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Inhibition of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by troglitazone: a peroxisome proliferator-activated receptor-γ (PPARγ)-independent, antioxidant-related mechanism

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Abstract

Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme of gluconeogenesis. Enhanced expression of the PEPCK gene in liver is present in most models of diabetes, and is thought to contribute to the increased hepatic glucose output seen in this disease. Recently, we showed that troglitazone, the first thiazolidinedione (TZD) used clinically, inhibits expression of the PEPCK gene in isolated hepatocytes. We have pursued the molecular mechanism whereby troglitazone exerts this inhibition. TZDs are known to bind and activate peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear receptor, which regulates expression of target genes. Initially, we examined the abilities of three other TZDs (rosiglitazone, englitazone, and ciglitazone) to inhibit expression of the PEPCK gene. Despite the fact that these agents are ligands for PPARγ, they displayed little if any inhibitory activity on the expression of this gene. GW1929 [*N*-(2-benzoyl phenyl)-l-tyrosine], another potent PPARγ ligand that is unrelated structurally to TZDs, had no inhibitory effect on PEPCK gene expression, while a natural PPARγ ligand, the prostaglandin metabolite 15-PGJ$_2$ (15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$), displayed only modest inhibitory activity. Treatment of hepatocytes with ligands for other isoforms of PPAR also had no significant effect on PEPCK gene expression. Troglitazone has an α-tocopherol (vitamin E) moiety that is not present in other TZDs, and treatment of hepatocytes with vitamin E led to an inhibition of PEPCK gene expression. These observations support the conclusion that troglitazone inhibits the expression of the PEPCK gene by a PPARγ-independent, antioxidant-related mechanism.

Keywords: Thiazolidinediones; Phosphoenolpyruvate carboxykinase; Troglitazone; Antioxidant; Hepatocytes; Gluconeogenesis; Diabetes

1. Introduction

TZDs are a relatively new class of anti-hyperglycemic drugs used in the treatment of type 2 diabetes [reviewed in Ref. 1]. They have no direct effect on insulin secretion, but rather enhance the insulin sensitivity of peripheral tissues which results in an improvement in both lipid and glucose homeostasis. While it is clear that a major action of TZDs is to potentiate the action of insulin, i.e. enhance insulin sensitivity, there is evidence to suggest that they can also have direct insulin-like effects. For example, TZDs have been shown to activate glycogen synthase in myocytes, and inhibit gluconeogenesis in hepatoma cells [2], both in the absence of insulin in the culture medium.

TZDs are thought to exert most of their effects by acting as ligands for PPARγ [reviewed in Ref. 3]. PPARs are members of the nuclear receptor superfamily, and act as transcriptional regulators. This mechanism of action is consistent with the ability of troglitazone to alter the expression of specific genes. The pharmacological evidence that PPARγ is the major target for TZDs is compelling, and includes the observations that: (i) the TZDs tested thus far bind and activate PPARγ at concentrations that parallel their effective antidiabetic dose [4–6], and (ii) the rank order of potency of the various TZDs is similar to their relative affinities for PPARγ [5,7].

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Abbreviations: BADGE, bisphenol A diglycidyl ether; DCF, dichlorofluorescein; DCFH, 5- (and 6-)carboxy-2′,7′-dichlorodihydrofluorescein; PEPCK, phosphoenolpyruvate carboxykinase; 15-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; PPAR, peroxisome proliferator-activated receptor; RPPO, ribosomal phosphoprotein PO; and TZD, thiazolidinedione.

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The anti-hyperglycemic actions of TZDs occur via their ability to stimulate glucose disposal and decrease glucose output simultaneously [8,9]. In liver, one of their most significant effects is to reduce the rate of gluconeogenesis [2,10]. Recently, our laboratory demonstrated that troglitazone, the first TZD to be used clinically, inhibits the expression of genes coding for PEPCK and glucose-6-phosphatase, both of which are rate-limiting enzymes of gluconeogenesis [11]. Inhibition of PEPCK gene expression occurred by a mechanism distinct from that whereby insulin, a physiological inhibitor of PEPCK gene transcription, exerts its effects. There are two PPAR binding sites in the PEPCK promoter [12], thus offering potential sites through which troglitazone could inhibit transcription of this gene. This assumes, however, that troglitazone inhibits this gene via its PPARγ ligand activity. In this study, we took a pharmacological approach to further examine the mechanism whereby troglitazone inhibits the expression of the PEPCK gene.

2. Materials and methods

2.1. Materials

Troglitazone was a gift from the Parke-Davis Pharmaceutical Co. Englitazone was a gift from Pfizer, and GW1929 [*N*-(2-benzoyl phenyl)-L-tyrosine] was a gift from GlaxoWellcome Laboratories. BADGE [2,2-bis (4-glycidyloxyphenyl) propane] was purchased from TCI America. The PPAR ligands 15-PGJ$_2$, LY171,883 [1-(2-hydroxy-3-propyl-4-(4-(1*H*-tetrazol-5-yl) butoxy) phenyl) ethane], WY14,643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid], and ciglitazone were purchased from Biomol Research Laboratories. Collagenase, α-tocopherol, and Williams E medium were obtained from Sigma-Aldrich Canada. DCFH-DA (DCFH-diacetate) was purchased from Molecular Probes.

2.2. Preparation of primary hepatocytes

Male Sprague–Dawley rats (150–250 g) were obtained from Charles River Laboratories. They were allowed access to food and water *ad lib.* and housed under a 12-hr light–dark cycle. Principles of laboratory care were followed, and all protocols were approved by the University of Saskatchewan Committee on Animal Care and Supply. Hepatocytes were isolated by the collagenase method [13], and cell viability, which was typically greater than 90%, was determined by the trypan blue exclusion method. Primary hepatocytes were cultured on collagen-coated 100-mm plates and incubated in Williams E medium (Sigma) containing 10% fetal bovine serum in a humidified atmosphere (5% CO_2) at 37°. Cells were checked for adherence after a 1-hr incubation, and the medium was replaced. After 4 hr, the medium was replaced, again, and the cells were treated for 8 hr in the presence of the indicated compounds.

2.3. Plasmids and DNA probes

The PEPCK cDNA was excised from the plasmid pPCK10 [14] by *Pst*I digestion. The cDNA insert for the RPPO control probe was released from p36B4 [15] by *Pst*I digestion. The ^{32}P-labelled cDNA probes were prepared by the random priming method using the instructions of the manufacturer (Boehringer Mannheim).

2.4. RNA extraction and northern analysis

Total RNA was extracted from cells using the TriZol reagent (Gibco-BRL) according to the instructions of the manufacturer. For northern analysis, 20 μg RNA was resolved by denaturing formaldehyde gel electrophoresis and transferred by capillary elution to nylon membranes (GeneScreen Plus, DuPont-New England Nuclear). Following elution, the membranes were UV-cross-linked at 254 nm (1200 mW/cm^2, Stratalinker 1800, Stratagene) to facilitate re-probing of the membranes. Membranes were prehybridized at 68° for 1 hr in buffer (5× SSPE [0.75 M NaCl, 50 mM NaH_2PO_4 (pH 7.4), 5 mM EDTA], 0.5% SDS, 5× Denhardt's solution, 10% dextran sulfate) containing 100 μg/mL of denatured salmon sperm DNA. The membranes were incubated with the DNA probes (1×10^7 cpm) for 16–20 hr at 68° in a rotary hybridization oven (TurboSpeed, Bio/Can Scientific). Following hybridization, the membranes were washed with 2× SSPE, 0.1% SDS at 68° for 30 min with two changes of buffer, followed by two washes with 1× SSPE, 0.1% SDS for 30 min. The membranes were exposed to x-ray film (Kodak XAR), and autoradiograms were scanned using a UMAX Astra 1220S scanner and matching software program (UMAX VistaScan 3.5.1). Signals were quantified with NIH imaging software. The northern blots shown are representative of three independent experiments.

2.5. DCFH measurement of oxidative stress

The DCFH ester readily crosses the cell membrane and is converted to the fluorescent DCF after it becomes oxidized by hydroxyl radicals [16]. It has been shown to be a reliable indicator of oxidative stress [17]. Primary hepatocyte cultures were pretreated with 0.1 μM DCFH-DA for 30 min, followed by the addition of α-tocopherol or TZDs to the culture medium and incubation for an additional 8 hr. The cultures were washed with ice-chilled buffer containing 50 mM Tris (pH 7.5) and 1% SDS, and cellular extracts were prepared as previously described [17] for the measurement of DCF. DCF was quantified using an Hitachi F-2000 fluorescence spectrophotometer, with excitation set at 490 nm and emission set at 515 nm. The amount of DCFH oxidized

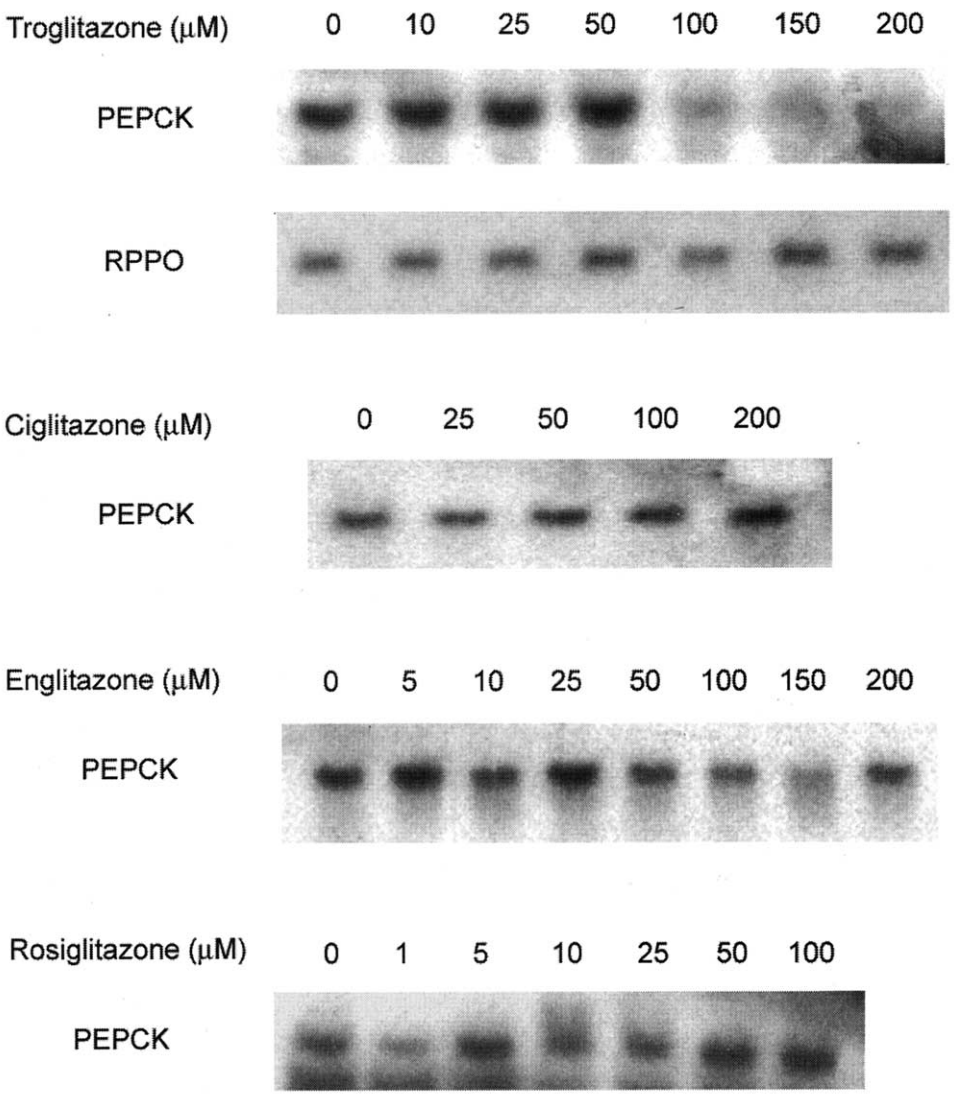

Fig. 1. Effects of troglitazone and other TZDs on the levels of PEPCK mRNA in hepatocytes. Isolated hepatocytes were treated with increasing concentrations of the TZDs shown for 8 hr. Then total RNA was isolated, and levels of PEPCK mRNA were determined by northern blot analysis. The membranes were subsequently stripped of the PEPCK probe and re-probed for RPPO mRNA levels. Since the levels of RPPO were unaffected by any of the compounds tested, only the RPPO blot for the troglitazone experiment is shown. The data shown are representative of three independent experiments performed.

(pmol DCF/mg protein) in control cells was assigned an arbitrary oxidative stress value of 1.0.

3. Results

We reported recently that troglitazone inhibits expression of the PEPCK gene in isolated hepatocytes [11]. This inhibition occurred at relatively high concentrations (~100 μM), which is well above the K_d of troglitazone for its presumed cellular target, PPARγ [7]. Based on this and a recent report on the ability of troglitazone to inhibit cholesterol synthesis through a PPARγ-independent mechanism [18], we hypothesized that there may be other cellular targets for troglitazone. To further define the mechanism whereby troglitazone inhibits expression of the PEPCK gene, we first examined the ability of other TZDs to inhibit PEPCK gene expression. The TZDs tested were rosiglitazone, which has a greater affinity for PPARγ relative to troglitazone [7], along with englitazone and ciglitazone, which are weaker ligands relative to troglitazone [6,19]. As shown in Fig. 1, treatment of isolated hepatocytes with troglitazone at concentrations of 100 μM or higher resulted in a reduction in the steady-state levels of PEPCK mRNA. Densitometric analysis of blots from three independent experiments indicated that the average percent inhibitory effect by the highest troglitazone concentrations tested was 90%. Ciglitazone had no effect on PEPCK mRNA levels, while englitazone treatment had only a modest inhibitory effect at higher concentrations. Rosiglitazone also had no significant, concentration-dependent inhibitory effect on PEPCK mRNA levels. Thus, various TZDs appear to have

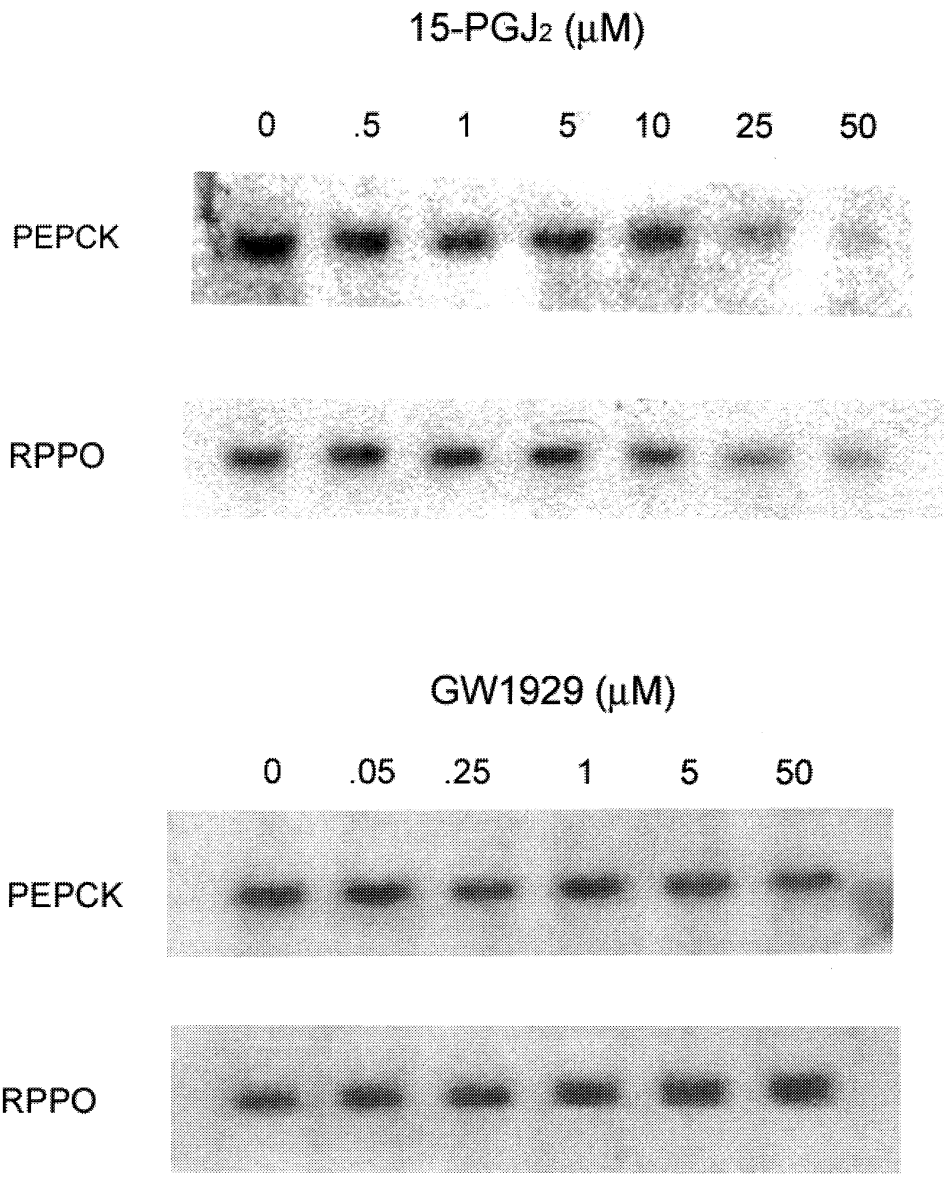

Fig. 2. Effect of non-TZD PPARγ ligands on the levels of PEPCK mRNA in hepatocytes. Isolated hepatocytes were treated with increasing concentrations of either 15-PGJ_2 or GW1929 for 8 hr. Then total RNA was isolated, and the levels of PEPCK mRNA and RPPO mRNA were determined by northern blot analysis. The data shown are representative of three independent experiments performed.

differing effects on PEPCK gene expression, which appear to be unrelated to their binding affinity for PPARγ.

We next tested PPARγ ligands that are structurally unrelated to TZDs for their ability to inhibit PEPCK gene expression. The prostaglandin metabolite 15-PGJ_2 has been shown to be a naturally occurring PPARγ ligand, with a binding affinity that is greater than that of troglitazone [7]. Incubation of hepatocytes with this prostaglandin reduced PEPCK mRNA levels at concentrations of 25 and 50 μM, although this activity appeared to be somewhat non-specific in nature since mRNA levels of the constitutively expressed RPPO gene were also decreased at higher concentrations of 15-PGJ_2. We also tested an *N*-aryl tyrosine agonist of PPARγ, GW1929, whose potency is approximately equivalent to that of rosiglitazone and two orders of magnitude greater than that of troglitazone [20]. Despite this agonist activity, however, this compound had no effect on PEPCK mRNA levels in hepatocytes (Fig. 2).

Another approach to examine the role of PPARγ in mediating the inhibitory effects of troglitazone on PEPCK gene expression is to use an antagonist for this nuclear receptor. Recently, the synthetic compound BADGE was characterized as a specific antagonist, with a K_d of 100 μM, due to its ability to antagonize the effects of PPARγ ligands [21]. We hypothesized that since troglitazone, a PPARγ agonist, inhibited PEPCK gene expression, the use of BADGE might lead to an induction in gene expression if a PPARγ-dependent mechanism was involved. However, in-

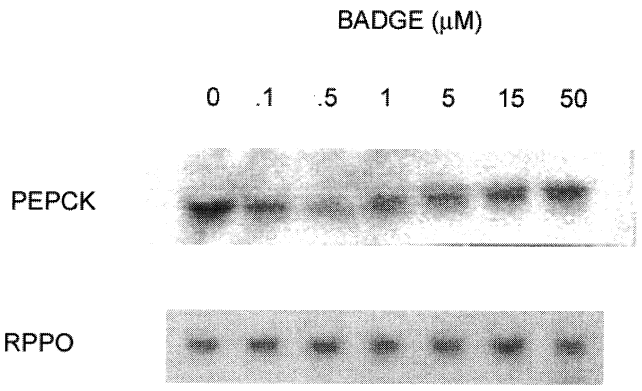

Fig. 3. Effect of BADGE, a PPARγ antagonist, on the levels of PEPCK mRNA in hepatocytes. Isolated hepatocytes were treated with increasing concentrations of BADGE for 8 hr. Then total RNA was isolated, and the levels of PEPCK mRNA and RPPO mRNA were determined by northern blot analysis. In this case, the RPPO mRNA levels were assessed on a separate blot. The data shown are representative of three independent experiments performed.

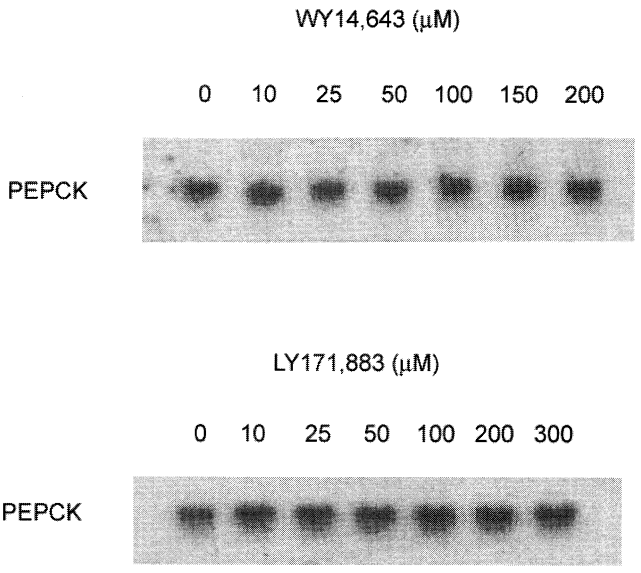

Fig. 4. Effects of ligands for other PPAR isoforms on the levels of PEPCK mRNA in hepatocytes. Isolated hepatocytes were treated with increasing concentrations of either WY14,643 or LY171,883 for 8 hr. Then total RNA was isolated, and the level of PEPCK mRNA was determined by northern blot analysis. Corresponding blots of RPPO mRNA are not shown since their levels were not affected by either treatment. The data shown are representative of three independent experiments performed.

cubation of hepatocytes with BADGE led to a small decrease in PEPCK gene expression at low concentrations, while higher concentrations had little effect (Fig. 3).

Several PPAR isoforms exist, and while relatively specific ligands have been discovered for each isoform, most ligands identified to date have some degree of cross-reactivity [22]. Thus, the possibility exists that troglitazone exerts its effects on PEPCK gene expression through an isoform other than PPARγ. To test this hypothesis, we examined the activity of other PPAR isoform ligands. WY14,643 is a ligand that has been shown to be specific for PPARγ [5,23,24], the most abundant isoform in liver [25]. WY14,643 had no effect on PEPCK gene expression (Fig. 4). The other ligand tested was LY171,883, a potent ligand for all three PPAR isoforms [26]. Incubation of rat hepatocytes with this compound had no effect on PEPCK gene expression (Fig. 4).

Out of the various PPAR ligands tested, the specific ability of troglitazone to inhibit PEPCK gene expression suggests that this compound has some unique chemical property that distinguishes it from the other ligands. One such feature, which is not preserved in any of the other TZDs nor present in any of the other ligands tested above, is a partial α-tocopherol (vitamin E) moiety (Fig. 5). We proceeded to test the ability of this compound to alter PEPCK gene expression. Treatment of rat hepatocytes with α-tocopherol caused a decrease in PEPCK mRNA levels (Fig. 6). Densitometric analysis of blots from three independent experiments indicated that the average percent inhibitory effect by the highest α-tocopherol concentrations tested was 65%.

It has been suggested that troglitazone, due to its α-tocopherol moiety, can act as an antioxidant [27]. Since troglitazone and α-tocopherol both displayed the ability to inhibit PEPCK gene expression, we went on to examine the effects of α-tocopherol, troglitazone, and rosiglitazone on the level of oxidative stress in rat hepatocytes. For this, we employed the DCFH assay, which has been shown to be a good indicator of oxidative stress [17]. Initially, concentration–response curves were obtained for all of the compounds (data not shown), and the concentrations that gave optimal responses were used to generate the data shown in Fig. 7. The data in Fig. 7 show that whereas troglitazone was a potent antioxidant in primary hepatocytes, comparing favorably with that of vitamin E, rosiglitazone had no significant antioxidant activity. These data, combined with the observation that α-tocopherol and troglitazone both inhibited the expression of the PEPCK gene whereas rosiglitazone did not, support the conclusion that a decrease in cellular oxidative stress in the hepatocyte leads to the inhibition of PEPCK gene expression.

4. Discussion

TZDs are the most recently introduced compounds to be used to treat type 2 diabetes. This drug family has gained a significant amount of attention, since they are the only compounds to date that target the primary defect, insulin resistance. One of their antidiabetic activities that our laboratory has been interested in is their ability to ameliorate hyperglycemia, which appears to be achieved by an increase in glucose utilization [28,29] and a decrease in hepatic gluconeogenesis [2,10,30]. Flux through the latter metabolic pathway is elevated in type 2 diabetes [31]. TZDs appear to inhibit gluconeogenesis through a direct mecha-

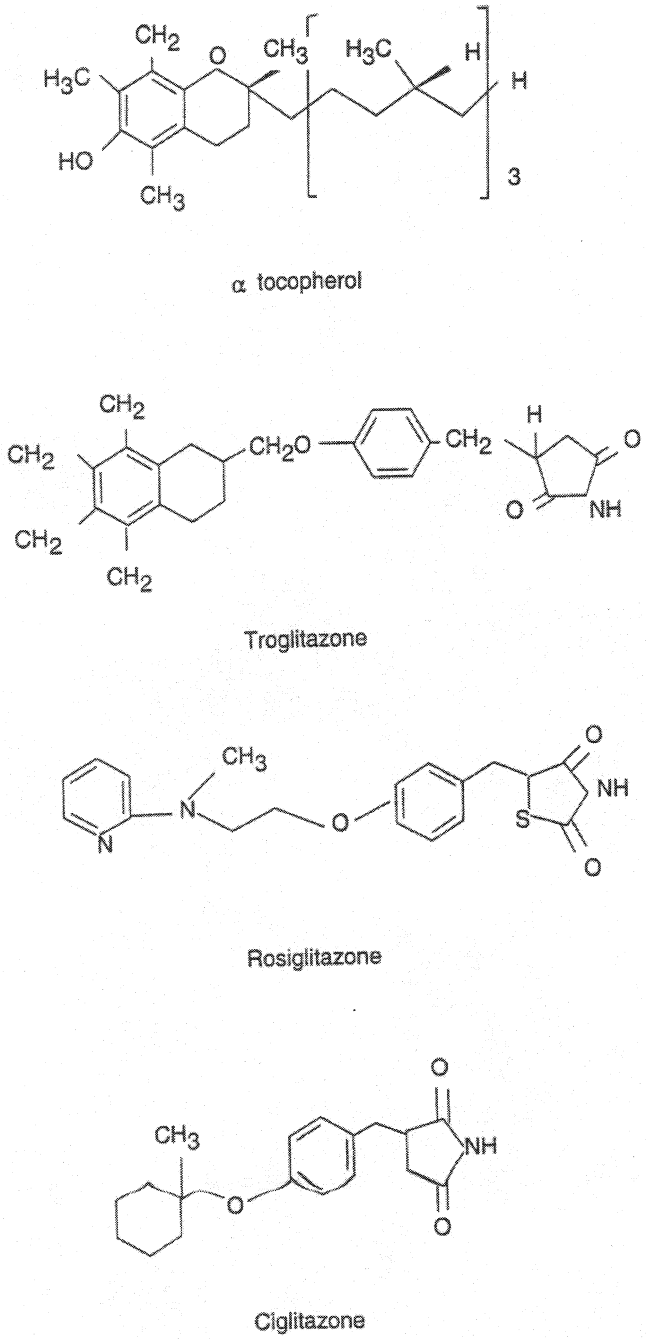

Fig. 5. Chemical structures of the TZDs used in this study and their comparison with α-tocopherol (vitamin E).

nism, i.e. insulin-independent, since the inhibition can be observed in isolated hepatocytes in the absence of insulin in the culture medium [2,30,32].

Precisely how TZDs inhibit this metabolic pathway is unclear but there are likely multiple targets within the cell. Moreover, the molecular targets may differ depending on which specific TZD is employed. For example, troglitazone decreases the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase in the livers of genetically diabetic *db/db* mice treated with this compound [33]. We have

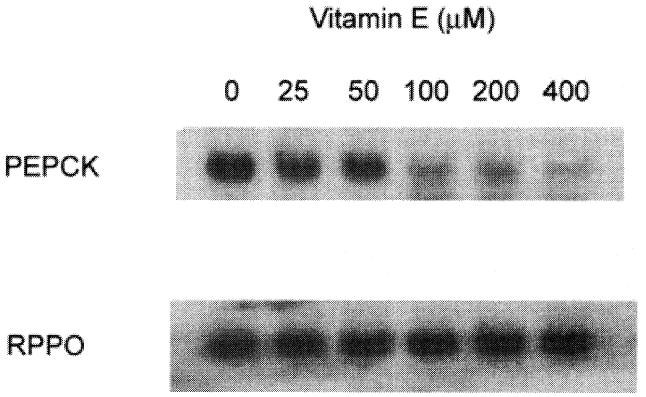

Fig. 6. Effect of vitamin E on the level of PEPCK mRNA in hepatocytes. Isolated hepatocytes were treated with increasing concentrations of vitamin E for 8 hr. Then total RNA was isolated, and the levels of PEPCK mRNA and RPPO mRNA were determined by northern blot analysis. The data shown are representative of three independent experiments performed.

shown recently that troglitazone also inhibits expression of the genes coding for glucose-6-phosphatase and PEPCK in isolated hepatocytes [11]. However, other TZDs, as shown in the present study, have essentially little if any ability to inhibit PEPCK gene expression. We speculate that TZDs that lack the ability to inhibit PEPCK gene expression perhaps target genes coding for other rate-limiting enzymes of this pathway, and/or directly inhibit activity of the enzymes themselves. It is also interesting to note that TZDs induce, rather than inhibit, PEPCK gene expression in adipose tissue [34], apparently through a PPARγ-dependent mechanism [12]. Thus, our data indicate that TZDs can display tissue-specific effects on individual genes.

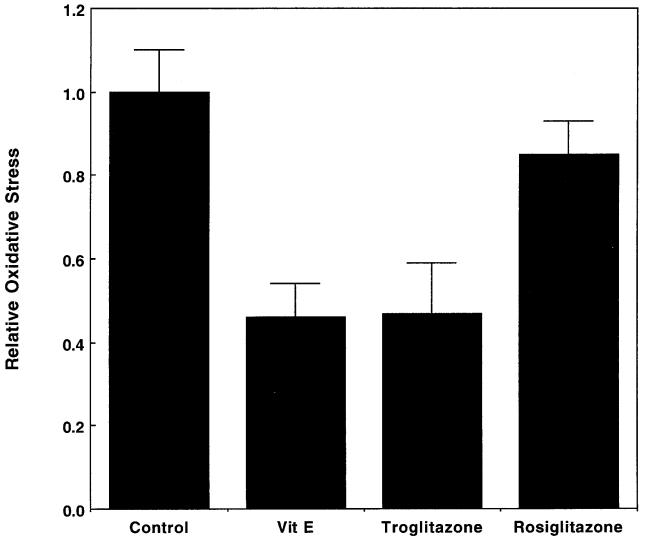

Fig. 7. Effects of different TZDs on the level of oxidative stress in isolated hepatocytes. Hepatocytes were loaded with the DCFH probe for 30 min, and then treated with vitamin E, troglitazone, or rosiglitazone for 8 hr. The level of oxidative stress was determined as described in "Materials and methods." The value for control cells was assigned an arbitrary value of 1.0. Values are the means ± SEM of three independent experiments.

The mechanism or signaling pathway used by troglitazone whereby it inhibits PEPCK gene expression is unclear, but our analysis to date has ruled out certain possibilities and implicated others. Based upon our observation that troglitazone inhibits the expression of this gene [11], we hypothesized that it might do so via a pathway utilized by insulin, which is the physiological inhibitor of PEPCK gene transcription. However, our analysis, which included the use of specific inhibitors of insulin-signaling pathways, suggested that the mechanism for the troglitazone effect did not parallel that used by insulin [11]. Moreover, while insulin is able to overcome the cAMP-stimulated expression of this gene, troglitazone is unable to do so [11], providing further evidence that it exerts its effects through a mechanism distinct from that of insulin, at least in hepatocytes. It is possible that *in vivo* troglitazone inhibits PEPCK gene expression by a second, indirect mechanism that involves its ability to enhance insulin sensitivity [1,3].

In the present paper, we provide pharmacological evidence that the mechanism is also unrelated to its activity as a PPARγ agonist. This evidence includes the observation that other TZDs, including rosiglitazone, which is a higher affinity agonist of PPARγ relative to troglitazone, have little, if any, effect on PEPCK gene expression. Additionally, we showed that a non-TZD agonist of PPARγ, GW1929, also had no effect on its expression. LY171,883, an agonist of all three PPAR isoforms, also showed a similar lack of activity. This apparent PPARγ-independent activity of troglitazone was, in fact, suggested by our previous study examining the concentration-dependent effect of troglitazone on the expression of gluconeogenic genes. In that study, we observed inhibition of expression at relatively high concentrations ($\sim$100 μM) of this compound, which is significantly higher than its K_d for PPARγ [7]. Interestingly, the biochemical and metabolic effects of troglitazone that have been observed generally fall into two categories. There are those that are observed at lower concentrations of troglitazone, and thus consistent with its PPARγ binding affinity [e.g. Ref. 29]. Conversely, others occur at relatively high concentrations [e.g. Ref. 30], suggestive of a PPARγ-independent effect, although in most studies this was never fully investigated. One study, however, that examined the mechanism whereby troglitazone inhibits cholesterol biosynthesis also used a pharmacologic approach to provide evidence that this effect occurred through a PPARγ-independent mechanism [18].

From a chemical perspective, troglitazone differs from other TZDs in that it contains a vitamin E moiety (Fig. 5). Vitamin E is a well-documented antioxidant, and troglitazone has been reported to have a scavenging effect on reactive oxygen species [27], which we confirmed in hepatocytes (Fig. 7). Since troglitazone, but not rosiglitazone, inhibited expression of the PEPCK gene, we hypothesized that the antioxidant activity of troglitazone formed the basis for its inhibitory activity. This could explain the lack of inhibitory activity of other TZDs, such as rosiglitazone, which possesses significantly less antioxidant activity than troglitazone (Fig. 7). In further support of this hypothesis, we showed that incubation of hepatocytes with vitamin E resulted in the reduction of PEPCK mRNA levels (Fig. 6).

Precisely how antioxidants lead to the inhibition of PEPCK gene expression is not known, although it is widely appreciated that the redox state of the cell does regulate the expression of certain genes [reviewed in Ref. 36]. There are two transcription factors, Activator Protein-1 (AP-1) and NF-κB, known to mediate redox regulation [34]. Generally, antioxidants reduce the activity of these transcription factors, while an increase in oxidative stress leads to their activation [35]. Interestingly, the PEPCK gene promoter has a binding site for AP-1, and AP-1 transactivates this promoter [36]. Therefore, the fact that antioxidants inhibit PEPCK gene expression and also inhibit the activity of AP-1 suggests a possible mechanism whereby the antioxidant effect is mediated. Studies are currently underway to determine the *cis*-element in the promoter that mediates the antioxidant effect.

Regardless of the mechanism whereby antioxidants exert their effects on the PEPCK gene, the fact that they inhibit its expression is entirely consistent with what is known about the relationship that exists between type 2 diabetes and oxidative stress. Oxidative stress is increased *in vivo* in the diabetic state, and there is increased production of oxygen-free radicals [37–39]. Roles for oxidative stress in β-cell dysfunction [40] and atherosclerosis [41] have also been proposed. More importantly, there is evidence that oxidative stress can cause or lead to peripheral insulin resistance, a hallmark of type 2 diabetes. Rudich *et al.* [42] showed that exposing 3T3-L1 adipocytes to H_2O_2 significantly reduced insulin-stimulated glucose transport, lipogenesis, and glycogen synthase activation. Expression of GLUT-4 (mRNA and protein) was also reduced as oxidative stress increased. A second study by this group showed that oxidative stress altered the redistribution of Insulin Receptor Substrate-1 (IRS-1) and phosphatidylinositol-3-kinase (PI3K) in the cell that occurs upon insulin stimulation; this resulted in a 90% reduction in insulin-stimulated protein kinase B phosphorylation and activation [43]. Hansen *et al.* [44] also showed that incubation of cells with H_2O_2 dramatically inhibited glucose transport and insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1, as well as inhibited activation of downstream signals such as PI3K and mitogen-activated protein kinase (MAPK). These studies, when coupled with the observation that hyperglycemia increases the level of H_2O_2 in cells [45], offer support for the hypothesis that oxidative stress is involved in the development of insulin resistance. Moreover, a number of studies have shown that antioxidant treatment ameliorates or reverses a number of altered physiological and metabolic parameters that occur as a result of type 2 diabetes. For example, vitamin E therapy prevents glucose-induced changes in protein kinase C and nitric oxide, and ameliorates poor glycemic control in GK rats (a model of type 2 diabetes) [46].

Laight *et al.* [47] demonstrated that the insulin resistance in BB/ZDF rats, another model of type 2 diabetes, can be ameliorated by vitamin E therapy.

Our data provide evidence that the antioxidant-related properties of troglitazone may be an important aspect of its overall antidiabetic activity. However, the newest members of the TZD family that have replaced troglitazone for use clinically have been modified in a manner that has reduced or eliminated their antioxidant properties. We propose that future rational design of TZD analogues should take into account the beneficial effects that antioxidants have on ameliorating a wide variety of physiological and metabolic perturbations associated with insulin resistance and diabetes.

Acknowledgments

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