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Long-Term Constitutive Androstane Receptor Activation By 2,4,6-Triphenyldioxane-1,3 Improves Glucose Metabolism in High-Fat Diet Rats

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Abstract

Recent studies suggested that Constitutive Androstane Receptor (CAR, NR1I3) is involved in energy metabolism (lipogenesis and gluconeogenesis), showing anti-diabetic and anti-obesity effects. The aim of this study was to examine the long-term effect of *cis* isoform of 2,4,6-triphenyldioxane-1,3 (*cis*TPD), a highly effective CAR activator in rat liver, on glucose metabolism and homeostasis in normal diet and high-fat diet rats.

Activation of CAR by *cis*TPD significantly reduces fasting blood glucose levels in both normal diet and high-fat diet rats. In *cis*TPD-treated normal diet and high-fat diet rats, the hepatic expression of PEPCK and G6Pase genes and protein levels were significantly inhibited, consistent with the decreased fasting glucose in these animals. Moreover, *cis*TPD treatment improves glucose tolerance in high-fat diet rats. The metabolic benefits of CAR activation by *cis*TPD may have resulted from the effect of inhibition of gluconeogenesis.

It is reasonable to conclude that CAR may be a target to prevent or suppress high level of glucose and improve glucose homeostasis and *cis*TPD may be a novel potential therapeutic tool for the regulation of gluconeogenesis.

Keywords: Glucose level; Gluconeogenesis; CAR; PEPCK; G6Pase

Introduction

The liver has a central role in maintaining glucose and energy homeostasis, which are tightly controlled by various metabolic and nutritional factors. Gluconeogenesis is largely responsible for overproduction of glucose in type 2 diabetes patients; whereas glycogenolysis is either unchanged or even reduce [1]. Regulation of gluconeogenesis is crucial for the maintenance of glucose homeostasis. Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are major enzymes involved in gluconeogenesis. These enzymes are highly activated during fasting and are suppressed in the fed state or by insulin [2]. The regulation of PEPCK and G6Pase genes at the transcriptional level involves a cross-talk between networks of transcription factors. Previous studies indicate that several transcription factors, including Hepatocyte Nuclear Factor-4α (HNF-4α) and Forkhead box O1 (FOXO1, also known as FKHR), may contribute to PEPCK and G6Pase genes expression [3-5].

The constitutive androstane receptor (CAR, NR1I3) was initially characterized as a xenosensor that regulates responses to xenochemicals. CAR is a key regulator of genes encoding several major types of drug metabolizing enzymes and transporters [6]. Recent studies suggested that CAR is also involved in energy metabolism via glucose and lipid metabolic pathways including PEPCK, G6Pase, SREBP-1, FAS and SCD-1, showing anti-diabetic and anti-obesity effects [7,8]. CAR-mediated repression of gluconeogenic genes seems to involve several molecular mechanisms. It was suggested that CAR inhibits gluconeogenic genes expression through HNF-4α inhibition, achieved by the competition of CAR with HNF-4α for binding to the DR1 motif in the promoter region of PEPCK and G6Pase genes and to the common co-activators peroxisomal proliferators activated receptor-γ coactivator 1α (PGC-1α) and Glucocorticoid receptor interacting protein 1 (GRIP-1) [9]. Also, it has been reported that CAR can physically bind to FoxO1 and suppress its transcriptional activity by preventing binding to the Insulin Response Sequence (IRS) in the gluconeogenic gene promoters [10]. It is tempting to speculate that pharmacological modulation of CAR may be beneficial in managing metabolic diseases. Thus, development of CAR activators is desirable as potential therapeutic tools for the treatment and prevention of some metabolic disorders.

In our previous studies, effect of the *cis* isoform of 2,4,6-triphenyldioxane-1,3 (*cis*TPD) were investigated [11,12]. *cis*TPD is highly effective activator of CAR and its target genes in rat liver, but it is not able to activate CAR in mice [11]. We have demonstrated that the maximal effect of *cis*TPD in rat liver occurred with a 10 mg/kg body weight i.p. injection (ED50 = 5.1 mg/kg body weight) [12] and a single 10 mg/kg body weight i.p. injection of *cis*TPD evoked significant decrease in the expression of key hepatic gluconeogenic genes, PEPCK and G6Pase, through a diminished association of HNF4α and FOXO1 with their specific binding sites in the gene promoters [13]. The aim of the present study was to examine the long-term effect of *cis*TPD on glucose metabolism and homeostasis in normal diet and High-Fat Diet (HFD) rats.

Materials and Methods

Chemicals

The *cis*TPD was synthesized according to Griengl and Geppert [14].

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The compound was identified by NMR spectroscopy and was shown to be 97-98 % pure by LC/MS analysis. All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Experimental animals

Male Wistar rats (150-200 g) were supplied by the Institute of Clinical Immunology SB RAMS (Novosibirsk, Russia). Animals were acclimated for one week and allowed free access to food and water. All experimental procedures were approved by the Animal Care Committee of the Institute of Molecular Biology and Biophysics SB RAMS. The rats were randomly divided into four groups. Five rats were used in each group. Control group, rats were fed on normal diet (5% fat of calories) during 8 weeks. HFD group, rats were received high-fat diet (45% fat of calories) during 8 weeks. TPD group, rats were fed on normal diet and i.p. injected with 10 mg/kg body weight of *cis*TPD in corn oil for 8 weeks as a single weekly dose. HFD+TPD, rats were received HFD during 8 weeks and i.p. injected with 10 mg/kg body weight of *cis*TPD in corn oil as a single weekly dose. After 8 weeks, all groups of rats were fasted for 16 h and the fasting blood glucose analysis was carried out using OneTouch Select glucometer (LifeScan, Inc.).

Glucose tolerance test (GTT)

For GTT, rats were fasted for 16 h before receiving an i.p. injection of D-glucose at 2 mg/kg body weight. Blood glucose was measured by tail bleed at 0, 20, 40, 60 and 120 min post glucose dose with OneTouch Select glucometer (LifeScan, Inc).

cDNA synthesis and real-time PCR

After 8 week *cis*TPD treatment livers were collected from each rat. Tissue for protein and RNA preparation was stored in –80°C freezer after snap-frozen in liquid nitrogen. The sample collection was conducted in fasting (16 h) condition. Total RNA was isolated from rat livers using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of the RNA was determined by measuring the absorbance at 260 and 280 nm with a correction for background at 320 nm, and the integrity was examined by visualizing the 18S and 28S rRNA bands on a denaturating agarose (1%) gel. One microgram of total RNA was used for synthesis of single-stranded cDNA. First strand cDNA synthesis was carried out using of the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Expression levels of the genes were measured by real-time PCR using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). Real-time PCR was carried out on an IQ5 Real-Time PCR system (Bio-Rad Laboratories). Signal were normalized to the "house-keeping" gene 18S rRNA as an endogenous internal control. The following gene-specific oligonucleotide primers were used for CYP2B1, CYP2B2, PEPCK, G6Pase and 18S rRNA: CYP2B1 F: 5'-TCATCGACACTTACCTTCTGC-3' and R: 5'- GTGTATGGCATTT-TACTGCGG-3'; CYP2B2 F: 5'- TCATCGACACTTACCTTCTGC-3' and R: 5'- AGTGTATGGCATTTTGGTACG-3'; PEPCK F: 5'-CAG-GAAGTGAGGAAGTTTGTGG-3' and R: 5'- ATGACACCCTCCTCCT-GCAT-3'; G6Pase F: 5'-GGCTCACTTTCCCCATCAGG-3' and R: 5'- ATCCAAGTGCGAAACCAAACAG-3'; 18S F: 5'-CCCAGTAAGT-GCGGGTCATA-3' and R: 5'-GGCCTCACTAAACCATCCAA-3'. The optimal annealing temperature for each primer set was determined prior to the analysis of experimental samples. Triplicate real-time PCR reactions were run for each sample. The following standard real-time PCR conditions were used: one cycle of 95°C for 3 min and 40 cycles of 95°C for 15 s, 58°C (the primer specific annealing temperature) for 20 s, and 72°C for 20 s, and optical data were collected at 75°C for 10 s. After the PCR experiments, the dissociation curve was established using the built-in melting curve program to confirm the presence of a single PCR product, which were then confirmed by gel electrophoresis. The fold change in the target gene, normalized to the level of 18S rRNA, was calculated based on PCR efficiency (E) and Ct.

Preparation of whole-cell liver protein extracts

For preparation of whole-cell liver protein extracts livers were rinsed in cold phosphate-buffered saline and suspended in lysis buffer 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM DTT, and 1 mM EDTA supplemented with the Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). The livers were homogenized. Homogenates were incubated on ice for 30 min and centrifuged at 5000 g for 10 min to remove insoluble precipitates. Supernatants were used as whole-cell liver protein extracts. Whole-cell liver protein extracts were collected and stored at -80°C. The protein concentrations in whole-cell liver protein extracts were determined using the Bradford method with bovine serum albumin as a standard.

SDS-PAGE electrophoresis and Western-blot

Sixty micrograms of the whole-cell liver proteins per lane were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were stained with Ponceau S to verify loading and transfer efficiency. Immunodetection was performed with polyclonal anti-PEPCK (sc-32879, Santa Cruz Biotechnology), anti-G6Pase (sc-25840, Santa Cruz Biotechnology) or anti-human b-actin (Sigma-Aldrich) antibodies. Beta-actin was used as loading control. The bands were visualized using the Visualizer Spray and Glow ECL Western Blotting Detection System (Millipore).

Data analysis

The data are presented as the mean ± SD. ANOVA followed by Tukey's post hoc test was used to multiple comparison tests. A p-value < 0.05 was considered statistically significant.

Results

We employed four rat groups including normal diet-fed or HFD-fed rats with or without *cis*TPD treatment. As expected, expression of CAR target genes, CYP2B1 and CYP2B2, were increased by long-term treatment with *cis*TPD in normal diet (3.7- and 2.3-fold, respectively) and HFD rat livers (3- and 2-fold, respectively) (Figure 1). To investigate how long-term CAR activation by *cis*TPD influences on glucose metabolism; we monitored blood glucose levels in rats in response to 8 weeks treatment. Long-term *cis*TPD treatment significantly decreased fasting blood glucose levels compared with vehicle-treated animals (1.3-fold) (Figure 2). Moreover, we examined the effect of long-term CAR activation in high-fat diet rats. Rats were fed with high-fat diet for 8 weeks and concurrently treated with *cis*TPD (10 mg/kg, once a week) or vehicle. Fasting blood glucose level was significantly increased by HFD compared with control group of rats (1.4-fold) (Figure 2). It was shown that treatment with *cis*TPD in HFD rats decreased the level of fasting blood glucose to control group level (Figure 2). Moreover, as shown in Figure 3, *cis*TPD significantly inhibited the gain of body weight of HFD rats after 8 weeks of treatment. By the end of the 8 week, the *cis*TPD-treated HFD group had 1.25-fold lower body weight compared with the HFD group.

The reduced fasting blood glucose level in *cis*TPD treated rats indicates that gluconeogenesis is suppressed by CAR activation. We

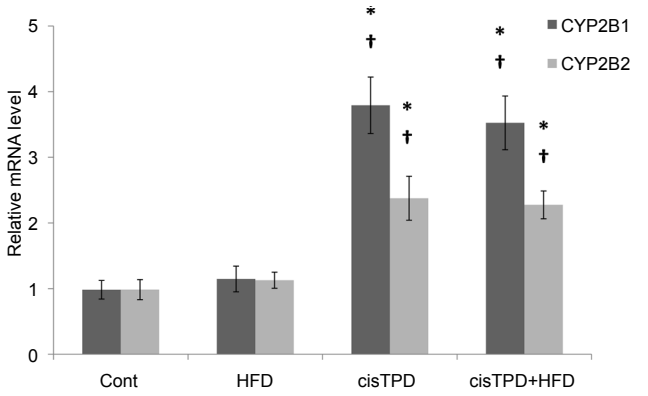

Figure 1: Effect of long-term *cis*TPD treatment on CYP2B1 and CYP2B2 genes expression in normal diet and HFD rat livers. Total RNAs were prepared and subjected to RT-PCR for measurement of mRNA. Fold change was expressed by taking the corresponding value of the control rats as one. Bars mean ± S.D (n=5). *Indicate a significant difference from control animals (p< 0.05); †Indicate a significant difference from HFD animals (p< 0.05).

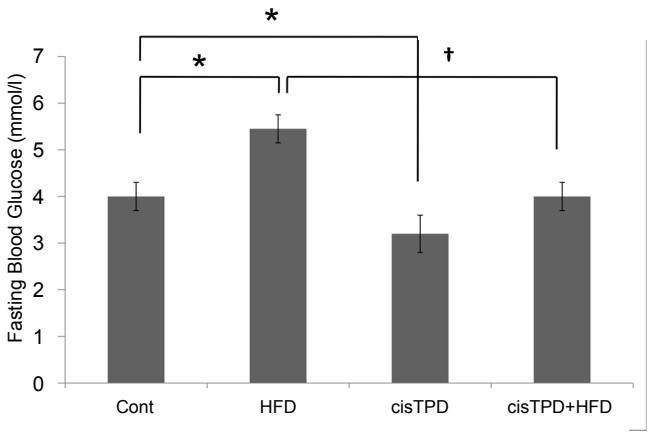

Figure 2: Fasting blood glucose levels in rats at the end of an 8-week normal diet and HFD and *cis*TPD treatment. Bars mean ± S.D (n=5). *Indicate a significant difference from control animals (p< 0.05); †Indicate a significant difference from HFD animals (p<0.05).

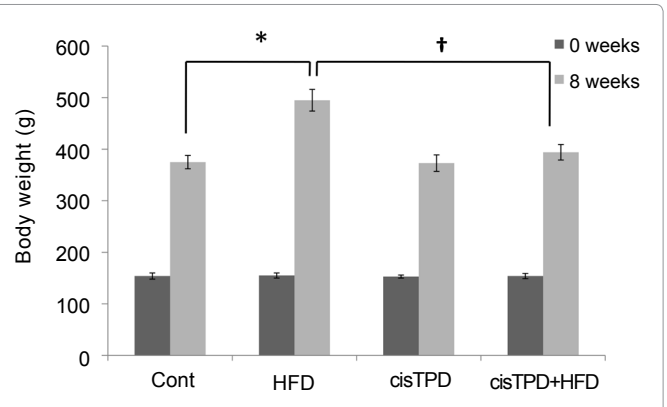

Figure 3: Effect of long-term *cis*TPD treatment on body weight. Bars mean ± S.D (n=5). *Indicate a significant difference from control animals (p< 0.05); †Indicate a significant difference from HFD animals (p< 0.05).

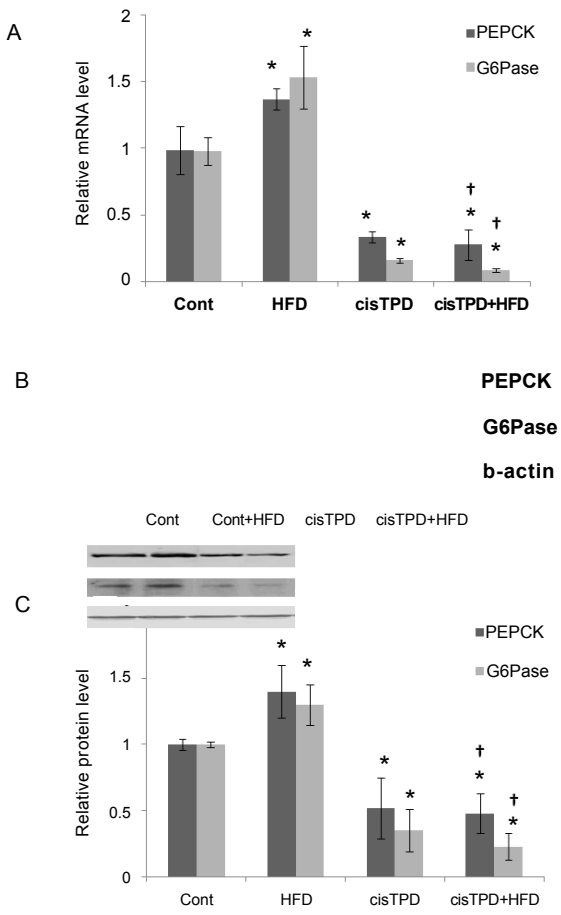

Figure 4: Effect of long-term *cis*TPD treatment on PEPCK and G6Pase genes expression and protein levels in normal diet and HFD rat livers. A) Total RNAs were prepared and subjected to RT-PCR for measurement of mRNA. Fold change was expressed by taking the corresponding value of the control rats as one. Bars mean ± S.D (n=5). *Indicate a significant difference from control animals (p< 0.05); †Indicate a significant difference from HFD animals (p<0.05). B) Representative Western blots of PEPCK, G6Pase and b-actin. Whole-cell extracts were prepared from rat livers and subjected to Western blot analysis using anti-PEPCK, anti-G6Pase or anti-b-actin antibodies as described under "Materials and methods". C) Relative intensity. The protein bands were analyzed by the computerized densitometric program "Total Lab". The intensities of the signals were determined from the areas under the curves for each peak and data were graphed. Beta-actin was used as internal control to which PEPCK and G6Pase were normalized. Fold change was expressed by taking the corresponding value of the control as one. Bars mean ± S.D (n=5). *Indicate a significant difference from control animals (p< 0.05); †Indicate a significant difference from HFD animals (p< 0.05).

examined the effect of long-term CAR activation by *cis*TPD on hepatic gluconeogenesis under fasting condition. In *cis*TPD-treated rats, the hepatic expression of PEPCK and G6Pase genes (3- and 6-fold, respectively) and protein levels (1.8- and 2.6-fold, respectively) were significantly inhibited (Figure 4), consistent with the decreased fasting glucose in these animals (Figure 2). In HFD rat livers, PEPCK and G6Pase mRNA (1.4- and 1.6-fold, respectively) and protein levels (1.4- and 1.3-fold, respectively) were increased when compared with control rats (Figure 4). On the other hand, PEPCK and G6Pase mRNA (5- and 19-fold, respectively) and protein levels (3- and 6-fold, respectively) were decreased upon 8 weeks of *cis*TPD treatment in HFD rat livers (Figure 4). These results suggest that *cis*TPD may have anti-hyperglycemia effect.

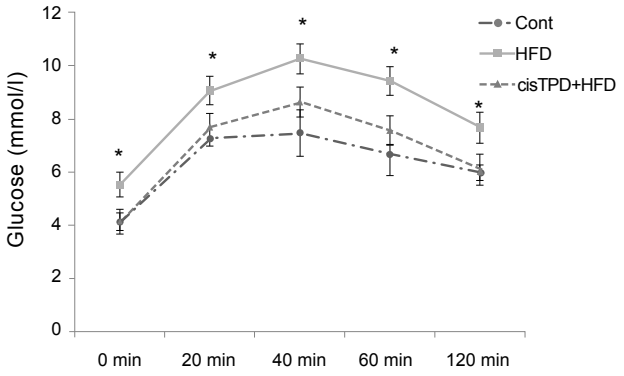

Figure 5: Treatment of HFD rats with *cis*TPD for 8 weeks improved glucose tolerance. Rats were fed on normal diet (Cont) or high-fat diet (HFD) during 8 weeks and treated with *cis*TPD as a single weekly dose (*cis*TPD+HFD). After 16 h of fasting, i.p. glucose tolerance test was performed. Glucose levels at each time point were measured. Bars mean ± S.D (n=5). *Indicate a significant difference from *cis*TPD+HFD group ($p < 0.05$).

To investigate how *cis*TPD influences glucose homeostasis, GTT was performed. For GTT, rats were fasted 16 h before receiving an i.p. injection of D-glucose at 2 mg/kg body weight. Blood glucose was measured by tail bleed at different time points. Blood glucose levels increased during GTT in all groups of animals (Figure 5). Treatment of HFD rats with *cis*TPD for 8 weeks significantly improved glucose tolerance compared with HFD rats treated with vehicle (Figure 5). Taken together, these results indicate that CAR activator *cis*TPD may improve insulin sensitivity through suppression of hepatic gluconeogenesis.

Discussion

Activation of CAR by TCPOBOP decreases the expression of gluconeogenic genes and blood glucose level [8], indicating that CAR agonist or activator could be a novel potential therapeutic tool for the glucose level regulation. In the present study, we investigated the long-term effect of *cis*TPD, a highly effective CAR activator in rat liver [11,12] on the glucose metabolism in rats. In our previous study we have shown that single *cis*TPD treatment repressed the expression of the gluconeogenic genes PEPCK and G6Pase in rat liver [13]. In the present study, we showed that the long-term activation of CAR by *cis*TPD produces the decrease of fasting blood glucose level in rats. Reduced fasting blood glucose level in *cis*TPD-treated rats indicates that gluconeogenesis is suppressed by CAR activation.

Consistent with our previous study [13], we found that the expression of the gluconeogenic genes PEPCK and G6Pase was repressed by long-term *cis*TPD treatment. In our previous study using chromatin immunoprecipitation, we have demonstrated that *cis*TPD prevents the binding of FoxO1 to insulin response sequences in the PEPCK and G6Pase gene promoters in rat liver. FoxO1 is a member of the forkhead family transcription factors and regulates glucose metabolism in the adult liver [15,16]. FoxO1 regulates the expression of PEPCK and G6Pase genes by direct binding to its target DNA sequence and also through the interaction with nuclear receptors. FoxO1 activity is regulated by phosphorylation and acetylation. Insulin induces phosphorylation of FoxO1 through the PI3K-Akt signaling pathway. Phosphorylated FoxO1 is excluded from the nucleus, and thereby its transcriptional activity is attenuated [17-19]. Kodama et al. [10] reported that following activation, CAR binds to FoxO1 and prevents its binding to the IRS, which could result in transcriptional repression of IRS-regulated genes. Moreover, in our previous study, we have shown that *cis*TPD-activated CAR inhibited HNF-4α transactivation by competing with HNF-4α for binding to the HNF-4α-binding element (DR1-site) in the gluconeogenic gene promoters [13]. HNF-4α is a key transcriptional activator for a large number of genes in hepatocytes [3]. HNF-4α is central to glucose metabolism because it is a key positive regulator for basal hepatic expression of PEPCK and G6Pase genes [20].

Many studies have reported that rats fed with high-fat diet develop obesity and insulin resistance. It is suggested that the HFD might be a better way to initiate the insulin resistance which is one of the important features of type 2 diabetes [21]. Because HFD is strongly associated with typical characteristic of type 2 diabetes, we examined the effect of *cis*TPD on glucose homeostasis in HFD rats after 8 weeks of compound treatment. Our data suggest that long-term *cis*TPD treatment ingibits glucose production in HFD rats. The inhibition is related to suppression of the PEPCK and G6Pase expression. Moreover, glucose homeostasis was improved in *cis*TPD treated HFD rats, as shown by GTT. Our results with *cis*TPD are consistent with another recent study showing that TCPOBOP treatment prevented obesity and improved insulin sensitivity in HFD mice [7]. Gao at al. [7] suggested that the metabolic benefits of CAR activation may have resulted from the combined effect of inhibition of lipogenic genes (including Srebp-1c, Acc-1, Fas and Scd-1), very low density lipoprotein secretion and export of triglycerides, and gluconeogenesis.

In considering all of the data, it is reasonable to conclude that CAR may be a target to prevent or suppress high level of glucose and improve glucose homeostasis. Although results of future studies must be the final arbiter of this issue, it is likely that CAR activation will be considered as a target for the treatment of diabetes, especially for treatment of poorly controlled type 2 diabetes, since this disease associated with an increased rate of hepatic glucose production, obesity and insulin resistance, and *cis*TPD as a novel potential therapeutic tool for the regulation of gluconeogenesis.

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