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# Peroxisome proliferator-activated receptor δ downregulates the expression of the receptor for advanced glycation end products and pro-inflammatory cytokines in the kidney of streptozotocin-induced diabetic mice

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ABSTRACT

Activation of peroxisome proliferator-activated receptor δ (PPARδ) plays board beneficial effects in treating metabolic syndrome. The aim of this study is to examine whether PPARδ alters the expression of the receptor for advanced glycation end products (RAGE) and downstream pro-inflammatory cytokines in diabetic nephropathy. Streptozotocin-induced diabetic mice (STZ mice) were injected with a PPARδ agonist, L-165041 (5 μM/kg, intraperitoneal) once daily for 10 days and high glucose-treated cultured HEK cells were also used. After L-165041 treatment, serum TNFα, IL-6 and IL-1 levels were significantly decreased in STZ mice. RAGE mRNA and protein expression were both decreased by L-165041 in kidney tissues of STZ mice. The high glucose incubation increased NF-κB, RAGE and IL-6 expressions in HEK293 cells. These effects were inhibited by L-165041 and specific RAGE siRNA transfection. This study demonstrated that PPARδ may play a beneficial role in preventing diabetic nephropathy. Its downstream signaling may include RAGE and NF-κB pathway. Target on PPARδ will provide new meaningful therapies to patients with diabetic nephropathy.



## 1. Introduction

Diabetic nephropathy is one of the leading causes of end-stage renal disease. Development of diabetic nephropathy is associated with a considerable increase in morbidity and mortality (Kern et al., 2001). Hyperglycemia-induced oxidative stress and advanced glycation end products (AGEs) have been implicated in the onset and progression of diabetic nephropathy (Pradhan et al., 2001; Rotimi et al., 2010). The actions of AGEs are mediated through both a non-receptor-mediated pathway and a specific receptor for AGEs (RAGE). The RAGE has been proposed to play a key role in the development of diabetic renal changes in experimental diabetic animals (Yamamoto et al., 2001).

It has been found that PPARδ activation induces various physiologic and pathophysiologic activities, including effects on reproduction, mast cell immunity, bone formation, skin and brain development, wound healing, and tumorigenesis (Guan and Breyer, 2001). Unlike PPARα or PPARγ, PPARδ seems to be ubiquitously expressed at low levels in almost all tissues examined. In the kidney, PPARδ exists in every part of the nephron with higher levels in glomerulus, cortical collecting ducts, and inner medullary collecting ducts (IMCD), and with lower levels in the outer medullary collecting ducts (Guan et al., 1997). The biological role and function of PPARδ remain relatively unclear. PPARδ may play an important role in renal metabolic adaptation to fasting and refeeding, which suggests its involvement in metabolic kidney diseases such as diabetic nephropathy (Guan, 2004). However, the underlying mechanism is still unclear.

In the present study, we demonstrated the relationship among PPARδ, AGE, and RAGE. The effects of PPARδ in kidney tissue of the streptozotocin-induced diabetic mice and the possible signaling mechanisms between PPARδ and RAGE in the glucose-stimulated kidney cells were investigated.

## 2. Materials and methods

### 2.1. Streptozotocin-induced diabetic mice protocol

Male BALB/c mice aged 5 weeks were divided into three groups: streptozotocin-induced diabetic mice (STZ mice, $n = 5$); STZ mice treated by PBS (STZ + PBS, $n = 5$); and STZ mice treated by a PPARδ agonist, L-165041 (STZ + L-165041, $n = 5$). Mice were injected with STZ (250 mg/kg, intraperitoneal) in citrate buffer (pH 4.5). Blood glucose levels were determined 7 days after STZ injection and only mice with blood glucose concentrations more than 16 mmol/L were used in the following study. The mice in STZ + PBS and

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STZ + L-165041 groups started to receive PBS or L-165041 injection (5 μM/kg, intraperitoneal) on the day of hyperglycemia, respectively. The treatment of L-165041 was modified according to the method of Dr. Pang (Pang et al., 2009). L-165,041 displays ⩾100-fold selectivity for PPARδ compared with other PPAR receptors, and has biological actions on insulin and glucose metabolism (Berger et al., 1999). All mice were killed after treatment for 10 days. The kidneys were harvested and weighed. The investigations conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. The STZ and L-165041 were bought from Sigma.

### 2.2. Cell Culture and reagents

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified eagle medium (DMEM) from Gibco (Grand Island, NY) containing 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The experiments were performed by using HEK293 after 2-4 passages. The effects of 1 μM PPARδ agonist (L-165041) from Sigma were evaluated by treating HEK293 before exposure to high glucose concentrations (25 mM) (Liang et al., 2010).

### 2.3. Measurement of tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-1 (IL-1)

The serum was collected from diabetic mice after different treatment and the supernatants of cultured cells were treated by high glucose for 18 h with or without L-165041. The TNF-α, IL-6 and IL-1 levels in mouse serum or culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits (Peprotech Inc., CA). Serum and supernatants were diluted 1:20 before measurement. The reaction products were measured at 450 nm wavelength with a microplate reader.

### 2.4. RNA and protein extraction

Total cellular RNA was isolated from kidney tissue or HEK293 cells using the single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. For preparing total protein extracts, cells were homogenized in modified RIPA buffer. The samples were centrifuged at 14,000*g* for 15 min, and the resulting supernatants were collected as total protein extracts. The protein concentrations were determined by a BioRad protein assay kit.

### 2.5. Western blot analysis

Total protein samples were mixed with sample buffer, boiled for 5 min, separated by 10% SDS–PAGE under denaturing conditions, and electroblotted to nitrocellulose membranes (Amersham Pharmacia Biotech, UK). The nitrocellulose membranes were blocked in blocking buffer, incubated with anti-RAGE or anti-PPARδ (Santa Cruz Biotechnology Inc., CA) antibodies, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies. Signals were visualized by enhance chemiluminescent detection.

### 2.6. Real-time PCR

The cDNA had a 10-fold dilution in nuclease-free water and was used for the Smart Quant Green Master Mix (Protech Technology Enterprise Co., TW): 2 μl of cDNA solution, 0.5 μmol/l primers, 5 mmol/l magnesium chloride, and 2 μl of Master SYBR-Green in nuclease-free water with a final volume of 20 μl. The primers used for PCR were: PPARδ: forward, 5′ACGCTATCCGTTTGGTCG3′, reverse, 5′CTCACGGGTGACAAAGCC3′; RAGE: forward, 5′AAGCCCC TGGTGCCTAATGAG3′, reverse, 5′CACCAA TTGGACCTC CTCCA3′; GAPDH: forward, 5′CGACCACTTTGT CAAGCTCA3′, reverse, 5′AGGG GTCTACATGGCAACTG3′. The initial denaturizing phase was 5 min at 95 °C followed by an amplification phase as detailed below: denaturation at 95 °C for 10 s; annealing at 55 °C for 10 s; elongation at 72 °C for 15 s; detection at 79 °C and for 45 cycles. Amplification, fluorescence detection, and post-processing calculation were performed using the ABI StepOne apparatus. Individual PCR product was analyzed for DNA sequence to confirm the purity of the product.

### 2.7. Cell viability MTT assay

Cell viability ($1 \times 10^4$ cells per well in a 96-well plate) was evaluated using a colorimetric MTT assay measuring reduction powder (R&D Systems Inc., Minneapolis, MN). Following incubation of cells for different treatment, 10 μl MTT solution was added to each wells containing 100 μl of medium and the cells were incubated for a further 4 h at 37 °C. After the cells were washed three times with PBS (pH 7.4), 100 μl of 10% SDS in 0.01 M HCl solubilization solution was then added to dissolve the water-insoluble formazan salt. The absorbance of each well was measured by spectrophotometer reader at 570 nm.

### 2.8. RNA interference and NF-κB luciferase reporter assay

HEK cells were transfected using Liporanax (AMBO Life. Co., TW) according to the manufacturer's instructions. Inhibition of RAGE mRNA was achieved by using siRNA. HEK cells were transfected by 100 nM/ml annealed siRNA oligonucleotides against RAGE (sc-36374, Santa Cruz Biotechnology Inc., CA). For negative control, siRNA against green fluorescent protein (siGFP) was used (Dharmacon Inc., Lafayette, CO). After incubation with siRNA at 37 °C overnight, HEK cells were incubated with high glucose for 18 h, and subjected to further analysis. The pNF-κB luciferase plasmid was purchased from Promega (Madison, WI). Luciferase activities were measured using a dual luciferase system (Promega).

### 2.9. Statistical analysis

The data were expressed as mean ± S.E.M. A Student's test was used for comparing parametric variables between the two groups, while ANOVA with repeated measurement design was used for time course changes. Statistical significance was evaluated by Turkey test (GraphPad Software Inc., San Diego, CA). A *p*-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. PPARδ agonist attenuated serum pro-inflammatory cytokines in STZ mice

TNFα, IL-6, and IL-1 levels in the serum of STZ mice were determined by ELISA. The STZ mice treated by PBS did not show significant change of cytokine levels (Fig. 1). STZ mice treated by L-165041 for 10 days showed significant decrease of serum TNFα (Fig. 1A), IL-6 (Fig. 1B), and IL-1 (Fig. 1C) levels. These data demonstrate that L-165041 decreases the STZ-induced serum pro-inflammatory cytokines in STZ mice.

### 3.2. Effects of L-165041 on PPARδ, RAGE expression and NF-κB activation in the kidney of STZ mice

After 10-day treatment by L-165041, we sacrificed STZ mice and collected kidney tissues for further study. Administration of