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Role of glucose and insulin in thiazolidinedione-induced alterations in hepatic gluconeogenesis

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Abstract

Previous studies from our laboratory as well as from others have suggested that the thiazolidinediones have the capacity to act as insulinomimetic agents, especially in the liver. In order to further characterize this insulinomimetic action, we evaluated the effect of troglitazone, a representative thiazolidinedione, on lactate- and glucagon-stimulated gluconeogenesis, in the presence or absence of insulin (10 nM) in isolated rat hepatocytes. The antigluconeogenic effect of troglitazone under basal (lactate-stimulated) conditions was found to be due to an elevation in the fructose 2,6-bisphosphate content, which was, however, not mediated by an activation of 6-phosphofructo 2-kinase. Troglitazone (125 and 250 μM) in the absence of insulin, produced a dose-dependent reduction in glucagon-stimulated gluconeogenesis, thereby suggesting an insulinomimetic effect. In addition, troglitazone (125 and 250 μM), in combination with insulin, produced an additive inhibition of gluconeogenesis during glucagon-stimulated conditions. However, unlike insulin, the metabolic mechanism responsible for these effects (in the presence or absence of insulin) does not involve fructose 2,6-bisphosphate.

Keywords: Troglitazone; Fructose 2,6-bisphosphate; Hepatocyte; Insulin; Thiazolidinedione

1. Introduction

Type 2 diabetes, formerly known as non-insulin-dependent diabetes mellitus, is characterized by peripheral insulin resistance and excessive hepatic glucose production, thus contributing to a state of hyperglycemia (Cerasi, 1995; DeFronzo et al., 1992; Kuzuya et al., 1991; Suter et al., 1992; Yki-Jarvinen, 1994). The major group of drugs used to treat type 2 diabetes are the sulfonylureas (e.g. glyburide, tolbutamide) which primarily act as insulin secretagogues to increase the availability of insulin to peripheral tissues. However, these agents do not directly address the primary physiological abnormalities associated with type 2 diabetes, those being defects in hepatic glucose production and a decrease in tissue sensitivity to insulin. Thus, research has continued in an effort to investigate antidiabetic agents which directly address the primary physiological problems associated with type 2 diabetes and possess a much-reduced potential to produce adverse effects.

The thiazolidinediones, which are structurally unrelated to the sulfonylureas, are a relatively new class of antidiabetic agents which directly address the physiological anomalies associated with type 2 diabetes. They selectively enhance or mimic certain actions of insulin, producing a gradual reduction in hyperglycemia in type 2 diabetic patients (Day, 1999; Spiegelman, 1998). Troglitazone was the first thiazolidinedione compound that became clinically available for both polytherapy and monotherapy management of type 2 diabetes mellitus in patients with significant insulin resistance and an inadequate response to or ability to tolerate combination therapy with metformin. Various studies have shown that troglitazone lowers plasma glucose in patients with non-insulin-dependent diabetes mellitus as well as in insulin-resistant obese and/or diabetic rodent models in which sulfonylureas are ineffective (Colca and Morton, 1990; Eldershaw et al., 1995; Fujiwara et al., 1991; Lee et al., 1994). This agent exerts potent glucose- and insulin-lowering effects in several obese, hyperinsulinemic, diabetic animal models such as the KK mice, C57BL6J-ob/ob mice and Zucker fatty rats, although it fails to decrease plasma glucose in insulin-deficient diabetic or normal animals (Fujiwara et al., 1988, 1995). Troglitazone has been shown to decrease gluconeogenesis

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in diabetic mice (Horikoshi et al., 1990), in HepG2 hepatoma cells (Ciaraldi et al., 1990) as well as in the perfused rat liver (Preininger et al., 1999). In addition, it has been suggested that the plasma glucose lowering effect of the drug may be related to an enhancement of glycolysis in the liver (Murano et al., 1994). More recently, in studies conducted in rat mesangial cells, troglitazone has been reported to enhance glucose uptake via an upregulation of glucose transporter 1 with an acceleration of glycolysis (Asano et al., 2000). The hypoglycemic action of troglitazone is suggested to involve an enhancement of insulin action both in vivo as well as in vitro (Ciaraldi et al., 1990; Fujiwara et al., 1995; Lee et al., 1994; O'Rourke et al., 1997).

Troglitazone has been reported to reduce fasting hyperglycemia in the Goto-Kakizaki rat, a non-obese and normolipidemic rodent model of type 2 diabetes, by inhibiting hepatic glucose production, independently of its action on peripheral insulin sensitivity or hyperlipidemia (O'Rourke et al., 1997). Studies conducted with another thiazolidinedione derivative, pioglitazone, in the perfused rat liver have reported acute, insulin-independent effects on hepatic glucose metabolism involving both stimulation of glycolysis as well as inhibition of gluconeogenesis (Nishimura et al., 1997). Our laboratory has also demonstrated that under certain metabolic conditions, troglitazone and englitazone (another representative thiazolidinedione, not approved for clinical use) inhibit lactate- and glucagon-stimulated gluconeogenesis in the complete absence of insulin in isolated rat hepatocytes (Raman et al., 1998) and in the isolated perfused rat liver (Adams et al., 1998), respectively, thereby suggesting an "insulinomimetic" effect. However, the biochemical mechanism(s) by which troglitazone suppresses hepatic gluconeogenesis is unclear. Also, the precise role of insulin in the effects of troglitazone on hepatic glucose metabolism remains controversial.

The present study was therefore undertaken to evaluate the role of glucose and insulin on the antigluconeogenic action of troglitazone in isolated rat hepatocytes. Experiments were conducted under basal-(lactate) and hormonally modulated (glucagon) conditions and we examined the hypothesis that an alteration in hepatocyte fructose 2,6-bisphosphate concentration (a key bifunctional gluconeogenic/glycolytic regulator) is responsible, in part, for the troglitazone-induced suppression of hepatic gluconeogenesis.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (150–200 g) from Harlan Sprague Dawley (Indianapolis, IN) were used for all experiments. Depending on the study protocol, animals either had access to food and water ad libitum or were fasted for 20–24 h prior to hepatocyte isolation. Animals were maintained on a 12:12-h light/dark cycle (lights on at 0600 h). All experimental protocols were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation.

2.2. Reagents

Collagenase (type I) was purchased from Worthington Biochemical (Freehold, NJ). Troglitazone was supplied by Parke-Davis (Ann Arbor, MI). All enzymes and coenzymes were purchased from Boehringer Mannheim (Indianapolis, IN). [U-^{14}C] L-lactate was obtained from New England Nuclear (Boston, MA). Fructose 2,6-bisphosphate used as a standard was obtained from Fluka Chemical (Ronkonkoma, NY). Crystalline porcine glucagon and insulin were obtained from Sigma (St. Louis, MO). All other reagents and chemicals used were of analytical grade and purchased from Sigma.

2.3. Isolation of hepatocytes

Hepatocytes were prepared by collagenase perfusion of the liver using a modification of the method described by Seglen (1976). Isolated cells were suspended in Krebs-Henseleit-Bicarbonate (KHBC) buffer containing 3% bovine serum albumin and oxygenated with 95% O_2/5% CO_2. The viability of hepatocytes was assessed by trypan blue exclusion and lactate dehydrogenase leakage. Only cell preparations with greater than 90% viability were used for experimentation.

2.4. Hepatocyte incubation protocol

Samples of cell suspension (2.0 ml) containing 40–80 mg of cells (wet weight) were shaken (100–120 strokes/min) in stoppered 25 ml polycarbonate erhlenmeyer flasks at 37°C with different concentrations of troglitazone (62.5–250 μM) or the corresponding vehicle (DMSO 0.05%) for 30 min in the presence or absence of 10 mM glucose in vitro. Following this preincubation, the cells were further incubated in the presence of insulin (10 nM) or the corresponding vehicle (KHBC) for a period of 6 min. This was followed by the addition of 2:0.2 mM [U-^{14}C] L-lactate/pyruvate (0.1–0.15 μCi/μmol) as the gluconeogenic precursor and the cells were incubated for an additional 30 min in the presence or absence of glucagon (0.3 nM). Aliquots of cell suspension (0.25 ml) were collected 15 min following incubation of the cells with the gluconeogenic stimulants. These were immediately frozen in liquid N_2 and subsequently stored at −70°C for the determination of fructose 2,6-bisphosphate concentrations,

carried out not more than 24 h later. Cellular fructose 2,6-bisphosphate was measured in aliquots of the alkali extracts by the ability of this metabolite to activate Ppi:fructose-6-phosphate-1-phosphotransferase, using the method described by Van Schaftingen et al. (1982). Subsequently, cell incubations were terminated at end point by the addition of 20% $HClO_4$. The acidic medium was neutralized and gluconeogenesis was estimated by the amount of ^{14}C-glucose formation from ^{14}C-lactate by utilizing ion exchange chromatography (Clark et al., 1974; Pilkis and Claus, 1977). Results are expressed as μmol of ^{14}C-lactate converted to glucose or nmol of fructose 2,6-bisphosphate produced per gram wet weight of hepatocytes during the corresponding incubation period.

2.5. Assay of 6-phosphofructo 2-kinase enzyme activity

Aliquots (0.350 ml) of cell suspension were collected 8 min following the addition of [U-^{14}C] L-lactate/pyruvate (with or without glucagon), pipetted into cryovials and immediately frozen in liquid N_2. Samples were kept at −70°C until the enzymatic assay was carried out, not more than 36 h later. The active form of hepatocyte 6-phosphofructo 2-kinase was measured as previously described, with some minor modifications (Bartrons et al., 1983). Briefly, frozen aliquots of the cell suspensions were thawed by shaking them in an equal volume of an ice-cooled buffer containing 20 mM potassium phosphate buffer, 100 mM KCl and 10 mM EDTA (pH = 7.1). The resulting mixture was centrifuged at 12,000 × *g* for 10 min at 4°C. Twenty microliter portions of the resulting supernatant were incubated for 20 min at 25°C in the presence of 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 5 mM potassium phosphate, 2 mM $MgCl_2$, 100 mM KCl, 5 mM MgATP, 1 mM fructose 6-phosphate and 3.5 mM glucose 6-phosphate (pH = 6.6), in a final volume of 0.250 ml. The reaction was stopped by the addition of 0.250 ml of 0.1 M NaOH, and the fructose 2,6-bisphosphate formed was determined in the resulting mixture as described above. Corresponding blanks were subtracted for the presence of fructose 2,6-bisphosphate at zero time point. One unit of enzymatic activity is defined as the amount of enzyme that transforms 1 μmol of substrate per minute under the assay conditions.

2.6. Statistical analyses

Results from all experiments are expressed as the mean ± standard error of the mean (S.E.M.). Statistical signifi-

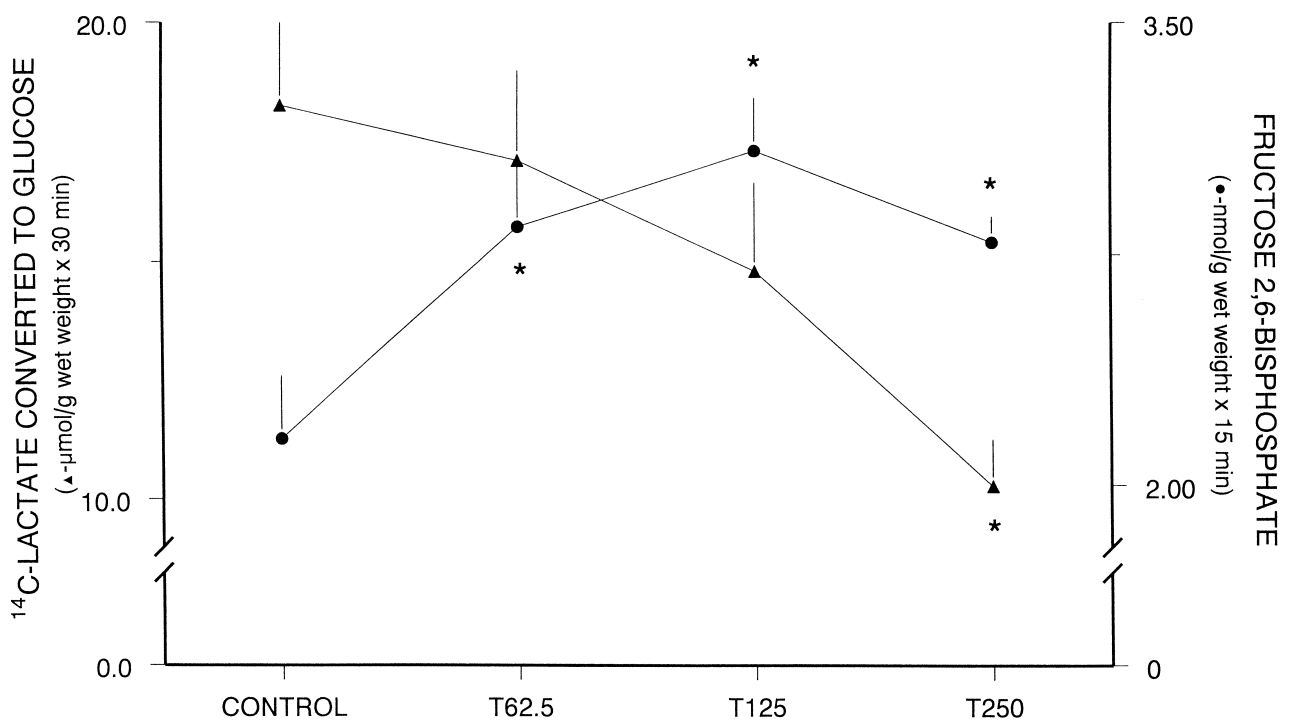

Fig. 1. Correlation between troglitazone-mediated effects on lactate-stimulated gluconeogenesis and fructose 2,6-bisphosphate content in hepatocytes isolated from 20–24 h fasted rats. Hepatocytes (40–80 mg wet weight) were incubated in oxygenated KHBC in the presence or absence of troglitazone (T62.5–T250 μM) for a period of 30 min. This was followed by the addition of 2:0.2 mM [U-^{14}C] L-lactate/pyruvate (CONTROL) as the gluconeogenic precursor. Aliquots of the cell suspension were collected 15 min after the addition of the gluconeogenic precursor for determination of fructose 2,6-bisphosphate concentration. The cells were further incubated for an additional 15 min and aliquots of cell suspension were collected at end point for the determination of ^{14}C-glucose. Results are expressed as μmol of ^{14}C-lactate converted to glucose or nmol of fructose 2,6-bisphosphate produced per gram wet weight of hepatocytes (mean ± S.E.M.). Values represent four independent experiments (from four different hepatocyte isolations) each conducted in triplicate. * $p < 0.05$ vs. control.

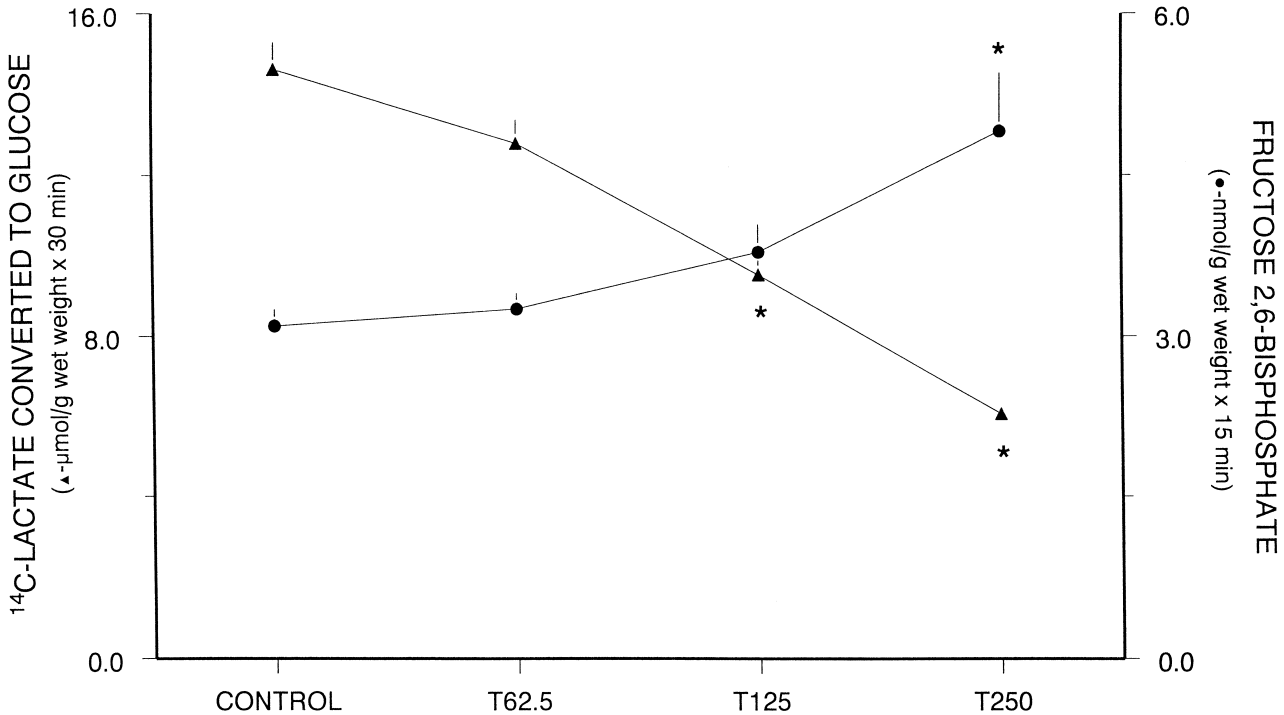

Fig. 2. Correlation between troglitazone-mediated effects on lactate-stimulated gluconeogenesis and fructose 2,6-bisphosphate concentration in 20–24 h fasted rat hepatocytes incubated in the presence of 10 mM glucose in vitro (see Fig. 1 for experimental details). Values are expressed as described in the legend for Fig. 1 and represent mean ± S.E.M. Values represent four independent experiments (from four different hepatocyte isolations) each conducted in triplicate. * $p < 0.05$ vs. control.

cance at the 95% confidence level was determined according to a one-way analysis of variance (ANOVA) and the Student–Newman–Keuls analyses was used as the post hoc test.

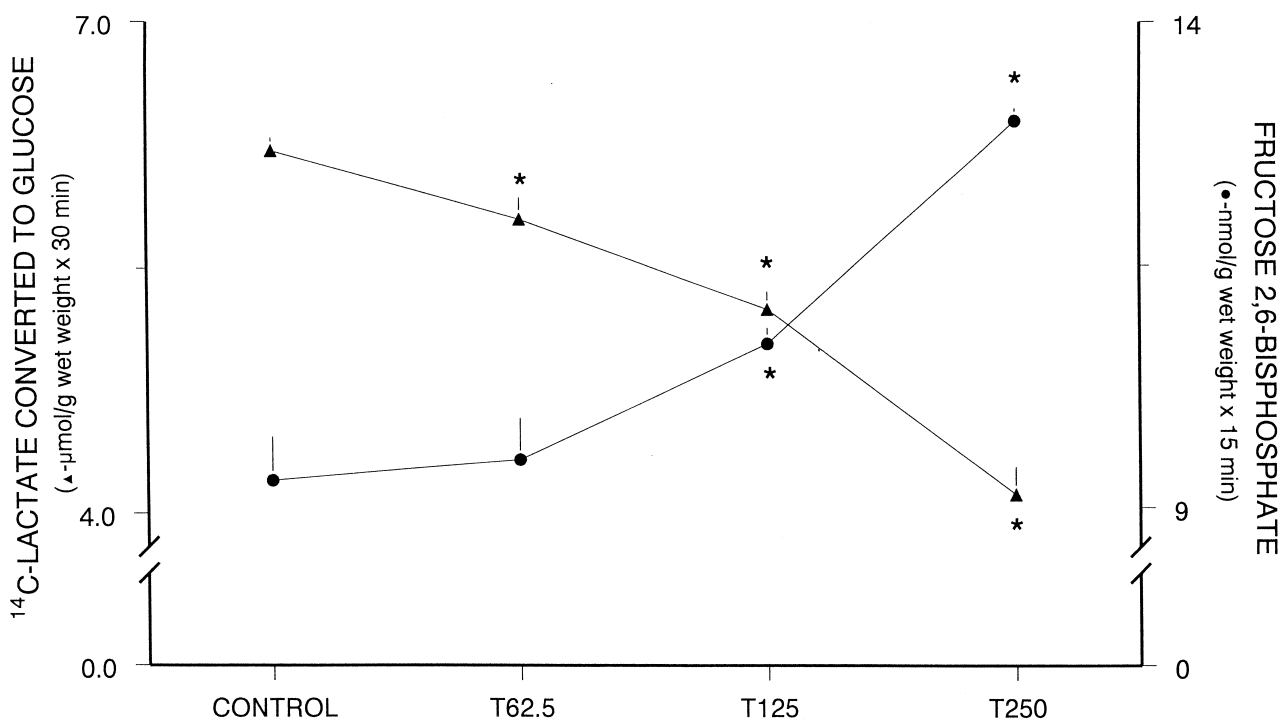

Fig. 3. Correlation between troglitazone-mediated effects on lactate-stimulated gluconeogenesis and fructose 2,6-bisphosphate content in hepatocytes isolated from fed rats (see Fig. 1 for experimental details). Values are expressed as described in the legend for Fig. 1 and represent mean ± S.E.M. Each value represents four independent experiments (from four different hepatocyte isolations) each conducted in triplicate. * $p < 0.05$ vs. control.

3. Results

3.1. Effect of troglitazone on hepatic gluconeogenesis under basal (lactate-stimulated) conditions

In hepatocytes isolated from 20–24 h fasted rats, troglitazone (250 μM) significantly inhibited hepatic gluconeogenesis by 43.8%, using 2:0.2 mM [U-^{14}C] L-lactate/pyruvate as the gluconeogenic precursor. Lower concentrations of the drug (62.5 and 125 μM), however, failed to produce any significant inhibitory effects. Under these conditions, troglitazone (62.5–250 μM) significantly elevated fructose 2,6-bisphosphate concentrations by 32.2%, 43.6% and 29.8%, respectively, as compared to the control treatment group. This elevation in the hepatocyte fructose 2,6-bisphosphate levels correlated in an inverse fashion with the inhibitory effect of the drug on lactate-stimulated hepatic gluconeogenesis (Fig. 1).

The effect of glucose in vitro on the glucose-lowering action of troglitazone observed in the fasted rat hepatocytes was then investigated. Hepatocytes isolated from 20–24 h fasted rats were incubated with different concentrations of troglitazone in KHBC plus 10.0 mM glucose in vitro (compared to no glucose as shown in Fig. 1). Under these conditions, troglitazone (125 and 250 μM) produced a significant reduction in lactate-stimulated hepatic gluco-

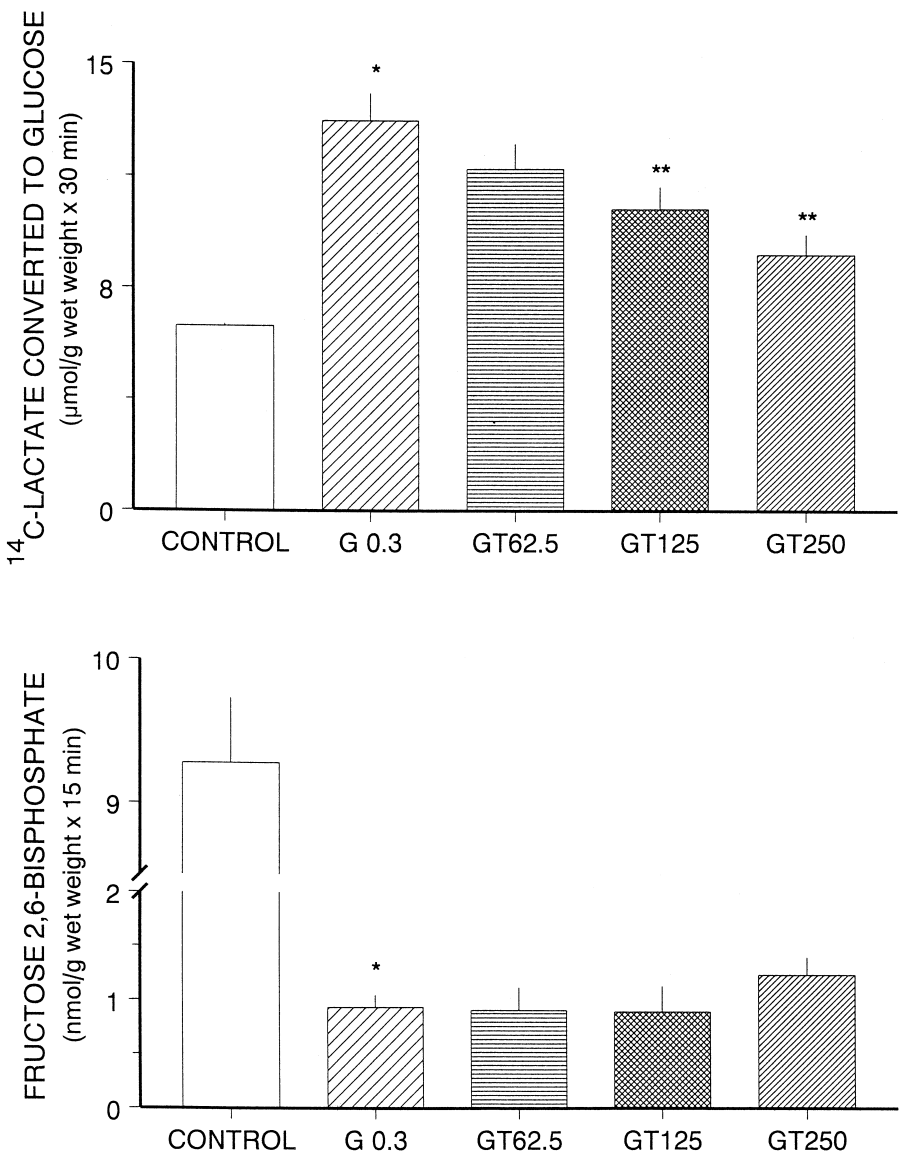

Fig. 4. Effect of troglitazone on glucagon-stimulated gluconeogenesis in hepatocytes isolated from fed rats: gluconeogenesis (top panel), fructose 2,6-bisphosphate (bottom panel). Hepatocytes (40–80 mg wet weight) were incubated in oxygenated KHBC (in the presence of 10 mM glucose in vitro) with or without troglitazone (T62.5–T250 μM) for a period of 30 min. The gluconeogenic precursor, 2:0.2 mM [U-^{14}C] L-lactate/pyruvate (CONTROL) was then added in the presence or absence of glucagon (G 0.3 nM) and the incubations were continued for an additional 30 min. Aliquots were collected at the indicated time points for the determination of ^{14}C-glucose and fructose 2,6-bisphosphate. Values represent four independent experiments (from four different hepatocyte isolations) each conducted in triplicate. $^{*}p < 0.05$ vs. control; $^{**}p < 0.05$ vs. glucagon (G 0.3).

neogenesis (34.9% and 58.5%, respectively). This antigluconeogenic effect of troglitazone was greater than that observed in fasted hepatocytes in the absence of glucose (Fig. 1). Specifically, in 20–24 h fasted rat hepatocytes incubated with glucose, the presence of glucose enhanced the antigluconeogenic effects of troglitazone (250 μM) during lactate-stimulated gluconeogenesis from 43.8% (observed under conditions of no glucose) to 58.5% (with 10.0 mM glucose in vitro). Under these experimental conditions, the hepatocyte fructose 2,6-bisphosphate concentration was significantly increased by 58.5%. This elevation in the intracellular metabolite concentration correlated with the suppressive effects of troglitazone on hepatic glucose production. Thus, an inverse correlation was established between the antigluconeogenic effects of troglitazone (250 μM) and the hepatocyte fructose 2,6-bisphosphate content (Fig. 2). Lower concentrations of troglitazone (62.5 and 125 μM), however, failed to produce any statistically significant effects on the hepatocyte fructose 2,6-bisphosphate levels.

The role played by the metabolic state of the donor rat in the antigluconeogenic effects of troglitazone was then

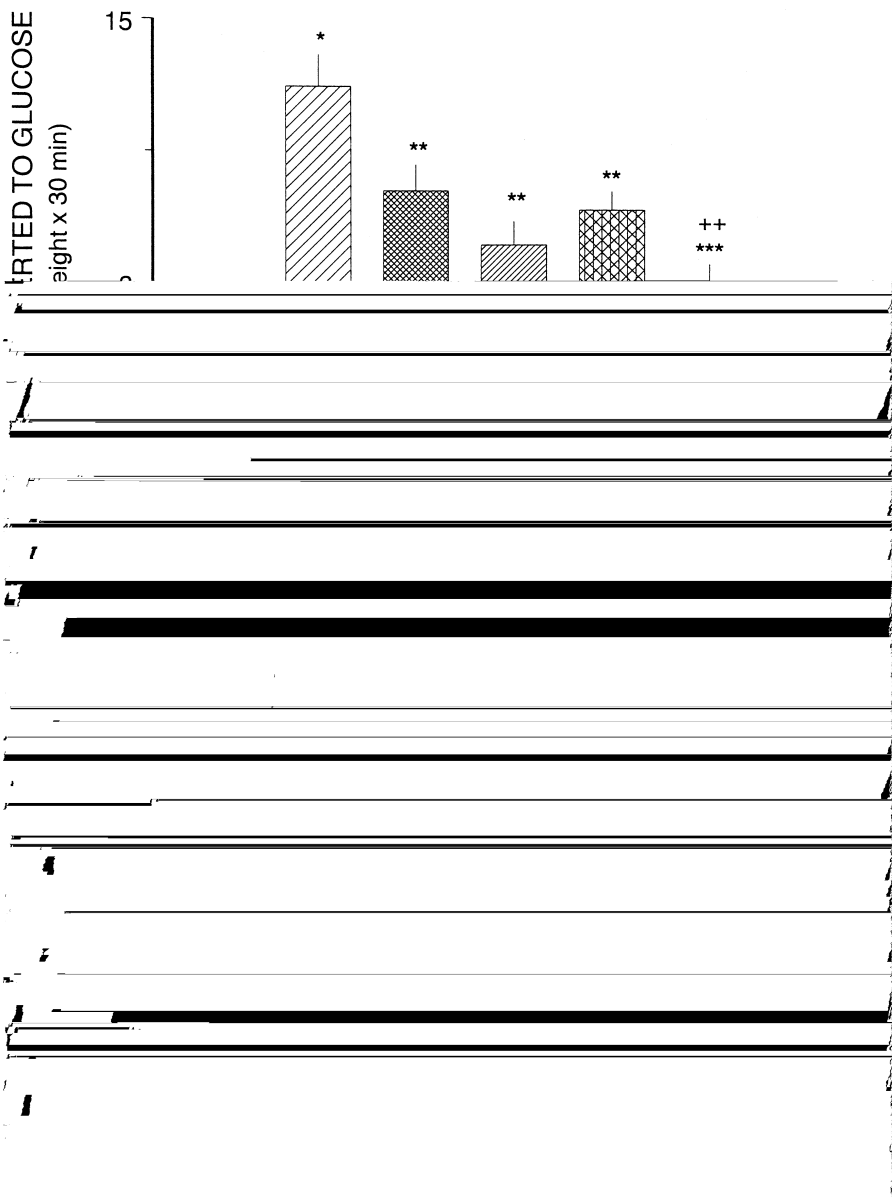

Fig. 5. Effect of troglitazone on hepatic glucose metabolism during glucagon modulation in the presence of insulin in fed rat hepatocytes: gluconeogenesis (top panel), fructose 2,6-bisphosphate (bottom panel). Hepatocytes were incubated in oxygenated KHBC (in the presence of 10 mM glucose in vitro) with or without troglitazone (T125 and T250 μM) for 30 min. Insulin (I 10 nM) was then added and the cells were further incubated for 6 min. This was followed by the addition of the gluconeogenic precursor, 2:0.2 mM lactate/pyruvate in the presence or absence of glucagon (G 0.3 nM). Aliquots were collected at the specified time points for the determination of ^{14}C-glucose and fructose 2,6-bisphosphate. Values represent four independent experiments (from four different hepatocyte isolations) each conducted in triplicate. $^{*}p < 0.05$ vs. control; $^{**}p < 0.05$ vs. G 0.3; $^{***}p < 0.05$ vs. GI 10; $+p < 0.05$ vs. GT 250; $++p < 0.05$ vs. GT 125.

Table 1
Effect of troglitazone on 6-phosphofructo 2-kinase (PFK-2) activity during lactate-stimulated gluconeogenesis in fed rat hepatocytes

Treatment	6-Phosphofructo 2-kinase activity (mU/g wet weight of cells)
CONTROL	2.012 ± 0.171
T62.5	2.020 ± 0.300
T125	2.049 ± 0.285
T250	2.112 ± 0.345

Hepatocytes (40–80 mg wet weight) were incubated in oxygenated KHBC(in the presence of 10 mM glucose in vitro) with or without troglitazone (T62.5–T250 μM) for a period of 30 min. This was followed by the addition of 2:0.2 mM [U-^{14}C] L-lactate/pyruvate (CONTROL) as the gluconeogenic precursor. Aliquots of the cell suspension were collected min after addition of the gluconeogenic precursor for the determination of PFK-2 activity. Results are expressed as mUnits of PFK-2 (6-phosphofructo 2-kinase) per gram wet weight of hepatocytes. Values represent mean ± S.D. from two independent experiments (from two hepatocyte isolations) each conducted in triplicate.

investigated. In order to do this, fed rats were used as the donor animals compared to the fasted rats utilized in the previously described studies. In hepatocytes isolated from fed rats (and incubated with 10 mM glucose in vitro), troglitazone inhibited hepatic glucose production in a concentration-dependent manner. Under these conditions, troglitazone (62.5–250 μM) significantly decreased lactate-stimulated gluconeogenesis by 6.8%, 15.7% and 33.8%, respectively. This antigluconeogenic effect of the drug was accompanied with an increase in the intracellular fructose 2,6-bisphosphate concentration (15.2% and 40.0% at 125 μM and 250 μM, respectively), thus resulting in an inverse correlation between gluconeogenesis and fructose 2,6-bisphosphate content (Fig. 3).

3.2. Effect of troglitazone on hepatic gluconeogenesis under hormonal modulation

Glucagon (0.3 nM) stimulated lactate-induced gluconeogenesis by 95% in fed rat hepatocytes, and this was associated with an 88% decrease in fructose 2,6-bisphosphate concentrations. Under these conditions of glucagon modulation, troglitazone (125 and 250 μM) significantly decreased glucagon-stimulated gluconeogenesis by 22.5% and 34.3%, respectively, in hepatocytes isolated from fed rats (Fig. 4, top panel). However, this inhibitory effect of troglitazone did not correlate with significant changes in the hepatocyte fructose 2,6-bisphosphate concentrations (Fig. 4, bottom panel).

Experiments were subsequently conducted to evaluate the role of insulin in the antigluconeogenic effect of troglitazone in fed rat hepatocytes under glucagon modulation. Under these conditions, insulin (10 nM) by itself decreased glucagon-stimulated gluconeogenesis by 26.7%. Troglitazone (125 and 250 μM), when used in combination with insulin, inhibited glucagon-stimulated hepatic gluconeogenesis by 43.5% and 56.7%, respectively (Fig. 5, top panel). This glucose lowering action of a combination of troglitazone and insulin was observed to be much higher than that produced when troglitazone was used alone, in the absence of any exogenous insulin. Specifically, when fed rat hepatocytes were exposed to troglitazone (250 μM) in the presence of a physiological concentration of insulin in vitro, the inhibitory effect of the drug on glucagon-stimulated gluconeogenesis was significantly increased from 34.3% to 56.7%. Similarly, with a lower concentration of the drug (125 μM), the glucose-lowering effects of troglitazone in the presence of insulin in vitro was found to increase significantly from 22.5% (observed in the absence of exogenous insulin) to 43.5% . Insulin (10 nM) by itself significantly elevated (1.17-fold) intracellular fructose 2,6-bisphosphate concentration in fed hepatocytes during glucagon stimulation. However, a combination dose of troglitazone and insulin did not produce any further increase in the hepatocyte fructose 2,6-bisphosphate concentrations (Fig 5, bottom panel).

In order to elucidate the underlying mechanism responsible for the elevations in the fructose 2,6-bisphosphate concentrations observed earlier during lactate-stimulated gluconeogenesis, further experiments were conducted to examine the effect of troglitazone on hepatocyte 6-phosphofructo 2-kinase activity. Hepatocytes isolated from fed rats were incubated with different concentrations of troglitazone, using 2:0.2 mM lactate/pyruvate and glucagon (0.3 nM) as the gluconeogenic stimulants, in the presence or absence of insulin (10 nM). Glucagon resulted in a significant decrease in the 6-phosphofructo 2-kinase activ-

Table 2
Effect of troglitazone on 6-phosphofructo 2-kinase (PFK-2) activity during glucagon modulation in the presence or absence of insulin in fed rat hepatocytes

Treatment	6-Phosphofructo 2-kinase activity (mU/g wet weight of cells)
CONTROL	2.012 ± 0.171
G 0.3	1.001 ± 0.087[a]
GT 62.5	1.055 ± 0.182
GT 125	1.065 ± 0.244
GT 250	1.026 ± 0.170
GI 10	1.392 ± 0.185[b]
GIT 125	1.399 ± 0.218[b,c]
GIT 250	1.492 ± 0.296[b,d]

Hepatocytes were incubated as described in the legend for Table 1 with or without troglitazone (T125 and T250 μM) for 30 min. Insulin (10 nM) was then added and the cells were further incubated for 6 min, followed by the addition of the gluconeogenic precursor, 2:0.2 mM lactate/pyruvate in the presence or absence of 0.3 nM glucagon (G 0.3). Aliquots were collected at specified time points for the determination of PFK-2 activity. Values are expressed as described in the legend for Table 1 and represent the mean ± S.D. from two independent experiments (from two hepatocyte isolations) each conducted in triplicate.

[a] $p < 0.05$ vs. control.
[b] $p < 0.05$ vs. glucagon (G 0.3).
[c] $p < 0.05$ vs. GT 125.
[d] $p < 0.05$ vs. GT 250.

ity (50%), which correlated with the glucagon-induced lowering of the hepatic fructose 2,6-bisphosphate concentrations. Under these conditions, troglitazone (62.5–250 μM) did not produce any significant effects on the hepatocyte 6-phosphofructo 2-kinase activity during both lactate- (Table 1) as well as glucagon-stimulation (Table 2). However, unlike troglitazone, insulin (10 nM) produced a significant increase in 6-phosphofructo 2-kinase activity, similar to previously reported findings, which in turn correlated with the insulin-induced elevations in fructose 2,6-bisphosphate levels.

4. Discussion

The present study demonstrates that the nutritional and metabolic state of the hepatocyte plays a key role in the degree of antigluconeogenic action of troglitazone, which is similar to our previously reported findings (Raman et al., 1998). Previous studies have shown that after a period of 20–24 h of starvation, the liver glycogen stores of the rat are almost completely depleted (Barzilai et al., 1995; Newsholme and Stork, 1973; Rigoulet et al., 1987). Under such conditions of stimulated glycogen breakdown and negligible glycogen synthesis, introduction of the gluconeogenic substrate, lactate, leads to a stimulation of the gluconeogenic pathway (Newsholme and Stork, 1973; Rigoulet et al., 1987). We have demonstrated that under these conditions of stimulated glucose synthesis, troglitazone (250 μM) significantly decreased lactate-stimulated gluconeogenesis in fasted rat hepatocytes. Simultaneous to this reduction in lactate-stimulated hepatic gluconeogenesis, there was a significant elevation in fructose 2,6-bisphosphate concentration.

When hepatocytes isolated from 20–24 h fasted rats were incubated with troglitazone in the presence of glucose in vitro, the inhibitory effect of the drug on hepatic gluconeogenesis was much greater than that observed in fasted hepatocytes (with no glucose in vitro). These findings demonstrate that in hepatocytes isolated from fasted rats, the presence of glucose in vitro significantly potentiated the antigluconeogenic effect of troglitazone, thereby suggesting a possible synergistic or additive effect. We have further demonstrated that under these conditions, the inhibitory effect of troglitazone observed on hepatic gluconeogenesis inversely correlated with an increase in the hepatocyte fructose 2,6-bisphosphate concentrations. It is possible that in fasted rat hepatocytes, glucose-mediated elevations in the intracellular fructose 2,6-bisphosphate levels may have a triggering effect on the ability of troglitazone to further increase the fructose 2,6-bisphosphate concentrations, either by an activation of the synthetic enzyme, 6-phosphofructo 2-kinase or by down regulation of the degradative enzyme, fructose 2,6-bisphosphatase, thereby facilitating a further increase in the glucose-induced elevations in the hepatic fructose 2,6-bisphosphate content. Alternatively, it is possible that in the fasted rat hepatocytes incubated with glucose in vitro, troglitazone may cause a further lowering of the glucose-induced decrease in phosphoenolpyruvate carboxykinase activity. Such an interaction of troglitazone with exogenous glucose, possibly occurring at some early time point of the incubation period, may be responsible for the potentiating effect of glucose observed with the drug on hepatic gluconeogenesis. It is important to note that this potentiating effect with troglitazone was not simply an additive effect with glucose, since there was no statistically significant difference between the controls, with or without glucose in vitro (15 vs. 18 μmol of glucose/g wet weight × 30 min). Instead, glucose seems to work with the drug, possibly modifying the enzyme to a state where troglitazone becomes more efficacious.

In hepatocytes obtained from fed rats, troglitazone produced a concentration-dependent reduction in lactate-stimulated hepatic gluconeogenesis. This antigluconeogenic effect of troglitazone resulted in an inverse correlation with the hepatocellular fructose 2,6-bisphosphate concentrations. The metabolic status of hepatocytes is dramatically different between cells obtained from fasted rats and those obtained from fed rats. This is primarily a result of alterations in the hormonal as well as metabolic profile of these rats in vivo (Pilkis and Claus, 1991). Our results demonstrate that incubation of fed hepatocytes with troglitazone causes a further increase in the intracellular fructose 2,6-bisphosphate concentrations, which are significantly elevated in fed hepatocytes as compared to fasted cells. Thus, findings from the present study suggest that under basal (lactate) conditions (fed or fasted), troglitazone decreases hepatic gluconeogenesis, at least in part, by increasing hepatocellular fructose 2,6-bisphosphate concentrations. Alterations in the fructose 2,6-bisphosphate levels observed during these short term exposure studies, however, were not found to be due to a direct enzymatic activation of 6-phosphofructo 2-kinase, the enzyme responsible for the synthesis of fructose 2,6-bisphosphate. Several alternate mechanism(s) can be suggested for the increase in the hepatocyte fructose 2,6-bisphosphate levels observed with troglitazone. It is possible that troglitazone may cause a direct inhibition of the bisphosphatase domain of 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase, thereby resulting in an increase in the fructose 2,6-bisphosphate levels. Alternatively, it could be suggested that troglitazone exposure may cause an increase in the hepatic pool of hexose 6-phosphates (glucose 6-phosphate, fructose 6-phosphate), possibly through inhibition of glucose 6-phosphatase (Davies et al., 1999). Fructose 6-phosphate is known to strongly inhibit fructose 2,6-bisphosphatase, thereby resulting in an increased accumulation of fructose 2,6-bisphosphate and a subsequent inhibition of hepatic gluconeogenesis. Furthermore, an increment in hepatocyte fructose 6-phosphate concentration could result in an increased substrate availability for 6-phosphofructo 2-kinase,

leading to an increase in the sensitivity of the enzyme and an increased production of fructose 2,6-bisphosphate. Such a possibility is also supported by the findings of Murano et al. (1994) who examined the effect of troglitazone on the enzyme kinetics of 6-phosphofructo 2-kinase studied as a function of fructose 6-phosphate concentration. These investigators demonstrated that troglitazone increases the sensitivity of 6-phosphofructo 2-kinase but does not have any appreciable effect on the maximal activity of the enzyme. Additionally, a decrease in cyclic AMP levels caused by troglitazone could lead to an inactivation of fructose 2,6-bisphosphatase, thereby facilitating an elevation in the fructose 2,6-bisphosphate concentrations.

Results from the hormonal studies demonstrate that glucagon (0.3 nM) significantly increased lactate-stimulated gluconeogenesis in hepatocytes isolated from fed rats, which is in agreement with previously reported findings (Claus and Pilkis, 1976; Hue and Bartrons, 1984; Johnson et al., 1972; Pilkis et al., 1975). Under these conditions, troglitazone (125 and 250 μM) produced a significant inhibition of glucagon-stimulated gluconeogenesis in the complete absence of any exogenous insulin, thereby suggesting an "insulinomimetic" effect. A number of previous studies have also demonstrated that troglitazone has direct, insulinomimetic properties, including both activation and inhibition of gene expression (Ciaraldi et al., 1990; De Vos et al., 1996; Ibrahimi et al., 1994; Kurebayashi et al., 1997; Marx et al., 1998; Tafuri, 1997; Teboul et al., 1995). However, these glucose-lowering effects of troglitazone observed during glucagon-modulation did not correlate with significant alterations in the hepatocyte fructose 2,6-bisphosphate levels.

Several alternate enzymatic mechanism(s) can be speculated to be responsible for the observed inhibitory effects of troglitazone during glucagon-stimulated gluconeogenesis, such as inhibition of phosphoenolpyruvate carboxykinase or fructose 1,6-bisphosphatase or activation of pyruvate kinase. The involvement of phosphoenolpyruvate carboxykinase is supported by previous studies where it has been shown that the elevated activity of phosphoenolpyruvate carboxykinase in diabetic mice and rats returned to the normal level after treatment with another representative thiazolidinedione, pioglitazone (Hofmann et al., 1992, 1995). An alternate possibility is that troglitazone may reduce the extent of phosphorylation of pyruvate kinase during glucagon-stimulated gluconeogenesis. Such effects would potentiate pyruvate kinase activity, thereby favoring glycolysis while decreasing glucagon-stimulated gluconeogenesis. There are no reports in the literature regarding thiazolidinedione-induced activation of pyruvate kinase. However, in the perfused rat liver, englitazone stimulates glycolysis from dihydroxyacetone indirectly suggesting a possible effect on pyruvate kinase (Adams et al., 1998). Additionally, in accordance with previously reported findings in the C57BL/KsJ-db/db mice (Fujiwara et al., 1995; Aoki et al., 1999), troglitazone may inhibit a glucagon-stimulated increase in the fructose 1,6-bisphosphatase activity, resulting in an increased accumulation of fructose 1,6-bisphosphate, and an allosteric activation of pyruvate kinase.

Insulin (10.0 nM), when used in conjunction with glucagon (0.3 nM), produced significant suppressive effects on hepatic glucose production, similar to earlier findings (Claus and Pilkis, 1976; Hue and Bartrons, 1984; Johnson et al., 1972; Pilkis et al., 1975). Under these conditions, troglitazone, in combination with insulin, produced an additive inhibition of glucagon-stimulated hepatic gluconeogenesis. In accordance with existing literature (Monge et al., 1986; Pilkis and Claus 1991; Pilkis et al., 1983, 1988), this inhibitory effect of insulin on glucagon-stimulated gluconeogenesis resulted in a marked elevation in the hepatocyte fructose 2,6-bisphosphate levels. However, we observed that troglitazone, in combination with insulin, did not have any effect (additive or potentiating) on the hepatocyte fructose 2,6-bisphosphate concentrations, when compared to the insulin treatment group by itself. Therefore, the present study demonstrates that in hepatocytes under hormonal modulation (in the presence or absence of insulin in vitro), the glucose-lowering action of troglitazone is not due to a modulation in the hepatocyte fructose 2,6-bisphosphate concentrations, thereby suggesting that there exists different metabolic mechanism(s) by which troglitazone and insulin decrease glucagon-stimulated hepatic gluconeogenesis. It is possible that troglitazone shares a common enzymatic site with insulin (not mediated by alterations in fructose 2,6-bisphosphate concentrations) to intervene with the actions of glucagon on hepatic gluconeogenesis. It can be speculated that troglitazone may cause an activation of cAMP phosphodiesterase, phosphatase 2A or some other phosphatases resulting in a reduction in the intracellular cAMP levels. Such effects of troglitazone could lead to a stimulation of the glycolytic enzyme, pyruvate kinase and/or an inhibition of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, similar to that caused by insulin. A combination of such effects of acute troglitazone and insulin exposure at these enzymatic sites could be responsible for an additive inhibition of glucagon-stimulated gluconeogenesis.

It should be noted that great care was taken in the selection of the particular dose of troglitazone utilized in these studies. Previously reported in vitro studies have used troglitazone in concentrations ranging from 1 to 1000 μM (Ciaraldi et al., 1990; Fulgencio et al., 1996; Murano et al., 1994; Preininger et al., 1999). While clinical studies suggest that an oral administration of 400 mg troglitazone results in peak peripheral blood concentrations of 1.2 μM (Murano et al., 1994), it must be emphasized that drug concentrations in the portal blood are higher than those in peripheral blood. Moreover, troglitazone has also been reported to exhibit ~99.9% binding to plasma proteins in vivo (Shibukawa et al., 1995). Therefore, based on these findings, we suggest that inclusion of 3% bovine serum

albumin in the incubation buffer containing troglitazone would lead to a significant protein binding of the drug, thereby resulting in a significant lowering of the free drug concentration available, which would be physiologically appropriate. In addition, in the present study, troglitazone did not have any inhibitory effects on the hepatocyte ATP concentrations (data not shown), thus implicating that the reduced free drug concentration available in this study does not alter the hepatocyte mitochondrial bioenergetics, resulting in hepatotoxicity.

In summary, results from the present study demonstrate that an elevation in hepatic fructose 2,6-bisphosphate concentration is responsible, at least in part, for the antigluconeogenic effects of troglitazone observed in the absence of any exogenous insulin during lactate-stimulated conditions. Such findings would suggest an insulin-independent or insulinomimetic effect of the drug, which is in agreement with earlier findings (Ciaraldi et al., 1990; Davies et al., 1999; De Vos et al., 1996; Ibrahimi et al., 1994; Kurebayashi et al., 1997; Marx et al., 1998; Raman et al., 1998; Tafuri, 1997; Teboul et al., 1995). Another important finding of this study is that there exists different metabolic mechanism(s) (other than fructose 2,6-bisphosphate) by which troglitazone and insulin decrease glucagon-stimulated gluconeogenesis. Therefore, in conclusion, it can be stated that results from the present study provide useful information about the thiazolidinedione class of antihyperglycemic agents, troglitazone being a representative member. These findings can be further utilized and extended to a better understanding of the underlying antigluconeogenic mechanism of action of two other recently approved thiazolidinedione compounds, pioglitazone and rosiglitazone, both of which are clinically available at present.

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