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Hypoglycemic agent YM440 suppresses hepatic glucose output via gluconeogenesis by reducing glucose-6-phosphatase activity in obese Zucker rats

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Abstract

Using a glucose clamp, we had shown that YM440, (*Z*)-1,4-bis{4-[(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)methyl]phenoxy}but-2-ene, reduced the increased hepatic glucose output in obese Zucker rats. We further examined effects of YM440 on ^{14}C-incorporation from [^{14}C]bicarbonate into blood glucose via gluconeogenesis, and on gluconeogenic enzymatic activities. Fed obese Zucker rats showed a 4-fold increase of ^{14}C-incorporation into blood glucose compared to that in lean rats. Glucose-6-phosphatase and fructose-1,6-bisphosphatase activities in obese rats were increased 1.4-fold and 1.6-fold compared with lean rats. YM440 (300 mg/kg for 2 weeks) decreased ^{14}C-incorporation into blood glucose by 29% in obese rats. Glucose-6-phosphatase but not fructose-1,6-bisphosphatase activity was reduced by YM440 and closely correlated with ^{14}C-incorporation into blood glucose, indicating a key role for glucose-6-phosphatase in hepatic glucose output. These results suggest that the increased gluconeogenesis in obese rats is mainly due to the increased activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase and that YM440 suppresses hepatic glucose output by reducing glucose-6-phosphatase activity.

Keywords: Glucose output, hepatic; Gluconeogenesis; Glucose-6-phosphatase; Fructose-1,6-bisphosphatase; YM440; Glucose, blood

1. Introduction

Hepatic glucose production is the net result of the breakdown of glycogen (glycogenolysis) and synthesis of new glucose molecules from lactate, amino acids and glycerol (glucogeneonesis) in liver. In type 2 diabetes, gluconeogenesis is a main cause of the elevated hepatic glucose output, contributing 50–60% of the released glucose (Tayek and Katz, 1996; Hundal et al., 2000). The rate of gluconeogenesis is regulated by the activity of the key gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase. Insulin inhibits gluconeogenesis by repressing the mRNAs that encode gluconeogenic enzymes (Pilkis and Granner, 1992). It is well established that obese Zucker rats are animal models of insulin resistance characterized by hyperglycemia, hyperinsulinemia, hyperlipidemia, glucose intolerance and obesity (Zucker, 1972; Zucker and Antoniades, 1972; Bray and York, 1972; Bach et al., 1981; Jeanrenaud et al., 1985). It is reported that the rate of hepatic glucose output in obese Zucker rats is higher than that in lean Zucker rats (Jeanrenaud et al., 1985; Rohner-Jeanrenaud et al., 1986; Nakano et al, 1999) and that the increased glucose output could lead to hyperglycemia in these animals. However, the precise biochemical mechanism for this remains largely unknown.

YM440, (*Z*)-1,4-bis{4-[(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)methyl]phenoxy}but-2-ene, an insulin sensitizer with

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oxadiazolidinediones, ameliorated hyperglycemia in *db*/*db* mice (Shimaya et al., 2000). In obese Zucker rats, this agent ameliorates abnormalities in hepatic glycogen metabolism (Kurosaki et al., 2002) and also improves insulin sensitivity by inhibiting hepatic glucose output rather than by increasing peripheral glucose utilization (Nakano et al., 1999). The purpose of the study was to examine the role of gluconeogenic enzymatic activities in regulating hepatic glucose production in obese Zucker rats in a fed state. We measured the gluconeogenic enzymatic activities (phosphoenolpyluvate carboxykinase, fructose-1,6-bisphosphatase and glucose-6-phosphatase) and hepatic glucose output by tracer kinetics using [^{14}C]sodium bicarbonate. We also examined the mechanism by which YM440 ameliorated hepatic glucose production in these obese animals.

2. Materials and methods

2.1. Materials

YM440 was synthesized at Yamanouchi Pharmaceutical (Tokyo, Japan). [^{14}C]Sodium bicarbonate was purchased from Dupont/NEN (Boston, MA, USA) and [α-^{32}P]UTP was from Amersham Japan (Tokyo, Japan). Other reagents were of analytical grade from commercial sources.

2.2. Animals

Male obese Zucker (*fa*/*fa*) rats and their lean littermates (Fa/−) (10–11 weeks of age) were obtained from Charles River Laboratories (Kingston, NY, USA) and allowed free access to laboratory chow (CE-2, 341 kcal/100 g, CLEA Japan, Tokyo, Japan). The animals were kept under a 12:12-h light–dark cycle. All procedures were performed according to the regulations of the ethical committee for animal studies at Yamanouchi Pharmaceutical and the experimental protocol was approved by the committee. The rats at age 14–15 weeks were orally given YM440 once a day at a dose of 300 mg/kg for 14 days. YM440 was suspended in a 0.5% methylcellulose solution (a volume of 5 ml/kg). The vehicle was administered to control rats. In separate studies using obese Zucker rats, YM440 decreased hyperglycemia in a dose-dependent manner (30, 100 and 300 mg/kg) but this agent did not significantly improve glucose tolerance at up to 100 mg/kg. Therefore we used 300 mg/kg of YM440 in this study.

2.3. Determination of gluconeogenic activity

Gluconeogenic activity was measured by the method described previously (Shikama and Ui, 1978). Briefly, a solution of $NaH^{14}CO_3$ (3 μmol and 20 μCi/100 g in saline) was administered intravenously into the femoral vein under pentobarbital anesthesia (45 mg/kg, i.p). Blood samples (0.1 ml) were taken from the tail vein at intervals. Collected blood was hemolyzed in 1.5 ml of distilled water and then deproteinized by the addition of $Ba(OH)_2$ and $ZnSO_4$ before centrifugation at 3000 rpm for 15 min. The glucose concentration in 0.1 ml of supernatant was measured by the glucose oxidase method (Glucose mono test, Roche, Tokyo, Japan). The radioactivity of [^{14}C]glucose in the supernatant, which was not absorbed by Dowex 50-X8 (H^+ form) and AG1-X8 (formate form), was determined in a liquid scintillation counter. For the liver biopsy, the abdominal cavity was opened under pentobarbital anesthesia, and a portion of the liver was rapidly excised and frozen in liquid nitrogen 45 min after [^{14}C]bicarbonate administration. The frozen tissues were kept at −80 °C until used for assays.

2.4. Determination of gluconeogenic and glycogenic enzymatic activities

2.4.1. Glucose-6-phosphatase activity

Part of a frozen liver was thawed to prepare a microsomal suspension for glucose-6-phosphatase assay (Aoki et al., 1999, 2000). The liver was homogenized in 50 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000×*g* for 20 min and the 20,000-g supernatant was again centrifuged at 105,000×*g* for 60 min. The resulting sediment was suspended in a homogenizing buffer and treated with 0.2% deoxycholate to free glucose-6-phosphatase from membranous constraints. Glucose-6-phosphate hydrolysis requires the coupled function of at least three integral proteins of the endoplasmic reticulum: (1) a glucose-6-phosphate phosphohydrolase; (2) a glucose-6-phosphate translocase; and (3) an inorganic phosphate translocase (Herling et al. 1998). Since the activity of glucose-6-phosphatase was determined by the amount of the inorganic phosphate released from glucose-6-phosphate in the fraction of disrupted microsome by the detergent (Nordlie and Arion, 1966), the glucose-6-phosphatase activity shown in this study mainly represents the activity of glucose-6-phosphate phosphohydrolase.

2.5. Fructose-1,6-bisphosphatase and phosphofructokinase activities

For the fructose-1,6-bisphosphatase and phosphofructokinase enzymatic analysis, frozen liver samples were homogenized in 50 mM Tris, pH 7.4, 1 mM EDTA, 5 mM $MgSO_4$, 150 mM KCl and 1 mM dithiothreitol (Fujiwara et al., 1995). The homogenate was then centrifuged at 27,000×*g* for 60 min, and the supernatant was used for measurement of fructose-1,6-bisphosphatase and phosphofructokinase activities. Fructose-1,6-bisphosphatase was determined using 20 mM triethanolamine, pH 7.5, 2 mM $MgCl_2$, 40 mM $(NH_4)_2SO_4$, 0.15 mM

Table 1
Effects of YM440 on body weight, plasma insulin and blood glucose levels in lean and obese Zucker rats

	No. of animals	Body weight (g)	Plasma insulin (ng/ml)	Blood glucose (mg/dl)
Lean Zucker rats				
Vehicle	7	394±10	6.95±0.93	124±14
YM440	6	396±10	3.88±0.38	95±3
Obese Zucker rats				
Vehicle	6	613±25[a]	28.7±4.4[a]	249±27[b]
YM440	7	624±12	22.9±3.9	204±25

Male lean and obese Zucker rats were treated orally with either vehicle or YM440 (300 mg/kg) for 14 days. Values are means±S.E.M.
[a] $P<0.001$ vs. vehicle control (Tukey's multiple range test).
[b] $P<0.01$ vs. vehicle control (Tukey's multiple range test).

fructose-1,6-bisphosphate, 0.5 mM NADP, 0.1 mM EDTA, 1 U/ml glucose-6-phosphate dehydrogenase and 1 U/ml glucose phosphate isomerase. Phosphofructokinase was determined using 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 10 mM dithiothreitol, 30 mM $(NH_4)_2SO_4$, 0.2 mM NADP, 2 mM ATP, 0.8 U/ml aldolase, 0.8 U/ml glycerol-3-phosphate dehydrogenase, 2.3 U/ml triosephosphate isomerase, 0.04 mM fructose-2,6-phosphate, 4 mM fructose-6-phosphate and 1 mM EDTA. Another aliquot was used for determination of the protein content with the DC protein assay kit (Bio-Rad Japan, Tokyo, Japan).

2.6. Determination of phosphoenolpyluvate carboxykinase mRNA levels

For the assay of phosphoenolpyluvate carboxykinase mRNA expression, the ribonuclease protection assay (RPA) was performed using an RPA II kit (Ambion, Austin, TX). Rat partial cDNA of phosphoenolpyluvate carboxykinase contained the sequence corresponding to bp 1911–2260 (349 nt). The plasmid was linearized and an [α-^{32}P]UTP-radiorabeled cRNA probe was made using T7 polymerase from the Riboprobe Gemini II kit (Promega, Madison, WI). Sample RNA (8 μg) was mixed with an [α-^{32}P]-labeled cRNA probe riboprobe and hybridized at 50 °C for 12 h in the hybridization buffer supplied. The samples were then incubated with ribonuclease solution [1:100 dilution mixture of an equal volume of ribonuclease A (250 U/ml) and T1 (15,000 U/ml)] at 37 °C for 60 min. The RNA fragment protected from ribonuclease was analyzed by 4.5% Urea-PAGE on a gel containing 8 M urea. The dried gel was exposed to an imaging plate for 30 min at room temperature and the mRNA level was determined by estimation of the intensity of photostimulated luminescence with a BAS2000 (Fuji Film, Tokyo, Japan).

2.7. Other analytical methods

The amounts of glycogen and [^{14}C]glycogen were analyzed using ethanol precipitation methods (Seifter et al., 1950; Saitoh and Ui, 1975). Plasma insulin concentrations were determined by radioimmunoassay, using the "Rat insulin assay system" (Amersham Pharmacia Biotech, Tokyo, Japan).

2.8. Data analysis

The data were analyzed by one-way or two-way analysis of variance. When differences were statistically significant ($P<0.05$), Tukey's multiple range test was used to compare values between experimental groups. Differences were accepted as significant at the $P<0.05$ level.

3. Results

3.1. Effects of YM440 on body weight, blood glucose and plasma insulin

Body weight and blood glucose and plasma insulin levels were significantly higher in obese Zucker rats than in lean Zucker rats (Table 1). The body weight of obese rats was about 1.5-fold that of lean rats but YM440 had no effect on body weight. Although YM440 reduced the levels of blood glucose and plasma insulin by 23% and 44% in lean rats and by 18% and 20% in obese rats, these effects did not reach the level of statistical significance.

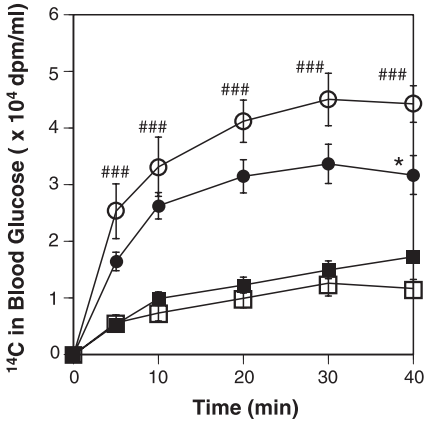

Fig. 1. Effect of YM440 on incorporation of ^{14}C from bicarbonate into blood glucose. [^{14}C]Bicarbonate was injected intravenously at 0 min. At the indicated time, blood was collected and ^{14}C levels in blood glucose were determined as shown in Materials and methods. Lean Zucker rats treated with vehicle (□) or 300 mg/kg of YM440 (■) and obese Zucker rats treated with vehicle (○) or 300 mg/kg of YM440 (●). Data are means±S.E.M. Data were analyzed by two-way analysis of variance. When differences were statistically significant ($P<0.05$), Tukey's multiple range test was used to compare values with those from lean Zucker rats or the vehicle control group. *$P<0.05$ vs. the vehicle control. $^{###}P<0.001$ vs. lean Zucker rats.

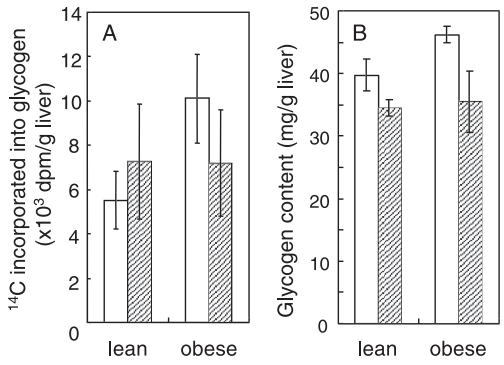

Fig. 2. Effect of YM440 on incorporation of ^{14}C from bicarbonate into liver glycogen (A) and liver glycogen content (B). Liver was excised 45 min after [^{14}C]bicarbonate loading. Liver glycogen content and the incorporation of ^{14}C from bicarbonate into liver glycogen in lean and obese Zucker rats treated with vehicle (open bar) or 300 mg/kg of YM440 (hatched bar) were determined as shown in Materials and methods. Data are means±S.E.M.

3.2. Effects of YM440 on incorporation of ^{14}C from [^{14}C]bicarbonate into blood glucose and hepatic glycogen

The incorporation of ^{14}C from [^{14}C]bicarbonate into blood glucose was increased about 4-fold in obese Zucker rats compared with that in lean rats. YM440 significantly decreased ^{14}C-incorporation into blood glucose by 29% 40 min after [^{14}C]bicarbonate administration (Fig. 1). In contrast, YM440 had no effect on ^{14}C-incorporation into blood glucose in lean Zucker rats. Since neither the incorporation of ^{14}C into hepatic glycogen nor the hepatic glycogen content was significantly different between lean and obese rats treated with either vehicle or YM440 (Fig. 2A and B), it is highly likely that the amount of [^{14}C]glucose in blood was well correlated with gluconeogenic activity in rats and that YM440 lowered the elevated gluconeogenic activity in obese Zucker rats.

3.3. Effects of YM440 on the activity of gluconeogenic enzymes

In order to examine the mechanism for the increased gluconeogenic activity in obese Zucker rats and its suppression by YM440, the mRNA levels or activities of the key gluconeogenic and glycolytic enzymes were determined (Table 2). Liver weight (% of body weight) was increased by 70% in obese compared with lean rats and YM440 reduced it significantly by 20%. The mRNA level of phosphoenolpyluvate carboxykinase, the first enzyme in the gluconeogenic pathway which converts oxaloacetate to phosphoenolpyruvate, was not different in lean and obese Zucker rats, and YM440 had no effect on it. The second key step in the gluconeogenic pathway is the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, which is regulated by the balance of activity between fructose-1,6-bisphosphatase and phosphofructokinase. The activity of fructose-1,6-bisphosphatase was increased by 61% in obese compared with lean Zucker rats, but YM440 did not reduce it significantly. The activity of phosphofructokinase, a glycolytic enzyme, did not vary among the groups. The final key step in the gluconeogenic pathway is that by which glucose-6-phosphatase dephosphorylates glucose-6-phosphate to glucose. The activity of glucose-6-phosphatase was significantly increased by 41% in obese Zucker rats compared with lean rats, and YM440 significantly reduced the activity.

3.4. Correlation of gluconeogenic activity with blood glucose level or glucose-6-phosphatase activity

As shown in Fig. 3, blood glucose levels have a tendency to correlate with the amount of ^{14}C in blood glucose. In addition there was a significant correlation between ^{14}C in blood glucose and glucose-6-phosphatase activity. These findings indicate that the increased gluconeogenic activity in obese rats may be responsible for the hyperglycemia in

Table 2
Effects of YM440 on mRNA expression level of phosphoenolpyruvate carboxykinase, and on the activities of fructose-1,6-bisphosphatase, phosphofructokinase and glucose-6-phosphatase, and liver weight

Treatment	Phosphoenolpyruvate carboxykinase mRNA level (arbitrary unit)	Fructose-1,6-bisphosphatase activity (nmol/min/mg protein)	Phosphofructokinase activity (nmol/min/mg protein)	Glucose-6-phosphatase activity (nmol/min/mg protein)	Liver weight	
					(g)	(% of body weight)
Lean Zucker rats						
Vehicle	3.04±0.84	25.1±0.8	3.95±0.30	245±2	13.0±0.4	3.30±0.05
YM440	2.05±0.15	21.5±2.5	4.58±0.18	252±4	13.0±0.3	3.28±0.06
Obese Zucker rats						
Vehicle	2.10±0.21	40.4±4.1[a]	5.20±0.23	345±8[a]	34.4±2.0[a]	5.61±0.13[a]
YM440	2.01±0.14	35.3±3.5	5.34±0.09	288±12[b]	27.8±1.7[c]	4.47±0.28[b]

A portion of liver was excised 45 min after [^{14}C]bicarbonate injection. Phosphoenolpyruvate carboxykinase mRNA expression level and the activities of fructose-1,6-bisphosphatase, phosphofructokinase and glucose-6-phosphatase were determined using the methods shown in Materials and methods. Data are means±S.E.M.

[a] $P<0.001$ vs. lean Zucker rats.
[b] $P<0.001$ vs. the vehicle control.
[c] $P<0.01$ vs. the vehicle control.

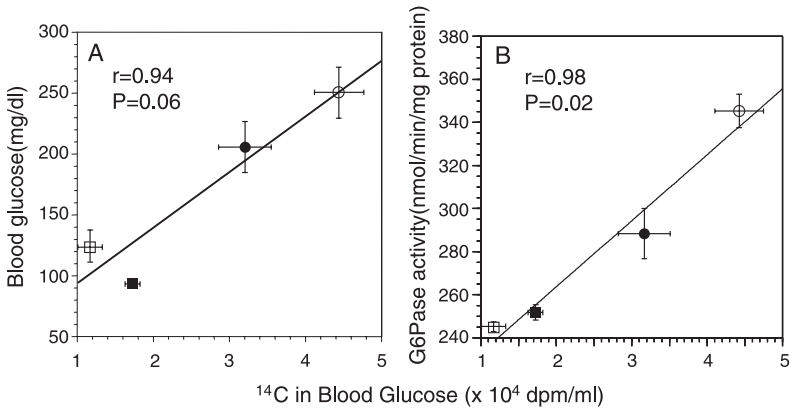

Fig. 3. Correlation of gluconeogenesis with blood glucose concentration (A) or glucose-6-phosphatase activity (B). The points represent the means±S.E.M. obtained from Fig. 1 and Tables 1 and 2. The ^{14}C in blood glucose at 40 min was obtained from Fig. 1. The blood glucose level was obtained from Table 1 and glucose-6-phosphatase activity from Table 2. Results for lean Zucker rats treated with either vehicle (□) or 300 mg/kg of YM440 (■), and obese Zucker rats treated with either vehicle (○) or 300 mg/kg of YM440 (●) are shown. A line was drawn by the least-squares fitting method.

obese Zucker rats. Thus, YM440 may ameliorate elevated hepatic gluconeogenesis by reducing glucose-6-phosphatase activity.

4. Discussion

Genetically obese Zucker rats exhibit mild hyperglycemia, hyperinsulinemia and hyperlipidemia, suggesting the existence of peripheral insulin resistance (Zucker, 1972; Zucker and Antoniades, 1972; Bray and York, 1972; Bach et al., 1981; Jeanrenaud et al., 1985). The primary causes of peripheral insulin resistance in this model are not fully understood, but it has been reported that facilitated gluconeogenesis increases hepatic glucose production and that the peripheral glucose uptake is impaired (Terrettaz and Jeanrenaud, 1983; Jeanrenaud, 1985). We reported previously that the high concentration of insulin in obese Zucker rats might be one determinant for abnormal lipid metabolism, such as serum triglycerides and non-esterified fatty acids (Noshiro et al., 1997). In addition, our prior study indicated that when a hyperinsulinemic euglycemic clamp method was used YM440 as well as troglitazone ameliorated insulin sensitivity in obese Zucker rats by decreasing the elevated hepatic glucose output (Nakano et al, 1999). To investigate the mechanism of action of YM440 on hepatic glucose output, we examined the effects of YM440 on gluconeogenic activity as measured by the incorporation of ^{14}C from [^{14}C]bicarbonate into blood glucose and hepatic glycogen. During the initial stage of gluconeogenesis in liver, pyruvate is carboxylated with $^{14}CO_2$ to yield oxaloacetate, which is in rapid equilibrium with malate, citrate and aspartate. Labeled oxaloacetate is then converted to phosphoenolepyruvate. Labeled phosphoenolepyruvate is converted into glucose-6-phosphate in the gluconeogenesis pathway. Glucose-6-phosphate is converted into glucose by glucose-6-phosphatase or glycogen through glucose 1-phosphate and UDP-glucose.

The amount of [^{14}C]glucose in blood was increased about 4-fold in obese Zucker rats compared with that in lean rats and had a tendency to correlate with blood glucose levels (Figs. 1 and 3A). YM440 decreased ^{14}C-incorporation into blood glucose by 20–37% over 40 min after [^{14}C]bicarbonate administration. However, YM440 had no effect on ^{14}C-incorporation into blood glucose in lean Zucker rats. Fujiwara et al. (1995) demonstrated that diabetic KK mice increased the amount of [^{14}C]glucose in blood about 2-fold compared with that in normal ddY mice and that troglitazone treatment for 7 days decreased [^{14}C]glucose in blood by 20–40%. Since the incorporation of ^{14}C into hepatic glycogen was not significantly different between lean and obese rats treated with either vehicle or YM440 in this study (Fig. 2A), it is highly likely that the amount of [^{14}C]glucose in blood was well correlated with gluconeogenic activity in rats and that YM440 decreased the elevated gluconeogenic activity in obese Zucker rats.

In order to examine the mechanism of elevated gluconeogenic activity in obese Zucker rats, we measured hepatic glycolytic/gluconeogenic key enzymatic activities (phosphofructokinase, glucose-6-phosphatase, fructose-1,6-bisphosphatase and phosphoenolpyluvate carboxykinase). In obese Zucker rats, the activity of fructose-1,6-bisphosphatase and of glucose-6-phosphatase was significantly increased but neither the mRNA level of phosphoenolpyluvate carboxykinase nor the activity of phosphofructokinase was changed compared with that in the lean control (Table 2). The activity of fructose-1,6-bisphosphatase and that of glucose-6-phosphatase in obese Zucker rats was increased by 61% and 41%, respectively, as compared with lean rats. These results are consistent with the findings of others that activities of fructose-1,6-bisphosphatase and glucose-6-phosphatase are significantly increased in fed *db*/*db* mice (Aoki et al., 1999) and obese Zucker rats (Taketomi et al., 1975). Phosphoenolpyluvate carboxykinase activity was not changed in fed obese rats (Perez et al., 1998). Aoki et al.

(1999) also showed that troglitazone and dehydroepiandrosterone decreased the activities of fructose-1,6-bisphosphatase and glucose-6-phosphatase in fed *db*/*db* mice. In contrast to these findings, YM440 decreased glucose-6-phosphatase activity in obese Zucker rats but not fructose-1,6-bisphosphatase activity. We do not know the reasons for the difference in the inhibitory effects of these agents on fructose-1,6-bisphosphatase activity. However, one possibility is the marked difference in plasma glucose concentrations in the two studies (557 mg/dl in *db*/*db* mice vs. 249 mg/dl in obese Zucker rats). Further study will be needed to clarify the cause.

Since glucose-6-phosphatase activity was correlated positively with ^{14}C in blood glucose in obese and lean Zucker rats with or without YM440 treatment (Fig. 3) but fructose-1,6-bisphosphatase or phosphoenolpyluvate carboxykinase activity was not (data not shown), the elevated activity of gluconeogenesis in obese Zucker rats could be attributed mainly to an increase in glucose-6-phosphatase activity. This conclusion could be supported by the observation of Trinh et al. (1998) that a modest overexpression of hepatic glucose-6-phosphatase activity (approximately a 1.6- to 3-fold increase) in rats, assayed with recombinant adenoviruses containing glucose-6-phosphatase cDNA, resulted in metabolic abnormalities, including glucose intolerance, hyperinsulinemia, decreased hepatic glycogen content and increased muscle triglyceride store compared with those in non-transfected rats. These changes are similar to those found in obese Zucker rats or early-stage type 2 diabetic patients.

In contrast, glucose-6-phosphatase knockout mice showed hypoglycemia, marked glycogen storage in the liver and kidney, and hyperlipidemia (Lei et al., 1996). Herling et al. (1998) demonstrated that in fed rats, glucagon-induced glycogenolysis results in hyperglycemia for nearly 2 h and that intravenous infusion of S-3483, an inhibitor of hepatic glucose-6-phosphatase, prevents a hyperglycemic peak and subsequently caused a further lowering of blood glucose. The hepatic content of glycogen and glucose-6-phosphatase was substantially increased after S-3483 treatment.

However, our previous study showed that YM440 decreased levels of fasting plasma insulin and blood glucose and decreased the hepatic glycogen content by 50% compared with values from untreated obese Zucker rats (Kurosaki et al., 2002). These results indicate that, in contrast to glucose-6-phosphatase knockout and an inhibitor of hepatic glucose-6-phosphatase, YM440 normalized glucose intolerance and hepatic glycogen metabolism. These findings suggest that the mechanism of action of YM440 is different from that of glucose-6-phosphatase knockout and an inhibitor of hepatic glucose-6-phosphatase, and that the effect of YM440 on glucose-6-phosphatase activity was not a primary effect but a secondary one after improvement of insulin sensitivity in obese animals.

Although the mechanism by which YM440 suppresses glucose-6-phosphatase activity remains unclear, it likely involves the lowering of glucose-6-phosphatase expression levels, based on the following reasons. It took at least 1–2 weeks to see a maximal reduction in blood glucose by YM440 in *db*/*db* mice (Shimaya et al., 2000) or KK/Ta mice, and an amelioration of glucose intolerance in obese Zucker rats (Kurosaki et al., 2002). Massillon et al. (1997) demonstrated that triglyceride infusion in vivo induced the gene expression of hepatic glucose-6-phosphatase. Since treatment with YM440 for 2 weeks significantly reduced the elevated plasma triglyceride level in obese Zucker rats (Kurosaki, unpublished data), we speculate that YM440 primarily reduces plasma triglyceride levels followed by suppression of hepatic glucose-6-phosphatase expression. This explanation may be supported by the fact that hepatic glucose-6-phosphatase activity did not change after treatment with YM440 in lean Zucker rats (Table 2).

Based on the fact mentioned above that the decrease in ^{14}C in blood glucose in YM440-treated obese rats was mainly due to a decrease in glucose-6-phosphatase activity, it is suggested that the amount of ^{14}C-incorporation into blood glucose via gluconeogenesis is modified by several other factors. One possibility is the change in liver weight. Liver weight (% of body weight) in obese rats was increased by 70% compared with that in lean rats (Table 2) and YM440 decreased not only the specific activity of glucose-6-phosphatase activity (nmol/min/mg protein) but also liver weight. This agent decreased liver weight by 20%. The hepatic hypertrophy in obese rats was accompanied by an increase in liver triglyceride content (Shin et al., 1997) and glitazones, such as rosiglitazone, reduced the liver triglyceride content in obese rats (Murakami et al., 1998). Although in this study we did not determine effects of YM440 on the contents of triglycerides and other components in the liver, it is possible that the reduction in liver weight can be attributed partly to the decreased gluconeogenic activity in obese animals. A second possibility is the change in the levels of substrates for gluconoegenesis, such as lactate. Plasma lactate level is one of the determinant factors for gluconeogenic activity and it was increased about 2-fold in obese rats compared with lean rats (van de Werve and Jeanrenaud, 1987). It was reported that troglitazone reduced plasma lactate levels by 15–30% in obese rats (Fujiwara et al., 1988; Kusano and Abe, 2000). Since we did not measure plasma levels of lactate in this study, it remains unknown whether or not YM440 reduces the amount of ^{14}C-incorporation from [^{14}C]bicarbonate into blood glucose by decreasing lactate levels in plasma. Further study will be needed to clarify the effect of YM440 on substrate levels for gluconeogenesis and on the levels of intermediary metabolites of glucose in liver, especially fructose-1,6-bisphosphate, fructose-6-phosphate and glucose-6-phosphate.

It is known that thiazolidinedione analogues such as troglitazone and pioglitazone activate peroxisome proliferator-activated receptor-γ (PPARγ), which is a key regulator for adipocyte differentiation (Berger et al., 1996; Willson et

al., 1996; Shimaya et al., 2000) and fat deposition (Shimaya et al., 2000). YM440, an oxazolidinedione analogue, is a less potent PPARγ agonist compared with troglitazone and pioglitazone and has no effect on body weight and fat deposition in *db*/*db* mice (Shimaya et al., 2000). Since YM440 improves hyperglycemia in several diabetic animal models including *db*/*db* mice and obese Zucker rats, YM440 may be a useful hypoglycemic agent in the treatment of obese type 2 diabetes.

In conclusion, gluconeogenic activity in obese Zucker rats was increased based on the elevation of glucose-6-phosphatase and fructose-1,6-bisphosphatase activities and was reduced by YM440. Glucose-6-phosphatase but not fructose-1,6-bisphosphatase activity was reduced by YM440 and closely correlated with gluconeogenic activity. These results suggest that glucose-6-phosphatase plays a pivotal role in regulating gluconeogenesis in obese Zucker rats.

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