Suppression of Hepatic Gluconeogenesis in Long-Term Troglitazone Treated Diabetic KK and C57BL/KsJ-db/db Mice

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The orally effective antidiabetic agent Troglitazone (CS-045) exerts hypoglycemic effects in various insulin-resistant obese and/or diabetic animals. Since increased hepatic gluconeogenesis is a major cause of hyperglycemia in these diabetic animals, we evaluated the effect of long-term Troglitazone treatment on hepatic gluconeogenesis. Troglitazone was administered for 7 days to normal ddY mice, diabetic KK mice, diabetic C57BL/KsJ-db/db mice, and its heterozygote, db/+ mice, as a 0.1% or 0.2% food admixture. Troglitazone significantly decreased plasma glucose in diabetic KK and db/db mice, but not in normal ddY and db/+ mice. ^{14}C incorporation into blood glucose from $NaH^{14}CO_3$ was measured to assess hepatic gluconeogenesis in diabetic KK and normal ddY mice. Hepatic gluconeogenesis was significantly increased in diabetic KK mice ($P < .01$) as compared with normal mice, and was significantly suppressed ($P < .05$) after 7 days of Troglitazone treatment (~200 mg/kg/d). Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were significantly decreased but fructose-1,6-bisphosphate (FBP) was not significantly increased in the liver of diabetic db/db mice treated with Troglitazone for 7 days (~80 mg/kg/d) as compared with control db/db mice. These changes in G6P, F6P, and FBP corresponded with the activity of fructose-1,6-bisphosphatase (Fru-1,6P_2ase) and 6-phosphofructo-1-kinase (6-PF-1K), which determined the content of F6P and FBP. Namely, Fru-1,6P_2ase was significantly decreased in Troglitazone-treated db/db mice as compared with control mice, whereas 6-PF-1K activity was not affected by Troglitazone treatment. Fructose-2,6-bisphosphate (F2,6P_2), an allosteric modulator of Fru-1,6P_2ase and 6-PF-1K, was not altered by 7 days of Troglitazone treatment in db/db mice. In contrast, in the liver of nondiabetic db/+ mice, in which Troglitazone did not decrease plasma glucose, G6P, F6P, and FBP were not significantly changed by Troglitazone treatment. These results suggest that long-term Troglitazone treatment suppresses hepatic gluconeogenesis at the regulatory step between FBP and F6P by decreasing Fru-1,6P_2ase activity in these diabetic mice.

ALONG WITH IMPAIRED insulin secretion and peripheral insulin resistance,[1,2] increased hepatic glucose production is a major cause of hyperglycemia in patients with non–insulin-dependent diabetes mellitus (NIDDM). Increased hepatic gluconeogenesis predominantly contributes to increased hepatic glucose production in patients with NIDDM[3,4] and diabetic animals.[5,6]

Troglitazone (CS-045), [(±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-2,4-thia zolidinedione)], is a new orally effective antidiabetic agent that decreases plasma glucose in insulin-resistant obese and/or diabetic rodent models[7,8] in which sulfonylureas are ineffective. Additionally, Troglitazone has recently been shown to exert a hypoglycemic effect in patients with NIDDM.[9,10] Troglitazone has been demonstrated to decrease hepatic glucose production in patients with NIDDM[10] and to decrease gluconeogenesis in Hep G2 cells[11] and perfused rat liver.[12] However, the biochemical mechanism by which Troglitazone suppresses gluconeogenesis still remains unclear. The aim of the current study is to assess the long-term effect of Troglitazone on hepatic gluconeogenesis in diabetic KK and db/db mice and in normal mice.

Hepatic gluconeogenesis is regulated by glycolytic and/or gluconeogenic enzyme activities, in addition to the supply of gluconeogenic substrates from systemic blood.[13] To determine further the site at which Troglitazone suppresses gluconeogenesis, we measured the levels of hepatic glycolytic intermediates to estimate overall changes in gluconeogenic/glycolytic enzyme activity by crossover analysis in control and Troglitazone-treated db/db mice. In addition, we measured glycolytic and gluconeogenic enzyme activities on the crossover point we found.

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MATERIALS AND METHODS

Animals

Male KK mice were purchased from Tokyo Experimental Animals (Tokyo, Japan) at 6 weeks of age. These animals were housed in individual cages and maintained on a moderately high-calorie and high-fat laboratory chow (375.6 kcal/100 g; carbohydrate:protein:lipid, 46.4:27.7:8.8), Oriental CMF (Oriental Yeast, Tokyo, Japan), and water ad libitum. They were used for experiments when their body weight was greater than 40 g after 3 months of age. Hyperglycemia and hyperinsulinemia were well developed at the body weight and age range used for this experiment.[14]

Male C57BL/KsJ-db/db mice and their lean heterozygote littermates (db/+) were obtained from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. They were caged in groups of five and given a normal-calorie laboratory chow, MM-1, and water ad libitum. They were used for experiments at 4 to 6 months of age.

Normal male ddY mice, the control group, were purchased from Japan SLC (Shizuoka, Japan) and maintained on normal laboratory chow (MM-1; Funabashi Firm., Funabashi, Japan) and water ad libitum.

Drug Administration and Liver Sampling

Troglitazone was administered to animals as 0.1% and 0.2% food admixtures. The maximum hypoglycemic effect of Troglitazone was obtained by these doses, and there were no significant changes in food intake.[7,8] Drug dosages used are indicated in the tables and figures.

For determining hepatic glycolytic intermediates, fructose-2,6-

bisphosphate ($F2,6P_2$), fructose-1,6-bisphosphatase (Fru-1,$6P_2$ase), and 6-phosphofructo-1-kinase (6-PF-1K), mice were killed by decapitation and liver specimens were immediately frozen with a pair of frozen tongs that were precooled with liquid nitrogen. Frozen liver samples were kept at −80°C until they were used for assays.

Determination of Gluconeogenic Activity

Gluconeogenic activity was measured by the method described by Shikama and Ui[15] and Rous.[16] $NaH^{14}CO_3$ (20 μCi/10 g body weight) was intravenously administered to fed mice via a tail vein. Since gluconeogenesis is increased only in the fed state in KK mice,[6] we selected the fed state for measuring gluconeogenesis.

Blood samples (25 μL) were obtained by tail-vein bleeding 10 minutes after $NaH^{14}CO_3$ administration. Collected blood was hemolyzed in 1 mL distilled water and then deproteinized by the addition of 0.1 mL 5% $Ba(OH)_2$ and 0.1 mL 5% $ZnSO_4$ before centrifugation at 3,000 rpm for 5 minutes. The glucose concentration in 0.2 mL of the supernatant was then measured using a commercial kit (Glucose C-test; Wako, Tokyo, Japan). Another 0.8 mL of this supernatant was placed into the tube containing anion-exchange resin (AG-8X, formate form, 200-400 mesh; Dow Chemical, Midland, MI). After shaking the tube occasionally for 20 minutes, the supernatant was transferred into another tube containing cation-exchange resin (AG50W-X8, H+ form, 200-400 mesh; Dow Chemical). Radioactivity (^{14}C-glucose) in the final supernatant was measured in a liquid scintillation counter (TRI-CARB 460; Packard, Meriden, CT) using ACSII solvent (Amersham, Amersham, UK).

Determination of Hepatic Glycolytic Intermediates

Levels of glycolytic intermediates were measured by the method described by Bergmeyer.[17] A portion of the frozen liver was powdered with a porcelain mortar and pestle precooled with liquid nitrogen. Powdered liver was weighed, and 30% ice-cold perchloric acid was added for deproteinization. This tissue specimen was ground rapidly, and then 4 mmol/L EDTA was added before centrifugation. A 1-mL aliquot of the supernatant was neutralized with 30% potassium hydroxide at 0°C and centrifuged. The resulting supernatant was used for measurement of glycolytic intermediates.

The assay consisted of a combination of specific enzyme reactions to transfer one intermediate to another, and the indicator reaction used NADH or NADPH.

Determination of $F2,6P_2$

The level of $F2,6P_2$ was measured according to the method reported by Van Schaftingen et al.[18] A portion of the liver samples was homogenized in 50 mmol/L NaOH and kept at 80°C for 5 minutes. The extracts were cooled and neutralized at 0°C by ice-cold 1 mol/L acetic acid in the presence of 20 mmol/L HEPES, followed by centrifugation for 10 minutes. The supernatants were used for determination of $F2,6P_2$.

Measurement of Fru-1,$6P_2$ase and 6-PF-1K Activities

For determination of Fru-1,$6P_2$ase and 6-PF-1K, a portion of the liver sample was homogenized in 50 mmol/L Tris buffer containing 1 mmol/L EDTA, 5 mmol/L $MgSO_4$, 150 mmol/L KCl, and 1 mmol/L dithiothreitol, pH 7.4. The homogenate was then centrifuged at 27,000× *g* for 60 minutes, and the obtained supernatant was used for measurement of Fru-1,$6P_2$ase and 6-PF-1K activities.

Fru-1,$6P_2$ase was determined as described by Marcus et al[19] using 20 mmol/L triethanolamine buffer containing 2 mmol/L $MgCl_2$, 40 mmol/L $(NH_4)_2SO_4$, 0.15 mmol/L F1,$6P_2$, 0.5 mmol/L NADP, 0.1 mmol/L EDTA, 1 U/mL G6P dehydrogenase, and 1 U/mL glucose phosphate isomerase, pH 7.5. 6-PF-1K was determined by the method reported by Kemp[20] using 50 mmol/L Tris buffer containing 5 mmol/L $MgCl_2$, 50 mmol/L, KCl, 10 mmol/L dithiothreitol, 30 mmol/L $(NH_4)_2SO_4$, 0.2 mmol/L NADH, 2 mmol/L adenosine triphosphate, 0.8 U/mL aldolase, 0.8 U/mL glutamic acid dehydrogenase, 2.3 U/mL triosephosphate isomerase (TPA), 40 U $F2,6P_2$, 4 mmol/L F6P, and 1 mmol/L EDTA, pH 8.0. Another aliquot was used for determination of protein content using biuret methods.

Materials

Troglitazone was synthesized by our Product Development Laboratories (Lot No. NR-105 and NR-210). $NaH^{14}CO_3$ was purchased from New England Nuclear (Boston, MA). Most enzymes, glycolytic intermediates, and $F2,6P_2$ used for measurements were purchased from Sigma Chemical (St Louis, MO).

Analytical Methods

Plasma glucose was determined with a glucose analyzer (Shimadzu CL-760, Kyoto, Japan). An Hitachi U-2000 spectrophotometer (Tokyo, Japan) was used to measure the absorbance change of NADH or NADPH for determining glycolytic intermediates, enzymes, and $F2,6P_2$.

Data Analysis

Data are expressed as the mean ± SEM, and statistical significance was analyzed using Student's *t* test (paired *t* test). *P* less than .05 was considered significant.

RESULTS

Hypoglycemic Activity of Troglitazone in Normal ddY and db/+ Mice and Diabetic KK and db/db Mice

Table 1 shows plasma glucose levels after 7 days of Troglitazone treatment in normal ddY and db/+ mice and diabetic KK and db/db mice. Troglitazone significantly decreased plasma glucose in KK and db/db mice, but it did not affect plasma glucose in ddY and db/+ mice.

Overall Gluconeogenic Activity

Figure 1 shows overall hepatic gluconeogenic activity in diabetic KK mice and normal ddY mice with or without Troglitazone treatment. Gluconeogenesis was significantly increased ($P < .01$) in KK mice as compared with normal ddY mice. Seven days of Troglitazone treatment significantly decreased gluconeogenesis ($P < .05$) in diabetic KK

Table 1. Hypoglycemic Activity of Troglitazone in Normal ddY and db/+ Mice and Diabetic KK and db/db Mice

Treatment Group	Plasma Glucose (mg/dL)			
	ddY	KK	db/db	db/+
Control	232 ± 1	391 ± 52	497 ± 65	163 ± 10
Troglitazone	239 ± 14	244 ± 25*	201 ± 51†	161 ± 7

NOTE. Troglitazone was administered for 7 days as a 0.1% (wt/wt) food admixture to db/db (~80 mg/kg/d) and db/+ (~120 mg/kg/d) mice, and as a 0.2% food admixture to KK (~200 mg/kg/d) and ddY (~200 mg/kg/d) mice. Each value represents the mean ± SEM (n = 4 ~ 5).

*$P < .05$ *v* control.

†$P < .01$ *v* control.

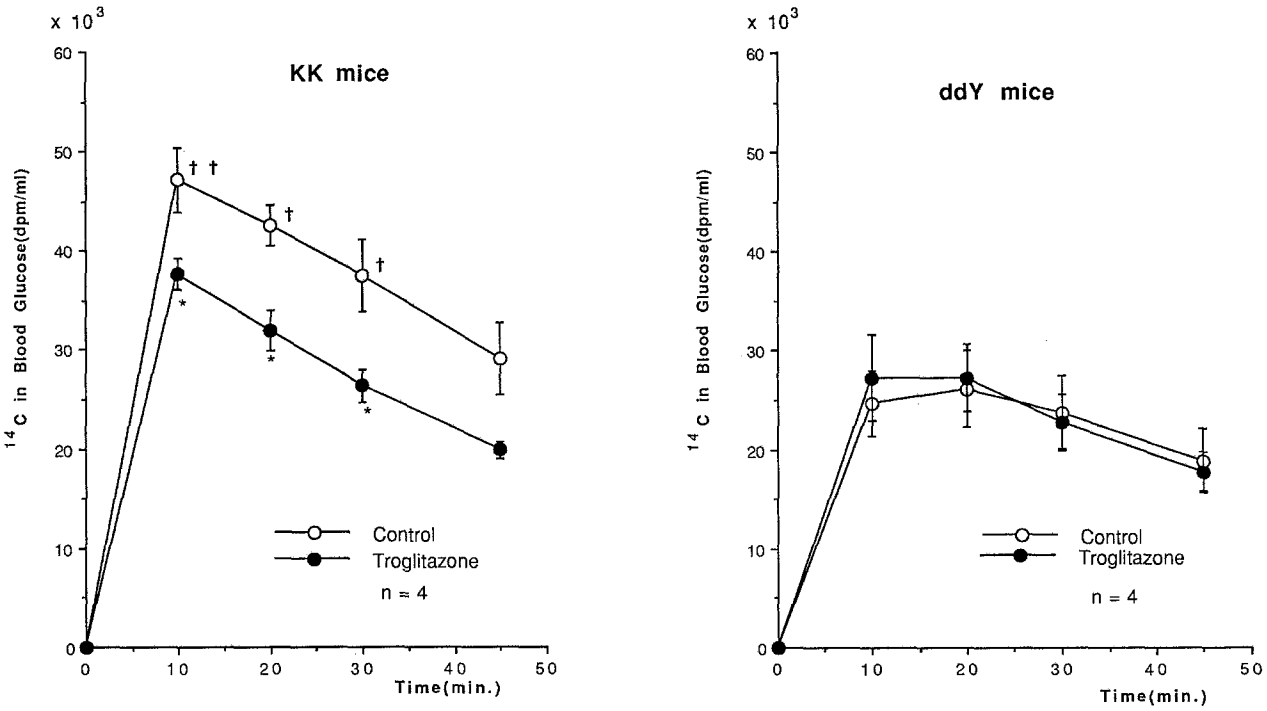

Fig 1. Effects of Troglitazone on gluconeogenesis in normal ddY mice and diabetic KK mice. Troglitazone was administered as a food admixture (~200 mg/kg) for 7 days. Values are the mean ± SE (n = 4). ††$P < .01$, †$P < .05$ *v* ddY control. *$P < .05$ *v* KK control.

mice, but it did not affect gluconeogenesis in normal ddY mice.

Glycolytic Intermediates in Liver

In the liver of db/db mice, glucose-6-phosphate (G6P) and F6P tended to increase (G6P, 516 ± 69 *v* 383 ± 8 nmol/g; F6P, 111 ± 18 *v* 80 ± 9; n = 4), but not significantly. In contrast, fructose-1,6-bisphosphate (FBP) was significantly decreased (34 ± 3 *v* 51 ± 5 nmol/g, $P < .05$, n = 4) in db/db mice as compared with lean db/+ mice, reflecting changes of enzyme activity located between FBP and F6P. These results suggest that hepatic gluconeogenesis was increased at the regulatory step between FBP and F6P in diabetic db/db mice.[21]

In the liver of db/db mice treated with Troglitazone, G6P and F6P were significantly decreased and FBP tended to increase, but not significantly, as compared with levels in the control group (Table 2). A negative crossover was observed at the step between FBP and F6P by crossover analysis (Fig 2). These data suggest that Troglitazone suppresses gluconeogenesis at this step. On the other hand, in the liver of db/+ mice, in which Troglitazone did not decrease plasma glucose (Table 1), G6P, F6P, FBP, and triose phosphate were not significantly changed by Troglitazone treatment (Table 3).

Activity of Fru-1,6P_2ase and 6-PF-1K

Since we found a negative crossover between F6P and FBP, we measured the enzyme activity of Fru-1,6P_2ase and 6-PF-1K, which determine the content of F6P and FBP. As shown in Table 4, 7 days of Troglitazone treatment significantly decreased Fru-1,6P_2ase activity but did not affect 6-PF-1K activity.

F2,6P_2 Content

Because F2,6P_2 is a modulator of both Fru-1,6P_2ase and 6-PF-1K,[20] we measured F2,6P_2 content in the liver of control and 7-day Troglitazone-treated db/db mice. As shown in Table 4, Troglitazone treatment did not change F2,6P_2 content.

DISCUSSION

Troglitazone is a new orally effective antidiabetic agent that exerts a hypoglycemic effect in insulin-resistant obese and/or diabetic rodent models[7,8] in which sulfonylureas are

Table 2. Effects of Troglitazone on Glycolytic Intermediates in the Liver of C57BL/KsJ-db/db Mice

Treatment Group	G6P (nmol/g)	F6P (nmol/g)	FBP (nmol/g)	TP (nmol/g)	3PG + 2PG (nmol/g)	PEP (nmol/g)	Pyr (nmol/g)	Lac (μmol/g)
Control (n = 5)	772 ± 13	259 ± 12	47 ± 8	65 ± 12	144 ± 33	315 ± 22	457 ± 30	5.47 ± 0.10
Troglitazone (n = 4)	596 ± 49*	146 ± 18†	65 ± 7	104 ± 24	198 ± 39	345 ± 27	538 ± 33	5.34 ± 0.25

NOTE. Troglitazone was administered as a 0.1% (wt/wt) food admixture (~80 mg/kg/d). Data are the mean ± SEM.

Abbreviations: TP, triose phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate.

*$P < .05$ *v* control.

†$P < .01$ *v* control.

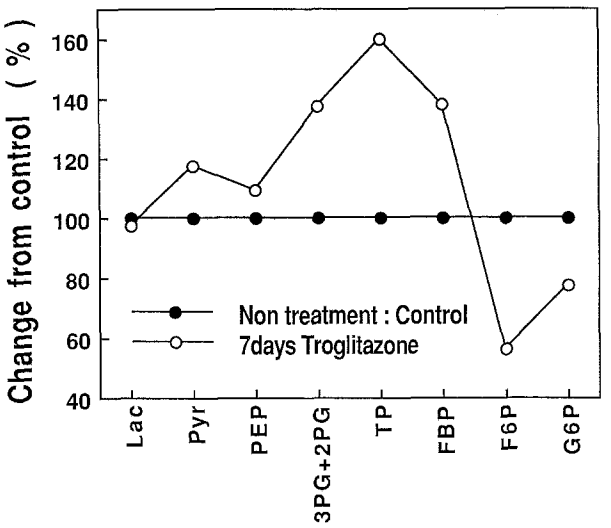

Fig 2. Crossover analysis of hepatic glycolytic intermediates in diabetic db/db mice treated with Troglitazone. (●) Control; (○) Troglitazone. Glycolytic intermediates were determined 7 days after Troglitazone administration as a food admixture (~80 mg/kg/d). Results are expressed as a percentage of the value (100%) determined in liver of control db/db mice. Absolute values and statistical analysis are shown in Table 2. Lac, lactate; Pyr, pyruvate; PEP, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; TP, triose phosphate.

ineffective. Recently, Troglitazone has also been shown to exert a hypoglycemic effect in patients with NIDDM.[9,10]

Increased insulin action is considered responsible for the hypoglycemic effect of Troglitazone, because long-term Troglitazone treatment increased the number of insulin receptors and improved postbinding defects in insulin target tissues.[7] Recent studies have demonstrated that Troglitazone decreased hepatic glucose production in patients with NIDDM[10] and decreased gluconeogenesis in Hep G2 cells[11] and perfused rat liver.[12] However, the biochemical mechanism by which Troglitazone suppresses gluconeogenesis remains unclear.

Hepatic gluconeogenesis was significantly increased in diabetic KK mice as compared with normal ddY mice (Fig 1), indicating that enhanced gluconeogenesis plays an important role in hyperglycemia in these diabetic animals.[5,6] Since Troglitazone significantly suppressed this enhanced hepatic gluconeogenesis (Fig 1), suppression of gluconeogenesis is one of the hypoglycemic mechanisms of Troglitazone in diabetic KK mice. On the other hand, Troglitazone had no effect on gluconeogenesis in normal ddY mice (Fig 1). This is consistent with the fact that blood glucose levels did not change after Troglitazone administration in normal animals.[7]

Troglitazone decreased plasma glucose to the normal range, but did not suppress gluconeogenesis to normal levels in diabetic KK mice. These results suggest that another hypoglycemic mechanism was operative with Troglitazone treatment. An increase in peripheral glucose uptake and/or a decrease in hepatic glycogenolysis may be responsible for the hypoglycemic effects of Troglitazone.

In db/db mice, another diabetic animal model, increased hepatic gluconeogenesis also contributes to hyperglycemia,[5] similar to KK mice. Troglitazone decreased plasma glucose (Table 1) and gluconeogenesis in db/db mice. To determine further the site at which Troglitazone suppresses gluconeogenesis, we measured the levels of hepatic glycolytic intermediates to estimate overall changes in gluconeogenic/glycolytic enzyme activity by crossover analysis in diabetic db/db mice.

In the liver of db/db mice, F6P increased and FBP decreased as compared with levels in control lean mice, indicating that gluconeogenesis increased at this step. Similar changes in F6P and FBP were observed in perfused liver of db/db mice.[21] These changes in F6P and FBP in the liver of db/db mice may indicate insulin resistance, because F6P decreased and FBP increased in the liver when insulin was administered to normal rats.[22] Troglitazone treatment decreased F6P and increased FBP in the liver of db/db mice (Table 2) and produced a negative crossover between F6P and FBP (Fig 2). These results suggest that Troglitazone suppresses gluconeogenesis at this step in these mice. This idea is reinforced by the observation that Troglitazone did not affect G6P, F6P, FBP, and triose phosphate (Table 3) in db/+ mice in which Troglitazone did not decrease plasma glucose.

Fru-1,6P_2ase is a rate-limiting gluconeogenic enzyme that converts FBP to F6P, and 6-PF-1Ka rate-limiting glycolytic enzyme that converts F6P to FBP.[13] Therefore, we measured the levels of Fru-1,6P_2ase and 6-PF-1K, the determinants of F6P and FBP content. Troglitazone treatment significantly decreased Fru-1,6P_2ase activity but did not affect 6-PF-1K activity (Table 4). This result indicates that one mechanism by which Troglitazone suppresses

Table 4. Effect of Troglitazone on FBPase, PFK, and F2,6P_2 Activity in the Liver of C57BL/KsJ-db/db Mice

Treatment Group	FBPase (nmol/min/mg protein)	PFK (nmol/min/mg protein)	F2,6P_2 (nmol/g tissue)
Control (n = 5)	441.9 ± 28.6	20.55 ± 2.18	44.8 ± 4.3
Troglitazone (n = 4)	344.0 ± 11.9*	21.28 ± 1.39	39.1 ± 3.1

NOTE. Troglitazone was administered as a 0.1% (wt/wt) food admixture (~80 mg/kg/d). Data are the mean ± SEM.

Abbreviations: FBPase, fructosebisphosphatase; PFK, phosphofructokinase.

*$P < .02$ *v* control.

Table 3. Effects of Troglitazone on Glycolytic Intermediates in the Liver of db/+ Mice

Treatment Group	G6P (nmol/g)	F6P (nmol/g)	FBP (nmol/g)	TP (nmol/g)	3PG + 2PG (nmol/g)	PEP (nmol/g)	Pyr (nmol/g)	Lac (μmol/g)
Control (n = 6)	537 ± 37	188 ± 14	29 ± 7	118 ± 11	619 ± 20	301 ± 17	428 ± 19	5.13 ± 0.51
Troglitazone (n = 6)	462 ± 33	140 ± 17	34 ± 3	139 ± 11	727 ± 35	370 ± 37	403 ± 36	5.18 ± 0.20

NOTE. Troglitazone was administered as a 0.1% (wt/wt) food admixture (~120 mg/kg/d). Data are the mean ± SEM.

Abbreviations: TP, triose phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate.

gluconeogenesis is to suppress Fru-1,6P_2ase activity in the regulatory step between F6P and FBP.

Since F2,6P_2, a modulator of both Fru-1,6P_2ase and 6-PF-1K, decreases Fru-1,6P_2ase,[23,24] we measured F2,6P_2 content in the liver of control and Troglitazone-treated db/db mice. As shown in Table 4, Troglitazone treatment did not change F2,6P_2 content. This finding suggests that the decrease in Fru-1,6P_2ase activity was not due to an increase in F2,6P_2.

The mechanism by which Troglitazone suppresses Fru-1,6P_2ase remains unclear. Since insulin suppresses Fru-1,6P_2ase activity[25,26] and Troglitazone enhances insulin action,[6] it appears that Troglitazone suppresses Fru-1,6P_2ase secondarily to enhanced insulin action. However, it is possible that Troglitazone alone suppresses Fru-1,6P_2ase directly. We previously observed that Troglitazone suppressed gluconeogenesis from lactate but not from fructose in the absence of insulin in perfused rat liver.[12] This observation suggests that Troglitazone alone suppresses Fru-1,6P_2ase directly in liver.

It has been reported that pioglitazone, which has a thiazolidine ring in its structure similarly to Troglitazone, decreased glucose-6-phosphatase and increased glucokinase but did not affect Fru-1,6P_2ase in the liver of diabetic Wistar Fatty rats.[27] These findings suggest that Troglitazone and pioglitazone may have different hypoglycemic mechanisms in the liver, despite sharing the same thiazolidine ring.

In summary, the current results indicate that (1) long-term Troglitazone treatment suppresses overall hepatic gluconeogenesis in insulin-resistant diabetic mice, (2) the decrease in Fru-1,6P_2ase activity may be associated with the decrease in gluconeogenesis in Troglitazone-treated db/db mice, and (3) the decrease in Fru-1,6P_2ase did not result from an increase in F2,6P_2, but may be due to an enhancement of insulin action and/or a direct effect of Troglitazone.

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