Pyrrolidine dithiocarbamate enhances hepatic glycogen synthesis and reduces FoxO1-mediated gene transcription in type 2 diabetic rats

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Diabetes mellitus is a congenital or acquired metabolic disease characterized by defects in pancreatic insulin production and/or in insulin action on peripheral tissues (2). Hyperglycemia is the main consequence of this disease. Persistent high level of blood glucose is considered to be a key factor in the development of several chronic complications, such as renal failure, microvasculopathy, and peripheral neuropathy, that contribute to the high morbidity and mortality observed in diabetes (15). A better control of blood glucose reduces diabetic complications sharply (5). Under normal conditions, the secretion of insulin is increased after ingestion of a meal, which enables glucose entry into target tissues via facilitative glucose transporters. The conversion of glucose to glycogen in skeletal muscle and liver prevents the occurrence of postprandial hyperglycemia (28). Although muscle is often considered the major site of insulin-stimulated glucose disposal (35), the liver also contributes significantly to glucose removal (39). When glucose is given orally, the liver may dispose of as much as one-third of the glucose load (6, 17). During the starvation or fasting state the level of insulin decreases, whereas those of glucagon and glucocorticoids rise, thereby leading to reduced glucose uptake by peripheral tissues and increased hepatic gluconeogenesis and glucose output to prevent hypoglycemia (24). Because the liver plays such a key role in the maintenance of glucose homeostasis (29), it is important to identify pharmacological agents that could regulate hepatic glucose metabolism.

Pyrrolidine dithiocarbamate (PDTC) is a clinically tolerated synthetic low-molecular-weight thiol compound with a variety of biochemical and cellular activities, including heavy metal chelation and inhibition of redox-sensitive enzyme activities (7). PDTC is widely used as an inhibitor of the transcription factor nuclear factor-κB (NF-κB) in cellular and animal models of inflammation (30, 32). Various studies have demonstrated that PDTC can counteract the toxic effects of free radicals and interfere with the generation of proinflammatory cytokines (9, 21). There is an accumulating body of evidence showing that PDTC has beneficial effects on several vascular diseases (19, 25) and that it lowers blood glucose levels in diabetic rats significantly (38) through mechanisms that have not been fully elucidated. In this study, we investigated whether PDTC exhibits antidiabetic properties in relation to hepatic glycogen synthesis and forkhead box O1 (FoxO1)-dependent transcription of genes encoding key gluconeogenic enzymes in a rat model of type 2 diabetes mellitus (T2DM). This model developed originally by Reed et al. (27) combines a low dose of streptozotocin (STZ) in rats fed a high-fat diet (HFD). A T2DM pattern emerges whereby the feeding of HFD produces insulin resistance and subsequent treatment with a low dose of STZ causes an initial β-cell dysfunction, followed by hyperglycemia and high blood triglyceride levels in Sprague-Dawley rats (27). Our results showed that the HFD-fed/STZ-treated rats were sensitive to the glucose-lowering effects of PDTC and that dysregulated expression of key hepatic gluconeogenic enzyme genes was normalized by the treatment with PDTC.

Materials and Methods

Animals, induction of experimental diabetes, and treatment schedule. Male Wistar rats (180–210 g) aged 8 wk were maintained in a controlled environment (20–25°C, 45–55% relative humidity) on a 12:12-h light-dark cycle. After acclimatization to their environment, the rats were randomly divided into the normal control (NC) group...
(n = 11) fed ad libitum with normal chow for 8 wk and the HFD group (n = 22) fed ad libitum with a diet containing 42% fat (wt/wt) for 8 wk. Animals were obtained from the Animal Center of Hebei Medical University. The normal chow (10.3, 65.5, and 24.2% of calories derived from fat, carbohydrate, and protein, respectively) and HFD (42% fat, 40% carbohydrate, and 18% protein, in terms of calories derived) were purchased and/or obtained from the Animal Center of Hebei Medical University. Both the NC and HFD rats (n = 8/group) were randomly subjected to an oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) to confirm that high-fat feeding induced insulin resistance. Subsequently, HFD rats received an intraperitoneal (ip) injection of a single dose of STZ (27 mg/kg body wt; Sigma-Aldrich, St. Louis, MO) to induce type 2 diabetes (27). The rats in the NC group were treated with the same volume of citric acid buffer only. Blood glucose levels were measured in non-fasted HFD animals 3 days after the beginning of STZ treatment and were found to be ≥16.7 mmol/l. Once type 2 diabetes was established, the diabetic rats were divided into the diabetes group (DM; n = 11) and the DM + PDTC group (n = 11), with the latter group receiving an ip administration of PDTC (50 mg/kg body wt · day−1; Sigma-Aldrich) that was mixed with physiological saline, whereas rats in the NC and DM groups were treated with saline only. One week later, the rats were fasted overnight and subjected to OGTT and ITT (n = 5/group). The other rats were euthanized, and their livers were removed and either snap-frozen in liquid nitrogen or fixed with 4% paraformaldehyde (PFA) for further analysis.

**Determination of liver glycogen.** Liver glycogen content was assayed by the anthrone reagent method (34) according to the manufacturer’s instructions (Jiancheng Biotech, Nanjing, China). Briefly, 1 g of frozen liver tissue was minced and dropped in 3 ml of 30% KOH, boiled for 20 min, and diluted with ice-cold water. Aliquots of the KOH

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**Fig. 1.** The oral glucose tolerance test (OGTT; A–D) and insulin tolerance test (ITT; E and F) in 8-wk-old male rats after being fed normal diet (NC) or maintained on high-fat diet (HFD) for 8 wk. For experimental details, see MATERIALS AND METHODS. Blood glucose (A) and plasma insulin (C) levels were measured after OGTT; blood glucose levels were measured after ITT (E). Each point represents the mean ± SE (n = 8/group). Area under the curve (AUC) data for OGTT glucose (B), OGTT insulin (D), and ITT glucose (F) were calculated. Repeated-measures ANOVA (A, C, and E) or t-test (B, D, and F) was performed. *P < 0.05; **P < 0.01 vs. NC.
digest were mixed slowly with the anthrone reagent (1:2, vol/vol) and boiled for 10 min for the development of color. The optical density was read spectrophotometrically within 2 h at 620 nm. Contents of glycogen in tissue samples were determined from glycogen standards (Sigma-Aldrich) run with each assay and normalized to tissue weight of each individual sample.

**Western blot analysis.** Prechilled pestle and mortar were used to grind frozen liver tissue into a fine powder, which was then lysed in RIPA buffer (25 mM HEPES, 134 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM vanadate, 0.5% sodium deoxycholate, and 100 mM NaF supplemented with protease inhibitors). The crude lysates were centrifuged at 10,000 g for 20 min at 4°C, and the protein concentration in the clarified extracts was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Equivalent amounts of protein were combined with SDS sample buffer and boiled at 100°C for 5 min. The samples (100 µg) were then subjected to SDS-PAGE on 10% gels and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were blocked with 3% BSA in TBS-T and incubated with rabbit anti-FoxO1 (1:100), anti-Akt, and anti-phospho-Akt (Ser273) (1:1,000; Cell Signaling Technology, Beverly, MA); anti-phospho-FoxO1 (Ser256), anti-GSK-3β, and anti-phospho-GSK-3β (Ser9) (1:1,000; Santa Cruz Biotechnology). Anti-β-actin (1:1,000; Santa Cruz Biotechnology) was used as an internal control.

**Tissue preparation, histology, and immunohistochemistry.** Liver tissues were fixed in 4% PFA, embedded in paraffin, and sectioned. Sections were used for periodic acid shift (PAS) and immunohistochemical staining. For immunohistochemistry, the sections were analyzed by the use of PowerVision Two-Step Histostaining Reagent (Zhongshan, Beijing, China). Briefly, sections (5 µm) were deparaffinized with turpentine and rehydrated through a graded ethanol series. Endogenous peroxidases were blocked by incubation in 3% H2O2 for 15 min. Sections were heated at 98°C for 15 min in 10 mM sodium citrate buffer (pH 6.0) to retrieve antigen and then incubated with anti-phospho-GSK-3β (Ser9; 1:100) overnight at 4°C. The immunocomplex was detected with a peroxidase-conjugated secondary antibody and visualized by a diaminobenzidine solution. The sections were then counterstained with hematoxylin and mounted with DPX mounting medium.

**Real-time PCR analysis of phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and β-actin mRNA.** Total RNA was extracted from liver tissues with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Four micrograms of RNA was subjected to reverse transcription using the Rotor-Gene 3000 (Corbett Life Science; Qiagen, Brisbane, Australia) according to the manufacturer’s protocol. Four micrograms of RNA were run in duplicate. Each run included a buffer blank and no template control. Gene expression levels were calculated by the cycle threshold (Ct) 2-ΔΔCt method and normalized to β-actin mRNA expression.

**Immunofluorescence microscopy.** PFA-fixed liver tissues were cryoprotected in 30% sucrose for 48 h, embedded in TissueTek freezing medium, frozen on dry ice, and then cut into 5-µm-thick sections. Sections were blocked with 10% goat serum in PBS-T and incubated with rabbit anti-FoxO1 (1:100) overnight at 4°C. Immunoreactive bands were visualized with FITC-conjugated anti-rabbit secondary antibody (1:500; Santa Cruz Biotechnology) for 1 h at room temperature. Nuclei were counterstained with 4',6-diamino-2-phenylindole (Invitrogen). The stained sections were observed under an inverted confocal laser fluorescence microscope (LSM-510; Carl Zeiss, Jena, Germany), and the images were processed with the Zeiss LSM Image Browser. As a negative control, the primary antibody was omitted.

**Statistical analysis.** The statistical analysis was performed with SPSS 13.0 statistical software (SPSS, Chicago, IL). All results were expressed as means ± SE. Comparisons between different groups were performed by employing either a one-way ANOVA followed by the Fisher’s least significant difference (LSD) post hoc test or Student’s t-test.
repeated-measures ANOVA for the OGTT and ITT data. The AUC comparisons between two groups were evaluated by Student’s t-test. A P value /H11021 0.05 was considered statistically significant.

RESULTS

HFD-fed rats exhibit reduced insulin sensitivity. Rats that were maintained on a HFD for 8 wk weighed significantly more than the control group fed a normal diet (NC) (361.91 ± 5.88 vs. 313.18 ± 3.87 g, respectively,  P /H11021 0.01). Although fasting blood glucose levels were not altered by HFD, there were significant alterations in the rates of blood glucose clearance (Fig. 1A) and plasma insulin levels (Fig. 1C) during OGTT in HFD rats. In the NC rats, the levels of blood glucose and plasma insulin increased rapidly after glucose ingestion, peaking at 30 min, followed by a gradual decline before returning to nearly preprandial concentrations at 2 h. In contrast, the insulin concentrations were higher basally in the HFD group, and the change from basal in the two groups looked remarkably similar. The glucose concentrations were also similar until the last two time points. These results indicate that the failure of insulin to rise more in the HFD group allowed the development of hyperglycemia late in the OGTT. The mean values of the AUCglucose (Fig. 1B) and AUCinsulin (Fig. 1D) in the HFD group were significantly higher than in the NC group (  P /H11021 0.05 and  P /H11021 0.01, respectively). ITT was then performed to verify whether HFD was able to induce insulin resistance in this experimental model. In Fig. 1E, HFD-fed rats showed higher glycemia after insulin administration compared with control rats, and the ITT AUC was significantly larger in the HFD group (  P /H11021 0.01; Fig. 1F).

Table 1. Plasma lipid profiles in NC, DM, and DM + PDTC groups

<table>
<thead>
<tr>
<th>Group</th>
<th>TC, mmol/l</th>
<th>TG, mmol/l</th>
<th>LDL-C, mmol/l</th>
<th>FFA, mEq/l</th>
</tr>
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<tbody>
<tr>
<td>NC</td>
<td>2.18 ± 0.11</td>
<td>0.66 ± 0.07</td>
<td>1.00 ± 0.06</td>
<td>1.27 ± 0.13</td>
</tr>
<tr>
<td>DM</td>
<td>8.79 ± 1.83*</td>
<td>1.12 ± 0.32*</td>
<td>5.65 ± 1.34*</td>
<td>1.82 ± 0.14*</td>
</tr>
<tr>
<td>DM + PDTC</td>
<td>7.93 ± 1.23*</td>
<td>0.70 ± 0.10#</td>
<td>4.93 ± 0.46*</td>
<td>1.65 ± 0.19*</td>
</tr>
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</table>

Values are means ± SE;  n = 8. NC, normal controls; DM, rats made type 2 diabetic by a single intraperitoneal injection of streptozotocin; DM + PDTC, DM with intervention of pyrrolidine dithiocarbamate; TC, total cholesterol; TG, triglycerides; LDL-C, LDL cholesterol; FFA, free fatty acids. After treatment with saline or PDTC for 1 wk, rats were fasted overnight and then euthanized. *  P < 0.05 vs. NC; #  P < 0.05 vs. DM by 1-way ANOVA, followed by the Fisher’s least significant difference post hoc test.
PDTC restored fasting blood glucose and hepatic glycogen deposition in the HFD-fed, STZ-treated rats. To explore possible beneficial effects of PDTC in type 2 diabetes, insulin-resistant, HFD-fed rats received a single dose of STZ to induce T2DM, as described in MATERIALS AND METHODS. HFD-fed, STZ-treated rats received daily ip injection of either saline (DM) or PDTC (DM + PDTC) for 1 wk. The fasting blood glucose levels at the end of the treatment were found to be significantly higher in the DM group than those in the NC group \( (P < 0.01; \text{Fig. 2A}) \). In addition, the plasma levels of triglycerides, total cholesterol, LDL cholesterol, and FFA were elevated significantly in DM rats compared with the NC group (Table 1), which confirmed previous observations (27, 37). In contrast, after a 1-wk PDTC treatment, fasting blood glucose levels showed a significant reduction compared with the DM group \( (P < 0.01; \text{Fig. 2A}) \), indicating that the HFD-fed, STZ-treated rats were sensitive to the glucose-lowering effects of PDTC. Although there was significant reduction in triglyceride levels after PDTC injection in DM rats (to a level that was comparable with the NC group), PDTC failed to signifi-
cantly alter the levels of total cholesterol, LDL cholesterol, and FFA (Table 1).

OGTT was then performed, and the results showed that glucose and insulin levels rose rapidly and then returned to basal levels by 2 h in PDTC-treated DM rats, whereas in DM rats basal glucose and insulin levels were already significantly higher than their DM counterparts and remained elevated for the duration of the test (Fig. 3, A and C). OGTT AUC glucose (Fig. 3B) and AUC insulin (Fig. 3D) were found to be not significant between the two experimental groups. An ITT test was performed to assess insulin-mediated glucose utilization, and the results established that PDTC significantly improved glucose clearance by insulin in DM rats (Fig. 3E) along with a reduction in ITT AUC (P < 0.01; Fig. 3F). Taken together, these results demonstrated that fat-fed, STZ-treated rats (DM group) were not insulin deficient and were hyperglycemic in the fasting state. Moreover, it would appear that PDTC improved insulin sensitivity in this HFD/STZ rat model.

To assess glycogen deposition in liver tissue, quantitative glycogen analysis was carried out by applying the anthrone technique (Fig. 2B) and by performing PAS staining (Fig. 4A). Glycogen content per gram tissue protein in isolated liver declined significantly for the DM group when compared with NC controls (P < 0.01; Fig. 2B). By contrast, a 1-wk PDTC treatment resulted in a significant recovery of glycogen content compared with DM rats (P < 0.01). Furthermore, DM animals exhibited lower PAS-positive staining compared with NC controls, whereas PDTC treatment restored PAS staining (Fig. 4A).

PDTC increased phosphorylation of Akt and GSK-3β in the liver of diabetic rats. Because of the essential function of the Akt/GSK-3β signaling in the regulation of glycogen synthesis (11), Western blotting technique was used to analyze the phosphorylation status of Akt and GSK-3β in liver extracts from fasted animals. Phosphorylation levels for Akt (Ser473; Fig. 5A) and GSK-3β (Ser3; Fig. 5B) were significantly lower in the DM group than in the NC group (P < 0.01). By contrast, administration of PDTC to DM rats normalized the phosphorylation of Akt and GSK-3β (P < 0.01). Furthermore, a similar trend in GSK-3β phosphorylation was observed by immunohistochemistry (Fig. 4B). Therefore, phosphorylation-mediated activation of the Akt/GSK-3β axis may represent a possible mechanism underlying the increased glycogen deposition observed in DM + PDTC vs. DM rats.

Inhibition of FoxO1 activity by PDTC correlated with reduction in PEPCK and glucose-6-phosphatase (G-6-pase) mRNA levels in diabetic rats. The reduction in fasting blood glucose after PDTC treatment of DM rats suggested suppression of gluconeogenesis. FoxO1 is a key transcriptional regulator in the control of hepatic gluconeogenesis whose activity is inhibited by phosphorylation on Ser256 (12). To explore whether the lowering of fasting blood glucose by PDTC would correlate with a reduction in FoxO1 activity, the effect of PDTC on FoxO1 phosphorylation and expression was assessed by Western blot analysis. It was found that FoxO1 phosphorylation (Ser256) was reduced significantly in the liver of DM rats compared with the NC group (P < 0.01; Fig. 5C). By contrast, administration of PDTC to DM rats increased significantly FoxO1 phosphorylation levels, approaching those of the NC group (P < 0.01). Enrichment of FoxO1 in the nuclei of DM hepatocytes was observed; conversely, PDTC treatment of DM rats resulted in increased nuclear export of FoxO1 (Fig. 4C).

Owing to the fact that PDTC administration was associated with upregulation of phosphorylated (inactive) FoxO1 in DM rat livers, we therefore assessed the expression of two FoxO1-targeted genes, Pepck and G6pase, that have been identified as key regulators of hepatic gluconeogenesis (3, 16). The fasted DM rats showed significantly higher PEPCK mRNA (Fig. 6A) and G-6-Pase (Fig. 6B) mRNA expressions compared with the NC rats (P < 0.01); however, a 1-wk PDTC treatment of DM rats normalized the dysregulated induction of both mRNAs in fasted animals (P < 0.01).

**DISCUSSION**

Our study highlights a novel and important role of PDTC in modulating hepatic glucose metabolism in HFD-fed, STZ-treated rats. This rat model developed a decade ago has been found to closely mimic the natural progression of the disease and metabolic characteristics typical of T2DM in humans (27, 31, 37). Here, PDTC was found to increase hepatic glycogen deposition and decrease fasting blood glucose, whereas it

![Fig. 6. Effects of PDTC on mRNA levels of key gluconeogenic enzymes in the liver of diabetic rats.](http://ajpendo.physiology.org/)
reduced FoxO1 transcriptional activity in DM rats. The conversion of glucose into glycogen is a major pathway for the removal of blood glucose by the liver during the postprandial state (1). Moreover, hepatic glycogen synthesis and breakdown play important roles in the modulation of blood glucose levels (28). Glycogen synthase (GS) is a key biosynthetic enzyme whose activity is inhibited by phosphorylation catalyzed by GSK-3β. In response to insulin stimulation, Akt phosphorylates GSK-3β, causing its inhibition and subsequent activation of GS (11). Our results show that a 1-wk PDTC treatment elicited significant increases in the levels of phosphorylated Akt and GSK-3β in liver of fasted DM animals and correlated with higher hepatic glycogen content compared with the DM group. From the result of hepatic glycogen content and the OGTT and ITT analyses, we postulate that PDTC contributes to an increase in insulin sensitivity in the liver of HFD-fed, STZ-treated rats.

As indicated earlier, phosphorylation level for Akt (Ser473) was significantly lower in the DM group than in the NC group, and administration of PDTC normalized the altered phosphorylation of Akt in DM rats. The mechanism for the increased Akt signaling in response to PDTC could involve enhanced expression and/or phosphorylation of the insulin receptor, insulin receptor substrates (e.g., IRS-1 and IRS-2), and phosphoinositide (PI) 3-kinase. Moreover, PDTC may suppress expression and/or activity of protein phosphatases acting on the PI 3-kinase/Akt pathway (8, 10). It has become apparent that PDTC increases the generation of glutathione disulfide, a marker of thiol oxidation (4), and promotes protein S-glutathionylation in intact cells (40, 42). It follows that PDTC may stimulate reversible oxidative modification of active-site cysteines of protein phosphatases (8). Work is underway to identify the underlying mechanisms of increased Akt activation by PDTC in HFD-fed, STZ-treated rats.

Given the significant increase in hepatic phosphoactive Akt levels and the decrease in fasting blood glucose in PDTC-treated DM rats, we investigated whether FoxO1 activity could be negatively regulated by PDTC. The transcription factor FoxO1 binds to insulin-responsive elements in the promoter of PEPCK and G6pase, two key target genes involved in hepatic gluconeogenesis (23, 26). In the fasted state the liver is primarily responsible for maintaining glucose levels through gluconeogenesis, whereas insulin suppresses hepatic gluconeogenic enzyme expression postprandially. Moreover, FoxO1 is susceptible to inhibition and nuclear export due to its phosphorylation on Ser256 by Akt in response to insulin (reviewed in Ref. 12). We found that PDTC treatment reduced PEPCK and G-6-Pase mRNA levels, with a concomitant increase in phosphorylation and nuclear export of FoxO1 in DM livers. Because the activation and DNA-binding ability of FoxO1 is regulated by posttranslational modifications such as ubiquitination, phosphorylation, acetylation, and O-GlcNAcylation (14, 20), it is possible that PDTC induces modifications in FoxO1 that may alter its susceptibility to phosphorylation, although these are yet to be investigated.

PDTC brought about an improvement in virtually every other metabolic parameter examined, with little or no improvement in the lipid data. These results are in part contradictory to those of Ebenezer et al. (9), in which PDTC was found to lower total and VLDL cholesterol, whereas it raised HDL cholesterol in rats fed a HFD. In the Ebenezer et al. (9) study, homozygous Zucker fatty (fa/fa) and heterozygous (fa/+) rats were used, whereas Wistar rats were chosen for our study. In addition, in the study of Ebenezer et al. (9), rats were allowed free access to water containing PDTC (150 mg/kg) for a total of 10 wk. In contrast, our protocol called for ip administration of PDTC (50 mg·kg body wt·1·day–1) for 1 wk. Therefore, differences in rat strain and route/dose/duration of PDTC treatment could explain the divergence in the data between Ebenezer’s group and ours.

Diabetes-associated glucotoxicity is detrimental to pancreatic β-cells, causing impaired insulin secretion and induction in FAS expression, leading to apoptosis. Maedler et al. (18) demonstrated that activation of the IL-1β/NF-κB pathway contributes to pancreatic β-cell apoptosis in response to chronic hyperglycemia. There is no doubt that addition of STZ to HFD-fed rats induces some form of oxidative stress and exacerbates β-cell dysfunction. Because of its ability to inhibit NF-κB and the production of inflammatory cytokines (22, 33), it is likely that PDTC preserves pancreatic β-cell mass and function through a decrease in intraislet inflammatory cytokine production (e.g., IL-1β) in this HFD/STZ model. Studies have also shown that PDTC treatment reduces IL-6-mediated STAT3 activation and expression of acute-phase response proteins in HepG2 cells (13, 42). This small thiol compound can also activate the transcription factor HSF1 and expression of target genes, many of which are involved in cellular protection against apoptosis (36). Thus, the reciprocal effect of PDTC on the activity of a number of key transcription factors is consistent with its powerful pharmacological functions. We are currently performing cDNA microarray analyses to identify pathways affected by PDTC that are dysregulated in HFD-fed, STZ-treated rats. These results will be reported elsewhere.


10. E416 PDTC ACTION IN FAT-FED, STREPTOZOTOCIN-TREATED RATS


