NOTE

Troglitazone Improves Insulin-Stimulated Glucose Utilization Associated with an Increased Muscle Glycogen Content in Obese Zucker Rats

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Abstract. Recent studies have demonstrated that troglitazone has the capacity to improve insulin resistance. The present study was undertaken to determine the effect of troglitazone on in vivo insulin action, the activities of the pyruvate dehydrogenase (PDH) complex and 3-hydroxyacyl-CoA dehydrogenase (3-HADH) in muscle, and muscle GLUT-4 and glycogen content in obese and lean Zucker rats. Rats were fed a normal chow diet with and without troglitazone as a food admixture (0.2%) for 3 weeks. In vivo insulin action was measured by the sequential euglycemic clamp technique at two different insulin infusion rates (6 and 30 mU/kg BW/min). At the basal (fasting) state and after the clamp studies, the activities of PDH complex and 3-HADH, and the amounts of GLUT-4 and glycogen contained in the red gastrocnemius muscles were determined. Troglitazone treatment produced a significant rise in the metabolic clearance rate of glucose (MCR) during the 6-mU/kg BW/min insulin clamp study (19.5 ± 3.9 vs 9.9 ± 1.5 ml/kg BW/min, mean ± SE, P < 0.05) in obese rats, but not in lean rats. Troglitazone significantly increased the muscle glycogen content after the clamp study, compared to non-treated rats, in obese rats (9.9 ± 0.5 vs 6.5 ± 0.4 mg/g tissue, P < 0.05) and has the tendency to increase the activity state of PDH complex in obese and lean rats at the fasting state. However, no effect of the drug on muscle GLUT-4 content was found. These results indicate that troglitazone may improve insulin sensitivity associated with increased muscle glycogen content.

Key words: Troglitazone, Insulin sensitivity, Glycogen, Pyruvate dehydrogenase complex, 3-Hydroxyacyl-CoA dehydrogenase

THE major pathophysiological characteristics of non-insulin dependent diabetes mellitus (NIDDM) are insulin resistance and impaired insulin secretion. Insulin resistance is recognized as particularly important in the development and progress of NIDDM via premature beta-cell exhaustion caused by its demands on insulin secretion [1, 2]. Subjects with insulin resistance often suffer from dyslipidemia and hypertension and have an increased risk of atherosclerosis [3], hence, the improvement of insulin resistance could be of clinical importance. Although physical exercise therapy has been shown to have a beneficial effect for the treatment of NIDDM [4, 5], the long-term continuation of this therapy in daily life is very difficult for NIDDM patients to achieve [6]. This situation has made the development of new therapeutic agents that ameliorate insulin resistance and has been a major goal of ongoing research. Recently, the thiazolidinedione compound troglitazone has attracted considerable attention due to its ability to improve insulin resistance [7–9].
ed to have efficacy in improving peripheral glucose utilization, reducing hepatic glucose production, decreasing plasma insulin levels, and lowering fasting and postprandial plasma glucose concentrations [10-12]. Increasing tyrosine kinase activity in skeletal muscle of insulin-resistant animals was found after troglitazone treatment [13]. This study was undertaken to determine whether troglitazone could prevent the development of insulin resistance in Zucker obese rats, and to confirm whether troglitazone could increase glycogen content, glucose oxidation, and fatty acid oxidation in muscle. In vivo insulin action was measured by use of a two step hyperinsulinemic euglycemic clamp technique in conscious animals, and the activities of the pyruvate dehydrogenase (PDH) complex (which reflect glucose oxidation) and 3-hydroxyacyl-CoA dehydrogenase (3-HADH; an enzyme of the β-oxidation) were examined.

**Materials and Methods**

**Animals**

Obese and lean male Zucker rats (Chubu Kagakushizai, Nagoya, Japan) were obtained at 6 weeks of age and maintained in a 12:12-hr reversed light-dark environment at an ambient temperature of 23°C with access to food and water ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of Nagoya University, and this study was approved by the animal subjects committee of the Research Center of Health, Physical Fitness and Sports at Nagoya University.

After a 1-week acclimation period, obese and lean rats were divided into two groups, and each group was fed one of the following diets for 3 weeks: 1) normal chow diet (powdered rodent diet MF; Oriental Yeast Co. Ltd., Chiba, Japan) and 2) normal chow diet plus troglitazone (kindly supplied by Sankyo Company, Tokyo, Japan). Troglitazone was given as a food admixture (0.2%), freshly mixed in small amounts every 2-3 days and stored at 4°C. The drug dosage used here has been shown to yield maximal efficacy in previous studies [7, 8]. The animals' body weight and food intake were measured daily.

After 2 weeks on the respective diets, half of the rats in each group were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). Thereafter, a midline ventral incision was then made in the neck, and the right jugular vein and left carotid artery were cannulated with Silascon SH tubing (No. 00, Kaneka Medix, Osaka, Japan). The catheters were tunneled subcutaneously to the back of the neck and flushed with 200 μl of saline containing heparin (100 U/ml) and sodium penicillin G (5,000 U/ml). They were then filled with a viscous solution of polyvinylpyrrolidone (PVP-30, Sigma, St. Louis, MO, USA) and capped with a piece of polyethylene tubing melted and sealed at one end. The other rats received no operations and were continued on the same diets.

**Euglycemic clamp study**

One week after surgery, a two-step hyperinsulinemic euglycemic clamp procedure was performed on each rat after an overnight fast to assess whole body insulin action. The rat was placed in a restraining cage to which it was accustomed, and extension tubing was attached to the jugular catheter by an adapter for the continuous infusion of insulin (Actrapid MC, Novo Nordisk, Denmark) and glucose. The carotid catheter was used for blood sampling. A primed infusion was delivered at a rate of 6 mU/kg BW/min for 90 min and then at an increased rate of 30 mU/kg BW/min (maximal stimulation) for an additional 90 min. The plasma glucose concentration was kept constant at the basal level with a variable infusion of 20% (w/v) glucose solution, based on the plasma glucose concentration measured every 10 min. Additional blood samples were collected just before insulin infusion and at 90 min and 180 min after starting insulin infusion for the determination of plasma insulin concentration. The glucose disposal rate (GDR) in mg/kg BW/min was calculated every 10 min during the clamp study. The metabolic clearance rate of glucose (MCR) was then obtained by dividing GDR by the corresponding plasma glucose concentration in order to reduce the influence of the varying plasma glucose concentrations. The means of MCR values from 60 to 90 min and from 150 to 180 min for the two-step sequential euglycemic clamp procedure were regarded as an index of whole body insulin action since a plateau in the glucose infusion rate was achieved during these
times [14, 15].

Measurement of muscle enzyme assay (PDH complex and 3-HADH)

The rats used for the clamp study and the non-operated rats were sacrificed following overnight fast by cervical dislocation, and the red gastrocnemius muscles were removed, freeze-clamped, and stored for subsequent analyses.

Since extraction of PDH complex from muscle has recently been described in detail [16, 17], only the general procedure is outlined here. Frozen muscle was powdered at liquid nitrogen temperature and approximately 50 mg of the powdered muscle was weighed and homogenized in an extraction buffer containing 50 mM HEPES (pH 7.4 with KOH), 3% Triton X-100, 2 mM EDTA, 5 mM dithiothreitol, 0.5 mM thiamine pyrophosphate, 2 mM dichloroacetate (PDH kinase inhibitor), 2% bovine serum, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 0.1 mg/ml trypsin inhibitor, and 0.02 mg/ml leupeptin. Homogenate was divided into two portions for assay of actual and total activities. For measurement of the actual activity (activity state), 50 mM potassium fluoride (phosphatase inhibitor) was immediately added, and the supernatant for the assay was obtained by centrifugation at 12,000 x g for 10 min. For measurement of the total activity, the complex in the supernatant was fully activated using the broad-specificity phosphoprotein phosphatase in the presence of 10 mM MgCl2 at 30°C for 20 min. The activity of PDH complex was measured radiochemically at 30°C using [1-14C]pyruvate as substrate. One unit of PDH complex catalyzed the formation of 1 μmol of carbon dioxide/min. The 3-HADH activity was assayed by the method of Bradshaw and Noyes [18].

GLUT-4 protein and glycogen content in muscle

Muscle samples were homogenized (1: 20) in 20 mM N-2 hydroxyethylpiperazine- N’-2-ethanesulfonic acid, 1mM EDTA, and 250 mM sucrose (pH 7.4) (HES) buffer with a Polytron homogenizer by three 15-s bursts at high speed. Sample homogenates were diluted with equal portions of Laemmli sample buffer. An aliquot of each sample containing 75 μg protein was then subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on a 12.5% resolving gel. Protein determinations were performed using essentially the same method described in our previous report [19].

Muscle glycogen content was measured as previously described [20]. Glycogen concentration was determined spectrophotometrically at 490 nm using the H2SO4-phenol reaction.

Analytical procedures

Plasma glucose concentration was determined with a YSI 23A glucose analyzer (Yellow Springs Instrument Co, Yellow Springs, OH, USA). Samples were immediately separated into plasma and cell portions in a centrifuge at 4°C and stored at −70°C until analyses. The cell fraction after plasma separation was suspended in saline and returned to the rat so as not to reduce the hematocrit level. Plasma insulin was assayed with a radioimmunological assay kit (Phadeseph Insulin RIA, Pharmacia AB, Stockholm, Sweden).

All values are presented as means ± SEM. Statistical analysis was performed using two-way analysis of variance followed by Fisher’s PLSD test. P values less than 0.05 were considered significant.

Results

Body weight and food intake

For both obese and lean Zucker rats, the body weights and daily food intakes of the chow-fed and the chow-plus-troglitazone-fed groups were comparable throughout the study period. Although body weights and food intake transiently decreased in all rats after surgery for cannulation, they returned to normal levels after 4 days. Final body weights on the experimental day tended to be smaller than on the last day because the rats had been fasted overnight.

Whole body insulin action

Mean plasma glucose and insulin concentrations in obese and lean rats before and during the euglycemic clamp studies are presented in Table 1. As expected, plasma glucose levels tended to be greater in obese than lean rats, although not to a significant extent.
Obese rats showed a significant elevation in basal plasma insulin concentrations. Troglitazone treatment did not influence basal plasma glucose or insulin levels in either obese or lean rats. During the clamp studies there were no significant differences in plasma insulin concentrations among the four groups, though the concentrations were slightly higher in the obese rats. The plasma glucose levels in all rats were maintained at basal levels during clamp studies.

A plateau GDR was achieved in each animal for the last 30 min during the 6- and the 30-mU/kg BW/min insulin infusions. In the obese rats, troglitazone treatment resulted in a significant rise in MCR compared with the chow-fed during the 6-mU/kg/min insulin infusion (Fig. 1). During the 30-mU/kg BW/min insulin infusion, there was no significant difference in the MCR, though it tended to be higher in the troglitazone-treated animals. In the lean rats, however, troglitazone treatment did not elicit a significant alternation in MCR compared with that in the chow-fed group during either the 6- or the 30-mU/kg BW/ insulin infusion (Fig. 1). MCRs during both the 6- and the 30-mU/kg/min insulin infusions in the troglitazone-treated obese rats were significantly lower than those in the troglitazone-treated and chow-fed lean rats (P < 0.01). Troglitazone did not increase the MCRs in the obese rats to the levels reached in the lean rats.

### Table 1. Plasma glucose and insulin concentrations before and during the sequential euglycemic clamp procedure

<table>
<thead>
<tr>
<th>Insulin infusion rate (mU/kg/min)</th>
<th>Weight (g)</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (μU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>6.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Obese Zucker rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>384 ± 6</td>
<td>84 ± 1</td>
<td>83 ± 2</td>
</tr>
<tr>
<td>Troglitazone (5)</td>
<td>407 ± 14</td>
<td>79 ± 4</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>Lean Zucker rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (5)</td>
<td>243 ± 11</td>
<td>71 ± 3</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>Troglitazone (5)</td>
<td>243 ± 11</td>
<td>68 ± 3</td>
<td>62 ± 2</td>
</tr>
</tbody>
</table>

Control: normal chow diet  
Troglitazone: normal chow diet plus troglitazone  
Means ± S.E.

Fig 1. Metabolic clearance rate of glucose (MCR) for the last 30 min during the two-step sequential euglycemic clamp procedure at the 6- and the 30-mU/kg BW/min insulin infusions in obese and lean Zucker rats. Control, normal chow diet. Troglitazone, normal chow diet plus troglitazone. Values with different letters are significantly different. a vs. b, p < 0.05. a vs. c, p < 0.001. b vs. c, p < 0.001. d vs. e, p < 0.01.
Activities of PDH complex and 3-HADH (Table 2)

The total activity of the PDH complex tended to increase after the two-step hyperinsulinemic clamp study, with significant differences found in the obese chow-fed rats and in the lean troglitazone-treated rats. A significant increase in the activity state of the muscle PDH complex after the hyperinsulinemic clamp was observed in the lean rats without troglitazone treatment. The activity state in the troglitazone-treated rats both obese and lean was greater than in the chow-fed control rats, although the differences were not statistically significant.

In both the obese and lean rats, 3-HADH activities in muscle at the basal state and after the clamp study did not differ between the troglitazone-treated and chow-fed groups.

GLUT-4 protein and glycogen content

The GLUT-4 protein content in muscle was not different among the groups (Table 2). The two-step hyperinsulinemic euglycemic clamp significantly increased the glycogen content in muscle in both groups of obese rats and in lean troglitazone-treated rats.
A significant elevation of muscle glycogen content after the clamp study was found only between obese troglitazone-treated and chow-fed rats.

Discussion

It is well known that Zucker obese rats exhibit severe skeletal muscle insulin resistance characterized by a pronounced decrease in insulin-stimulated glucose uptake [21]. The locus of this insulin resistance is thought to reside in the glucose transport process [22, 23]. In the present study, troglitazone resulted in increased insulin-stimulated glucose uptake, insulin sensitivity, and muscle glycogen content in Zucker obese rats. Troglitazone is a novel thiazolidinedione derivative that appears to lower blood glucose concentrations primarily by enhancing insulin action rather than by altering insulin secretion [7]. The precise mode of action of this agent remains unknown, but it seems to affect insulin signaling at various points in the signaling pathway [7, 13]. In vitro and animal studies demonstrated that troglitazone enhanced glucose utilization through increases in the conversion of glucose to glycogen in Hep G2 cells [24], in glycogen synthase activity [7, 24], and in human insulin receptor kinase activity in rat fibroblasts [13], and prevention of the inhibitory effect of tumor necrosis factor-α (TNF-α) on the insulin action on 3T3-1 cells [25]. Another study showed that pioglitazone, one of the thiazolidinedione agents, increased GLUT-4 mRNA and GLUT-4 protein levels in adipocytes and muscle of KKAY mice [26]. Recently, thiazolidinedione derivatives have been shown to bind to and activate nuclear peroxisome proliferator-activated receptor γ (PPARγ), which regulates the expression of genes involved in adipocyte differentiation [27, 28]. More recently, Okuno et al. [29] have reported that the primary action of troglitazone may be to increase the number of small adipocytes in white adipose tissue, presumably via PPARγ, leading to normalization of TNF-α expression.

In the present study, either insulin sensitivity or muscle glycogen content was enhanced by troglitazone administration without an increase in muscle GLUT-4 protein in Zucker obese rats. MCRs in Zucker obese rats with or without troglitazone treatment were significantly lower than those in Zucker lean rats in spite of the fact that muscle glycogen content in obese rats tended to be greater compared with lean rats. Moreover, a decrease in glycogen content by troglitazone may trigger augmentation of insulin-stimulated glucose uptake in hindlimb muscle [30]. MCR is not always dependent upon muscle glycogen content. It is also unclear if the increased muscle glycogen content is a cause or a consequence of improved insulin sensitivity. However, it was reported that troglitazone per se enhanced glycogen synthase I activity in BC3H-1 myocytes [24]. One possibility deduced from the results in Zucker obese rats is that troglitazone may stimulate insulin-induced glucose uptake in the skeletal muscle, where most of the absorbed glucose during the euglycemic clamp has been converted to glycogen, by causing translocation of the GLUT-4 protein to the plasma membrane via activation of tyrosine kinase activity of insulin receptors.

The glucose transported into skeletal muscle, where more than 80% of the infused glucose during the euglycemic clamp is taken up, can be either stored as glycogen or undergo glycolysis, and then is either oxidized in the tricarboxylic acid cycle, or is released as lactate, alanine or pyruvate. More than 60% of glucose is phosphorylated into glycogen during the euglycemic clamp [31], and the rest of the glucose may be oxidized mainly through the PDH complex. The activity state of the PDH complex reflects glucose oxidation. Inactivation of the PDH complex in rat skeletal muscle was demonstrated by diabetes and food deprivation [33]. Re-feeding [33] and insulin infusion [34] activate the muscle PDH complex. In fact, Okuno et al. [30] demonstrated that both insulin and troglitazone increased glycolysis in perfused rat hindlimb. In the present study, we were only able to duplicate the results from previous reports in Zucker lean rats. The euglycemic clamp did not affect the activity state of PDH complex in Zucker rats with or without troglitazone treatment. However, at the basal state, troglitazone administration resulted in a rise in the activity state of PDH complex both in the obese and lean rats. Troglitazone treatment may increase the activity state at the basal (fasting) state. A tendency of increased total activity of PDH complex was also observed in Zucker obese and lean rats. A 180-min hyperinsulinemic glucose infusion (the euglycemic clamp) may induce this phenomenon.

Troglitazone is also reported to reduce hepatic
triglyceride production and enhance the rate of triglyceride removal [7]. Recently, Inoue et al. [35] showed a decrease in liver mitochondrial palmitic β-oxidation rate by troglitazone in fructose-fed rats. In this study, muscle 3-HADH activity, an enzyme in β-oxidation, was almost identical in all groups, suggesting that troglitazone may not affect β-oxidation in muscle, contrary to that in the liver as previously reported [7, 35]. There was also no effect of troglitazone treatment on fasting insulin concentrations. As Yoshioka et al. [36] reported a significant decrease in fasting insulin 4 weeks after troglitazone treatment, the length of the treatment in this study (3 weeks) may be involved in the ineffectiveness of the troglitazone treatment on fasting insulin levels. In conclusion, troglitazone can improve insulin sensitivity in Zucker obese rats which is associated with an increase in muscle glycogen content, and in the activity of the PDH complex in the fasting state.

References


