

PPAR α/γ dual agonist tesaglitazar attenuates diabetic nephropathy in db/db mice

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Abstract

Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors and play a central role in insulin sensitivity, lipid metabolism, and inflammation. Both PPAR α and PPAR γ are expressed in the kidney and their agonists exhibit renoprotective effects in type 2 diabetes. In the present studies we investigated the effect of PPAR α/γ dual agonist tesaglitazar on diabetic nephropathy in type 2 diabetic db/db mice. Treatment of db/db mice with tesaglitazar for 3 months significantly lowered fasting plasma glucose and HOMA-IR levels, but had little effect on body weight, adiposity or cardiac function. Treatment with tesaglitazar was associated with reduced plasma insulin and total triglyceride levels and increased plasma adiponectin levels. Notably, tesaglitazar markedly attenuated albuminuria and significantly lowered glomerulofibrosis, collagen deposition, and TGF β 1 expression in renal tissues of db/db mice. In cultured mesangial cells and proximal tubule cells where both PPAR α and PPAR γ were expressed, tesaglitazar treatment abolished high glucose-induced total collagen protein production and type I and IV collagen gene expression. Collectively, tesaglitazar treatment not only improved insulin resistance, glycemic control and lipid profile, but also markedly attenuated albuminuria and renal glomerular fibrosis in db/db mice. These findings support the utility of dual PPAR α/γ agonists in treating type 2 diabetes and diabetic nephropathy.

INTRODUCTION

Type 2 diabetes mellitus is characterized by hyperglycemia, insulin resistance, and progressive loss of pancreatic β -cell function during the disease process (1). In recent years, synthetic ligands of α and γ subtypes of peroxisome proliferator activated receptors (PPARs) have been reported to have beneficial effects in type 2 diabetes mellitus (2-4). The fibrate class of PPAR α agonists are hypolipidemic agents and also suppresses atherogenic processes including vascular inflammation, plaque instability and thrombosis (5). On the other hand, PPAR γ agonists including various thiazolidinediones, improve glycemic control by enhancing insulin sensitivity in peripheral insulin sensitive tissues and also have been shown to be potent anti-diabetic, antifibrotic and anti-inflammatory agents in patients with type 2 diabetes (6). Importantly, both PPAR α and PPAR γ agonists exert renal protective effects in type 2 diabetic animals (7-10). These beneficial actions of PPAR α and PPAR γ agonists support the idea that combination of PPAR α and PPAR γ agonist may have additive or synergistic benefits on metabolic control and vascular complications in type 2 diabetes mellitus. Moreover, several studies report that PPAR α agonist may mitigate the weight gain in rodent model of insulin resistance without affecting food intake (11; 12). These observations suggest the possibility that dual activation of PPAR α and PPAR γ may eliminate the undesirable side effect of PPAR γ agonist especially weight gain.

Recently, a number of PPAR α/γ dual agonists have been or remain under evaluation for their efficacy in animal models of insulin resistance and in type 2 diabetic patients (13-18). However, most studies have focused on the lipid lowering effect and metabolic alterations, and little is known about renal protective effect of PPAR α/γ dual agonist in diabetic nephropathy. In present studies, we examined the effects of a PPAR α/γ dual agonist, tesaglitazar, on renal function and histological changes in a type 2 diabetic

db/db mouse model.

RESEARCH DESIGN AND METHODS

Animal studies

Eight-week old male C57BKS/J db/db mice were purchased from the Jackson Laboratory. Mice were maintained on a standard rodent diet until 5 months of age when they were treated with tesaglitazar. Because the primary aim of the study was to evaluate the efficacy of tesaglitazar in fully developed diabetic nephropathy instead of the preventive role of the drug in the progression of diabetic nephropathy, we started to administer drugs at 5 months of age. One group (n=5) of mice was gavaged with tesaglitazar (a gift from Dr. Eric Lundholm, AstraZeneca) at 1 μ mol/kg/day suspended in 0.5% methylcellulose. This dose of tesaglitazar is sufficient for normalization of hyperglycemia and hypertriglyceridemia in ob/ob mice and increased the whole body insulin sensitivity in obese Zucker rat (13). The second group (control group, n=5) was gavaged with vehicle alone. During the experiment, food consumption, water intake, urine volume and plasma insulin levels were measured every month. Body weight, fasting plasma glucose concentration and HbA1C level were measured every 2 weeks. HbA1C, plasma glucose and insulin levels, blood urea nitrogen (BUN), electrolytes, hematocrit, and serum creatinine, triglyceride and cholesterol levels were analyzed as previously reported (8; 19; 20). Lipoprotein profile was measured by a FPLC system at the Lipid/Lipid Peroxidation Core at Vanderbilt University. Plasma adiponectin levels were measured by RIA kit (Linco Research) Homeostasis model assessment index (HOMA-IR) was calculated using fasting glucose (mmol/L) x fasting insulin (mU/L) / 22.5. Urinary albumin concentration was measured using ELISA method (Albuwell kit, Exocell Inc.).

Light microscopy, immunohistochemistry and islet area measurement

The kidney tissues embedded in paraffin were cut into 4 μm thick slices and were stained with Periodic Acid-Schiff (PAS). Immunohistochemistry was performed for type IV collagen. A semi-quantitative score for a sclerosis index (SI) was used to evaluate the degree of glomerulosclerosis on PAS-stained sections. Sclerosis was defined as collapse and/or obliteration of the glomerular capillary tuft, accompanied by hyaline material and/or an increase in the matrix. The severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, sclerosis of 25 to 50%, 51 to 75%, and >75% of the glomerulus, respectively. A whole-kidney average SI was obtained from specimens by averaging scores from all glomeruli on each section in each group. Histologic examination was carried out by a pathologist in a blinded manner, according to previously described methods (21). More than 100 glomeruli were analyzed in kidney sections of each mouse.

For immunohistochemical staining, renal tissues were cut into 4 μm thick sections and the slides were then incubated overnight at 4⁰C with primary antibody against rabbit polyclonal anti-type IV collagen antibody (1:150, Santa Cruz). For the evaluation of type IV collagen staining, glomerular fields were graded semi-quantitatively. Briefly, each score reflects both changes in the extent and the intensity of staining and graded on a five scale basis: Grade 0, very weak or absent staining and no localized increases of staining; Grade 1, diffuse, weak staining with 1% to 25% of the glomerulus showing focally increased staining; Grade 2, 26% to 50% of the glomerulus demonstrating focal, strong staining; Grade 3, 51% to 75% of the glomerulus stained strongly in a focal manner; Grade 4, more than 75% of the glomerulus stained strongly. From more than 60 glomeruli were counted under high power (x 400) and an average score was calculated. Each slide was scored by pathologist blinded

to the experimental conditions.

Total islet area was measured in PAS stained sections using a NIH image analysis system (Scion Image beta 4.02 Win) equipped with digital camera through Olympus microscope (IX 81, Olympus America Inc.). More than ten fields were examined under high power (x 400) and islet area was traced and total islet area was calculated and expressed as average score.

Culture of mouse mesangial and proximal tubule cells

Mesangial cells (MC) were isolated from PPAR α gene deficient (-/-) mice and db/db mice using standard sieving method and MCT cells, a murine line of proximal tubule cells (PTCs) were cultured as previously reported (22; 23). To evaluate the effect of tesaglitazar on collagen synthesis under high glucose condition, sub-confluent MC and MCT were serum-starved for 24 hours, and then tesaglitazar was administered at a final concentration of 30 μM to the culture media containing 30mM of glucose concentration. All experimental groups were cultured in triplicate and harvested at 24hours for extraction of total RNA and protein.

Expression of PPAR α and γ in cultured renal cells

Expression of PPAR α and PPAR γ in MCs and PTCs was determined using RT-PCR and immunoblots. Messenger RNA levels were detected using RT-PCR using the following primers: 5' GAATTTGCCAAGGCTATCCCA3' (sense primer for PPAR α), 5' ATGATGTCACAGAACGGCTTC 3' (antisense primer for PPAR α), 5' CCGAAGAACCATCCGATTGA 3' (sense primer for PPAR γ) and 5' CGGGAAGGACTTTATGTATGA 3' (antisense primer for PPAR γ). Immunoblots were utilized to determine protein expression levels of PPAR α and PPAR γ using a rabbit anti-PPAR α and a rabbit anti-PPAR γ antibody (SC-9000 & SC-7273, Santa Cruz), respectively. To determine the endogenous

PPAR transcriptional activity and effect of tesaglitazar on TGF β -induced transcriptional activation, PPRE-luciferase reporter and 3TP-luciferase reporter activity were measured in cultured MCs and PTCs as previously reported (24).

Analysis of gene expression by real-time quantitative PCR

Primers were designed from the respective gene sequences using the Primer 3 software, and templates secondary structures were examined and excluded using an mfold software program. The sequence of each primer was as follows: pro-collagen α 1 chain of type I collagen, forward: 5' CCA AAG GTG CTG ATG GTT CT 3', reverse: 5' ACC AGC TTC ACC CTT GTC AC 3'; pro-collagen α 1 chain of type IV collagen, forward: 5' GCT CTG GCT GTG GAA AAT GT 3', reverse: 5' CTT GCA TCC CGG GAA ATC 3'; TGF β 1, forward: 5' AGC CCG AAG CGG ACT ACT AT 3', reverse: 5' CTG TGT GAG ATG TCT TTG GTT TTC 3'; adiponectin, forward: 5' TGT TGG AAT GAC AGG AGC TGA A 3', reverse: 5' CAC ACT GAA GCC TGA GCG ATA C 3'; β -actin, forward: 5' GGA CTC CTA TGT GGG TGA CG 3', reverse: 5' CTT CTC CAT GTC GTC CCA GT 3'; The amplicon length of each gene was as follows: pro-collagen α 1 chain of type I collagen, 107bp; pro-collagen α 1 chain of type IV collagen, 102bp; TGF β 1, 96bp; adiponectin, 121bp; β -actin, 103bp. Quantitative gene expression was performed on a Bio-Rad iCycler system (Bio-Rad) using SYBR Green technology. Total mRNA was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed and the mRNA level of each sample was normalized to that of β -actin mRNA. The ratio of each gene to β -actin level (relative gene expression number) was calculated by subtracting the threshold cycle number (Ct) of the target gene from that of β -actin and raising 2 to the power of this difference. Ct values are defined as the number of PCR cycles in which the fluorescent signal during the PCR reaches a

fixed threshold.

Measurement of secreted collagen in cultured MCs and PTCs

Total soluble collagen was measured in culture supernatants by the SircolTM soluble collagen assay kit (Biocolor, Belfast, N.Ireland) following the manufacturer's instructions. The calibration curve was set up using the collagen standard provided by the manufacturer.

Statistical analysis

We used non-parametric analysis due to a few sample numbers. Results were expressed as mean \pm S.E. A Kruskal-Wallis test was used for comparison of more than two groups, followed by a Mann-Whitney U test for comparison using a microcomputer-assisted program with SPSS for Windows 10.0 (SPSS Inc.). A P<0.05 was considered statistically significant.

RESULTS

Physical parameters in experimental animals

Physical parameters between tesaglitazar treated and untreated group were compared (Table 1). Although water intake and urine volume were initially not different between two groups, their levels were decreased in the tesaglitazar treated group following one month of treatment. Interestingly, food intake and body weight did not differ throughout the study.

Effect of tesaglitazar on metabolic parameters in experimental animals

Tesaglitazar treatment markedly improved glycemic control. Within two weeks of treatment, fasting plasma glucose levels were significantly decreased, and this hypoglycemic effect persisted throughout the study (Figure 1A). In accordance with plasma glucose levels, HbA1C levels were also markedly improved in the tesaglitazar treated group (3.92 \pm 0.09 v.s.8.84 \pm 0.36%, p<0.01) (Figure 1B). In addition, at the end of studies plasma insulin levels were markedly

decreased in the tesaglitazar treated group compared with control group (tesaglitazar group, 1.98 ± 0.33 vs. control group, 6.37 ± 4.4 $\mu\text{g/ml}$, $p < 0.05$) (Figure 1C). Similarly, HOMA-IR indexes were dramatically improved after tesaglitazar treatment (Figure 1D).

Tesaglitazar treatment markedly decreased triglyceride levels, whereas HDL-cholesterol levels were significantly increased compared with untreated control group (Supplemental table 1). It was also noticed that total cholesterol and LDL-cholesterol levels were significantly increased following tesaglitazar treatment. Tesaglitazar treatment did not change heart and kidney weight (Supplemental table 2). As expected, liver was significantly heavier in the tesaglitazar treated group without significant histological change (Supplemental figure 1), consistent with the effect of hepatic PPAR α activation. Surprisingly, epididymal fat mass did not differ between two groups, whereas pancreatic weight was significantly reduced in the tesaglitazar treated group compared to the control group (Supplemental table 2).

Tesaglitazar did not alter plasma K^+ concentration, blood urea nitrogen (BUN), creatinine or hematocrit levels between two groups at the end of study (Supplemental table 3). However, plasma sodium (Na^+) and chloride (Cl^-) levels were significantly higher in the tesaglitazar treatment group.

Effect of tesaglitazar on pancreatic islet hypertrophy in db/db mice

Tesaglitazar treatment significantly reduced islet hypertrophy in C57BKS db/db mice (control group, 100 ± 26.3 vs. tesaglitazar group, 38.4 ± 10.2 , $p < 0.05$) (Figure 2).

Effect of tesaglitazar on adipose expression and plasma levels of adiponectin in db/db mice

To elucidate the possible mechanism by which tesaglitazar improves insulin resistance, adiponectin mRNA levels in adipose tissues and plasma adiponectin levels

were measured. As shown in figure 3, adiponectin gene expression was dramatically up-regulated by tesaglitazar treatment in the adipose tissues (Figure 3A), which was in agreement with significantly increased plasma levels of adiponectin (Figure 3B).

Effect of tesaglitazar on renal function and glomerulosclerosis

Baseline daily urinary albumin excretion was markedly increased in C57 BKS db/db mice at 5 months of age in both groups (control group, 241 ± 67 vs. pre-tesaglitazar group, 230 ± 65 $\mu\text{g/day}$). In the control group, albuminuria was persistently increased and at the end of the study albuminuria reached $659 \pm 247 \mu\text{g/day}$. However, tesaglitazar treatment markedly reduced urinary albumin excretion from 230 ± 65 to $98 \pm 47 \mu\text{g/day}$ (Figure 4A). In terms of renal function, tesaglitazar group trended to have slightly higher levels of both BUN and serum creatinine than the control group, but no statistical difference in serum creatinine levels was observed (control group, 0.52 ± 0.11 vs. tesaglitazar group, $0.92 \pm 0.28 \text{mg/dl}$, $p > 0.05$) (Supplemental table 3).

Consistent with the marked attenuation of albuminuria, glomerulosclerosis was significantly reduced in the tesaglitazar treatment group (Figure 4B & D). Furthermore, immunostaining score for type IV collagen, a major extracellular matrix component of fibrotic glomeruli, also demonstrated dramatic improvement in the tesaglitazar treated group (Figure 4C & E).

To further characterize the molecular mechanism of beneficial effect of tesaglitazar on the kidney, we quantified mRNA levels for profibrotic factors including type I collagen, type IV collagen and TGF β 1. Gene expression of all of these profibrotic molecules was down-regulated by tesaglitazar treatment (figure 5).

Endogenous PPAR α and PPAR γ transcriptional activity in cultured renal cells

To test the cytotoxic effect of tesaglitazar, an

