic mobility shift was detected in the blot using anti-GLUT4 antibody because of the difference in molecular weight between GLUT4 and GLUT4-HA. The total amount of GLUT4 increased 1.4-fold in the GLUT4-HA–transfected adipocytes compared with the pCIS2-transfected cells (Fig. 1, lower panel). Considering that the transfection efficiency estimated by immunofluorescence study was 5% (see RESEARCH DESIGN AND METHODS), the transfection resulted in an eightfold overexpression (11.4 ± 1.0)/0.05 of GLUT4-HA compared with the endogenous GLUT4 level per transfected cell.

**Effect of troglitazone on translocation of GLUT4-HA.** Next, we studied the dose-response relation between troglitazone and the cell surface level of GLUT4-HA in adipocytes. Transfected adipocytes were incubated with or without troglitazone for 24 h, and the cell surface level of GLUT4-HA was determined. Troglitazone (0–10⁻⁴ mol/l) increased the cell-surface GLUT4-HA in a dose-dependent manner (Fig. 2A). The level was increased by 1.5-fold (148 ± 8%) at the maximal concentration (10⁻⁴ mol/l) of troglitazone. To determine the time course, troglitazone was added to the medium of transfected adipocytes 6, 12, 18, or 24 h before the detection of cell-surface GLUT4-HA (Fig. 2B inset). The cell-surface level of GLUT4-HA was increased by troglitazone in a time-dependent manner (Fig. 2B).

To confirm that troglitazone did not increase the expression of GLUT4-HA, we evaluated the GLUT4-HA levels with a Western blot using whole-cell lysates treated with or without troglitazone for 24 h. The results of this experiment demonstrated that there was no detectable change in the GLUT4-HA levels caused by troglitazone (Fig. 3).

**Effect of troglitazone on insulin-stimulated translocation of GLUT4-HA.** We examined the effect of troglit-
troglitazone on insulin-stimulated translocation of GLUT4-HA. After being incubated with or without troglitazone (10^{-4} \text{mol/l}) for 24 h, the GLUT4-HA–transfected cells were further incubated with insulin (0–60 \text{nmol/l}) for 20 min, and the cell-surface GLUT4-HA was detected. The cell-surface–associated radioactivity was normalized to the lipid weight of the cells. In both A and B, specific counts (GLUT4-HA) observed for the basal samples are 1,570 ± 178 cpm and nonspecific counts (pCIS2) are 398 ± 32 cpm. Results are the means ± SE of five independent experiments in triplicate.

**DISCUSSION**

The purpose of the present study was to clarify the mechanisms underlying the antidiabetic effects of troglitazone using primary cultured rat adipocytes. The results comprise the first evidence that troglitazone enhances the translocation of GLUT4 in addition to increasing the amount of GLUT4 in rat adipocytes. These effects of troglitazone on adipocytes may contribute to the antidiabetic action of this compound.

It was difficult to determine the direct effect of troglitazone on the translocation of GLUT4 using the membrane fractionation method when the endogenous GLUT4 content was changed. In this study, to investigate the direct...
The effect of troglitazone on translocation of GLUT4, apart from the amount of GLUT4, we transfected GLUT4-HA into rat adipocytes. One advantage of our system was the ability to use GLUT4-HA as a reporter gene. Expression of GLUT4-HA was ~5% of the adipocytes subjected to electroporation. This system allowed us to overcome the potential difficulty of studying such a small population of cells using GLUT4-HA as a reporter gene (16). Therefore, we could study transiently transfected cells exclusively without interference from nontransfected cells. Another advantage is that 24 h of incubation decreases the endogenous GLUT4 content in rat adipocytes (20). Under this condition, GLUT4-HA is overexpressed compared with endogenous GLUT4. The decrease in endogenous GLUT4 increases the ratio of GLUT4-HA to total GLUT4 content. GLUT4-HA was overexpressed by eightfold compared with endogenous GLUT4 in the transfected adipocytes. Troglitazone increased endogenous GLUT4 levels by 1.4-fold (Fig. 5) but did not change the expression of GLUT4-HA in this transfection system. The ratio of GLUT4-HA to total GLUT4 (GLUT4-HA/total GLUT4) in the adipocytes treated with troglitazone was changed by only 4% compared with those without troglitazone treatment (from 8/[8 + 1] to 8/[8 + 1.4]). When we determined the direct effect on translocation of GLUT4-HA, the troglitazone-induced increase in endogenous GLUT4 (~140%) was overcome by 800% overexpression of GLUT4-HA. Thus, it was possible to examine the direct effect on translocation using GLUT4-HA as a reporter when endogenous GLUT4 increased.

The cell-surface level of GLUT4-HA was increased by troglitazone in a time- and dose-dependent manner (Fig. 2). Troglitazone (10^{-4} mol/l) caused a leftward shift of the dose-response curve between insulin and translocation of GLUT4-HA, without changing the maximal response (Fig. 4). Thus, troglitazone increases the insulin sensitivity of GLUT4 translocation in adipocytes. This suggested that the pathway of the signaling by troglitazone converges with that activated by insulin. We have recently demonstrated that troglitazone also increases translocation of GLUT4 in L6 myotubes (13). In L6 myotubes, it is supposed that troglitazone acts at a step beyond PI3K and PKB in which GLUT4 endocytosis is enhanced or reduces endocytosis of GLUT4. Further study is necessary to clarify the precise site of action of troglitazone with regard to the exocytosis and endocytosis of GLUT4. Long-term incubation was required for troglitazone to significantly increase the translocation of GLUT4 in adipocytes. In our recent study (13), inhibition of protein synthesis by cycloheximide completely eliminated the increase in translocation of GLUT4 by troglitazone in L6

FIG. 4. Effect of insulin on the troglitazone-induced increase in translocation of GLUT4-HA. GLUT4-HA–transfected cells were incubated with or without troglitazone (10^{-4} mol/l) for 24 h. After washing, the cells were further incubated with various concentrations of insulin (0–60 nmol/l) for 20 min. Then, the cell-surface levels of GLUT4-HA were determined using an antibody binding assay as described in RESEARCH DESIGN AND METHODS. The cell surface–associated radioactivity was normalized to the lipid weight of the cells. The values are the means ± SE of three independent experiments in triplicate. Specific counts (GLUT4-HA) observed for the basal samples are 1,600 ± 203 cpm and nonspecific counts (pCIS2) are 407 ± 52 cpm. Control cells (•) were able to recruit GLUT4-HA to the cell surface in an insulin dose-dependent manner (ED_{50} of 0.1 nmol/l). The insulin dose-response curve for cells treated with troglitazone (○) was significantly different from that of the control cells. *P < 0.05 comparing troglitazone-treated cells with their respective controls.

FIG. 5. Effect of troglitazone on protein levels of GLUT4 and GLUT1 in rat adipocytes. Adipocytes were preincubated with or without troglitazone (10^{-4} mol/l) in DMEM for 24 h. After incubation for 24 h, total cell lysates containing 6 or 15 μg total protein were subjected to SDS-PAGE and immunoblotted with antibodies against the COOH-terminus of GLUT4 or GLUT1, respectively. The panels show representative blots from three experiments. Densitometric scanning was used to determine the relative amount of GLUT4 protein. □, control; ■, troglitazone. *P < 0.05 vs. control.
myotubes. It is tempting to speculate that troglitazone affects the synthesis of some molecules involved in the translocation of GLUT4. We have detected a protein of 90 kDa associated with GLUT4-containing vesicles in adipocytes by SDS-PAGE, which was increased by troglitazone treatment (unpublished data). It has recently been reported that annexin II, which may be involved in vesicular transport, is increased by troglitazone treatment after 24 h in 3T3-L1 adipocytes (21). These molecules may be involved in the increase in translocation of GLUT4. Under in vivo conditions, it has been suggested that thiazolidinediones improve insulin resistance by enhancing adipocyte differentiation and increasing the number of small adipocytes in obese Zucker rats (6) and correct the deficit in glucose transporters in KK£y diabetic mice (7). In in vitro studies, it has been reported that thiazolidinediones increase glucose uptake in 3T3-F442A and 3T3-L1 adipocytes by increasing GLUT4 and/or GLUT1 content (9,10). In mature adipocytes, troglitazone has also been reported to increase GLUT4 mRNA levels (11). These in vitro and in vivo studies could not distinguish between the increases in glucose transporters associated with the enhancement of adipocyte differentiation and those resulting from the direct effects of the thiazolidinedione on mature adipocytes. In the present study, however, we confirmed the increase in GLUT4 protein levels in mature adipocytes. The size and morphology of the cells were not significantly changed by troglitazone treatment after 24 h (data not shown). Our results suggest that troglitazone has direct effects on mature adipocytes other than adipocyte differentiation.

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