Effects of CP-900691, A Novel Peroxisome Proliferator-Activated Receptor α Agonist on Diabetic Nephropathy in the BTBR ob/ob mouse

Bardia Askari¹,3, Tomasz Wietecha¹, Kelly L. Hudkins¹, Edward J. Fox¹, Kevin D. O’Brien², Jinkyu Kim¹, Tri Q. Nguyen¹, and Charles E. Alpers¹
¹Department of Pathology, University of Washington, Seattle, WA
²Department of Medicine; Division of Cardiology, University of Washington, Seattle, WA
³Department of Pharmaceutical and Biomedical Sciences, Touro College of Pharmacy, New York, NY

Abstract

Piperidine-based peroxisome proliferator-activated receptor alpha agonists are agents that are efficacious in improving lipid, glycemic, and inflammatory indicators in diabetes and obesity. This study sought to determine whether CP-900691 ((S)-3-[3-(1-carboxy-1-methyl-ethoxy)-phenyl]-piperidine-1-carboxylic acid 4-trifluoromethyl-benzyl ester) (CP), a member of this novel class of agents, by decreasing plasma triglycerides, could prevent diabetic nephropathy in the BTBR ob/ob mouse model of type 2 diabetes mellitus. 4-week old female BTBR WT and BTBR ob/ob mice received either regular chow or one containing CP (3 mg/kg/day) for 14 weeks. CP elevated plasma high-density lipoprotein, albuminuria and urinary excretion of 8-epi PGF\(_{2\alpha}\), a product of the non-enzymatic metabolism of arachidonic acid and whose production is elevated in oxidative stress, in BTBR WT mice. In BTBR ob/ob mice, CP reduced plasma triglycerides and non-esterified fatty acids, fasting blood glucose, body weight, and plasma interleukin-6, while concomitantly improving insulin resistance. Despite these beneficial metabolic effects, CP had no effect on elevated plasma insulin, 8-epi PGF\(_{2\alpha}\) excretion and albuminuria, and surprisingly, did not ameliorate the development of diabetic nephropathy, having no effect on the accumulation of renal macrophages, glomerular hypertrophy and increased mesangial matrix expansion. In addition, CP did not increase plasma high-density lipoprotein in BTBR ob/ob mice, while paradoxically increasing total cholesterol levels. These findings indicate that 8-epi PGF\(_{2\alpha}\), possibly along with hyperinsulinemia and inflammatory and dysfunctional lipoproteins, is integral to the development of diabetic nephropathy and should be considered as a potential target of therapy in the treatment of diabetic nephropathy.
Keywords

diabetic nephropathy; diabetes; obesity; mouse models; peroxisome proliferator activated receptor; isoprostanes; reactive oxygen species; insulin resistance

Diabetes is characterized by chronic hyperglycemia and dyslipidemia with the development of diabetes-specific complications in nerves, retina and kidney and other organs. Diabetic nephropathy (DN), one of major complications of diabetes mellitus, is the most common cause of end-stage renal disease in the U.S. and Europe (1). Approximately one-third of people with diabetes develop evidence of nephropathy, eventually constituting over half of those on dialysis. While progressive DN is most likely due to a combination of environmental and genetic influences, the exact pathophysiologic mechanisms remain to be elucidated. The onset and course of DN can be ameliorated in some patients by several interventions (i.e. changes in lifestyle, glycemic control and renin-angiotensin-aldosterone system (RAAS) inhibition). However, mortality and morbidity due to DN is increasing (2), making the development of more effective interventions a compelling task. Numerous studies suggest that hyperlipidemia is an independent risk factor in the development of DN (3) and elevated levels of plasma lipids have been associated in the progression of renal dysfunction (3, 4).

Peroxisome proliferator activated receptors (PPAR) are members of a family of ligand-activated transcription factors, consisting of three members (α, γ and β/δ) (5). The insulin-sensitizing thiazolidinedione (TZD) class of PPARγ ligands have well-established efficacy in the treatment of type 2 diabetes mellitus (T2DM) (6) and PPARα ligands, such as the fibrate class of oral hypolipidemics (i.e. fenofibrate), increase plasma high-density lipoprotein-cholesterol (HDL-C) and decrease plasma triglycerides (TGs) in people with hyperlipidemia (7). However, TZDs, despite ameliorating DN in animal models of diabetes and in people with T2DM (8, 9), are no more effective than other oral anti-diabetics (i.e. metformin) in this regard (10) and their cardiovascular side effects (increased risk of congestive heart failure and myocardial infarction), makes the use of these drugs in the treatment of DN a challenge. PPARα is mainly expressed in tissues with high rates of fatty acid metabolism, regulating the expression of genes that promote fatty acid synthesis and oxidation. PPARα ligands are observed to have a variety of beneficial effects in animal models of DN (11), and data from the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) and ACCORD (Action to Control Cardiovascular Risk in Diabetes) studies, both randomized, controlled clinical trials investigating the benefits of fenofibrate in preventing cardiovascular complications in people with type 2 diabetes, showed a reduction in rates of progression to albuminuria, indicating a therapeutic potential for such agents in the treatment of DN (12, 13).

A major limitation in elucidating mechanisms underlying DN has been the lack of relevant animal models, with most models developing some, but not all, features of DN (14-16). We have characterized DN in the Black and Tan, BRachyuric mouse with the leptin-deficiency mutation (BTBR ob/ob), possessing many features of advanced human DN (17). Use of this
The preclinical model allows us to test the mechanisms of altered disease pathophysiology and efficacy of specific interventions.

The purpose of the present study was to determine whether CP-900691 (CP), a novel, potent and selective PPARα agonist (18-20), by primarily decreasing plasma TGs, can prevent the development of DN. CP decreased body weight and plasma triglycerides and had pronounced hypoglycemic and anti-inflammatory effects, while upregulating fatty acid oxidation gene expression in BTBR WT and BTBR ob/ob mice. Treatment with CP increased total cholesterol in BTBR ob/ob mice, without having any effect on HDL-C and surprisingly, did not prevent DN, having no effect on plasma insulin levels, urinary excretion of 8-epi PGF2α (8-epi) and mesangial matrix expansion or macrophage accumulation. These results suggest that increases in renal 8-epi, associated with hyperinsulinemia and altered lipoproteins contribute to DN.

**Material and Methods**

**Animals**

The Institutional Animal Care and Use Committee of the University of Washington in Seattle approved all experimental protocols and procedures. The establishment and care of BTBR ob/ob mice has been previously described (17). We placed 4-week-old female, BTBR wildtype (BTBR WT, n=9) and BTBR ob/ob (BTBR ob/ob, n=9) mice on standard rodent diet (Bio-Serv, Frenchtown, NJ), an age where there were no observable changes in albumin-creatinine ratio (17) and no alterations in renal structure were observed (unpublished observation). This early age for starting treatment also reflects our unpublished experience that by 6 weeks of age some BTBR ob/ob mice will have albuminuria. CP (3 mg/kg/day, Pfizer, Groton, CT) was incorporated into the diet and administered ad libitum. The mice were treated for 14 weeks, for a total of 18 weeks of age, an age where the maximum renal damage was observed (17). In selecting a dose for evaluating the actions of CP in BTBR WT and BTBR ob/ob mice, we chose to study the administration of 3 mg/kg/day, a dose shown to be approximately 100X more selective for PPARα than fibric acid derivatives and where the maximum hypotriglyceridemic effect was observed, both in studies describing piperidine-derived compounds in mice (19) and reinforced by a studies using CP in other species (20). Mice were fasted for 6 hours prior to drawing of blood on the day of sacrifice. Post-sacrifice, the internal organs and the subcutaneous, retroperitoneal, epididymal and subscapular adipose tissues were excised and weighed. Portions were either snap-frozen with liquid N2 or fixed with 10% neutral-buffered formalin and embedded in paraffin wax. Frozen tissues were stored at −70°C until use. Body weights (BW) were measured weekly and lean body weight was calculated as the weight of mice after removal of all internal organs and adipose tissues. In addition, tibias were removed, cleaned and measured.

**Blood Chemistry and Insulin Tolerance tests**

Serum cholesterol, triglycerides (TG), non-esterified fatty acids (NEFA), insulin, adiponectin, interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were analyzed at the University of Washington Mouse Metabolic Phenotyping Center (MMPC) metabolic testing...
Insulin, adiponectin, IL-6 and TNF-α was measured via the Luminex Platform. HDL-C was measured indirectly by polyethylene glycol precipitation of non-HDL and measurement of total cholesterol levels in the supernatant. In rodents, PPARα agonists have hepatic and muscular side effects (21). Increases in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine kinase (CK) activity (Table 2) were associated with both DM and CP treatment, but no fibrosis or hepatocellular carcinomas (data not shown). ALT and AST activity were measured using the ALT (SGPT) Liquid and AST (SGOT) Liquid kits (Pointe Scientific, Inc, Canton, MI). CK was determined via the Stanbio CK, Liqui-UV (NAC) kit (Stanbio Labs, Boerne, TX) and according to the manufacturer’s instructions.

Blood glucose was monitored biweekly using the Freestyle Blood Glucose Monitor (Abbott Diabetes Care, Alameda, CA), beginning at 4 weeks. Insulin sensitivity was assessed one week prior to the end of the study in treated and untreated BTBR ob/ob mice using insulin tolerance tests (ITT), performed after a 4-hour fast by an intraperitoneal (IP) injection of diluted insulin (Humulin, 10 IU/g body weight). IP insulin (1IU/g body weight), a dose that effectively decreases fasting blood glucose in WT mice, was without effect in the BTBR ob/ob (unpublished observations). Blood was drawn via the saphenous vein at 0, 15, 30, 60 and 120 min post-injection for blood glucose measurements.

**Urinary Analysis**

Urine was collected from individual mice 6 h prior to sacrifice. Urinary albumin and creatinine were analyzed as previously described (17) and extrapolated to obtain a 24-hour rate. 8-epi and 8-hydroxy 2-deoxy-guanosine (8OH-dG) were measured using their respective ELISA kits (Cayman Chemical Co., Ann Arbor, MI), and according to manufacturer instructions.

**Immunohistochemical Analysis**

Kidneys and other organs were obtained from WT and BTBR ob/ob mice after 14 weeks of drug therapy. Tissue sections were fixed, embedded, immunostained and analyzed as previously described (17), as were glomerular mesangial expansion and matrix accumulation. Briefly, the mesangial area occupied by silver methenamine stained matrix was quantified, as was the total area positive for matrix, by computer image analysis (Image Pro Plus, Media Cybernetics, Rockville, MD), for each glomerular cross-section.

Mesangiolysis was determined by assessing the silver methenamine-stained glomeruli and scoring at least 50 glomeruli per section, scored present when lucency and dissolution of the normally compact silver-staining mesangial matrix was present and/or there were dilatation/ballooning of adjacent capillary loops.

Rat anti–Mac-2 (1:5000, Cedarlane, Hornby, Ontario, CA) and rabbit polyclonal anti-p57 (1:3000, Santa Cruz Biotechnology, Santa Cruz CA) were used to visualize glomerular macrophages and podocytes, as previously published (18). Quantification for Mac-2 and p57 were performed on digital images of immunostained tissues by counting, in a blinded manner, the number of Mac-2-positive cells and p57-positive podocytes per glomerular cross section in 50 glomeruli, per animal.
Gene Expression Analysis

Total RNA was extracted from sections of frozen tissues using the SV Total RNA Isolation miniKit (Promega, Madison, WI), treated with DNase (DNase I, 15m at 25°C) to eliminate contamination of genomic DNA and quantified via UV spectrophotometry (NanoDrop 3300, Thermo Scientific, Wilmington, DE). cDNA was then synthesized from RNA (500 ng), using the RevertAid™ First Strand cDNA kit (Fermentas, Glen Burnie, MD). Primer sequences for target genes were identified using PrimerBank and RTPrimerDB databases (supplemental Table 1) and purchased from Invitrogen (Carlsbad, CA). Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) was performed using Brilliant® SYBR® Green qPCR Master Mix (Strategene, Santa Clara, CA) and analyzed using the DNA Engine Opticon® 2 System (BioRad, Hercules, CA). Samples were analyzed in duplicate and normalized using 18S ribosomal subunit as reference gene. Relative mRNA expression was expressed using the ΔΔ cycle threshold (Ct) method. For analysis of random mutation, renal cortical tissues were digested for 1 hr in a buffer containing 0.2 mg/ml proteinase K (Sigma-Aldrich, St. Louis, MO), 0.75% SDS, 0.01 M Tris HCl, 0.15M NaCl and 0.005 M EDTA (pH 7.8). Total DNA was extracted using phenol-chloroform (1:1) followed by ethanol precipitation. DNA was then diluted and digested in a specialized Taqal buffer in the presence of 100 units Taqal (NEB, Ipswich, MA) and 1X BSA for 10 h, with the addition of 100 units of Taqal per hour. Random mutation was detected via qPCR, performed using a DNA Engine Opticon® 2 System. Amplicons were visualized with Brilliant® SYBR® Green qPCR Master Mix. Primers used for amplification are listed in Supplementary Table 1. qPCR were performed in 25-μl reactions containing 2X Brilliant SYBR Green qPCR Master Mix, 20 pmol forward and reverse primers and 2U uracil DNA glycosylase (UDG). The samples were amplified as follows: UDG incubation at 37°C for 10 m, 95°C for 10 m followed by 45 cycles of 95°C for 0.5 m, 60°C for 1 min and 72°C for 1.5 m. Samples were held at 72°C for 5 m and immediately stored at −20°C.

Statistical Analysis

All values are expressed as the mean ± standard errors mean (SEM) and analyzed using Graphpad Prism (GraphPad Software, La Jolla, CA). P<0.05 was considered as statistically significant.

Results

Body and Tissue Weights

To evaluate changes in growth and BW, we analyzed four-week old BTBR WT and BTBR ob/ob mice treated with CP for 14 weeks (Fig. 1A). Both total and lean BWs were increased in BTBR ob/ob mice over the 14 weeks (Fig 1B), an increase that was attenuated by CP treatment. We measured tibia lengths (TLs) as an index of growth. TLs were attenuated in BTBR ob/ob mice, and CP treatment caused a further decrease in both BTBR WT and BTBR ob/ob mice (Table 1).

Overall, tissue weights were increased in BTBR ob/ob, compared to BTBR WT mice. CP treatment induced hepatomegaly in both groups, similar to the effects of other PPARα...
agonists in rodents (21). Despite the increase in size and concomitant increase in plasma ALT/AST, histochemical analysis of hemotoxylin and eosin-stained livers demonstrated no obvious or significant injury (unpublished observations). Elevated kidney weights in the BTBR ob/ob mice were decreased with CP (Table 1). However, while CP treatment reduced adipose tissue weights in BTBR WT mice, it had no effect on epididymal, retroperitoneal and subscapular adipose tissues in the BTBR ob/ob mice.

**Blood Glucose and Insulin Resistance**

In order to see whether CP-induced decreases in BW had any effect on diabetes and insulin resistance, we analyzed fasting blood glucose and insulin tolerance. BTBR ob/ob mice were hyperglycemic and insulin resistant, as previously reported (17, 22) (Fig 2A, Table 2). In BTBR WT mice, CP decreased plasma insulin levels and had a modest hypoglycemic effect. In the BTBR ob/ob, CP attenuated hyperglycemia (Fig. 2A), without having any effect on plasma insulin (Table 2). In order to evaluate the effect of CP on insulin resistance (IR), ITT were performed in BTBR ob/ob mice. In untreated ob/ob mice, insulin administration was without effect, while in CP-treated mice insulin decreased blood glucose (Fig 2B). Analysis of the areas under the curve of blood glucose demonstrated significant improvement of IR (Fig. 2C).

**Serum Lipids and Blood Chemistry**

CP and other piperidine-derived PPARα agonists have demonstrated potent hypolipidemic and anti-inflammatory effects in rodents and other species (20, 21). In order to verify the efficacy of our treatment regimen, we measured serum lipids and indicators of systemic inflammation. BTBR ob/ob mice are hyperlipidemic, with elevated serum TG, NEFA and total cholesterol levels. In BTBR WT mice, CP treatment increased HDL-C, lowered serum TG, but not NEFA levels (Table 2). In the BTBR ob/ob mice, CP treatment decreased serum TG and NEFA without having any effect on HDL-C while concomitantly increasing total cholesterol levels (Table 2).

PPARα agonists have been shown to be mildly hepatotoxic and myopathic when administered to rodents (22). We therefore analyzed plasma ALT, AST and CK levels in BTBR WT and BTBR ob/ob mice. CP induced increases in ALT and AST levels in both BTBR WT and BTBR ob/ob mice. However, CP had no effect on CK levels in BTBR WT mice (Table 2) while normalizing the significantly increased CK levels seen in BTBR ob/ob mice (51.4 ± 16.8 U/L vs. 173.2 ± 11.4 U/L, P<0.05, BTBR WT vs. BTBR ob/ob).

CP treatment increased adiponectin levels in WT mice, without having any effects on IL-6 and TNF-α. BTBR ob/ob mice had lower adiponectin and higher IL-6 levels, indicating a systemic inflammation. Treatment with CP lowered IL-6, while having no effect on serum adiponectin. TNF-α values in BTBR ob/ob mice were below the limits of detection of the assay (Table 2).

**Hepatic Gene expression**

Previous studies have shown PPARα as a key regulator of the expression of hepatic genes associated with mitochondrial and peroxisomal β-oxidation (5). In mice, CP-related
piperidines compounds robustly induce canonical pathways involved in lipid metabolism (20). We therefore analyzed hepatic RNA isolated from CP-treated and untreated BTBR WT and BTBR ob/ob mice and quantified the expression of several of these PPARα-dependent genes. Mitochondrial and peroxisomal β-oxidation-associated genes, namely isoforms of carnitine palmitoyl acyltransferase (cpt1a, cpt1b and cpt2) and palmitoyl acyl CoA oxidase-1 (acox1), were increased in livers of BTBR ob/ob mice (Fig 3A-F). These changes were associated with increases in PPAR-γ (pparg), but not PPAR-α (ppara), expression (Fig 3G-H). In BTBR WT mice, CP-treatment had no effect on cpt1a, while increasing acox1, cpt1b and cpt2 expression. CP treatment in the BTBR ob/ob mice potentiated acox1, cpt1b and cpt2 expression, while attenuating the increases in pparg.

**Urinary Parameters**

In order to assess the efficacy of CP on DN, we analyzed urinary albumin, creatinine, glucose and 8-epi excretion in CP-treated and untreated BTBR WT and BTBR ob/ob mice. Daily urinary creatinine was elevated in BTBR ob/ob mice (0.565 ± 0.06 mg/24hr vs. 0.989 ± 0.2 mg/24hr, P<0.05, BTBR WT vs. BTBR ob/ob), and CP treatment had no effect on either BTBR WT (0.565 ± 0.06 mg/24hr vs. 0.445 ± 0.059, P=0.1501, BTBR WT vs. BTBR WT + CP) or BTBR ob/ob mice (0.989 ± 0.2 mg/24hr vs. 1.050 ± 0.1 mg/24hr, P=0.7756, BTBR ob/ob vs. BTBR ob/ob + CP). Surprisingly, CP-treated BTBR WT mice had pronounced albuminuria (Fig. 4B, C), despite having normal urine flow (Fig. 4A). BTBR ob/ob mice were polyuric (Fig. 4A), albuminuric (Fig. 4B-C) and glycosuric, measured as the % fractional excretion of glucose (% FEGlc) (0.49 ± 0.04 % vs. 2.71 ± 0.73, BTBR WT vs. BTBR ob/ob, P<0.01). In BTBR ob/ob mice, CP had an anti-diuretic effect (Fig. 4A), and abolished glycosuria (2.71 ± 0.73 % vs. 0.80 ± 0.06 %, BTBR ob/ob vs. BTBR ob/ob + CP, P<0.001). Despite these improvements, CP treatment had no effect on the albuminuria in BTBR ob/ob mice (Fig. 4B-C).

Isoprostane production is increased in pathological conditions associated with oxidative stress and is postulated to be a key component in the development and progression of the renal complications of diabetes (24-29). We analyzed the urinary isoprostane, 8-epi, production in order to evaluate the effects of CP. While 8-epi excretion was elevated in BTBR ob/ob mice, it was, similar to albuminuria, also elevated in CP-treated BTBR WT mice. However, CP had no effect on 8-epi excretion in the BTBR ob/ob mice (Fig. 4D).

**Renal Histology**

In order to characterize the effects of CP on the renal histopathology, we analyzed kidney:TL ratio, glomerular structure, macrophage accumulation and podocyte content in both BTBR WT and BTBR ob/ob mice. Kidney weight was increased in BTBR ob/ob mice, consistent with previous observations (17; Table 1) and treatment with CP significantly decreased kidney size in both groups. Both treated and untreated BTBR WT mice had normal appearing glomeruli (Fig. 5A-B) and glomerular volume (Fig. 5I) while the comparable appearance of untreated BTBR ob/ob mice show mesangiolysis, increased matrix deposition and distinct capillary ballooning (Fig. 5C), accompanied by glomerular volume expansion (Fig. 5I) and macrophage accumulation (Fig. 5G, K). Analysis of glomerular p57-positive cells in CP-treated and untreated BTBR ob/ob mice demonstrated
that CP treatment had no effect on glomerular podocyte content (5.35 ± 0.09 vs. 5.49 ± 0.08, BTBR ob/ob vs. BTBR ob/ob + CP, P=0.2258).

Renal Gene Expression and Mitochondrial DNA analysis

The metabolic burden of diabetes has been associated with mitochondrial dysfunction. We analyzed mRNA expression of genes associated with β-oxidation, namely acox1, cpt1a and cpt2, and genes associated with mitochondrial function and biogenesis, namely uncoupling protein 2 (ucp2) and sirtuin 1 (sirt1) (30, 31), from the renal cortex of treated and untreated BTBR WT and BTBR ob/ob mice. Unlike the liver, there were no changes in the expression of acox1, cpt1a and cpt2 (Fig. 6A-C) in BTBR ob/ob kidneys. However, ucp2 and sirt1 expression were increased (Fig. 6D, 6E). CP treatment increased acox1 and ucp2 in BTBR WT mice (Fig. 6A, 6D), but did not have any effects on acox1, cpt1a and cpt2 in the ob/ob. However, CP treatment abolished the elevated sirt1 expression in the BTBR ob/ob mice (Fig. 6D).

The tricarboxylic acid cycle and β-oxidation pathway are 2 major enzymatic pathways within the mitochondria, serving not only as sources of energy but of ROS production. When levels of ROS exceed mitochondrial antioxidant capacity, the resultant oxidative stress can damage mtDNA and result in the creation of base pair mutations and deletion of mutated base pairs (32). The Random Mutation and Deletion Capture Assay permit us a highly sensitive method of detection of these changes in mtDNA in the renal cortex of CP-treated WT and ob/ob mice. Mutation frequency was determined at TaqI restriction site (32). Analysis of mtDNA showed no differences in point mutation frequency (mutations per base pair (m.b.p)) in the renal cortex of BTBR ob/ob mice, compared to BTBR WT controls (3.5 ± 0.40 x 10^-6 m.b.p vs. 15.9 ± 11.9 x 10^-6 m.b.p, BTBR WT vs. BTBR ob/ob, P=0.343). CP had no effect on point mutations in either BTBR WT (3.5 ± 0.4 x 10^-6 m.b.p vs. 10.6 ± 4.9 x10^-6 m.b.p, BTBR WT vs. BTBR WT + CP, P=0.114) or in BTBR ob/ob (10.6 ± 4.92 x 10^-6 m.b.p vs. 4.4 ± 0.4 m.p.b, BTBR ob/ob vs. BTBR ob/ob + CP, P=0.5530). No differences were observed in either total mtDNA copy number in treated vs. untreated BTBR WT (1518 ± 142 vs. 1182 ± 142, BTBR WT vs. BTBR WT + CP, P=0.200) or BTBR ob/ob mice (1351 ± 116 vs. 1720 ± 92 BTBR ob/ob vs. BTBR ob/ob + CP, P=0.114), or in mtDNA deletion frequency (data not shown), nor any changes in urinary 8OH-dG in CP-treated or untreated BTBR ob/ob mice (data not shown).

Discussion

The fibrate class of PPARα agonists has been used safely to lower TG, increase HDL-C and improve cardiovascular outcomes in diabetic patients (12, 13), mainly by activating the expression of genes involved in lipid homeostasis (5). Data from the FIELD and ACCORD studies suggested beneficial effects of fibrates in DN, with fenofibrate usage associated with attenuation of microalbuminuria (12). In this study we tested the benefits of a prototypic member of a class of novel PPARα agonists in a well-characterized murine model of advanced DN, potentially delineating intrarenal and extrarenal mechanisms for its anticipated benefits. While PPARα agonists have been shown to be efficacious in ameliorating renal injury in animal models with mild or moderate structural changes of DN,
it remains unknown whether they can provide substantial benefit in more advanced disease. Our studies demonstrate beneficial effects of CP treatment for the overall diabetic state, but they are somewhat disappointing in that they did not significantly ameliorate DN within the time frame of CP intervention.

Our studies extend those previously reported for the BTBR \textit{ob/ob} mouse in demonstrating that the characteristic presence of hyperglycemia, IR, hypertriglyceridemia, inflammation and DN in this model. Additional novel findings were increases in circulating NEFA, IL-6 and decreases in adiponectin, which further characterize this strain as a comprehensive model of T2DM and obesity. In the liver, genes associated with \(\beta\)-oxidation were increased, along with PPAR\(\gamma\) expression. We also observed that in general, CP had a growth retarding influence in both BTBR WT and BTBR \textit{ob/ob} mice. This decrease in growth was not due to a lack of nutrition, as food intake (as measured by grams/cage) actually \textit{increased} in BTBR WT mice (unpublished observation). CP had no effect on food intake in BTBR \textit{ob/ob} mice, an unsurprising observation given the role of the \textit{ob} gene in control of satiety. The CP-induced decreases in growth in these mice are most likely due to general catabolic effects of PPAR\(\alpha\) activation (20), and by studies demonstrating PPAR\(\alpha\) expression in growth plate chondrocytes, whose activation closes the growth plate, hence inhibiting long bone growth (33).

CP treatment of BTBR \textit{ob/ob} mice attenuated BW, hyperglycemia, hypertriglyceridemia, systemic inflammation and IR. However, CP had no effect on plasma HDL-C, while increasing total cholesterol levels. Piperidine-derived PPAR\(\alpha\) agonists have anti-inflammatory properties, in part by decreasing pro-inflammatory cytokine expression and release (20). We analyzed the effect of CP on serum adiponectin, IL-6 and TNF-\(\alpha\), cytokines used as indicators of systemic inflammation, observing that elevated plasma IL-6 levels in BTBR \textit{ob/ob} mice were abolished by CP, without any effect on adiponectin. CP and other piperidine-derived PPAR\(\alpha\) agonists have demonstrated effects consistent with activation of PPAR\(\alpha\) (20, 21). While we do not have any direct evidence of CP directly activating PPAR\(\alpha\) in our study, the robust increases in the expression of hepatic genes associated with \(\beta\)-oxidation in CP-treated mice gives strong evidence that PPAR\(\alpha\) is indeed activated. This is supported by the decreases in inflammatory markers and the reduction in plasma glucose, as CP-activated PPAR\(\alpha\) seems to play anti-inflammatory role, mainly by decreasing acute phase response and gluconeogenic enzymes (20).

Studies of the effects of PPAR\(\alpha\) agonists in rodent models of diabetes and DN have shown varying degrees of benefit. For example, type 1 diabetic mice lacking the PPAR\(\alpha\) gene have diminished kidney function, increased glomerular damage and accelerated DN, indicating a protective role of PPAR\(\alpha\) (34). Additionally, fenofibrate treatment in Zucker diabetic fatty rats attenuated renal inflammation and tubular injury, evidenced by decreases in renal expression of monocyte chemotactic protein-1 and interstitial macrophage infiltration, ameliorating diabetes-associated kidney damage (35). Improvement of kidney damage and function was most striking in fenofibrate-treated C57 BLKS \textit{db/db} mice (36). Similar to our study, treatment of obese and diabetic mice caused hypoglycemia, amelioration of IR and attenuation of body weight. While the increases in plasma ALT/AST levels and increases in total cholesterol appear to be confounders in this study, it’s important to note that fenofibrate
also caused hepatomegaly and increases in total cholesterol levels that were not accounted for with the changes in HDL-C in C57 BLKS db/db mice (36). In addition, while CP increased ALT/AST in both BTBR WT and BTBR ob/ob mice, it did not cause any changes in glomerular histopathology in the BTBR-WT mice. CP also decreased CK levels in BTBR ob/ob mice, indicating a systemic benefit, leading us to conclude the increases in liver function parameters is something unique to rodents and not related to renal damage. This is supported by the observation that fenofibrate had beneficial effects on the renal function and histopathology associated with T2DM in C57 BLKS db/db mice. We hypothesize that the difference between this study and ours, outside of the obvious ones (treatment and study regimen, leptin receptor deficient vs. leptin-deficient mice), may be due to the persistent hyperinsulinemia present in the BTBR ob/ob mice. Insulin levels measured at the termination of the study (C57 BLKS db/db vs. BTBR ob/ob respectively) were approximately 4-5X higher in the BTBR strain, levels that are not significantly affected by CP treatment (despite the improvement of IR). When leptin-deficient (ob) is introduced into the BTBR genetic background strain, the resultant hyperglycemia and IR is more severe than when it is introduced to age and gender-matched C57BLKS mice (23). The BTBR strain has genetic deficiencies associated with both pancreatic β-cell function and peripheral insulin resistance (37-39), variables that were not affected by CP treatment. This chronic and unresolved hyperinsulinemic state, unaffected by systemic and metabolic parameters, may alter numerous signaling pathways associated with DN. Without any modification of this state, amelioration of DN may not be possible. Alternately, the 16-week-old C57 BLKS db/db mouse utilized by the prior study has only very mild structural changes of DN when compared to the marked changes of the 18-week-old BTBR ob/ob mouse (16). The advanced structural injuries of the BTBR ob/ob mouse may not be amenable to changes induced by PPARα agonists, despite improvements in diabetes-associated abnormalities.

Isoprostanes as contributors in the initiation and progression of DN

The increased risks for renal disease in diabetes are multi-factorial, with ROS being one of the primary drivers of disease progression (24-26). ROS can directly damage molecules and cells, and target those that have been irretrievably damaged for degradation, eventually leading to loss of function. In the kidney and depending on cell type, ROS can arise from a number of different mechanisms, such as glycolysis, nitric oxide synthase (NOS) dysfunction and the activation of the NADPH oxidases (24-26). Renal ROS production is balanced by a variety of antioxidant defense systems, (i.e. superoxide dismutase (SOD), catalase and thioredoxin, and their accessory proteins) and oxidative balance is integral to normal function. In the diabetic kidney, dysregulation of these pathways result in cell damage, eventually leading to DN. For example, decreased expression of cytosolic and extracellular SOD was observed in mice susceptible to DN, when compared to DN-resistant mice (40) and eNOS deficiency, perhaps leading to excess oxidative stress, can produce an accelerated nephropathy (41). Conversely, renal expression of the endogenous inhibitor of thioredoxin, the thioredoxin interacting protein, was increased in the streptozotocin-induced, type 1 diabetes (mRen-2) 27 mouse (42), an increase that was associated with DN. In both models, these changes led to increases in ROS production. We observed elevated ROS production in the glomeruli of BTBR ob/ob mice (18), and preliminary analyses of these
kidneys demonstrated decreased expression SOD (unpublished observation), indicating that dysregulation of antioxidant balance and resultant oxidative stress is the cause of kidney dysfunction. Therefore, CP, despite removing what are considered to be the main systemic drivers of ROS (elevated glucose, lipids and increased inflammation), by increasing elevated fatty acid oxidation in the BTBR ob/ob kidney, essentially replaces one source of ROS with another. Some of the effects of oxidative stress are mediated by the ROS-mediated modifications of arachidonic acid (AA) (28, 29, 43). These urinary metabolites of AA, such as the F2-isoprostanes (F2-Isops), have been used in numerous studies as an indicator of oxidative stress (29). The best-characterized member of the F2-Isops, 8-epi, is also purported to be a direct modulator of renal function, acting via the thromboxane A2 receptor (TP) (44), activation of which can lead to NOS uncoupling, renal hyperfiltration, albuminuria and glomerular damage (44-46). In fact, the selective TP receptor antagonist, S1886 (Terutobran), has been shown to attenuate renal damage in a rodent models of hypertension (47), type 1 (48) and type 2 diabetes (49). In this study, we show elevated renal excretion of 8-epi, but not 8OH-dG, a marker of oxidative-induced DNA damage and a putative risk factor for diabetes (50, 51). The increase in 8-epi can activate glomerular TP, and via sensitization of the tubuloglomerular feedback mechanism, increase glomerular filtration pressure and albumin excretion (44). The fact that CP increased 8-epi and albuminuria, without having any effects on urinary creatinine, in lean, non-diabetic control mice without any changes in glomerular histology supports the hypothesis that it is a direct effect of 8-epi on the glomerular filtration that results in albuminuria. Whether the production and release of renal isoprostanes in this model is involved in initiation and progression of DN remains to be elucidated. Another possible explanation underlying the albuminuric effects CP in BTBR WT mice involves the role of PPARα in the regulation of renal RAAS and intrarenal hemodynamics. It is possible that, in the kidney, PPARα may serve as a mediator of the RAAS. In the Tsukuba hypertensive mouse, a model of angiotensin II-dependent hypertension, administration of fenofibrate, contrary to most observations on the effect of fibrates in models of hypertension, worsened systemic blood pressure (52). The Tsukuba hypertensive mouse has high plasma renin activity, with elevated levels of angiotensin II and aldosterone. This observation is not consistent with a significant number of studies demonstrating an ant-hypertensive effect of PPARα activation (53-55). We’ve observed that BTBR ob/ob when compared to their their WT counterparts (18), which can be attributed to the lack of leptin (56). Does CP increase systemic or intrarenal pressure in the BTBR strain? It is possible that the activation with PPARα with CP can modulate vascular tone in renal arterioles and influence filtration pressure, leading to increase in albumin excretion. Additional studies, either using TP receptor antagonists or by isolating and analyzing a possible RAAS-PPARα axis in the BTBR strain will be required to provide definitive answers to these questions.

In summary, treatment with CP had many beneficial effects in the BTBR ob/ob mouse, reducing plasma triglycerides and many of the hallmarks of type 2 diabetes. Despite the improvements in metabolic parameters, CP did not ameliorate nephropathy, albuminuria, glomerular hypertrophy, mesangial matrix expansion, glomerular macrophage content, podocyte loss and renal ROS production. However, the failure of CP on preventing the development of DN, while disappointing, can gives us further insight on factors that may
mediate the renal complication of diabetes. Based on our results, we propose that
dysfunctional renal antioxidant mechanisms, one perhaps regulated by insulin-dependent
pathways, result in elevations in the production of renal F\textsubscript{2}-Isops. Additionally, the
production of these metabolites may serve as an important contributor to the renal
complication of diabetes in the BTBR ob/ob mouse, and therapies targeted to the treatment
of DN should focus on decreasing either the production or the action of renal F\textsubscript{2}-Isops.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| ACCORD       | Action to Control Cardiovascular Risk in Diabetes |
| ALT          | alanine aminotransferase |
| AST          | aspartate aminotransferase |
| BTBR         | Black and Tan, Brachyuric |
| BW           | body weight |
| CP           | CP-900691 |
| CK           | creatine kinase |
| DN           | Diabetic nephropathy |
| 8-epi        | 8-epi-PGF\textsubscript{2α} |
| 8OH-dG       | 8-hydroxy 2-deoxy-guanosine |
| FIELD        | Fenofibrate Intervention and Event Lowering in Diabetes |
| HDL-C        | high-density lipoprotein-cholesterol |
| IR           | insulin resistance |
| ITT          | insulin tolerance tests |
| IL-6         | interleukin 6 |
| MMPC         | Mouse Metabolic Phenotyping Center |
| NOS          | nitric oxide synthase |
| NEFA         | non-esterified fatty acids |
PPAR  peroxisome proliferator activated receptors
ROS   reactive oxygen species
SOD   superoxide dismutase
TZD   thiazolidinedione
TP    thromboxane A₂ receptor
TL    tibia length
TG    triglycerides
TNF-α tumor necrosis factor-α
T2DM  type 2 diabetes mellitus
RAAS renin-angiotensinaldosterone system

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Figure 1. CP-900691 reduces body weight in female BTBR WT and BTBR ob/ob mice
(A) Study design. Three-week old female BTBR WT and BTBR ob/ob mice were fully weaned onto standard chow diet for one week. The diet was then changed into standard chow with and without CP (3 mg/kg/day, closed arrow) for an additional 14 weeks. (B) Mean body weight during and at the end of the study are shown (BTBR WT, open circles, n=10; BTBR WT + CP, closed circles, n=9; BTBR ob/ob, open squares, n=9; BTBR ob/ob + CP, closed squares, n=9). Results are shown as means ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni post-test. *P < 0.05 vs. BTBR; #P < 0.05 vs. BTBR ob/ob.
Figure 2. CP-900691 normalizes blood glucose and improves insulin sensitivity in BTBR ob/ob mice

4 week-old BTBR WT and BTBR ob/ob mice were placed on standard chow diet with and without CP (3 mg/kg/day) for 14 weeks. (A) Blood glucose levels during the 14-week study are shown (BTBR WT, open bars, n=9; BTBR WT + CP, striped bars, n=9; BTBR ob/ob, closed bars, n=9; BTBR ob/ob + CP, cross-hatched bars, n=9). ITT were performed in 18-week-old treated and untreated BTBR ob/ob mice (as described in Methods) and (B) plasma glucose (BTBR ob/ob, open squares, n=9; BTBR ob/ob + CP, closed squares, n=9) and (C) area under the curve (AUC) were analyzed. Statistical analysis was performed using two-way (A, B) and one-way (C) ANOVA followed by Bonferroni (A, B) and Newman-Keuls (C) post-tests. Results are shown as means ± SEM (A, B) *P < 0.05 and **P < 0.01 vs. BTBR WT; #P < 0.05, ##P < 0.01 vs. BTBR ob/ob.

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Figure 3. CP-900691 increases in the expression of genes associated with β-oxidation in the RNA extracted from livers of BTBR WT and BTBR ob/ob mice.

RT-qPCR was used to determine the relative expression of genes associated with β-oxidation. BTBR WT; open bars, (n=4, both untreated and CP-treated mice), BTBR ob/ob; closed bars (n=4, both untreated and CP-treated mice). When statistically justified, gene expression values were log-transformed before statistical analysis was performed. Results are shown as means ± SEM. Statistical analysis was performed via one-way ANOVA followed by Newman-Keuls post-test. *P < 0.05 and **P < 0.01, vs. BTBR WT; #P < 0.05 vs. BTBR ob/ob.
Figure 4. CP-900691 alters albuminuria and urinary ROS excretion in BTBR WT, but not in BTBR ob/ob mice

BTBR WT and BTBR ob/ob were treated with or without CP for 14 weeks (BTBR WT, n=8; BTBR ob/ob, n=9; BTBR WT + CP, n=9; BTBR ob/ob + CP, n=9). Urinary volume (A), daily albumin (B), albumin:creatinine ratio (ACR) (C) and 8-epi concentrations (D) were measured as described in Methods (BTBR; open bars, BTBR ob/ob; closed bars). Results are shown as means ± SEM. Statistical analysis was performed via one-way ANOVA followed by Newman-Keuls post-test. *P < 0.01 vs. BTBR WT; #P < 0.05 vs. BTBR ob/ob.
Figure 5. CP-900691 does not ameliorate altered glomerular histopathological changes or elevated macrophage content in the BTBR ob/ob mice

Representative photomicrographs of normal-appearing glomeruli from CP-treated and untreated BTBR WT mice (silver methenamine staining, A, B). Glomerulus of BTBR ob/ob mouse at 18 weeks, showing mesangiolysis (double arrows) and capillary ballooning (single arrow) (C). Comparable glomeruli of CP-treated BTBR ob/ob mouse show mesangiolysis and capillary ballooning (double and single arrows, respectively) (D). Glomerular macrophage accumulation, as analyzed by the macrophage-specific antibody Mac-2 (E-H, K) in BTBR ob/ob mice, compared to BTBR WT (G vs. E, respectively). CP treatment has no effect on macrophage content in BTBR WT or BTBR ob/ob glomeruli (E-H).

Glomerular volume (I), mesangial matrix deposition (J) and macrophage content (K) in CP-treated BTBR WT (open bars; untreated BTBR WT, n=9, BTBR WT + CP, n=9) and BTBR ob/ob (closed bars; untreated BTBR ob/ob, n=9, BTBR ob/ob + CP, n=9) kidneys were quantified as described in Methods. Results are shown as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls post-test. *P < 0.05 vs. BTBR WT; †P < 0.05 vs. BTBR ob/ob.
Figure 6. CP-900691-induced effects in the expression of genes associated with β-oxidation and mitochondrial homeostasis in RNA extracted from the renal cortex of BTBR WT and BTBR ob/ob mice

RT-qPCR was used to determine the relative expression of genes associated with β-oxidation and mitochondrial homeostasis. BTBR WT, open bars (BTBR WT; n=4, BTBR WT + CP; n=4) BTBR ob/ob, closed bars (BTBR ob/ob; n=5, BTBR ob/ob + CP; n=5). Results are shown as means ± SEM. When statistically justified, gene expression values were log-transformed prior to statistical analysis. Statistical analysis was performed via one-way ANOVA followed by Newman-Keuls post-test. *P < 0.05 vs. BTBR WT; #P < 0.05 vs. BTBR ob/ob.
Table 1

Effect of CP-900691 on body and tissue weights in BTBR WT and BTBR ob/ob mice.

|                        | BTBR WT (n=9) | +CP-900691 (n=9) | P     | BTBR ob/ob (n=9) | +CP-900691 (n=9) | P     |
|------------------------|---------------|------------------|-------|------------------|------------------|-------|
| Total body weight (g)  | 28.9 ± 0.6    | 19.6 ± 0.5       | <0.001| 57.8 ± 1.7       | 49.9 ± 2.0       | <0.001|
| Lean bodyweight (g)    | 20.7 ± 0.5    | 10.7 ± 0.3       | <0.001| 36.7 ± 1.0       | 26.7 ± 1.3       | <0.001|
| Tibia Length (mm)      | 17.4 ± 0.2    | 16.2 ± 0.1       | <0.0001| 16.8 ± 0.1       | 15.9 ± 0.2       | 0.0002|
| Kidney (g/m)           | 24.4 ± 0.5    | 20.4 ± 0.5       | <0.0001| 46.6 ± 1.4       | 39.6 ± 2.3       | 0.0185|
| Liver (g/m)            | 97.7 ± 3.9    | 238.8 ± 5.1      | <0.0001| 213 ± 12.6       | 340 ± 10.8       | <0.0001|
| Epididymal (g/m)       | 31.9 ± 2.9    | 3.3 ± 0.7        | <0.0001| 168.1 ± 8.8      | 161.3 ± 14.8     | 0.6981|
| Retroperitoneal (g/m)  | 5.8 ± 0.4     | 1.0 ± 0.2        | <0.0001| 90.1 ± 5.6       | 81.1 ± 8.5       | 0.3918|
| Subcutaneous (g/m)     | 16.2 ± 1.2    | 8.6 ± 0.6        | <0.0001| 158.1 ± 11.4     | 121.6 ± 9.5      | 0.0262|
| Subscapular (g/m)      | 7.0 ± 0.7     | 3.0 ± 0.3        | <0.0001| 75.3 ± 8.5       | 58.4 ± 6.7       | 0.1504|

Total, lean and Tissue weights were weighed at week 18 of the study in CP-treated and untreated BTBR WT and BTBR ob/ob mice, normalized to tibia lengths and presented as grams/meter. Data is presented as mean ± SEM. Data was analyzed by Student’s t-test.
Table 2

Effect of CP-900691 on blood and serum markers BTBR WT and BTBR ob/ob mice.

|                  | BTBR WT (n=9) | +CP-900691 (n=9) | P    | BTBR ob/ob (n=9) | +CP-900691 (n=9) | P    |
|------------------|---------------|------------------|------|------------------|------------------|------|
| Glucose (mg/dL)  | 147 ± 4.4     | 125.9 ± 11.1     | 0.0713 | 373 ± 48.1      | 181 ± 10.9      | 0.0023 |
| Insulin (ng/ml)  | 0.50 ± 0.04   | 0.20 ± 0.06      | **0.0003** | 16.3 ± 3.9    | 10.2 ± 1.3      | 0.1355 |
| TG (mg/dL)       | 74.4 ± 6.8    | 39.9 ± 1.6       | **0.0001** | 124.3 ± 11.9   | 47.3 ± 9.2      | **0.0001** |
| NEFA (mEq/L)     | 0.49 ± 0.05   | 0.41 ± 0.03      | 0.2120 | 1.01 ± 0.23     | 0.43 ± 0.06     | **0.0224** |
| HDL-C (mg/dL)    | 71.8 ± 1.6    | 105.3 ± 1.9      | **0.0001** | 133.5 ± 11.4   | 129 ± 64        | **0.7277** |
| TG (mg/dL)       | 96.1 ± 2.6    | 183.6 ± 7.7      | **0.0001** | 205 ± 24.2     | 310.3 ± 22.6    | **0.0062** |
| Adiponectin (ng/ml) | 2.21 ± 0.18  | 3.31 ± 0.22      | **0.0012** | 1.59 ± 0.05    | 1.40 ± 0.13     | 0.1947  |
| IL-6 (pg/ml)     | 3.89 ± 1.1    | 2.45 ± 0.25      | 0.2240 | 34.49 ± 14.8    | 8.42 ± 3.3      | **0.0152** |
| TNF-α (pg/ml)    | 1.93 ± 0.2    | 2.30 ± 0.2       | 0.401 | N.D              | N.D             | ---    |
| ALT (U/L)        | 30.1 ± 3.9    | 74.2 ± 6.8       | **0.0001** | 47.9 ± 4.0     | 77.6 ± 3.4      | **0.0036** |
| AST (U/L)        | 44.2 ± 0.05   | 304.9 ± 36.3     | **0.0003** | 43.2 ± 3.4     | 107.4 ± 15.6    | < **0.0001** |
| CK (U/L)         | 51.4 ± 16.8   | 114.7 ± 29.9     | 0.1328 | 173.2 ± 11.4    | 43.7 ± 14.7     | **0.0046** |

TG; triglycerides, NEFA; non-esterified fatty acids, HDL-C; high-density lipoprotein cholesterol, TC; total cholesterol, IL-6; interleukin 6, TNF-α; tumor necrosis factor-alpha, ALT; alanine aminotransferase, AST; aspartate aminotransferase, CK; creatine kinase. Data is presented as mean ± SEM. Data was analyzed by Student’s t-test. N.D not determined; levels below the limits of detection of the assay.